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M. Sc. (Medical Biotechnology) **Silvia La Porta**

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Endothelial Tie1 regulates tumor angiogenesis, vascular abnormalization and metastatic dissemination

Referees:

Prof. Dr. Peter Angel

Prof. Dr. Hellmut G. Augustin

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To My Parents

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Table of Contents

List of Figures

List of Tables

Summary

The Angiopoietin/Tie system is one of the most important vascular-tissue specific signaling pathways, essential during embryonic vascular development and maturation, and key regulator of adult homeostasis. The Tie receptors, Tie1 and Tie2, and their secreted angiopoietin (Ang) ligands, Ang1 and Ang2, have been identified as the main factors of the system. Ang1 is primarily produced by perivascular cells and acts in a paracrine fashion as strong Tie2 agonist that mediates endothelial cell (EC) survival and maturation signals. Ang2, expressed and stored in EC, functions primarily as an antagonist of Tie2 and promotes vascular destabilization. Nevertheless, it can act as a partial agonist of Tie2 in a context-dependent manner. Tie1 and Tie2 are both expressed by EC and share high similarities in their overall domain structure. Whereas Tie2 acts as the primary signal transducing receptor, Tie1 function as a holoreceptor is not completely understood: It does not activate signaling pathways on its own but rather modulates the Tie2 kinase activity. Tie1 is highly expressed in activated endothelial cells during embryogenesis and during pathological conditions, such as tumor progression and metastasis, but it is downregulated in the adult quiescent endothelium. Thus, Tie1 global deficiency in mice leads to embryonic lethality at late gestation due to perturbed vessel integrity, while its specific deletion in the growing tumor vasculature results in decreased blood vessel density. The combination of high expression in the tumor endothelium and low expression in the adult vasculature makes Tie1 an interesting molecule and a potential therapeutic target.

Angiogenesis is one of the most critical steps during tumor growth and metastasis progression. Comparative tumor and metastasis experiments in wildtype and Tie1 endothelial-deficient mice were performed to mechanistically unravel the role of the orphan receptor during individual steps of tumor progression and metastasis. Analysis of the tumor vasculature demonstrated that Tie1 endothelial deletion induces no significant changes in the early steps of tumor growth but rather affects later steps of tumor progression. Endothelial-Tie1 deletion induces progressively normalization of primary tumor blood vessels accompanied by decreased microvessel density, increased mural cell coverage, improved vessel perfusion and reduced tumor cell intravasation into the blood stream as well as extravasation at secondary sites. These effects result in almost complete inhibition of post-surgical spontaneous lung metastasis and improved overall survival of Tie1-endothelial deleted mice. Mechanistically, Tie1 targeting in the primary tumor leads to vascular normalization and stabilization by increasing the proportion of Tie2-positive endothelial cells (stalk cells) and by potentiating the Ang1/Tie2 signaling axis which is primarily essential for the maintenance of endothelial quiescence and survival.

The data establish a remarkable contribution of the orphan receptor Tie1 to primary tumor angiogenesis and to individual steps of metastatic cascade. These findings contribute to the mechanism-guided validation of Tie1 as a therapeutic target to sharpen the balance between triggering vascular regression and promoting vascular normalization.

Zusammenfassung

Das Angiopoietin/Tie-System ist einer der wichtigsten Gefäß-spezifischen Signalwege. Es ist essentiell während der embryonalen Gefäßentwicklung und reifung, und ist ein wesentlicher Regulator der Homöostase im Erwachsenen. Die Tie-Rezeptoren, Tie1 und Tie2, und ihre sezernierten Angiopoetin (Ang)- Liganden, Ang1 und Ang2, wurden als Hauptfaktoren des Systems identifiziert. Ang1 wird primär von perivaskulären Zellen produziert und wirkt in parakriner Weise als starker Tie2-Agonist, der Endothel zellen Überlebens- und Reifungssignale vermittelt. Ang2 wird von Endothel zellen exprimiert und gespeichert und wirkt vorrangig als Antagonist des Tie2-Signalweges, der so eine Gefäßdestabilisierung fördert. Allerdings wirkt Ang2 kontextabhängig teilweise auch als Agonist. Tie1 und Tie2 werden beide von Endothel zellen exprimiert und haben hochgradige Ähnlichkeiten in ihrer Proteinstruktur. Während Tie2 den eigentlichen Signaltransduktionsrezeptor darstellt, ist die Funktion von Tie1 als Holorezeptor noch nicht vollständig geklärt: Nach dem heutigen Wissensstand aktiviert es selbst nicht direkt Signalwege, sondern interagiert und moduliert die Tie2-Kinase-Aktivität. Tie1 wird in aktivierten Endothel zellen während der Embryogenese und bei pathologischen Zuständen wie der Tumor progression und Metastasierung stark exprimiert, während der Expressionsstand im erwachsenen, ruhenden Endothel eher niedrig ist. Somit führt eine Tie1-Deletion in Mäusen zu embryonaler Letalität im späteren Trächtigkeitsstadium aufgrund der gestörten Gefäßintegrität. Eine spezifische Deletion von Tie1 im wachsenden Tumorendothel führt zu einer verminderten Blutgefäßdichte. Die Kombination einer erhöhten Expression im Tumorendothel und geringer Expression in den ruhenden Gefäßen macht Tie1 zu einem interessanten Molekül als potentielles therapeutisches Medikament.

Die Angiogenese ist eine der kritischsten Schritte während des Tumorwachstums und der Metastasen-Progression. Vergleichende Tumor- und Metastasenexperimente in Wildtyp- und endothelspezifischen Tie1-defizienten Mäusen wurden durchgeführt, um die Rolle des Orphan-Rezeptors Tie1 während einzelner Schritte der Tumor progression und Metastasenbildung mechanistisch zu entschlüsseln. Die Analyse der Tumorgefäße zeigte, dass eine endotheliale Tie1 Deletion keine signifikanten Veränderungen in den frühen Stadien des Tumorwachstums induziert, sondern eher die späteren Stadien der Tumor progression beeinflusst. Endothelspezifische Tie1-Deletion induzierte eine fortschreitende Gefäßnormalisierung der Blutgefäße im Primärtumor, begleitet von einer verminderten Gefäßdichte, einer erhöhten periendothelialen Gefäßabdeckung, einer verbesserten Gefäßperfusion, einer reduzierten Tumorzellintravasation in den Blutstrom sowie einer Extravasation an sekundären Positionen. Diese Effekte führten zu einer nahezu vollständigen Hemmung spontaner Lungenmetastasen nachdem der Tumor operativ entfernt wurde und einem verbesserten Gesamtüberleben in den Tie1-endothelial-deletierten Mäusen. Mechanistisch gesehen führt das Tie1-Targeting im Primärtumor zu einer vaskulären Normalisierung und Stabilisierung durch eine Erhöhung des Anteils an Tie2-positiven Endothel zellen (Stalkzellen) und dadurch zu einer Verstärkung der Ang1/Tie2-Signalachse, die primär für die Aufrechterhaltung des ruhenden Endothels und dessen Überleben von wesentlicher Bedeutung sind.

Die Daten erbringen einen bedeutenden Beitrag des Orphan Rezeptors Tie1 zur primären Tumorangiogenese und noch wesentlicher zu seiner Rolle in den einzelnen Schritten der metastatischen Kaskade. Diese Erkenntnisse tragen entscheidend zu einer Mechanismus-geführten Validierung von Tie1 als therapeutisches Target bei, das das Gleichgewicht zwischen Gefäßregression und der vaskulären Normalisierung fördern kann.

1. Introduction

1.1 Cancer Statistics

Tumorigenesis is a multi-step process characterized by genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Foulds, 1954). This process is formally analogous to Darwinian evolution, in which the succession of genetic changes confers growth advantage, leading to the progressive conversion of normal cells into cancer cells (Foulds, 1954; Nowell, 1976). Nevertheless, tumors are more than insular masses of proliferating cancer cells; they are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions between each other. This notion reveals that the biology of tumors must also encompass the contributions of the "tumor microenvironment" to tumorigenesis (Hanahan and Weinberg, 2011). As reported by the World Health Organization (WHO), cancer is one of the most prominent causes of mortality worldwide, with 8.2 million deaths from cancer (13% worldwide) and 14 million people affected every year. In the next two decades, 23.6 million cancer cases are expected (http://www.who.int/cancer/en/). The most common causes of cancer death in men are lung, prostate, colorectal, stomach and liver cancer. On the contrary, the most prevalent in women are colorectal, lung, breast, stomach and cervical cancer. A recent review of the fifth version of GLOBOCAN, the source of cancer incidence and mortality from 184 countries worldwide, indicated lung, breast, and colorectal cancer as the most commonly diagnosed cancers, while estimating lung, liver and stomach cancer as the most common causes of death (Ferlay et al., 2015). Although much progress has been made in the last decades against cancer, the overall incidence and mortality of cancer is estimated to rise to 12 million cancer deaths in 2030 (Fig. 1).

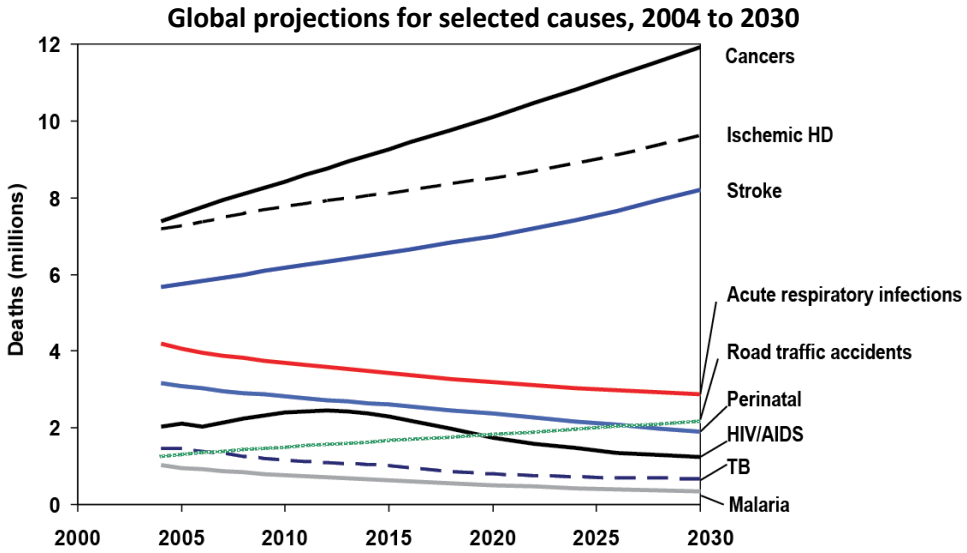

Figure 1: Global projections for selected causes, 2004 to 2030

The cancer mortality will dramatically increase in the following years. Taken from WHO, global burden of disease 2004, http://www.who.int/topics/global_burden_of_disease/en/).

Notably, cancer-related deaths are generally not caused by the primary tumor, rather than by the spread of tumor cells throughout the body, a process known as metastasis (Steeg, 2016). The diagnosis of metastatic disease indicates a terminal illness and causes approximately 90% of the human cancer deaths (Mehlen and Puisieux, 2006; Wirtz et al., 2011).

1.2 Cancer Progression

Cancer types are classified according to their site of origin: carcinomas have epithelial origin, sarcomas derive from connective tissue, leukemias and other hematological malignancies derive from blood-forming tissues, lymphomas originate from cells of the lymphatic immune system and myelomas arise from plasma cells. Albeit their different origins, all types of cancers share common essential alterations in the cell physiology that collectively dictate the malignant phenotype (Hanahan and Weinberg, 2011). This set of features is known as “hallmarks of cancer” and was first described by Hanahan and Weinberg in 2000. These characteristics include self-sufficiency in growth signals, insensitivity to anti-growth signals, ability to evade apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig. 2) (Hanahan and Weinberg, 2000).

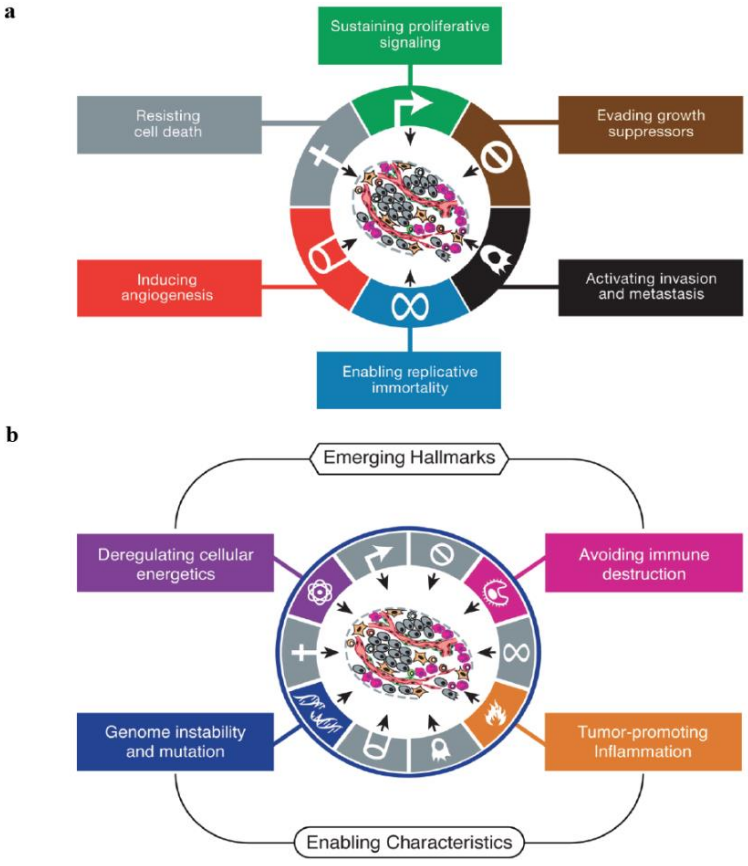

Figure 2: The Hallmarks of cancer

Reprinted with permission from (Hanahan and Weinberg, 2011). Cancer cells acquire **a)** six functional capabilities enhancing their tumorigenic performance; **b)** New emerging hallmarks and two additional enabling characteristics.

Recently, new emerging hallmarks have been added such as reprogramming energy metabolism, evading immune surveillance, tumor promoting inflammation, and genome instability and mutation (Hanahan and Weinberg, 2011).

1.3 Metastatic dissemination of cancer: the metastatic cascade

Activating invasion and metastasis has been proposed as a hallmark of cancer already in the last decade (Hanahan and Weinberg, 2000). The term metastasis derives from the Greek words μετά+ στάσις; where (μετά), *meta*, means "next" and στάσις (stasis), "placement". Therefore, metastasis means displacement and it represents a multistep process during which malignant cells disseminate from the tumor of origin to distant organs. It is the third most diffuse cause of mortality and the first cause of cancer morbidity and death (Mehlen and Puisieux, 2006; Wirtz et al., 2011). Although much progress has been made over the last years in order to understand the mechanism of cancer biology, new efforts are needed in cancer research to decipher the advanced stages of metastatic progression. Indeed, given the high incidence in the populations and the grave prognosis, new therapies are required to combat cancer.

In 1889, Stephen Paget's proposed that metastasis depends on cross-talk between selected cancer cells ("seeds") and specific organ microenvironment ("soil"), where the dependence of the seed on the soil is essential (Paget, 1989). In 1929, James Ewing challenged Paget's theory, claiming that metastatic dissemination occurs by purely mechanical factors that result from the anatomical structure of the vascular system (Fidler, 2003). Nowadays, metastatic progression is considered as a multistage event, critically dependent on the interaction of metastasizing cells with their microenvironment, either at the primary tumor site or at the site of metastasis. To be able to develop metastasis, cancer cells need to i) acquire motility and invade locally into the tumor adjacent tissue, ii) intravasate into blood vessels and lymphatic, iii) survive in the circulation, iv) extravasate into target organs, and v) colonize the tissue, supported by angiogenesis and micro-/macro-metastasis development (Fidler, 2003; Pantel and Brakenhoff, 2004) (Fig. 3).

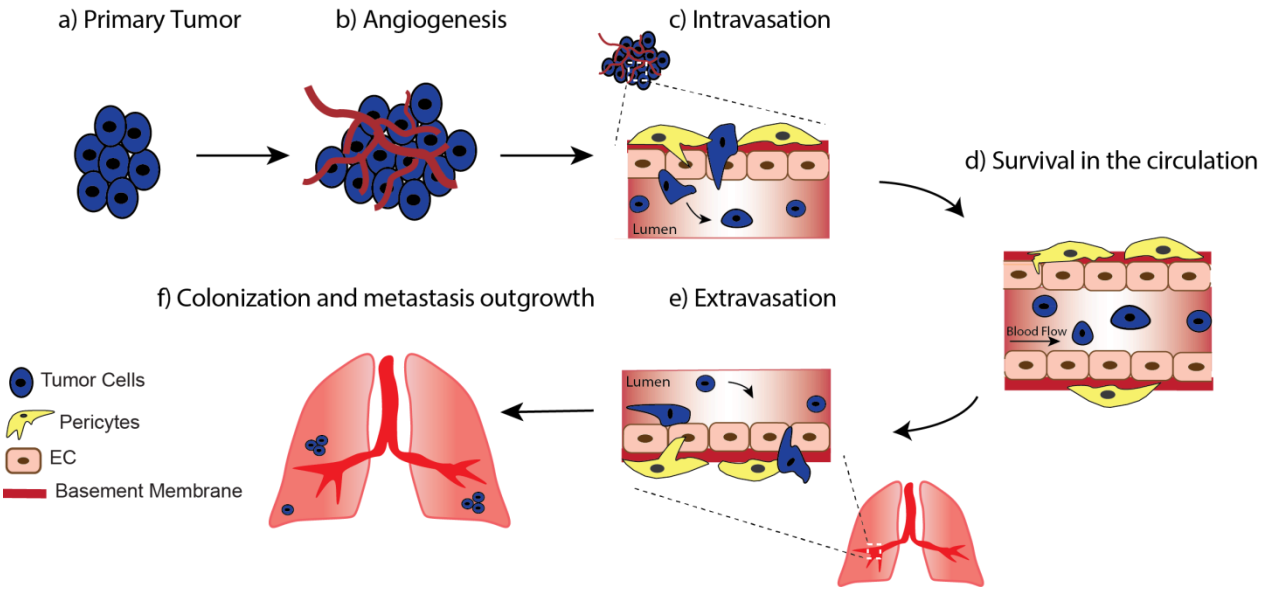

Figure 3: Schematic representation of the different steps of the metastatic cascade

a) Growth of tumor cells is progressive: in the beginning, neoplastic cells receive oxygen and supplies via diffusion from the host vasculature. **b)** When the tumor mass exceeds 1-2 mm in diameter, tumor cells start producing and releasing angiogenic factors that stimulate extensive tumor vascularization, important either for the delivery of oxygen and nutrients, and for the elimination of metabolic waste. **c)** Tumor cells either detach from each other or form small clusters, and then invade the surrounding tissue, reaching the blood or lymphatic capillaries and intravasating through the endothelial layer. **d)** In the circulation tumor cells encounter many difficulties and try to escape the immune system from being cleared out, so only few cells survive and reach capillary beds of distant organs. **e)** Here they attach to the inner vessel wall and extravasate into surrounding tissue. Once successfully transmigrated in the organ, the majority of tumor cells die while a small proportion stay in a non-proliferative "dormant state". **f)** Tumor cells proliferation and formation of metastatic colonies are the last steps of metastatic cascade.

1.3.1 Invasion

To metastasize to other organs, tumor cells need to migrate out from the primary tumor site. This motility is obtained either by the loss of cell adhesion or by an increase in factors that are involved in the degradation of the extracellular matrix (ECM). In particular, tumor cells need to lose cell-cell contacts, to anchor to the ECM and secrete metalloproteinases in order to cut the ECM and migrate through it, according to the gradient formed by the chemokines released from ECM degradation (Liotta, 1986). The gain of migratory capabilities requires a temporary and reversible phenomenon that is known as epithelial to mesenchymal transition (EMT) (Thiery, 2009). Because of their epithelial origin, carcinoma cells are originally non-motile cells, presenting tight cell-cell interaction and basal-apical polarization. During EMT, tumor cells lose their rigid epithelial morphology and transform in motile spindle-like mesenchymal morphology. This transition requires fundamental changes at the transcriptional level; for example, cell-cell adhesions are disrupted by repression of E-Cadherin and cytokeratin expression, while the expression of typical components of the mesenchymal cytoskeleton is induced, such as vimentin and α smooth muscle actin (αSMA). In addition, cell-basement membrane adhesions are reduced by downregulation of some integrins, whereas disseminating tumor cells start expressing typical fibroblast markers, such as N-cadherin, fibronectin and collagen type I (Hanahan and Weinberg, 2011).

Molecularly, EMT can be induced by various growth factors that are secreted in the tumor microenvironment like TGFβ (transforming growth factor β), HGF (hepatocyte growth factor), IGF (insulin-like growth factors), some members of the epidermal growth factors (EGF) and fibroblast growth factors (FGF). All these different upstream molecules converge in the same signaling pathways that involve the small GTPases RhoA and Rac1 (Ras-related C3 botulinum toxin substrate 1), Ras (Rat Sarcoma), PI3K (phosphoinositol-3 kinase), MAPK (mitogen-activated protein kinase), ILK (integrin-linked kinase) and the Jagged/Notch pathway (Thiery, 2002). The EMT transdifferentiation is limited to a restricted number of single cells at the invasive front: intravital imaging analysis has shown that just 1-5% of tumor cells display motile capabilities (Sahai, 2005). The acquired mesenchymal phenotype is reversible once disseminating tumor cells reach secondary sites and form metastases. Here, cells undergo the mesenchymal to epithelial transition (MET), whereby they lose their motile and spindle-like shape and regain a rigid epithelial phenotype to form solid epithelial-looking metastasis (Hugo et al., 2007; Tsai and Yang, 2013).

Besides EMT, tumor cells can invade distant tissues through collective cell migration. In this process tumor cells move in concert, without completely disrupting their cell-cell contacts, and express podoplanin at their site of invasion (McCaffrey et al., 2012; Wicki et al., 2006). Recently, the structural organization of the ECM has also been found to be involved in the tumor cell invasion. Rearrangement of collagen into a more linearized shape promotes tumor cell invasion, while a non-linearized and less stiff fibrillary collagen can impede tumor cell motility (Maller et al., 2013). In conclusion, tumor cells invasion can be initiated by different processes that induce changes in tumor and stroma cells, in order to remodel and restructure the ECM.

1.3.2 Intravasation

Intravasation occurs shortly after the successful invasion of tumor cells through the basement membrane and ECM, and refers to the process through which tumor cells enter the blood stream within or close to the primary tumor site. This is the rate-limiting event for tumor cells to give rise to distant metastasis or not. Intravasation requires the disruption of endothelial junctions for cancer cells to cross the endothelium, in a process known as transendothelial migration. Indeed, the newly formed tumor blood vessels generally present weak cell-cell junctions, that can be easily crossed by tumor cells to enter the blood stream (Weis and Cheresh, 2011).

It is still not clear if intravasation is a passive or an active mechanism. On one hand, it has been shown that a notable number of tumor cells shed through immature tumor vasculature and that a correlation exists between the number of large vessels diameter with the amount of tumor cells clumps (Butler and Gullino, 1975; Liotta et al., 1976), supporting the passive form of intravasation. On the other hand, the high production of tumor-cell intrinsic factors or factors produced by the stroma gives significant evidence for an active mechanism. For example, matrix metalloproteinases 1 (MMP1) is required for intravasation of tumor cells through the endothelial monolayer, via activating the protease-activated receptor 1 (PAR1), that mediates cytoskeleton rearrangements and remodeling of endothelial junctions

(Juncker-Jensen et al., 2013). Alternatively, a disintegrin and metalloproteinase 12 (ADAM12) on endothelial cells (EC) can induce cleavage of vascular-endothelial cadherin (VE-cadherin) and Tie2, causing the disruption of endothelial junctions (Frohlich et al., 2013). Notch signaling has also been postulated to contribute to intravasation: Notch receptor on tumor cells can bind to Notch ligands on EC and promote transmigration through the endothelial junctions (Sonoshita et al., 2011).

Moreover, cancer cells invasion through the stroma to the blood vessels can be promoted by tumor associated macrophages (TAM). They can attract neoplastic cells towards blood vessels by releasing EGF (Roussos et al., 2011; Wyckoff et al., 2004; Wyckoff et al., 2007) and tumor necrosis factor 1α (TNF1α) (Reymond et al., 2013), which induce the retraction of endothelial junctions, thus facilitating transendothelial migration. Promotion of vessel leakiness is another mechanism through which macrophages induce intravasation (Mierke, 2012). TAM have also been associated with increased hypoxia (Rezvani et al., 2011); generally the hypoxic microenvironment mediates the activation of pathways correlated to ECM remodeling, therefore impacting tumor cell transmigration.

In conclusion, intravasation into the blood stream is a complicated process that involves different cell types besides tumor cells. New tools and new *in vitro* assays are needed in order to better recapitulate the *in vivo* scenario, making it possible a clear comprehension of this process.

1.3.3 Survival in the circulation

Once into the blood stream, tumor cells are exposed to continuous stress, such as shear stress, absence of cell contacts or of ECM and immune surveillance. To successfully extravasate and colonize secondary sites, tumor cells need to overcome these obstacles. This explains the disparity between the millions of cells shed from a tumor every day and the relatively few clinically detectable metastases (Bockhorn et al., 2007; Cristofanilli, 2006). A notable amount of tumor cells, for example, undergoes apoptosis in the circulation induced by shear stress, and a significant number of cells are already apoptotic into the primary tumor but undergo passive shedding, without the involvement of active cell migration (Bockhorn et al., 2007). Tumor cells are protected by platelets against physical stress in the circulation and against lysis induced by cytotoxic immune cells (Labelle et al., 2011; Nieswandt et al., 1999). In order to improve their viability in the blood stream, tumor cells migrate in clusters (Duda et al., 2010). These clusters express plakoglobin, an epithelial cell junction molecule, that if inhibited can prevent metastasis (Aceto et al., 2014). Via interaction between selectin and glycoproteins, tumor cells can also acquire a rolling behavior on the inner surface of the blood vessels (Dimitroff et al., 2004). In particular, the integrins expressed on the surface of tumor cells bind to adhesion molecules on the EC surface, forming stable interactions. This event is crucial for subsequent extravasation and metastatic colonization (Garofalo et al., 1995).

1.3.4 Extravasation

Extravasation is an essential and rate-limiting process for metastatic spread. Shedded tumor cells transmigrate through the endothelium into distant organs in a way that is far less efficient if compared

to leukocytes extravasation: while the latter can reach secondary tissues in order of minutes, tumor cells can take up to 1-2 days (Strell and Entschladen, 2008). During extravasation, tumor cells start producing adhesion molecules, like CD44, integrins and galectin-3 (Hiratsuka et al., 2011; Miles et al., 2008; Murugaesu et al., 2014), promoting a direct contact with endothelial cells, and start secreting vasoactive compounds such as vascular endothelial growth factor (VEGF), angiopoietin-like 4 (Angptl4), HGF, angiopoietin 2 (Ang2), chemokine (C-C motif) ligand 2 (CCL2), stromal cell-derived factor1α (SDF1α) that induce vascular permeability or loosening of endothelial junctions, therefore favoring metastasis (Balkwill, 2012; Garcia-Roman and Zentella-Dehesa, 2013). ATP, released by tumor cell-activated platelets, is also an important factor that facilitates opening of endothelial barrier and therefore tumor cell extravasation (Schumacher et al., 2013). Clinical data have shown that high platelet counts in breast cancer patients associate with poor survival prognosis (Taucher et al., 2003). Another mechanism through which tumor cells promote extravasation is the production of substances that cause endothelial damage, such as reactive oxygen species (ROS), epiregulin, cyclooxygenase 2, MMPs etc (Gupta et al., 2007). In conclusion, the process of extravasation requires production and release in the microenvironment of new factors by tumor cells and their physical interaction with the endothelium.

1.3.5 Dormancy and survival at the secondary site

Once successfully transmigrated to a secondary site, tumor cells either die or stay in a viable but non-proliferative state, called “dormancy”. For the patients, this results in disease free-periods that can last even for 20-25 years, during which they do not develop metastasis (Ghajar et al., 2013; Karrison et al., 1999). Although dormancy is a status that frequently occurs in breast cancer, there is no detailed knowledge about the signals that induce tumor cells to transition from a dormant state to a proliferating state and vice versa. Studies have suggested that a large number of solitary cells that arrive at secondary site and fail to initiate cell division can survive for a long period of time in the organ. This pause in progression is partially due to the immortalization of disseminated cancer cells, acquired after gradual accumulation of mutations that induce loss of p53, retinoblastoma 1 (Rb1), cyclin-dependent kinase inhibitor 2a (p16), activation of RAS, BRAF and amplification of ERBB2 (Hanahan and Weinberg, 2000). Alternatively, it has been suggested that pre-angiogenic micro-metastases remain dormant because the tumor cell proliferation is balanced by an equivalent rate of cell death (Hahnfeldt et al., 1999; Holmgren et al., 1995). In conclusion, disseminated cells in micro-metastases or individual cells need to survive during this period of dormancy. The microenvironment can play an essential role in surveilling the growth of the lesions (Bragado et al., 2012).

1.3.6 Colonization and metastatic outgrowth

To successfully complete the metastatic cascade, extravasated tumor cells need to proliferate and to form metastatic colonies. As suggested by Paget with the “seed and soil” theory, tumor cells can only grow in a suitable microenvironment (Paget, 1989). Nevertheless, it is still not clear if the primary tumor releases factors that facilitate the formation of a pre-metastatic niche where tumor cells can seed, or if shedded tumor cells produce factors that remodel the microenvironment in secondary sites according to

their needs. Evidence has been provided that the primary tumor releases metalloproteinases 9 (MMP9) or other enzymes, such as lysyl oxidase (LOX), which can either act on endothelial cells of secondary sites (Kaplan et al., 2006) or induce recruitment of macrophages to distant organs (Erler et al., 2009), thereby promoting colonization. Tumor cells proliferation in the metastatic site occurs after activation of kinases such as focal adhesion kinase (FAK) and the extracellular signal regulated kinase (ERK) (Aguirre Ghiso et al., 1999), and it is driven by the formation of filopodia-like protrusions (Shibue et al., 2012). Recent work has also shown a role of exosome in the pre-metastatic niche formation (Costa-Silva et al., 2015). In conclusion, suitable tumor microenvironment is essential for tumor formation, progression and metastatic outgrowth.

1.4 The tumor microenvironment

The tumor microenvironment, also known as tumor stroma, surrounds the cancer cells and dynamically interacts with them, promoting primary tumor initiation, supporting cancer progression and endorsing metastatic outgrowth (Li et al., 2007; Pietras and Ostman, 2010). Tumor stroma is composed of ECM and other non-malignant cell types such as inflammatory cells, stromal myofibroblasts, cellular constituent of blood and lymphatic vessels such as endothelial cells, pericytes and smooth muscle cells (SMC) (Gerhardt and Semb, 2008; Pietras and Ostman, 2010; Sund and Kalluri, 2009; Wels et al., 2008). Tumor growth strictly depends on its vasculature, which provides the delivery of oxygen and supplies into the microenvironment. Tumor initiates as an avascular mass, where cancer cells grow along pre-existing vessels in order to satisfy their need of metabolites. When tumor outgrows the supplies, it triggers the “angiogenic switch” that induces the formation of a vascular network to secure tumor cell proliferation and growth (Folkman, 1971; Nyberg et al., 2008).

1.4.1 Vascular components

The vascular system controls the metabolic exchange between the blood and adjacent tissues, guaranteeing a constant delivery of oxygen and supplies. It is composed of EC and mesenchymal derived cells, such as pericytes and smooth muscle cells, surrounded by a basement membrane (BM). EC derive from hemangioblasts and/or angioblasts (Augustin et al., 1994; Coultas et al., 2005). They line together forming a monolayer along the inner vessel wall, creating a lumen that allows the passage of the blood. Blood vessels can have different size: capillaries, arterioles and venules are small vessels, while arteries and veins are large vessels. Smaller vessels are surrounded by the basal lamina and are covered by single pericytes. In the larger vessels, the endothelium is covered by a dense layer of SMC and pericytes that are essential for vessel stabilization (Fig. 4).

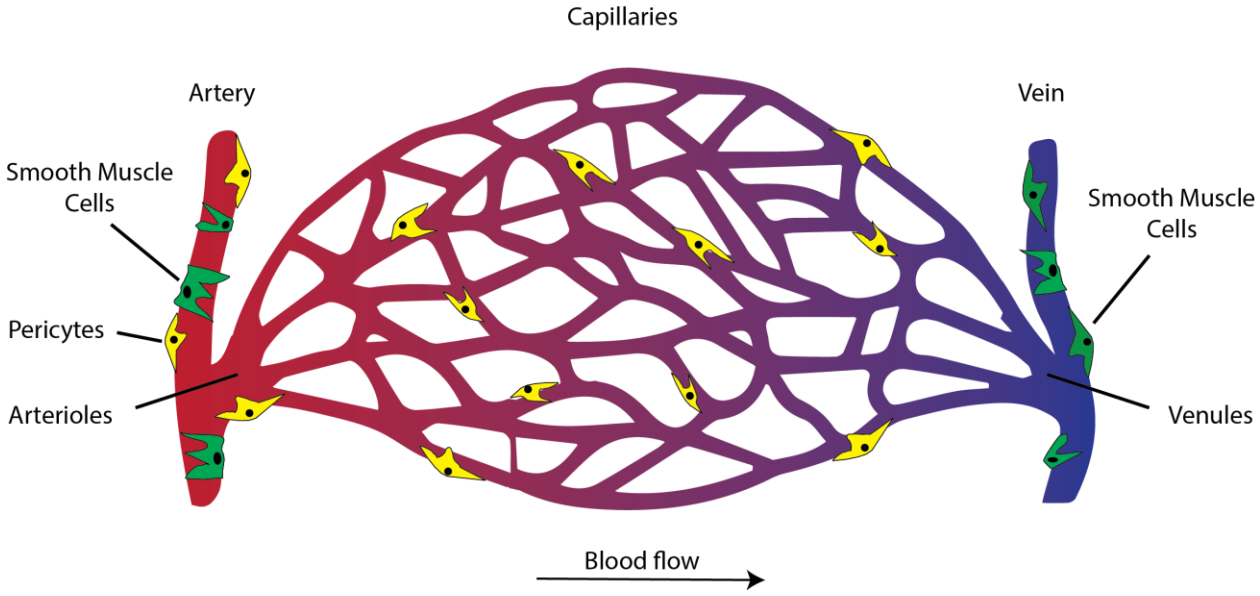

Figure 4: The vascular system

The hierarchical structure of the blood vascular system consists of arteries-arterioles-capillaries-venules-veins. Arterioles, capillaries and venules are surrounded by basal lamina and are loosely covered by perivascular cells. Larger vessels, such as arteries and veins, are densely covered by SMC and pericytes, in order to increase blood vessel elasticity, therefore promoting blood flow.

There are differences also between arteries and veins: the former have a thick and elastic endothelial wall in order to sustain the high blood pressure coming from the heart to distant organs; while the latter present a thinner wall and are less covered by mural cells (Aird, 2007a; Aird, 2007b; Garlanda and Dejana, 1997).

1.4.2 Tumor-vascular interaction

The diffusion limit for oxygen is 100-150 μm. Therefore, tumor cells need to be within a distance of maximum 200 μm from a functional vessel to survive (Torres Filho et al., 1994). When the microenvironment becomes hypoxic, tumor cells release pro-angiogenic factors like VEGF and platelet-derived growth factor (PDGF), which favor neovascularization via induction of the angiogenic switch. Nevertheless, the tumor microenvironment is also characterized by anti-angiogenic cytokines such as thrombospondin 1 (TSP1), angiostatin and endostatin, which inhibit angiogenesis, causing hypoxia in the tumor microenvironment, and lead to tumor cells death. The balance between pro- and anti-angiogenic molecules is controlled by different pathways. In particular, it has been demonstrated that RAS activation upregulates VEGF, while downregulating the angiogenesis inhibitor TSP1 (Rak et al., 1995; Sheibani and Frazier, 1996). On the contrary, p53, phosphatase and tensin homolog (PTEN) and mothers against decapentaplegic homolog 4 (SMAD4) have been shown to induce expression of TSP1 (Chandrasekaran et al., 2000; Dameron et al., 1994; Volpert et al., 1997). The direct or indirect interactions between cancer cells and endothelial cells can be rate-limiting for tumor growth.

1.5 Angiogenesis and blood vessel formation

Angiogenesis is the formation of new blood vessels from pre-existing ones and it differs from vasculogenesis, which is the process where blood vessels form *de novo.* The angiogenic cascade is a tightly regulated event composed of several steps, such as activation of endothelial cells by pro-angiogenic stimuli, degradation of the basal lamina, proliferation and migration of endothelial cells, lumen formation and recruitment of mural cells to stabilize the newly formed blood vessels (Risau, 1997). In particular, during hypoxic condition, hypoxia inducible factor 1α (HIF1α) is activated in endothelial cells, resulting in the expression of VEGF-A and its receptor (VEGFR2) (Tang et al., 2004). The production and release of VEGF-A create a gradient that is followed by endothelial cells in order to reach avascular areas and create new vessel sprouts. This occurs via tip cells formation and subsequent proliferation of the adjacent stalk cells (Gerhardt and Betsholtz, 2003; Ruhrberg et al., 2002). Subsequently, ECM and the basement membrane are degraded by activated metalloproteinases, allowing mural cells to migrate on the top of the blood vessels and proliferate (Folkman and D'Amore, 1996; Hughes, 2008). The interaction between EC and perivascular cells further promotes endothelial quiescence, via inducing expression of MMP inhibitors, so that ECM degradation is suppressed (Beck and D'Amore, 1997; Benjamin et al., 1998; Saunders et al., 2006).

During tumor growth, the demand for oxygen and nutrients and the need to eliminate metabolic waste from the tumor microenvironment induces a continuous release of proangiogenic molecules that foster the production of abnormal tumor vessels in their appearance and function (Baluk et al., 2005; Folkman, 1971; Jain, 2005; Nagy et al., 2010). Tumor vessels lose their hierarchical structure (arterioles-capillaries-venules-veins) and appear tortuous, disorganized, hyperpermeable and poorly functional. EC, indeed, lose their typical cobblestone shape and present loose interconnections, becoming occasionally multilayered. Mural cells are poorly attached to the EC, favoring a reduction in blood vessel functionality and an increase in permeability. This chaotic vasculature, therefore, leads again to insufficient supply of nutrients, oxygen and growth factors to the tumor mass, and re-stimulates the release of proangiogenic molecules, exacerbating the scenario (Goel et al., 2011; Jain, 2005). The hypoxic and acidic milieu that results from this non-productive angiogenesis, implies limitations in drug delivery and lowers the efficacy of radiotherapy (Vaupel et al., 2001).

1.5.1 Sprouting angiogenesis

Although there are several processes leading to angiogenesis, sprouting angiogenesis plays a major role during tumor blood vessels formation. This process has been studied in great detail, allowing the development of drugs that selectively target this mechanism. As mentioned before, sprouting angiogenesis involves degradation of the ECM and basement membrane to facilitate the migration of endothelial tip cells towards a gradient of proangiogenic stimuli. Two major signaling pathways orchestrate sprouting angiogenesis: VEGF-VEGFR and DLL4-Notch pathway (Hellstrom et al., 2007; Phng and Gerhardt, 2009; Suchting et al., 2007). VEGF act through three structurally related VEGF receptor tyrosine kinases, denoted VEGFR1 (Flt1), VEGFR2 (Flk1) and VEGFR3 (Flt). Binding of VEGF to its VEGFR2

or VEGFR3 induces receptor auto- or transphosphorylation and therefore activation of a signaling cascade that is involved in endothelial cell proliferation and migration. VEGFR1 is a decoy receptor, with weak kinase activity. It is highly expressed in stalk cells and it negatively regulates VEGFR-2 response (Koch and Claesson-Welsh, 2012). Dll4 is a Notch ligand, upregulated in tumor endothelial tip cells by VEGF (Claxton and Fruttiger, 2004; Liu et al., 2003). Dll4 induces expression of Notch in the adjacent stalk cells, which downregulate VEGFR2, upregulate VEGFR1, and do not respond anymore to VEGF stimuli, leading to a failure of sprouting in the stalk cell compartment. Interference with the Dll4-Notch expression could lead to hypersprouting, resulting in a non-perfused, chaotic and non-functional vasculature (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). In opposition, the other Notch ligand, Jagged1 (Jag1), has proangiogenic functions and it is expressed either by tumor endothelial cells or by cancer cells.

1.5.2 Vascular cooption

Several mechanisms contribute to tumor angiogenesis. Amongst these, vascular cooption is an angiogenic process that occurs during the early stages of tumor growth. In this mechanism, tumors obtain a blood supply by hijacking the existing vasculature, and tumor cells proliferate along the vessels of the host organ until the tumor milieu becomes hypoxic and induces the angiogenic switch. This phenomenon is observed in highly vascularized organs, such as lungs and brain (Donnem et al., 2013). Ang2 has been proposed as a critical mediator of vessel cooption since its expression is induced in the endothelium of coopted tumor vessels as well as in the late stage tumors (Holash et al., 1999). Contrarily, VEGF is highly induced only in late stage angiogenic tumors (Holash et al., 1999).

1.5.3 Intussusception

Intussusception is a mechanism wherein a pre-existing vessel splits into two daughter vessels, via formation of a trans-vascular bridge. In particular, two endothelial cells that are on opposite site of the lumen form “kissing contacts” resulting in a transluminal bridge. Therefore, there is a rearrangement of EC junctions and new perforations are formed (Burri et al., 2004). This variant of angiogenesis has been demonstrated in models of colon adenocarcinoma and breast cancer (Djonov et al., 2001; Patan et al., 1996). Intussusception has been correlated with low levels of VEGF. This process, therefore, might occur in anti-VEGF resistant tumors as a consequence of sprouting angiogenesis inhibition (Djonov et al., 2001).

1.5.4 Vascular mimicry

During vascular mimicry, tumor cells physically rearrange and form tubular, vessel-like structures. Recent studies have demonstrated that this process is driven by differentiation of cancer stem cells in vascular cell types, in specific cancers such as leukemia (Shen et al., 2008), breast cancer (Bussolati et al., 2009), ovarian cancer (Alvero et al., 2009) and glioblastoma (Ricci-Vitiani et al., 2010; Wang et al., 2010a).

1.6 VEGF-VEGFR2 system as anti-angiogenic target

The VEGF-VEGFR2 signaling axis is the most important pathway activated in the tumor microenvironment after the angiogenic switch. Therefore, several drugs targeting VEGF and its receptors have been developed. The use of a monoclonal antibody against VEGF successfully inhibited angiogenesis in several tumor models (Kim et al., 1993). Despite the promising results obtained in pre-clinical settings (Brekken et al., 2000; Holash et al., 2002), the survival of patients treated with either the monoclonal anti-VEGF antibody (Bevacizumab) or with Receptor Tyrosine Kinase Inhibitors (RTKI), was prolonged just by a few months (Allegra et al., 2011; de Gramont et al., 2012; Wood et al., 2000). The first anti-VEGF monoclonal antibody approved by the Food and Drug Administration (FDA) was Bevacizumab (Avastin®), a humanized murine antibody, that specifically binds the VEGF-A isoform (Ferrara et al., 2004). Avastin® treatment created a transient "normalization window" characterized by increased perivascular coverage and improved vessel perfusion and oxygenation (Winkler et al., 2004). Owing to this vessel normalization effect, anti-VEGF therapy was combined with chemotherapy and radiotherapy in order to improve drug delivery (Jain, 2005). Indeed, combinations of chemotherapy and Avastin® in a primary tumor treatment line increased the survival of patients with metastatic colorectal cancer (Hurwitz et al., 2004) and advanced lung cancer (Sandler et al., 2006), and the progression free survival (PFS) of metastatic breast cancer patients (Miller et al., 2007). However, Bevacizumab treatment was not successful in less vascularized cancer types such as gastric cancer and metastatic pancreatic cancer (Kindler et al., 2010; Van Cutsem et al., 2009). The BEATRICE clinical trial stated the failure of Bevacizumab as an adjuvant treatment in patients with triple-negative breast cancer, although it underlines the importance of VEGFR2 plasma levels as a prognostic biomarker (Cameron et al., 2013). In addition to monoclonal antibodies, several RTK inhibitors have been developed and successfully used in pre-clinical and clinical trials: Sunitinib, which targets VEGFR1 and VEGFR3, PDGFR α and ß and c-KIT (Abrams et al., 2003), or Sorafenib, which targets VEGFR2 and VEGFR3, and PDGFR α and ß have shown successful results in breast cancer (Escudier et al., 2007), renal cancer patients (Escudier et al., 2007) and hepatocellular carcinoma (Llovet et al., 2008).

Since VEGF is a molecule important for pathological as well as physiological angiogenesis, anti-angiogenic therapy is not restricted just to the tumor vessels, and it causes severe side effects such as cardiac defects, gastrointestinal perforation and venous thromboembolic events (Eskens and Verweij, 2006; Verheul and Pinedo, 2007). Given the frequent side effects associated to Bevacizumab therapy, FDA revoked its approval for breast cancer treatment in 2011 (Rose, 2011). In conclusion, the efficiency of the anti-angiogenic therapy depends mostly on the tumor type and on tumor-stage.

1.6.1 Anti-angiogenic therapy and vessel normalization

Tumor angiogenesis consists mostly of the production of an increased number of blood vessels to serve a growing mass. This process occurs in an uncontrolled way and the resultant vascular network is highly abnormal. Anatomically tumor vessels are dilated, tortuous, disorganized and highly permeable, with several branch points (Goel et al., 2011). Unlike the microvasculature of normal tissue, tumor

vasculature is characterized by areas in which the vessel density is high and areas where vessel density is reduced. At the cellular level, the EC in tumor vessels have an irregular disorganized morphology and are loosely connected to each other. In addition, perivascular cells, both pericytes and SMC, are loosely attached to the blood vessels or are completely absent, facilitating the movement of EC into the surrounding matrix to form new vessels, and increasing vessel permeability.

As mentioned before, the first rationale of anti-angiogenic therapy was to induce profound vascular regression, starving tumors to death or rendering them dormant (Folkman, 1971). The clinical response to anti-VEGF monotherapy was quite limited and was unable to induce vascular regression to cause significant tumor shrinkage (Cobleigh et al., 2003; Yang et al., 2003). However, combination of anti-angiogenic therapy with chemotherapy showed successful results due to the synergistic effects of the two therapeutic regimens (Jain, 2014). A non-maximal dose of anti-angiogenic drug induces normalization of the tumor vasculature, reverting the grossly abnormal structure and function towards a more normal state. This facilitates the delivery of chemotherapy and therefore ensures a better response to cytotoxic treatment (Jain, 2014).

In conclusion, a judicious dose of anti-angiogenic therapy creates a “normalization window” where immature and dysfunctional blood vessels are pruned away, whereas those remaining are actively fortified. This strategy limits also the damages to normal tissue vasculature, while promoting the delivery of systemically administered cytotoxic compounds (Fig. 5).

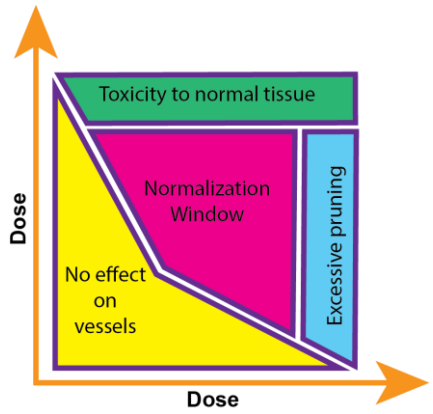

Figure 5: Schematic representation of normalization window

Modified from R. Jain review (Jain, 2014). The efficacy of therapies that combine anti-angiogenic and chemotherapeutic drugs depends on the dose and the delivery of each drug. Under specific dose of anti-angiogenic agents, a “normalization window” occurs, in which the addition of a chemotherapeutic gives the best outcomes. In this short time frame, there is passive pruning of immature vessels and normalization of the remaining ones, without affecting normal tissue vasculature. Furthermore, cancer cells become more vulnerable to cytotoxic therapies. The use of high dose anti-angiogenic drugs leads to normal-tissue toxicity and excessive pruning that compromises blood vasculature so that the delivery of the cytotoxic drugs is limited.

1.6.2 Resistance to anti-angiogenic therapy

The greatest challenge for anti-angiogenic therapy is that a large number of cancer patients are intrinsically refractory to anti-angiogenic drugs and respond minimally or do not respond at all to the therapy (Loges et al., 2010). Other cancer patients, who initially respond to the therapy, experience a survival benefit just in the range of months, without reaching a permanent cure (Kerbel, 2008). Furthermore, pre-clinical studies have shown that anti-angiogenic treatment suppresses tumor growth but simultaneously selects more invasive cancer cell clones, with high metastatic potential (Ebos et al., 2007; Paez-Ribes et al., 2009). However, these findings have not been confirmed by clinical data (Singh et al., 2012b).

Several pre-clinical studies focused their attention on the clarification of the mechanisms behind therapy refractoriness and evasive escape. Current hypotheses include i) alternative pathways of tumor vascularization, that are independent of sprouting angiogenesis, such as vascular cooption and intussusception (di Tomaso et al., 2011; Kienast et al., 2010), ii) activation of other signaling pathways that could compensate VEGF blockade, such as Ang-Tie, EGF-EGFR and FGF-FGFR (Vasudev and Reynolds, 2014), iii) pericyte recruitment on the endothelium, making the EC less responsive to VEGFR2 targeting (Erber et al., 2004) or iv) strong selective pressure on tumor cells that need to survive in an adverse and hypoxic tumor milieu, leading to the survival of the most resistant clones (Wong et al., 2015). Another reason of anti-angiogenic resistance is the v) recruitment of myeloid derived suppresser cells $Cd11b^+$ $Gr1^+$ to the tumor site (Ebos et al., 2007; Shojaei et al., 2007; Shojaei et al., 2009). Anti-angiogenic treatment stimulates the production of pro-inflammatory cytokines such as granulocytes stimulating factor (GCSF) and SDF1α, which induce the recruitment of the resistance mediating $Cd11b^+$ $Gr1^+$ cells. These cells produce Bv8 (Bombina variagata peptide 8), that stimulates tumor angiogenesis, rendering tumors resistant to anti-angiogenic treatments. In conclusion, the combination of anti-angiogenic therapy with drugs interfering with the mechanisms inducing resistance, could improve anti-angiogenic drug efficacy.

1.7 Ang/Tie system

The Angiopoietin (Ang)-Tie system, together with the VEGF/VEGFR signaling, represent the two pathways that are almost exclusively specific for EC. The Ang-Tie system is comprised of two receptors, Tie1 and Tie2 (or TEK), and four Angiopoietin ligands Ang1, Ang2, Ang4 and its mouse orthologue Ang3 (Dumont et al., 1992; Korhonen et al., 1992; Maisonpierre et al., 1993; Partanen et al., 1992; Sato et al., 1993), which together form the angiopoietin growth factor family. The angiopoietins and their endothelial Tie receptors are essential for blood and lymphatic vessel development, for the regulation of sprouting angiogenesis, vascular remodeling and endothelial maturation, and they play an important role in vascular permeability, inflammation and tumor vascularization (Augustin et al., 2009).

1.7.1 The structure of Tie receptors and angiopoietin ligands

Tie1 and Tie2, whose acronym stands for Tyrosine kinase with Ig and EGF homology domains (Partanen et al., 1992), are single transmembrane molecules with an extracellular ligand-binding domain and a split intracellular Tyr kinase domain. They are highly homologous receptors, sharing 76% and 33% similarities in their intracellular and extracellular domains, respectively (Schnurch and Risau, 1993). The extracellular domains are composed of two amino-terminal immunoglobulin (Ig)-like domains, three epidermal growth factor (EGF) homology domains, a third Ig-homology domain and finally three fibronectin type III domains that are adjacent to the transmembrane domain (Jones et al., 2001). The intracellular kinase domain can bind to different molecules after phosphorylation (Fig. 6a).

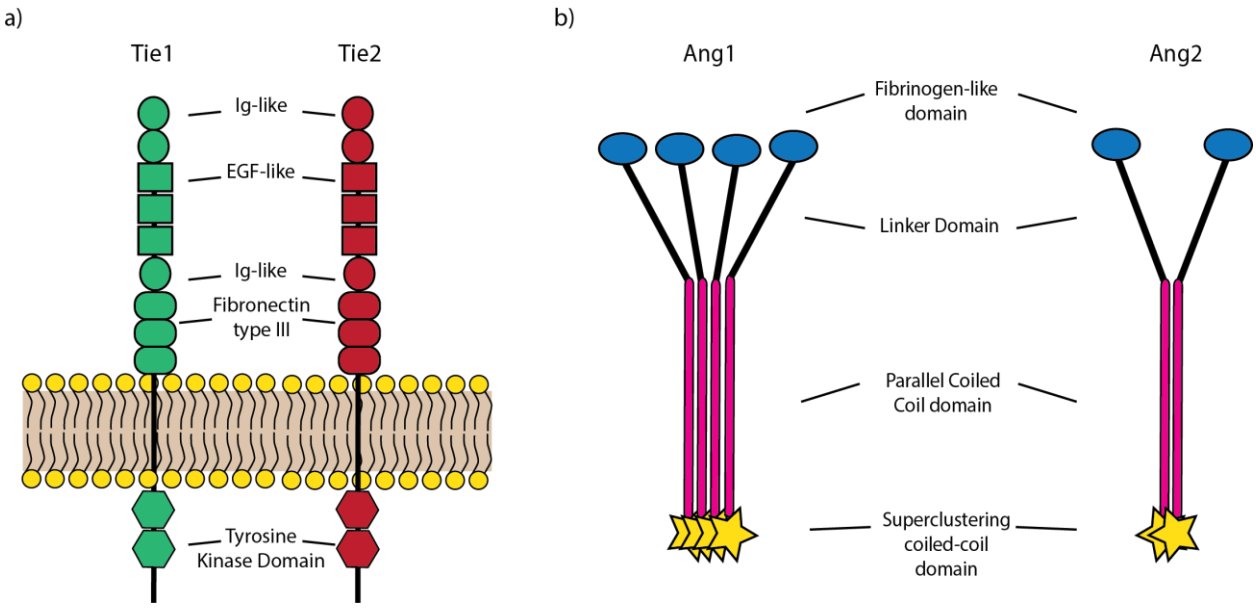

Figure 6: Structure of Tie receptors and angiopoietin ligands

a) Tie receptors are transmembrane receptor Tyrosine kinases, showing a similar overall domain structure. Their extracellular domain consists of two immunoglobulin-like (Ig) domains, followed by three EGF-like domains, a third Ig-like domain and three fibronectin type III domains. The transmembrane domain is followed by the intracellular domain, which consists of a split tyrosine kinase domain. **b)** The Angiopoiein ligands are secreted glycoproteins that comprise of an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. Ang ligand monomers oligomerize via the parallel coiled-coil domain and the superclustering domain. Ang1 primarily forms tetramers, while Ang2 exists mostly as dimers.

The four Angiopoietin ligands are secreted glycoproteins, sharing high homology in their amino acid sequence. They present a small amino-terminal domain that regulates ligand clustering (superclustering region), followed by a large coiled-coil motif, a short linker peptide and a carboxy-terminal fibrinogen-homology domain (Fig. 6b). The coiled-coil motif is essential for angiopoietin dimerization and oligomerization, while the fibrinogen-homology domain at the C-terminal is important for receptor binding (Davis et al., 2003; Procopio et al., 1999). Electron microscopy studies have demonstrated that both Ang1 and Ang2 can form multimers with trimeric, tetrameric and pentameric oligomers. While

Ang1 primarily forms tetramer or higher order multimers (Kim et al., 2005), Ang2 exists mostly as dimers. The oligomerization status of these ligands influences the receptor binding and activation. In particular, Ang1 multimeric structures can bind both ligand-binding sites of extracellular Tie2 homodimers and therefore strongly activate Tie2 receptor (Leppanen et al., 2017). Due to its dimeric conformation, Ang2 fails to bridge Tie2 extracellular domain homodimers, limiting receptors dimerization and activation (Leppanen et al., 2017). Therefore it acts either as antagonist of Ang1 mediated Tie2 signaling or as a partial agonist (Maisonpierre et al., 1997; Yuan et al., 2009).

1.7.2 Expression of Tie receptors and angiopoietin ligands

The endothelial-specific Tie receptors are expressed in blood and lymphatic endothelium and are ubiquitous expressed during mouse embryonic development (Dumont et al., 1992; Korhonen et al., 1994). In the adult endothelium, Tie2 is uniformly expressed and constitutively activated in stalk and phalanx cells, whereas it is transcriptionally downregulated upon EC activation in angiogenic tip cells (del Toro et al., 2010; Felcht et al., 2012). In particular, it has been shown that Tie2 cell surface presentation in tip cells is counter-regulated by Tie1 expression (Savant et al., 2015). In contrast, Tie1 is dynamically expressed upon exogenous activation. Its expression is induced by VEGF, turbulent shear stress in vessel bifurcations, and hypoxia, hinting at a positive role of Tie1 during sprouting angiogenesis (Chen-Konak et al., 2003; Porat et al., 2004). In the retina vasculature, Tie1 has a mosaic expression pattern in the angiogenic front, whereas it is negatively transcriptionally regulated under conditions of laminar flow and, therefore, in the quiescent endothelium (Savant et al., 2015). Yet, enhanced Tie1 expression has been reported in the vasculature of several primary tumors (Brown et al., 2000; Hatva et al., 1996; Hatva et al., 1995; Kaipainen et al., 1994; Lin et al., 1999; Salven et al., 1996) and it has been found to be correlated with metastatic melanoma progression (Kaipainen et al., 1994). Tie receptors are also expressed by circulating hematopoietic cells, including megakaryocytes, hematopoietic stem cells and osteoblasts in the bone marrow niche (Yano et al., 1997). In particular, Tie2 is expressed by a subpopulation of monocytes (Tie2-expressing monocytes) that are highly angiogenic in tumors (De Palma et al., 2005; De Palma et al., 2003).

Angiopoietin ligands have a distinct expression pattern. Ang1 is expressed by perivascular cells like SMC, pericytes and fibroblasts, but it can be expressed also by tumor cells (Stratmann et al., 1998; Sugimachi et al., 2003). In healthy adults, Ang1 is constitutively produced and released by peri-endothelial cells and activates Tie2 receptor in a paracrine manner. Ang1, but not Ang2, can bind the extracellular matrix (ECM) via its linker peptide motif (Xu and Yu, 2001). Ang2, on the contrary, is almost exclusively produced by endothelial cells. It is normally stored in granules called Weibel-Palade bodies and, after endothelial activation, is released and binds Tie2 in an autocrine manner (Fiedler et al., 2004). Under physiological conditions, Ang2 expression is limited to vessel remodeling regions, but during pathological condition such as tumor angiogenesis and sepsis, it is strongly upregulated (Orfanos et al., 2007). Inflammatory cytokines, including Ang2 itself, induce a rapid release of the ligand from the Weibel-Palade bodies, making Ang2 an important regulator of the fast endothelial response to inflammation,

permeability and coagulation (Fiedler and Augustin, 2006; Fiedler et al., 2006). Factors like shear stress, hypoxia and VEGF, transcriptionally regulate Ang2 expression (Mandriota and Pepper, 1998; Oh et al., 1999). Importantly, laminar flow regulated transcription factor Kruppel-like factor 2 (KLF2) suppresses Ang2 expression, contributing to the endothelial quiescence (Parmar et al., 2006).

1.7.3 Ang/Tie system: loss-of-function and gain-of-function studies

The functional role of the Ang-Tie system has been further investigated through the generation of knock-out and overexpressing mice for Angiopoietin ligands and Tie receptors. These studies have highlighted the importance of Tie1 and Tie2 as well as Ang1 and Ang2 during physiological angiogenesis, while demonstrating their dispensable role during vasculogenesis.

Tie2 global deletion (Tie2-KO) leads to embryonic lethality and the mice die between embryonic day (E) 10.5 and E12.5 due to impaired vascular network formation (Dumont et al., 1994; Sato et al., 1995). In particular, Tie2-KO mice develop normally through the early steps of primary capillary plexus formation but the vascular network fails to remodel and remains poorly organized with fewer endothelial cells and branches (Dumont et al., 1994). Further studies have shown also a direct role of Tie2 in hematopoiesis and heart development (Takakura et al., 1998). On the contrary, a hyperactive mutant form of Tie2, resulting from a missense mutation in the kinase domain of the receptor tyrosine kinase, causes venous malformations with dilated vessels (Vikkula et al., 1996), and its overexpression in the skin causes psoriasis-like phenotype characterized by epidermal hyperplasia, altered dermal angiogenesis and inflammation (Voskas et al., 2005).

Mice globally deficient for Tie1 (Tie1-KO) show no significant perturbation in the first steps of embryonic angiogenesis but they die at later stages of embryonic development between E13.5 and birth (Puri et al., 1995; Sato et al., 1995) due to edema and hemorrhage, caused by lack of vessel integrity. In contrast to Tie2-KO, Tie1-KO mice do not show any defect in hematopoiesis (Rodewald and Sato, 1996). Interestingly, during development Tie1 receptor is expressed on the venous lymph endothelial progenitor cells and lymphatic vessels. Indeed, mouse embryos with a hypomorphic Tie1 allele present abnormal lymphatic patterning and architecture, decreased lymphatic draining efficiency and dilated jugular lymph sacs. The severity of the phenotypes observed correlates with the expression levels of Tie1, confirming dosage dependence for Tie1 in lymphatic endothelial cells (LEC) integrity and survival (D'Amico et al., 2010; Qu et al., 2010). Recent studies have also shown an involvement of Tie1 in valve morphogenesis and collecting vessel development (Qu et al., 2015). The double deletion of Tie1 and Tie2 resemble the phenotypes observed in Tie2-KO mice, underlying the essential role of Tie2 in the endocardium (Puri et al., 1999).

Ang1 deficiency phenocopies the Tie2-KO phenotype and leads to embryonic lethality at E12.5 (Suri et al., 1996). This study further confirms the agonistic role of Ang1 on Tie2 receptor, underlying the pivotal position of Ang1/Tie2 axis in the maintenance of endothelial quiescence. Nevertheless, as discussed for Tie2 gain-of-function studies, overexpression of Ang1 leads to severe adverse effects. In particular, Ang1

hyperactivity in the skin leads to hypervascularization, with larger and highly branched vessels (Suri et al., 1998), as well as decreased vessel permeability (Thurston et al., 1999).

Ang2, on the other hand, has been described as an antagonist of Ang1/Tie2 signaling as its overexpression results in mid-gestational lethality, similar to Ang1 and Tie2 deficiency (Maisonpierre et al., 1997). Ang2-KO mice do not show any apparent defects and the pups are born normal (Gale et al., 2002), suggesting that this ligand is dispensable for normal embryonic development. Nevertheless, the mice show compromised vascular sprouting and remodeling in the postnatal-retina angiogenesis, with lack of hyaloid vessels regression after birth (Gale et al., 2002; Hackett et al., 2002). Furthermore, depending on the genetic background, Ang2-KO mice develop lymphatic defects, such as chylous ascites (Gale et al., 2002).

In summary, Ang/Tie signaling is a multifunctional pathway that controls the quiescent EC phenotype in the adults but also the remodeling process that occurs during vessel formation and maturation in active angiogenesis.

1.7.4 Ang1-induced Tie2 signaling

In the quiescent endothelium, Ang1 is constitutively released by perivascular cells and binds to Tie2 receptor, inducing its phosphorylation. The Ang1/Tie2 complex translocates to the cell-cell contacts and forms trans-endothelial complexes with Tie2 molecules from the neighboring cells. This leads to the recruitment of adaptor proteins like growth factor receptor-bound protein2 (GRB2) and p85, regulatory subunit of the phosphoinositide 3-kinase (PI3K). This complex activates the AKT survival-pathway that involves survivin, endothelial nitric oxide synthase (eNOS) while suppressing apoptotic pathways (Fig. 6a) (DeBusk et al., 2004; Kim et al., 2000b; Kwak et al., 1999; Papapetropoulos et al., 2000). Ang1-mediated Tie2 activation can also repress Ang2 expression: AKT phosphorylates and inactivates the forkhead transcription factor, FOXO1, which usually translocates to the nucleus and activates Ang2 expression (Daly et al., 2004). By contrast, when the AKT survival pathway is inhibited, FOXO1 is activated, and Ang2 expression is induced, causing endothelial destabilization (Tsigkos et al., 2006).

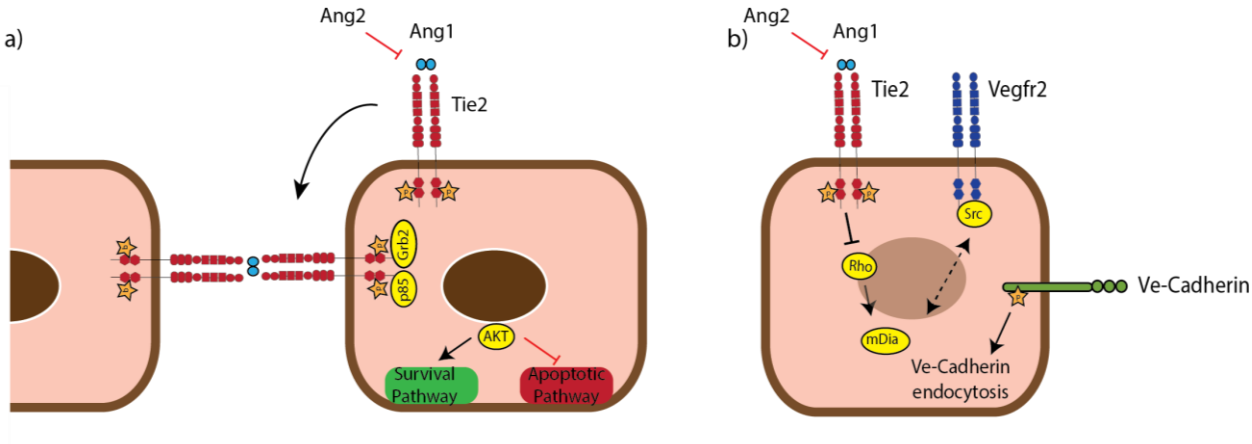

Figure 7: Angiopoietin-Tie signaling

a) In resting endothelial cells, Ang1 mediated phosphorylation of Tie2 leads to the recruitment of adaptor proteins, such as Grb2 and the p85 subunit of PI3K, which results in the activation of AKT signaling. AKT activates survival promoting pathways while downregulating apoptotic genes. **b)** Ang1 induced Tie2 signaling is also involved in the inhibition of vascular permeability. In presence of Ang1, Tie2 phosphorylation mediates the activation of mDia (mammalian diaphanous), which sequesters Src, preventing VE-Cadherin phosphorylation and internalization, and therefore increased permeability.

Ang1/Tie2 signaling is also directly involved in the regulation of EC permeability. In particular, Tie2 mediates the sequestration of the non-receptor Tyr kinase Src. Src is phosphorylated during VEGF-mediated permeability, causing the activation of Rac. Consequently, Rac phosphorylates the junctional protein VE-cadherin, thereby recruiting beta-arrestin-2, and leading to VE-Cadherin internalization from junctional complexes and promoting VEGF-dependent vascular permeability (Dejana et al., 2008; Gavard and Gutkind, 2006). Tie2 signaling promotes sequestration of Src by mDia (mammalian diaphanous) in a small GTPase, RhoA-dependent manner, leading to the anti-permeability response of the endothelium (Fig. 7b) (Gavard et al., 2008). Thus, in resting endothelial cells Ang1 has vascular protective effects, maintaining vessel integrity and limiting permeability. While in conditions of endothelial activation (in the absence of endothelial cell junctions), Ang1 induces endothelial cell migration and proliferation. In this scenario, Ang1 mediates translocation of Tie2 to cell-substratum contacts, triggering ERK1/2 signaling, which in turns leads to FAK (focal adhesion kinase) phosphorylation (Kim et al., 2000a) and therefore migration. This prepares the endothelium for an angiogenic response (Fukuhara et al., 2008).

Ang1/Tie2 pathway is also involved in the recruitment of perivascular cells (Augustin et al., 2009), which surround the vessel wall and are essential in promoting vascular maturation and maintaining vascular quiescence. Although the mechanisms are still not clear, it is well known that Ang1-mediated Tie2 activation leads to expression of endothelial heparin-binding epidermal-like growth factor (HB-EGF), which causes migration of SMC (Iivanainen et al., 2003). Similarly, HGF leads to Ang1 mediated recruitment of SMC to the vasculature (Kobayashi et al., 2006). Furthermore, serotonin has also been implicated to play a role in Ang1/Tie2 mediated SMC recruitment to the endothelium (Sullivan et al.,

2003). Platelet-derived growth factor B (PDGFB) is the best characterized cytokine expressed by endothelial cell and is important in pericyte recruitment during the formation of new blood vessels. During angiogenesis, sprouting endothelial cells secrete PDGFB (Heldin and Westermark, 1999), which signals through its receptor, PDGFRB, expressed by mural cells, resulting in proliferation and migration of perivascular cells during vascular maturation. However, no molecular evidence for a possible link between the Ang1/Tie2 and PDGFB/PDGFRB axis has been demonstrated so far.

1.7.5 Ang2-mediated Tie2 signaling: agonist or antagonist?

Unlike Ang1, which is the guardian of endothelial quiescence, Ang2 is important for sprouting angiogenesis, vessel destabilization, remodeling and vessel regression (Fiedler and Augustin, 2006). Ang2 cannot rapidly induce Tie2 phosphorylation, but in the presence of pro-angiogenic stimuli, it binds to Tie2 and acts as an antagonist of Ang1-mediated Tie2 signaling (Maisonpierre et al., 1997). During vessel destabilization, Ang2 stimulation results in a complex formation amongst Tie2, FAK and $a_v\beta_3$ integrin. The latter, is internalized, ubiquitinylated and degraded, driving endothelial cells into apoptosis (Adams and Alitalo, 2007).

Nevertheless, in stressed endothelial cells, Ang2 has been described to function as a Tie2 agonist. Its expression is rapidly induced by the transcription factor FOXO1 after inhibition of PI3K/ AKT pathway and it bolsters AKT activity so as to provide negative feedback on FOXO1-regulated transcription and apoptosis (Daly et al., 2006). The same group has provided evidence that Ang2 plays a protective role in tumor endothelial cells by activating Tie2, therefore underlying its agonistic activity also in a tumor setting (Daly et al., 2013). More recent reports have clarified the agonistic/antagonistic role of Ang2 in basal and inflammatory conditions (Kim et al., 2016; Korhonen et al., 2016). In pathogen-free conditions, autocrine Ang2 act as a Tie2 agonist, capable of promoting high phosphorylation of Tie2 and low FOXO1 activation. This agonistic activity requires the presence of the orphan receptor Tie1. On the contrary, upon inflammation, Ang2 is released from Weibel-Palade bodies but the cleavage of Tie1 prevents Ang2 agonistic activity, favoring its antagonistic action on Tie2, therefore leading to vessel destabilization (Kim et al., 2016; Korhonen et al., 2016). Thus, Ang2 mediated Tie2 signaling has a dual role in stabilizing the vasculature in physiological condition as well as destabilizing the endothelium and inducing remodeling in pathological settings.

1.7.6 Ang2 signaling pathway independent of Tie2

In the absence of Tie2, Ang2 can signal via integrins exerting angiogenic functions (Felcht et al., 2012). Although angiogenic tip cells upregulate Ang2 expression, they present low levels of Tie2 (del Toro et al., 2010; Felcht et al., 2012) and high levels of integrins. In this Tie2-low setting, Ang2 binds to $\alpha_v ß_3$, $\alpha_v ß_5$ and $\alpha_5 ß_1$ integrins and phosphorylates FAK on Tyrosin397, resulting in Rac activation. This results in endothelial cell migration and sprouting angiogenesis at the tip cell front (Felcht et al., 2012).

1.7.7 Ang/Tie system during pathology

Molecules composing the Ang/Tie pathway not only play a role in vascular development/remodeling and in the adult endothelium for sustaining endothelial quiescence, but also in pathological conditions such as inflammation, atherosclerosis and tumor angiogenesis. In an animal model of sepsis, Ang1 has been shown to attenuate LPS-induced lung injuries and to improve animal survival via reducing microvascular leakage (Witzenbichler et al., 2005). Ang1 has also a protective role against the development of allograft arteriosclerosis (Nykanen et al., 2003) and protects the endothelium against radiation induced endothelial cells apoptosis (Cho et al., 2004).

Similarly, the activation of Tie2 receptor mediated by the Tie2-agonistic synthetic peptide, Vasculotide, has been reported to improve the devastating outcome of sepsis (Kumpers et al., 2011). More efficient results have been obtained with the new antibody, ABTAA, which activates Tie2 while blocking Ang2. ABTAA protects the vasculature from septic damage and provides survival benefits in different septic models (Han et al., 2016). Tie2 activation can also prevent inflammation by interfering with the NFkB pathway (Hughes et al., 2003; Tadros et al., 2003).

While Ang1 and Tie2 have protective roles, Ang2 is mostly a pro-inflammatory cytokine, upregulated in response to several stimuli. In particular, Ang2 potentiates the inflammatory effects induced by TNFα by promoting the expression of adhesion molecules such as ICAM and VCAM. Indeed, Ang2-KO show an impaired inflammatory response when challenged with intraperitoneal injection of *Staphylococcus aureus* (Fiedler et al., 2006) and respond to hyperoxia induced acute-lung injuries with reduced oxidant-induced injury, cell death, inflammation, permeability alteration and mortality (Bhandari et al., 2006). During sepsis, Ang2 serum levels are increased up to 20 fold (Orfanos et al., 2007). Its expression increases also during neovascularization in different pathologies such as arthritis, psoriasis (Augustin, 2003), and in particular during tumor growth and metastasis. In tumor angiogenesis, Ang2 primarily antagonizes Tie2 receptor, causing endothelial destabilization and then, together with VEGF, drives tumor angiogenesis. Human patient sample studies have indicated its crucial role in angiogenesis of highly vascularized glioblastoma (Stratmann et al., 1998) and other preclinical and clinical studies have established its central role in the angiogenesis of several cancers such as gastric cancer (Etoh et al., 2001), ovarian cancer (Zhang et al., 2003) and melanoma (Siemeister et al., 1999). Overexpression of Ang2 in tumor cell lines has produced divergent results. On one hand, Ang2 promotes tumor growth leading to a more invasive behavior (Ahmad et al., 2001; Etoh et al., 2001; Hu et al., 2003; Yoshiji et al., 2005), while on the other hand Ang2 overexpression leads to disordered angiogenesis and inhibition of tumor growth (Ahmad et al., 2001; Etoh et al., 2001; Hu et al., 2003; Yu and Stamenkovic, 2001). Systemic overexpression of Ang2 leads to tumor regression in 24 hours (Cao et al., 2007), and its genetic deletion delays the tumor growth, affecting the early stages of tumor progression (Nasarre et al., 2009). Yet, Ang2 adenoviral administration can also have a protective role in atherosclerosis progression (Ahmed et al., 2009).

Although Ang2 shows distinct effects on the tumor vasculature, it acts synergistically with VEGF,

enhancing VEGF-induced angiogenesis (Holash et al., 1999; Lobov et al., 2002). Furthermore, VEGF can induce transcriptional activation of Ang2 promoting tumor angiogenesis (Zhang et al., 2003). Anti-angiogenic (AA) agents targeting the VEGF/VEGFR pathway fail to give generous benefits in survival of cancer patients mainly because they target unstable and immature blood vessels, leaving functional and normalized vessel behind (Gerald et al., 2013). Agents targeting Ang/Tie system affect later stages of remodeling and maturation. In order to target the synergistic effects of both tyrosine kinase systems and to affect different stages of tumor angiogenesis, a dual targeting approach has been successfully used in several pre-clinical studies, showing delayed tumor growth and prolonged survival in different glioblastoma model (Daly et al., 2013; Gerald et al., 2013; Hashizume et al., 2010; Kienast et al., 2013; Kloepper et al., 2016; Koh et al., 2010; Peterson et al., 2016). In addition to anti-VEGF antibodies, anti-Ang2 has also been shown to improve the therapeutic benefit upon combination with standard chemotherapy in a primary tumor setting (Brown et al., 2010; Leow et al., 2012). The targeting of Ang2 in association with low dose metronomic chemotherapy, in a post-surgical adjuvant scenario, reduced the incidence of bone metastasis and the growth of lung metastasis, improving mice survival (Srivastava et al., 2014). Nevertheless, despite this pre-clinical success, a recent clinical study identified limited benefit of co-targeting Ang2 and VEGF in colorectal cancer (Reuters, 2016).

1.8 Tie1 Receptor

1.8.1 Tie1 signaling

Tie1 was discovered in 1992, but no ligand has been identified yet, rendering its signaling still poorly understood. Endothelial cells express Tie1 as a doublet of 135 and 125 kiloDalton (kDa), representing the mature glycosylated cell surface receptor and intracellular non-glycosylated immature receptor, respectively (Partanen et al., 1992). Upon inflammatory conditions, the mature receptor can be cleaved into two fragments: a 100 kDa soluble fragment that contains the extracellular domain and is released upon shedding, and a 45 kDa fragment representing the transmembrane and intracellular domains which remain cell-associated. Tie1 cleavage is triggered by multiple factors, such as VEGF, tumor necrosis factor α (TNFα), LPS (lipopolysaccharide), phorbol ester PMA (phorbol-12-myristate-13-acetate) and other inflammatory cytokines that activate metalloproteases and induce Tie1 shedding at the E749/S750 peptide bond in the proximal transmembrane domain (Chen-Konak et al., 2003; Marron et al., 2007; Yabkowitz et al., 1999; Yabkowitz et al., 1997). The newly formed intracellular domain, can be further proteolitically cleaved by γ-secretase to give rise to an N-terminal truncated 42 kDa fragment, which is then degraded by proteasomal activity (Marron et al., 2007). Recent reports have shown that Tie1 ectodomain shedding is important for controlling angiopoietin functions *in vivo*. In physiological condition, full-length Tie1 sustains Ang2 agonistic activity on Tie2, but its ectodomain shedding, which occurs during inflammation, leads to a reduction in Tie2 phosphorylation, downregulation of Tie2 and upregulation of Ang2. This prevents the basic agonistic action of Ang2 and favors Ang2 antagonistic role on Tie2, initiating a positive feedback loop through FOXO1-driven Ang2 expression that leads to vascular remodeling (Kim et al., 2016; Korhonen et al., 2016). Other studies have shown that the proteolytic

ectodomain process of Tie1 increases the responsiveness of Tie2 to Ang1, and therefore Tie2 activation. Mechanistically the extracellular domain of Tie1 limits the ability of COMP-Ang1, a soluble chimeric form of human Ang1, to bind Tie2 (Marron et al., 2007). Both full length Tie1 and the truncated receptor lacking the ectodomain exist as pre-formed heterodimers that bind physically to Tie2, suggesting that also Tie1 endodomain contrbutes to the Tie1-Tie2 complex (Seegar et al., 2010; Singh et al., 2012a). A very elegant study has recently demonstrated that Tie1-Tie2 heterodimerization is mediated by intermolecular β-sheets formation between the membrane-proximal Fibronectin type III domain n3 of the two receptors (Leppanen et al., 2017). Interestingly, activation of Tie1 and Tie2 in heteromeric complexes depends on β1 integrin (Korhonen et al., 2016). In contrast to these reports, Seegar et al. have demonstrated that Tie1 and Tie2 form heterodimers via ectodomain electrostatic interactions, which inhibit Tie2 activation and clustering. According to their model, Ang1 binding to Tie2 promotes heterodimer dissociation, Tie2 clustering and signaling activation; Ang2 binding to Tie2, on the contrary, is not able to dissociate Tie1-Tie2 heterocomplexes, and therefore cannot induce Tie2 signaling, behaving as a competitive Tie2 antagonist (Seegar et al., 2010). Tie1, therefore, is considered as an inhibitor co-receptor for Tie2 signaling, which downregulates Ang1/Tie2 mediated AKT and MAPK phosphorylation *in vitro* (Yuan et al., 2007). *In vivo* studies have better clarified the context-dependent role of Tie1 on Tie2 signaling during post-natal retina vascularization. Functionally, Tie1 expression by angiogenic EC contributes to shaping the tip cell phenotype by negatively regulating Tie2 surface presentation. In contrast, Tie1 acts cooperatively to sustain Ang1/Tie2 signaling in remodeling stalk cells, while it is strongly downregulated in quiescent EC (Savant et al., 2015).

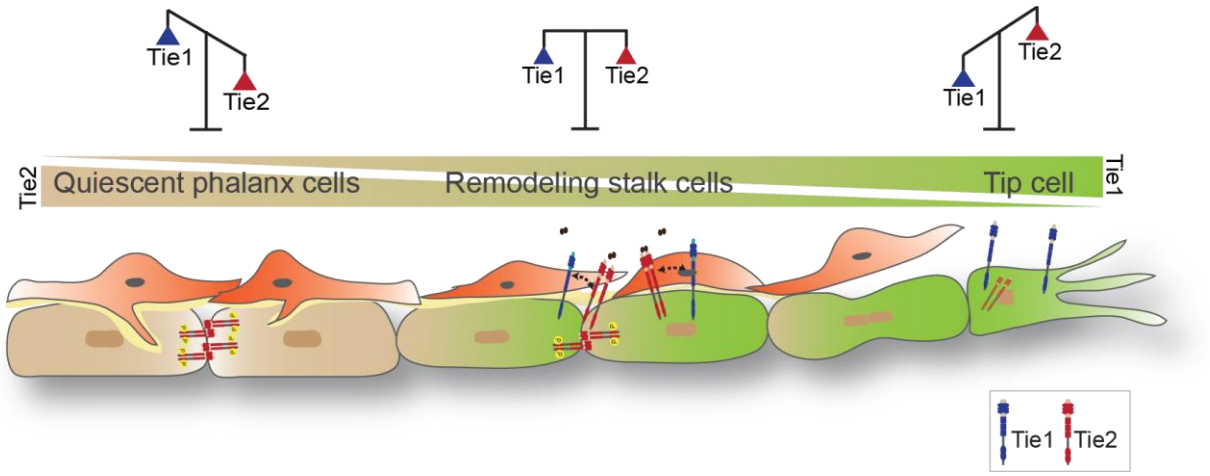

Figure 8: Representation of the context dependent role of Tie1 in regulating Tie2 signaling

Tie1 counter-regulates Tie2 cell surface presentation in tip cells and sustains Ang1-Tie2 signaling in stalk cells by forming heterocomplexes. In quiescent phalanx cells, Tie1 is downregulated. Figure modified from (Savant et al., 2015).

While Tie2 has a strong tyrosine kinase activity, Tie1 is not able to phosphorylate other proteins or to induce autophosphorylation *per se*. Interestingly, Tie1 phosphorylation can be induced by COMP-Ang1, in a Tie2 dependent manner (Saharinen et al., 2005; Savant et al., 2015; Yuan et al., 2007). In the

absence of Tie2, high concentration of COMP-Ang1, far from the physiological setting, can weakly phosphorylate Tie1. Native Ang1 and Ang4 can also induce Tie1 phosphorylation, but in a less efficient way than COMP-Ang1 (Saharinen et al., 2005; Savant et al., 2015). Chimeric Tie1 presenting the extracellular domain of CSF-1 (macrophage colony-stimulating factor 1 receptor) and its intracellular kinase domain has been shown to activate PI3K-AKT survival pathway and inhibit apoptosis (Kontos et al., 2002). This study suggests that Tie1 could potentially transduce signals similar to Tie2, but the absence of a specific ligand leaves the hypothesis that Tie1 could signal through additional mechanisms open. Recently, Reinardy et al. have investigated the role of a Threonine phosphorylation site (T794) within the juxtamembrane domain of Tie1. Mutation of T794 did not alter Tie1 or Tie2 tyrosine phosphorylation or downstream signaling, suggesting that this Threonine site could represent a novel Tie1 function independent from its RTK properties. In particular, under angiogenic stimuli, the small GTPase Rac1, activates PAK (p21-activated kinase), that in turn phosphorylates Tie1 on T794. Rac1-GTP associates with phospho-T794 and facilitates interactions with effector molecules that either stabilize Rac1 in its active GTP-bound form or mediate GTP hydrolysis and therefore Rac1 inactivation. Expression of a non-phosphorylated mutant of this site (T794A) in zebrafish significantly disrupted vascular development, resulting in fish with stunted and poorly branched intersomitic vessels (Reinardy et al., 2015).

1.8.2 Tie1 in pathological conditions

Tie1 is not only involved in physiological angiogenesis but also exerts critical functions in pathological blood vessel formation, such as inflammatory disease and tumor angiogenesis. Tie1 expression is induced in atherosclerosis-prone regions of the vasculature characterized by disturbed blood flow, and its attenuation reduces murine atherosclerotic lesions in a dose-dependent manner (Woo et al., 2011). It exerts pro-inflammatory functions also in other inflammatory diseases, such as rheumatoid arthritis and arthritis (Jin et al., 2008). It has even been proposed to be involved in the pathogenesis of Ebola Virus infection (Rasmussen et al., 2014). Recently, its involvement in LPS-induced acute lung inflammation has been investigated in detail. Upon inflammatory conditions, Tie1 extracellular domain is cleaved and limits the agonistic role of Ang1 on Tie2 while promoting Ang2 antagonistic action on Tie2, favoring vascular destabilization (Kim et al., 2016; Korhonen et al., 2016). In addition, the serum level of Tie1 ectodomain is significantly increased, further suggesting soluble Tie1 as a biomarker.

Tie1 is strongly expressed in acute myeloid leukemia, myelodysplastic syndrome and chronic myeloid leukemia. High-Tie1 level is a predictor of shorter survival in early chronic myeloid leukemia (Verstovsek et al., 2001; Verstovsek et al., 2002). Tie1 overexpression has also been detected in several solid tumors, such as breast cancer (Salven et al., 1996), colorectal adenocarcinoma (Ito et al., 2004), metastatic melanoma (Kaipainen et al., 1994), gastric carcinoma (Lin et al., 1999), and thyroid neoplasm (Ito et al., 2004). In breast cancer, Tie1 is not only increased in the vasculature, but it is also highly expressed in epithelial breast cancer cells and in ductal carcinoma *in situ* (Salven et al., 1996). Although Tie1 is not expressed in all microvessels of normal or malignant breast tissue, the number of Tie1-positive capillaries

is greater in malignant tissue than in normal breast tissue, strongly supporting Tie1 as a marker for tumor angiogenesis (Salven et al., 1996). In the colorectal adenocarcinoma, Tie1 expression is significantly correlated with histological grading, depth of tumor invasion, Duke's classification and lymphatic invasion (Ito et al., 2004). In gastric adenocarcinoma, Tie1 expression is a prognostic marker since its expression is inversely correlated to patients' survival (Lin et al., 1999). Furthermore, in thyroid tumorigenesis, Tie1 plays a role in the early phase of papillary carcinoma while it is dispensable for the progression of anaplastic carcinoma or follicular tumor (Ito et al., 2004).

The involvement of Tie1 in tumorigenesis has made it an interesting molecule to study during tumor angiogenesis. Using a conditional knock-out mouse, D'Amico et al. have demonstrated that Tie1 deficiency in tumor endothelium reduces tumor vascularization and tumor growth by decreasing endothelial cell survival in tumor microvasculature. This anti-angiogenic effect occurs via activation of the Notch pathway that inhibits sprouting angiogenesis (D'Amico et al., 2014). Thus, Tie1 as a potential target for anti-angiogenic therapy needs further investigation.

2. Aim of the thesis

The orphan Tie1 receptor is essential for vascular remodeling during embryonic development as well as in the post-natal angiogenesis. It is upregulated in the vasculature of several primary tumors and a recent study has promoted the receptor as a therapeutic target for controlling primary tumor growth by limiting blood vessels development. Angiogenesis is a rate-limiting process not only for primary tumor growth but also for metastatic dissemination, considered as a multistage event critically dependent on the interaction of metastasizing neoplastic cells and tumor microenvironment.

The aim of this thesis was to i) decipher the contribution of Tie1 to blood vessel formation in the early and late stage of tumorigenesis and to ii) dissect its pathological and mechanistic contribution to the main steps of the metastatic cascade, such as tumor cell intravasation, extravasation and colonization in distant organs. Finally, the last and most important is to iii) investigate new therapies targeting the Ang-Tie system and to propose Tie1 as a tumor-specific target.

3. Results

3.1 Endothelial Tie1 deletion during primary tumor growth induces necrosis and delays tumor growth at later stages

To understand the mechanistic contribution of endothelial Tie1 during tumor progression, Tie1 floxed mice were used in the present study. Given that homozygous constitutive Tie1 deletion is embryonic lethal, Tie1$^{flox/flox}$ mice were created by the lab of the Dr. Scott Baldwin using the Cre/loxP system to conditionally delete the first coding exon of Tie1 (Qu et al., 2010). Next, Tie1$^{flox/flox}$ mice were crossed with the inducible *Tg(Cdh5-cre/ERT2)1Rha (Cdh5Cre^{ERT2})* mice (Wang et al., 2010b) to specifically delete *Tie1* gene in the endothelial compartment after tamoxifen administration (Tie1^{iECKO}). Tie1$^{flox/flox}$ mice were used as controls (Wildtype, WT). For all the experiments, Tie1 deletion efficiency was evaluated by qPCR of total tissue RNA lysates. Mice with less than 60% Tie1 deletion were excluded from the analyses.

Two different tumor cell lines were inoculated subcutaneously into the left flank of syngeneic C57BL/6 WT and Tie1^{iECKO} mice: i) mouse Lewis Lung Carcinoma (LLC) cell line, derived from a tumor bearing lung of a C57BL/6 mouse (Bertram and Janik, 1980) and ii) mouse B16F10 melanoma cell line, developed in C57BL/6 mice (Fidler, 1975). In both models, Tie1 deletion was initiated two days after tumor cell inoculation (Fig. 9).

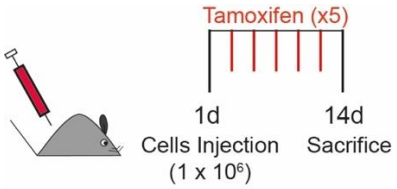

Figure 9: Experimental protocol for tumor cell injection

One million LLC or B16F10 were subcutaneously inoculated in the left flank of the mice. Five doses of tamoxifen (2mg/dose) were administrated into WT and Tie1^{iECKO} animals. Mice were sacrificed at day 14 after tumor cell inoculation.

For tumor growth experiments, LLC tumors were allowed to grow until ~1 cm^3 (day 18 post-inoculation) when mice were sacrificed. Tumor growth was monitored every three days using caliper measurements. The growth curves of WT and Tie1^{iECKO} tumors progressed similarly until tumors had reached almost 1 cm^3 in size (Fig. 10A). Thereafter, growth curves diverged, resulting in a significant reduction of tumor volume and tumor weight of Tie1 endothelial deleted tumors (Fig. 10B). To study the consequences of Tie1 deletion on the intratumoral microenvironment prior to overt tumor growth divergence, tumors were harvested at day 14 and tumor cell hypoxia and necrosis were analyzed. Hypoxic regions were identified by nuclear HIF1α staining, a subunit of the transcriptional factor HIF1, master regulator of cellular response to hypoxia. Quantification of HIF1α positive area per tumor area revealed a drastic increase of intratumor hypoxia (Fig. 10C, D) in Tie1^{iECKO} primary tumor. Consistently, tumor cell necrosis,

shown in the H&E staining as pink area, was significant higher in Tie1^{iECKO} mice compared to the WT controls (Fig. 10E, F), already 14 days post-LLC inoculation.

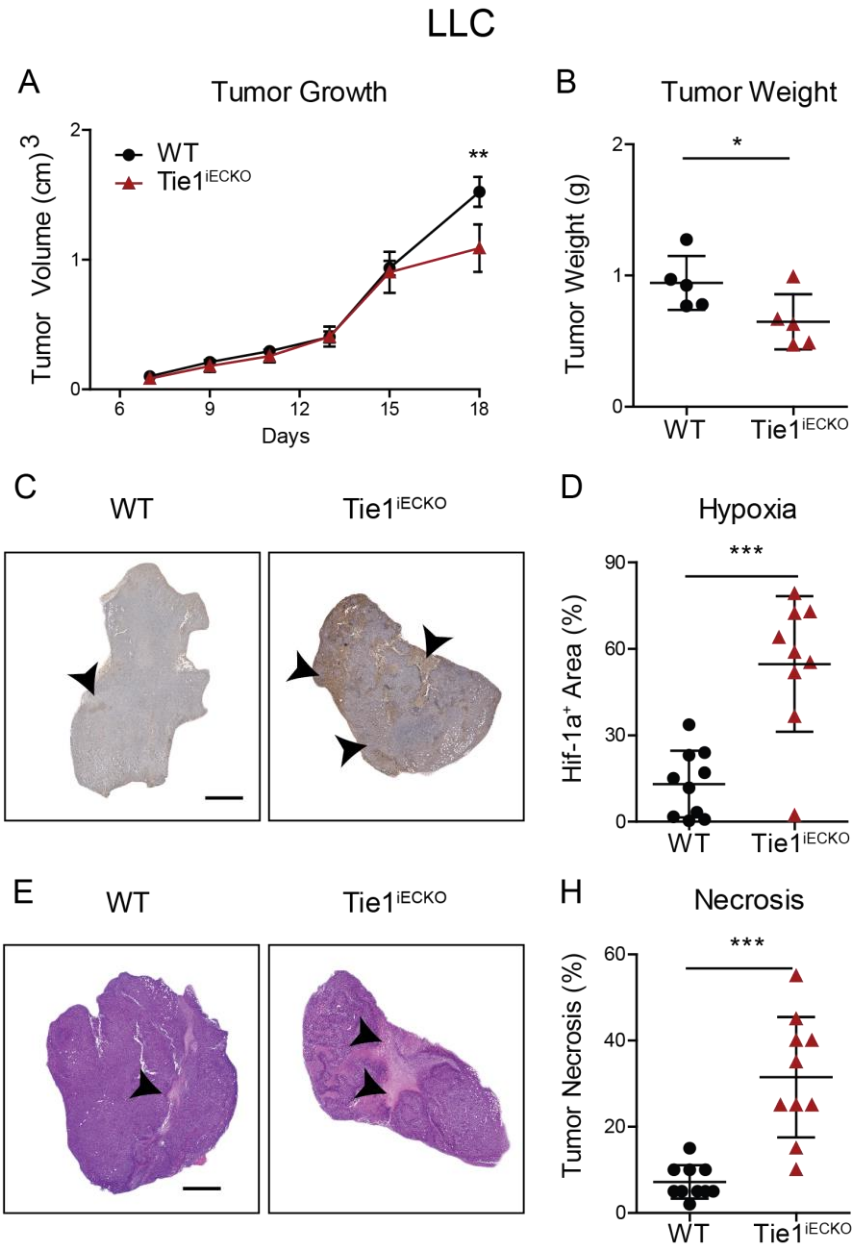

Figure 10: Tie1 loss influences later stages of tumor growth by promoting tumor hypoxia and necrosis

A) LLC tumor growth curve in WT and Tie1^{iECKO} mice (n=5; two-way ANOVA test, **, p<0.01. Data are expressed as mean ± SEM). **B)** LLC tumor weight n=5, Student's T-test, *, p<0.05). **C)** Bright-field microscope images of hypoxic area (stained with anti-HIF1α) from WT and Tie1^{iECKO} LLC tumors. Arrowheads indicate hypoxic areas. Scale bar: 1 mm. **D)** Quantification of HIF1α positive area from WT and Tie1^{iECKO} tumors (n=10 (WT) and 9 (Tie1^{iECKO}); two-tailed Student's *t*-test, ***, p<0.001). **E)** Bright-field microscope images of necrotic area (H&E staining, light pink, indicated by arrowheads) from WT and Tie1^{iECKO} LLC tumors. Scale bar: 1 mm. **F)** Quantification of the necrotic area from WT and Tie1^{iECKO} tumors (n=10 mice; two-tailed Student's *t*-test, ***, p<0.001).

Similarly, B16F10 melanoma cells were inoculated subcutaneously and allowed to grow until ~600 mm^3 (day 14 post-inoculation). The growth rate of B16F10 tumors was very heterogeneous amongst the animals (data not shown) but the intratumoral necrosis was more severe than in LLC tumors (Fig. 11A, B). Therefore, mice had to be sacrificed before a divergence between the tumor growth curves was evident in the two experimental groups.

In conclusion, endothelial Tie1 deletion induced severe hypoxia and necrosis in the tumor microenvironment, causing a tumor growth delay at later stages.

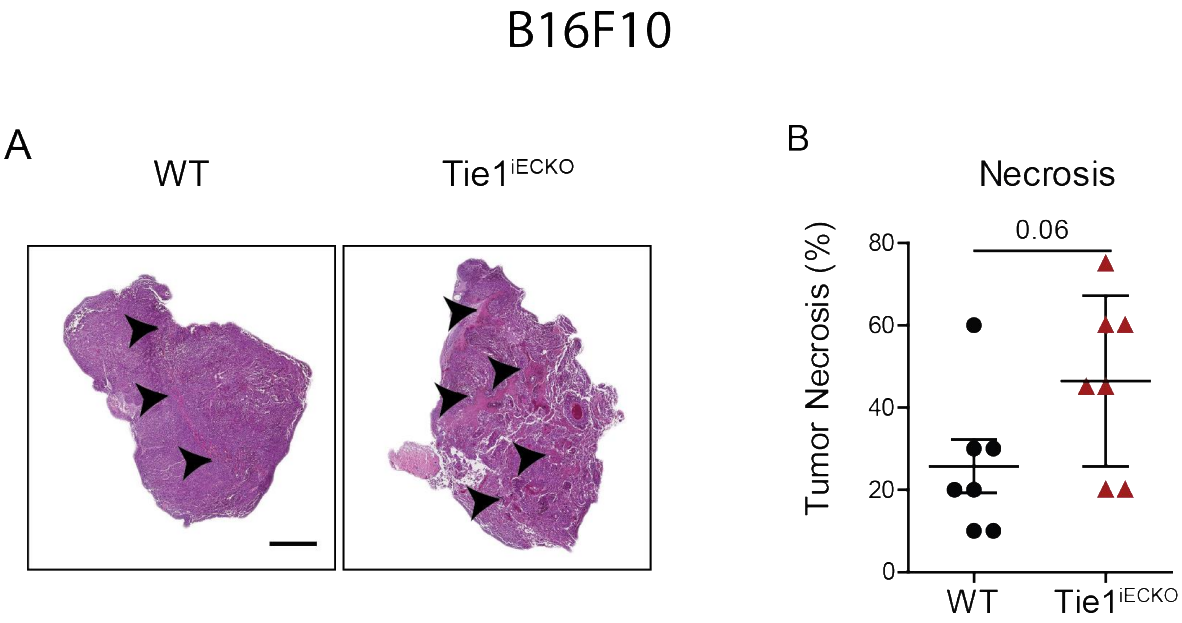

Figure 11: Tie1 deficiency increases necrosis in B16F10 primary tumor

A) Necrotic area (H&E staining, light pink, indicated by arrowheads) from WT and $Tie1^{iECKO}$ B16F10 tumors. Scale bar: 1 mm. **B)** Quantification of the necrotic area from WT and $Tie1^{iECKO}$ tumors (n=7 mice; two-tailed Student's *t*-test; p=0.06).

3.2 Loss of Tie1 during primary tumor growth inhibits angiogenesis and increases mural cell coverage without affecting vessel size

The strong effects of the orphan receptor deletion on primary tumor hypoxia and necrosis suggested that Tie1 loss may affect the tumor microvasculature. Therefore, LLC and B16F10 tumors were harvested and processed 14 days post-inoculation, when the difference in intratumor hypoxia and necrosis was already evident between the two experimental groups. Immunofluorescence CD31 vessel staining of LLC primary tumors revealed a large and significant decrease in microvessel area as well as density in the Tie1^{iECKO} tumors compared to the WT tumors (Fig. 12A-C).

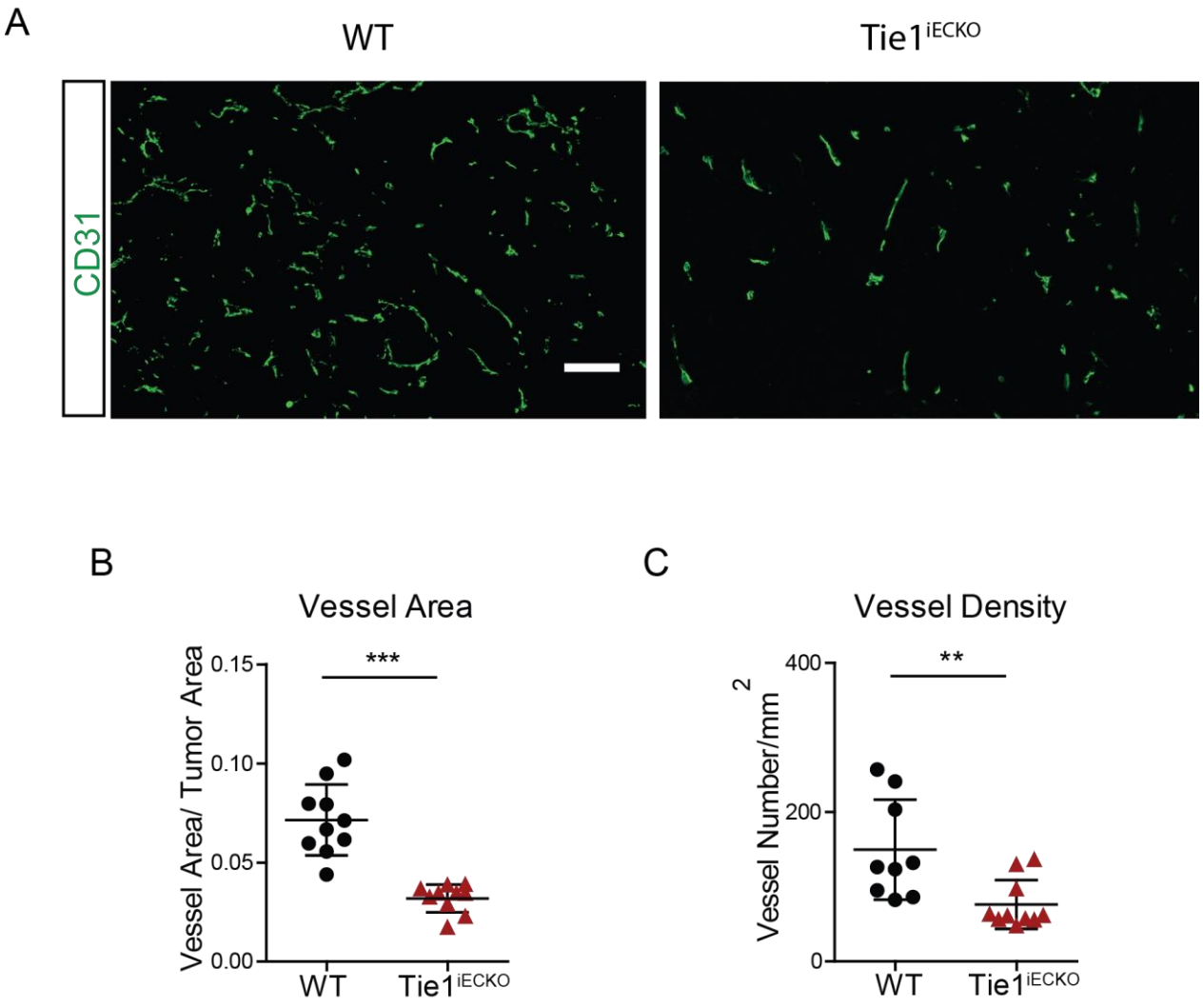

Figure 12: Tie1 endothelial deletion has an anti-angiogenic effect on LLC tumor vasculature

A) Representative microscope images of cryo sections stained with the pan-endothelial cell marker CD31 in LLC primary tumor. Scale bar: 100 μm. **B)** Quantification of vessel area and **C)** vessel density in LLC tumors from WT and Tie1^{iECKO} mice (n=9-10 (WT) and 10 (Tie1^{iECKO}); Mann-Whitney test, ***, p<0.001, **, P<0.01,).

To further characterize tumor vasculature in a Tie1 targeted setting, colocalization studies of CD31 and Desmin or CD31 and αSMA (markers for pericytes and smooth muscle cells, respectively) were performed. Interestingly, Tie1^{iECKO} tumor blood vessels displayed a significant increase of mural cells coverage (Fig. 13A-D) and presented a strong reduction in number of angiogenic sprouts per vessel (Fig. 13B, D arrowheads and E), indicative of a less active and more quiescent vasculature. Further dissection of vessels into different sizes, ranging from small vessels (50-500µm^2) to large vessels (>5000µm^2) showed no difference between the two experimental groups (Fig. 13F).

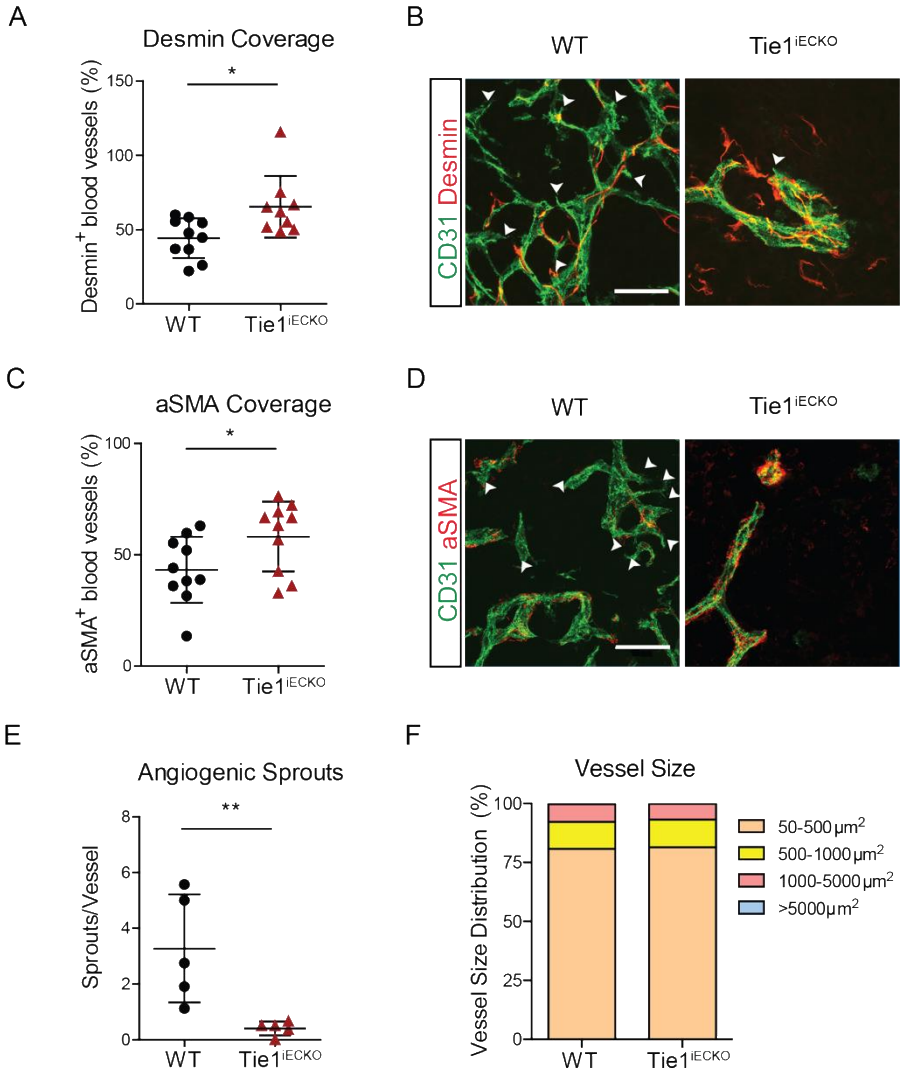

Figure 13: Tie1 endothelial deletion normalizes primary tumor vasculature in LLC tumors

A) Quantification of desmin-positive blood vessels in WT and Tie1^{iECKO} LLC primary tumors (n=10 (WT) and 9 (Tie1^{iECKO}); Mann-Whitney test, *, p<0.05). **B)** Confocal microscope images of blood vessels (stained with anti-CD31) and pericytes (stained with anti-desmin) from WT and Tie1^{iECKO} tumors. Arrowheads indicate angiogenic sprouts. Scale bar: 50 µm. **C)** Quantification of αSMA-positive blood vessels in LLC tumor vasculature (n=10; Mann-Whitney test, *, p<0.05). **D)** Confocal microscope images of blood vessels (stained with anti-CD31) and SMC (stained with anti-αSMA) from WT and Tie1^{iECKO} tumors. Arrowheads indicate angiogenic sprouts. Scale bar: 50 µm. **E)** Vessel sprouting quantification (n=5; Mann-Whitney test, **, p<0.01). **F)** Analysis of vessel size distribution within the vascularized area.

To validate these observations in a second tumor model, the same parameters were analyzed in B16F10 tumors. Similarly, a strong impairment in blood vessel area and density (Fig. 14A-C) as well as an increase in pericytes and smooth muscle cell coverage were evident after Tie1 endothelial deletion (Fig. 15A-D).

Taken together, these results indicate that tumors in Tie1^{iECKO} mice have fewer, more covered and more mature blood vessels than the tumors in WT mice. This phenotype resembles vessel normalization, during which chaotic, tortuous, leaky and poorly covered tumor vessels remodel into an organization that is similar to the normal vessel architecture (Jain, 2013).

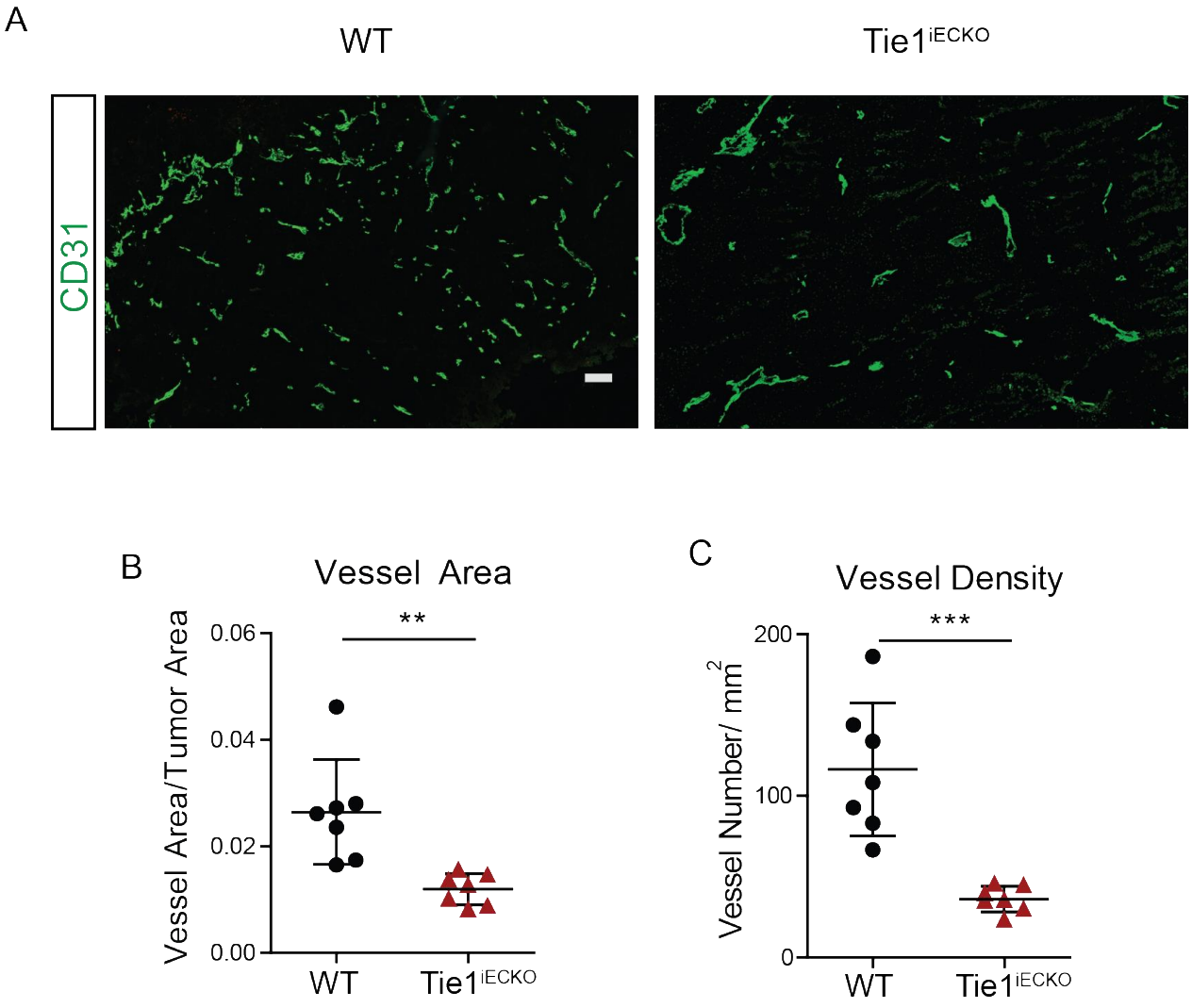

Figure 14: Tie1 deletion reduces tumor angiogenesis in B16F10 model

A) Representative images of WT and Tie1^{iECKO} tumors stained for the pan-endothelial marker CD31. Scale bar: 100 µm. **B)** Quantifications of vessel area and **C)** vessel density 14 days post-tumor cell inoculation (n=7; Mann-Whitney test, ***, p<0.001, **, p<0.01).

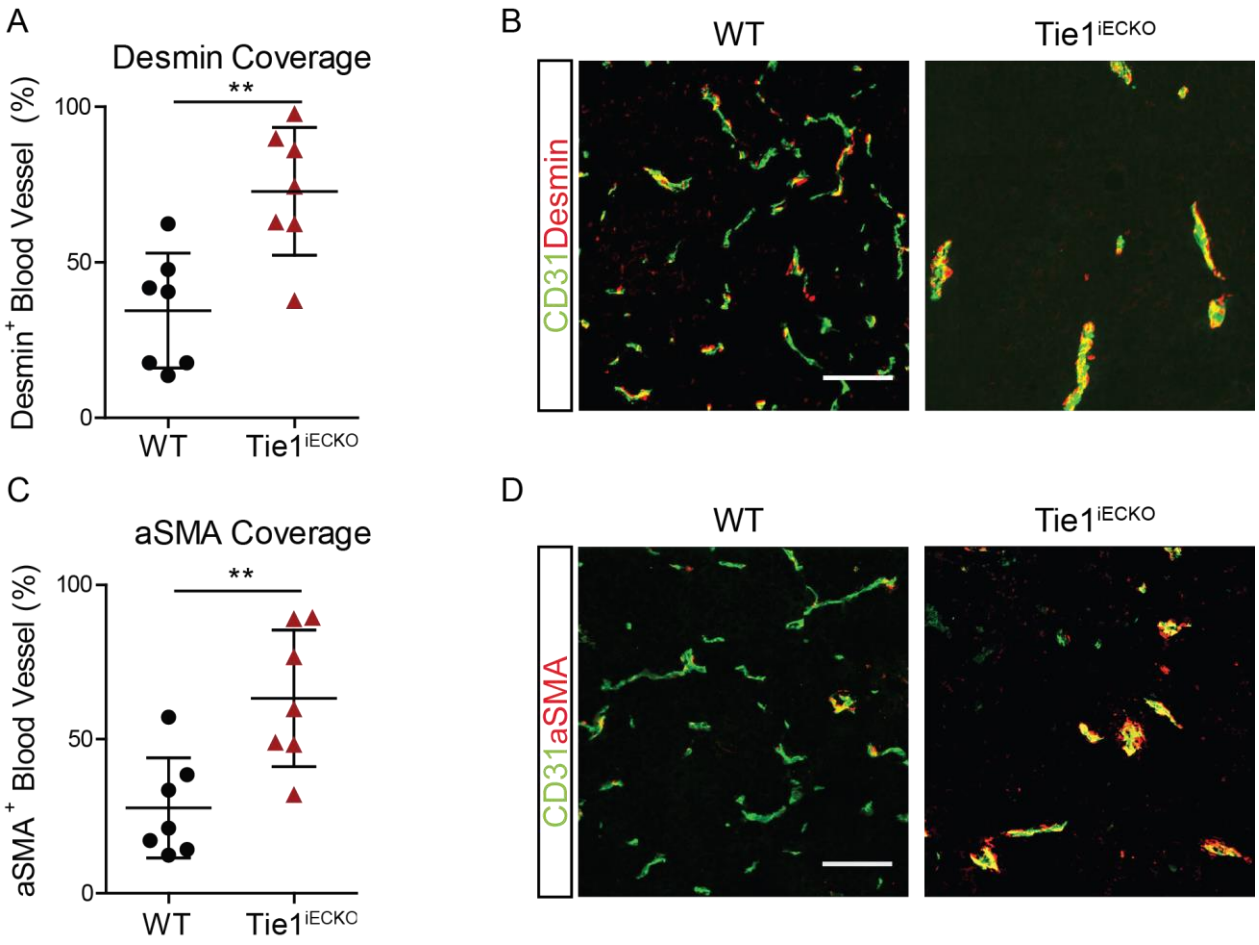

Figure 15: Tie1 loss increases perivascular coverage in B16F10

A) Quantification of desmin-positive area colocalized with CD31 (n=7; Mann-Whitney test, **, p<0.001), and **B)** representative microscope images. Scale bar: 100 µm. **C)** Quantification of αSMA-positive area colocalized with CD31 (n=7; Mann-Whitney test, **, p<0.01), and **D)** representative microscope images. Scale bar: 100 µm.

3.3 Tie1 deletion improves blood vessel perfusion and limits tumor cell intravasation *in vivo* and *in vitro*

Mature and mural cell covered blood vessels also show improved functionality including vessel perfusion and reduced vessel leakiness. Therefore, functionality experiments were performed. First, vessel perfusion was assessed by intravenously injecting fluorescent lectin that localizes to the luminal side of perfused vessels, and the colocalization between CD31 and lectin was quantified. A significant increase of lectin-perfused blood vessels was observed in Tie1^{iECKO} tumors compared to the WT tumors, suggesting an improvement in vessel functionality upon Tie1 deletion (Fig. 16A, B).

Second, vessel permeability was subsequently conducted by intravenously injecting 100 nm fluorescent-labelled beads and vessel leakiness was quantified by assessing the amounts of nano-beads extravasated from the vessels into the tumor tissue. No significant differences were observed between the two experimental groups (Fig. 16C, D).

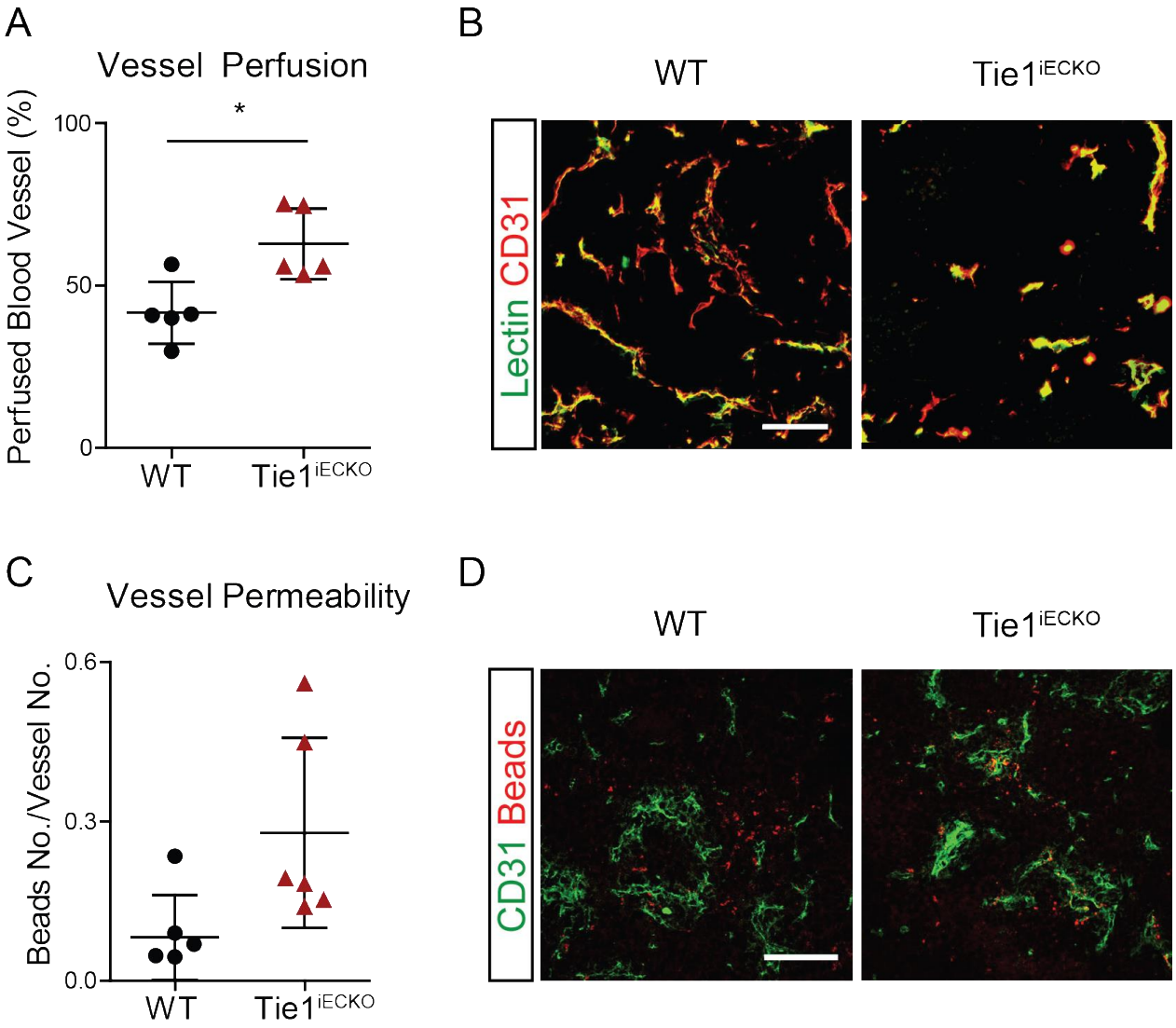

Figure 16: Tie1 endothelial deletion leads to improved vessel perfusion

A) Quantification of lectin perfused vessel area (n=5; two-tailed Student's *t*-test, *, p<0.05). **B)** Microscope images of blood vessels (stained with anti-CD31) perfused with lectin from WT and Tie1^{iECKO} tumors. Scale bar: 100 µm. **C)** Quantification of extravasated beads into the tumor tissue (n=5 (WT) and 6 (Tie1^{iECKO}); Mann-Whitney Test). **D)** Microscope images of blood vessels (stained with anti-CD31) and extravasated beads (red) from WT and Tie1^{iECKO} tumors. Scale bar: 100 µm.

Since the extravasation of nano-beads is a passive process that does not completely resemble the interaction between tumor cells and the endothelium, an *ex vivo* experiment, aimed at counting the number of tumor cell colonies derived from circulating tumor cells (CTC) present into the blood, was performed. Fluorescently-labelled LLC (LLC-RFP) were injected subcutaneously in WT and Tie1^{iECKO} mice and, the arterial blood of the tumor bearing mice was plated 14 days later into cell culture dishes (Fig. 17A). After 2 weeks in culture, outgrowing tumor cell colonies were counted under a fluorescent microscope: 7 out of 11 WT cultures developed colonies, whereas only 2 out of 10 Tie1^{iECKO} cultures did (Fig. 17B). This result suggested that, upon Tie1 endothelial deletion, a reduced number of tumor cells escape from the primary tumor site into the circulation. To confirm a direct involvement of Tie1 in tumor cell migration across the endothelium, an *in vitro* tumor cell transmigration assay was performed. HUVEC were initially transfected with non-coding siRNA (NC) or two different Tie1 targeting siRNAs (siTie1a and siTie1b) and then plated on transwell inserts. After 12 hours, fluorescently-labelled LLC were seeded on top of the HUVEC and were allowed to transmigrate through the endothelial monolayer for 8 hours (Fig. 17C). The number of LLC migrated across the Tie1 deficient monolayers was significantly reduced if compare to the number of LLC migrated through the control monolayer (Fig. 17D).

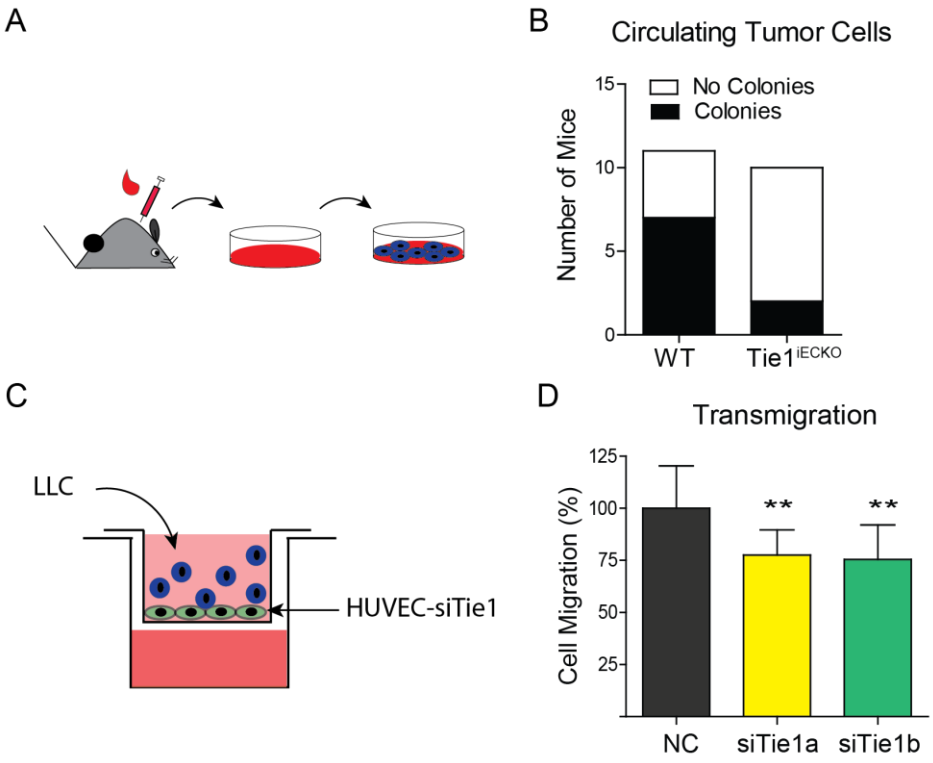

Figure 17: Tie1 loss reduces tumor cells transmigration in vivo and in vitro
A) Blood was drawn from WT and Tie1^{iECKO} tumor bearing mice 14 days post-LLC inoculation and the formation of tumor cells colonies was investigated. **B)** Number of blood samples that developed tumor cell colonies (n=11 (WT) and 10 (Tie1^{iECKO})). **C)** HUVEC were seeded on gelatin-coated transwell inserts and the PKH67-labeled LLC cells transmigrated in 8 h were counted. **D)** Percentage of transmigrated LLC through a layer of siTie1 HUVEC versus control; n= 3 independent experiments, each performed in triplicate, Mann-Whitney Test, **, $p<0.01$).

These results suggested that upon Tie1 deletion the endothelium is less prone to tumor cell migration. To check if this is related to an improvement of the integrity of the endothelial junctions, the level of an adherens junction marker, VE-Cadherin, was investigated. The expression level of *VE-Cadherin (Cdh5)* mRNA was increased in FACS sorted isolated tumor EC (Fig. 18A) as well as the amount of the protein in tumor sections was strongly augmented (Fig. 18B, C) in Tie1^{iECKO} mice, suggesting strengthening of adherens junctions amongst EC upon Tie1 loss.

Taken together, these data show that Tie1 deletion during primary tumor growth improves blood vessel functionality and decreases the number of tumor cells intravasated from the primary tumor site into the vasculature, and therefore limits the number of CTC in the circulation.

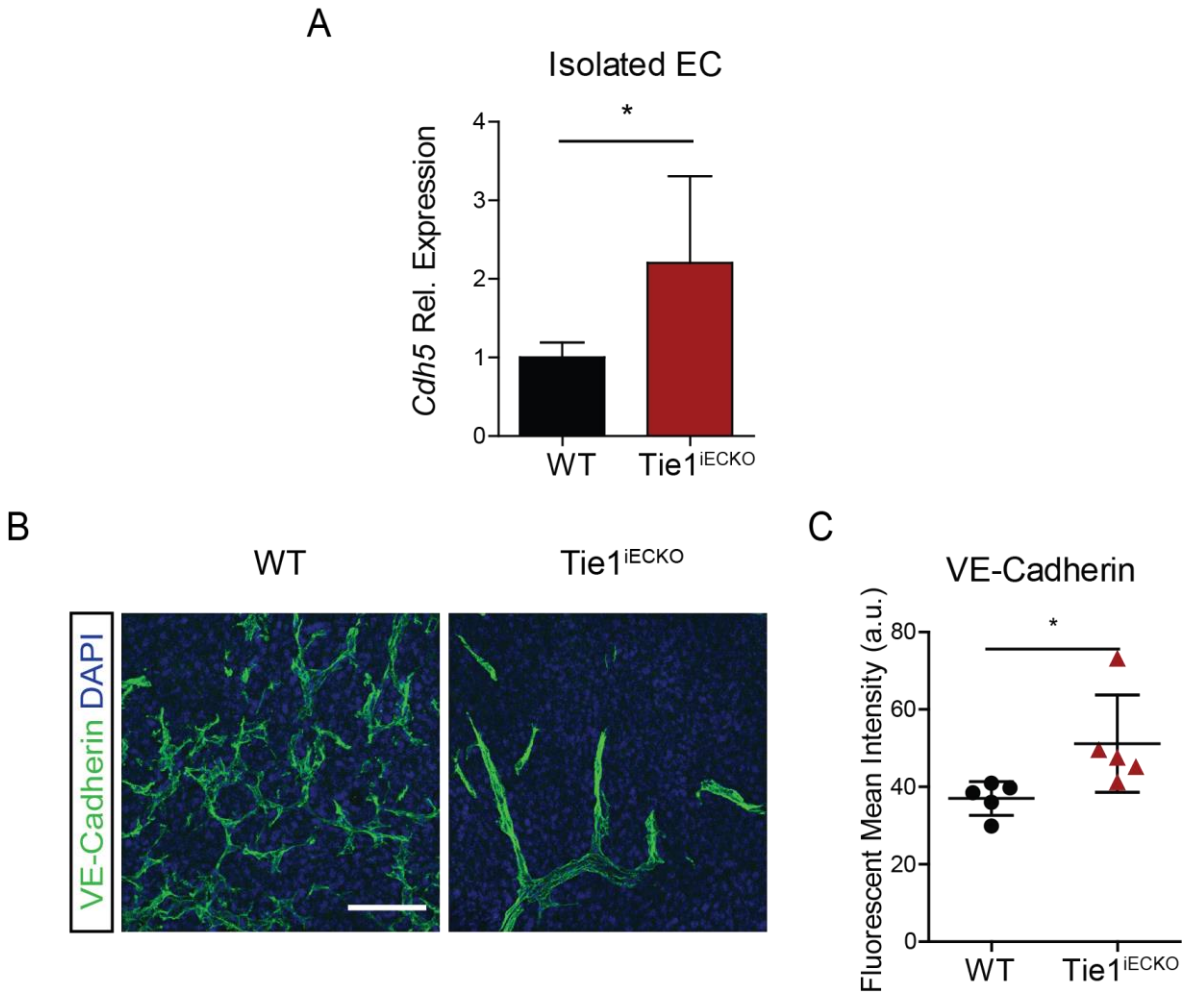

Figure 18: Tie1 endothelial specific deletion potentiate adherens junctions

A) Relative *Cdh5* expression in isolated tumor EC (n=4 (WT) and 5 (Tie1^{iECKO}); Mann-Whitney test, *, p<0.05). **B)** Confocal microscope images of blood vessels stained with VE-Cadherin and DAPI from WT and Tie1^{iECKO} tumors. Scale bar: 50 µm. **C)** Quantification of VE-Cadherin fluorescent mean intensity (arbitrary unit) (n=5, Student's T-test, *, p<0.05).

3.4 Endothelial Tie1 loss induces vessel normalization prior to necrosis progression

To investigate in more detail the role of Tie1 in determining the temporal relationship between angiogenesis, vascular abnormalizaiton and tumor growth, the vascular phenotypes were analyzed at earlier stages in a time-line experiment (days 9 and 12 post-LLC inoculation). On day 9, no major morphological changes in microvessel area and density as well as in desmin and αSMA-covered vessels were evident (Fig. 19A-D and Fig. 20A, B). Also, no difference in tumor cell necrosis between the two experimental groups was yet evident (Fig. 19E and Fig. 20D). Interestingly, there was already a minor, but significant improvement of microvessel perfusion in the $Tie1^{iECKO}$ group compared to control mice (Fig. 19F and Fig. 20C). Hence, Tie1 deletion does not significantly affect the vasculature during the initial phases of tumor growth, but influences primary tumor blood vessels, and subsequently tumor growth, only at later stages. Correspondingly, vessel area in tumors harvested on day 12 was significantly decreased (Fig. 19A, B and Fig. 20E, F), and mural cell coverage and perfusion were notably increased in $Tie1^{iECKO}$ tumors (Fig. 18C, D, F and Fig. 20E-G). Yet, no increase in tumor cell necrosis was detectable in $Tie1^{iECKO}$ tumors on day 12 (Fig. 18E Fig. 20H). These results demonstrate that, upon Tie1 deletion, tumor blood vessels acquire overtime a mature phenotype and improve their functionality at later stages, thereby preventing tumor cell escape.

In conclusion, the data indicate that EC Tie1 deletion has a direct effect on primary tumor vascularization prior to the onset of tumor necrosis.

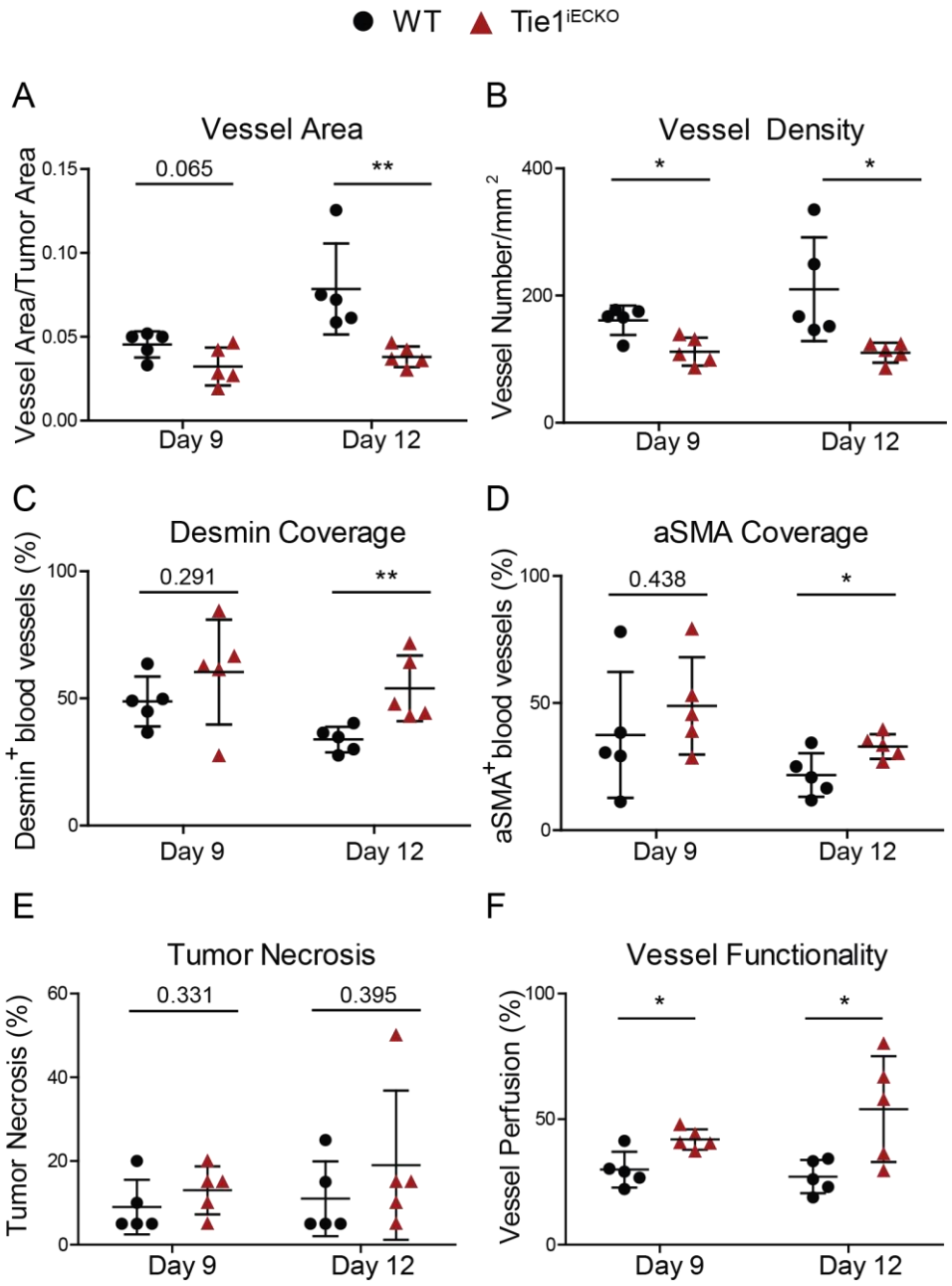

Figure 19: Endothelial Tie1 deletion influences later stages of primary tumor growth

A) Quantification of vessel area **B)** vessel density **C)** desmin-positive and **D)** αSMA-positive blood vessels, 9 and 12 days post LLC-inoculation (n=5; Mann-Whitney test, **, p<0.01; *, p<0.05). **E)** H&E quantification of primary tumor necrosis 9 and 12 days post-LLC injection (n=5; two-tailed Student's *t*-test). **F)** Quantification of lectin perfused CD31 area (n=5; Student's T-test, *, p<0.05).

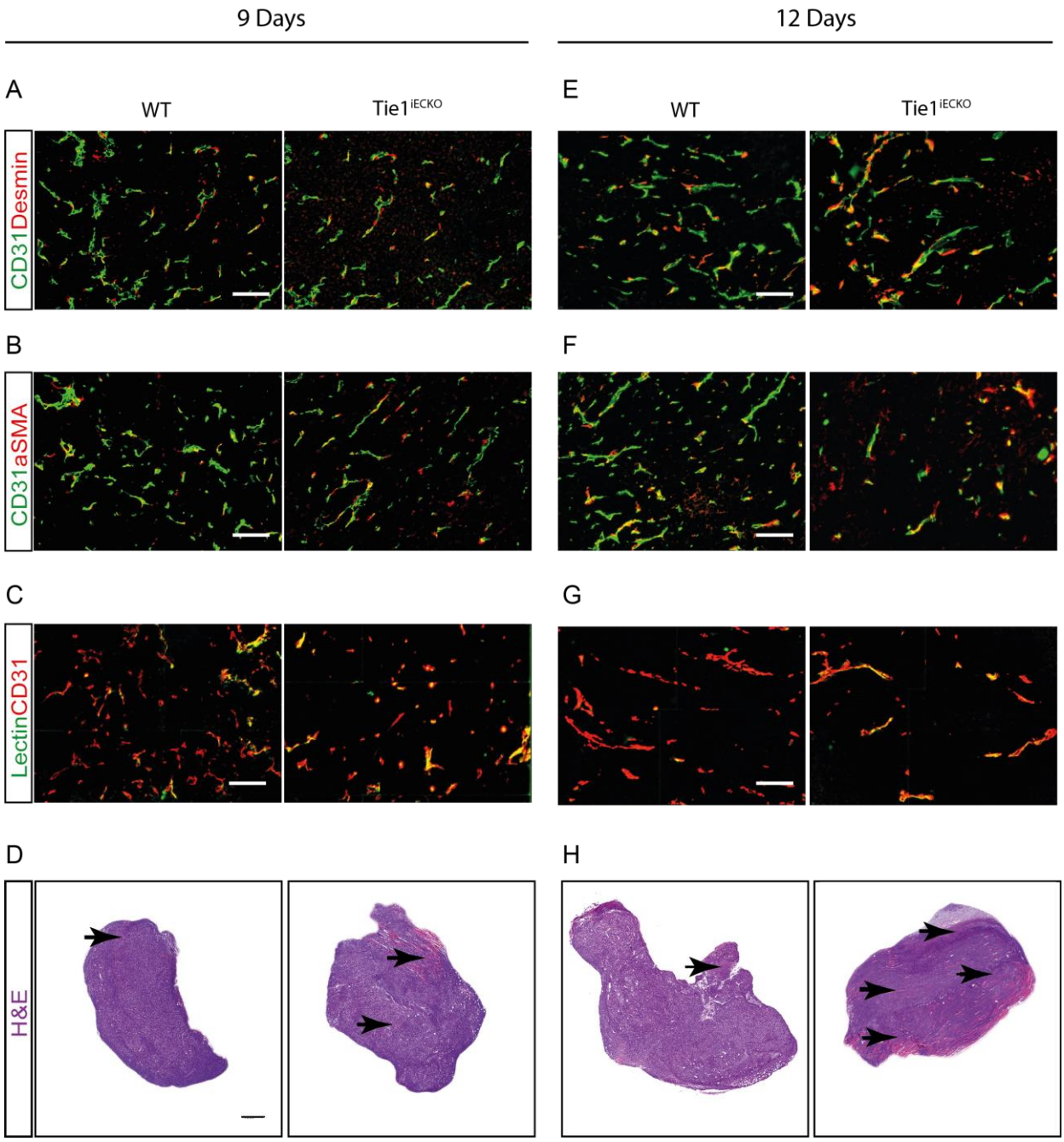

Figure 20 Tie1^{iECKO} induces vascular changes at later stages

Representative microscope images of **(A)** Desmin-positive, **(B)** αSMA-positive and **(C)** lectin-positive blood vessels 9 days post LLC-inoculation in WT and Tie1^{iECKO} mice. **(D)** Representative H&E staining from 9 days LLC primary tumors. Images of primary tumor blood vessels positive for **(E)** Desmin **(F)** αSMA and **(G)** lectin, 12 days post-inoculation. **(H)** Representative H&E staining from 12 days LLC primary tumors.

3.5 Endothelial Tie1 deletion during primary tumor growth inhibits spontaneous lung metastasis and prolongs survival

Normalized primary tumor vasculature and reduced CTC in the circulation are normally associated with a decrease of tumor cell seeding at secondary sites and, therefore, with a limited number of metastases (Jain, 2014). In order to investigate the effects of endothelial Tie1 on the initial steps of the metastatic cascade, a tumor resection model was selected, in which the primary tumor was surgically removed once it had reached ~0.8 cm^3 in size (day 14) (Pulaski and Ostrand-Rosenberg, 2001). This model closely mimics the clinical setting wherein patients generally survive the primary tumor burden due to surgery, but succumb to metastases that develop after surgery. Thus, LLC were subcutaneously inoculated in mice and Tie1 deletion was initiated during primary tumor growth via tamoxifen administration. Primary tumor was resected two weeks after tumor cells inoculation (Fig. 21A). Lungs were examined 3 weeks post-primary tumor removal for macro- and micro-metastases by immunohistological H&E staining (Fig. 21B). At this time point, 10 out of 14 (71%) WT mice presented metastatic foci while surprisingly only 1 out of 14 (7%) $Tie1^{iECKO}$ mice developed metastases (Fig. 21C). This result translated further into an improved overall survival of the $Tie1^{iECKO}$ mice compared to the WT controls. Indeed, 85% of the animals from the $Tie1^{iECKO}$ group were still alive 100 days post-surgery, whereas only 30% of WT mice survived during the same time (Fig. 21D).

In summary, these data suggest that targeted deletion of Tie1 in the endothelial compartment inhibits spontaneous metastases formation in the lungs and increases overall survival.

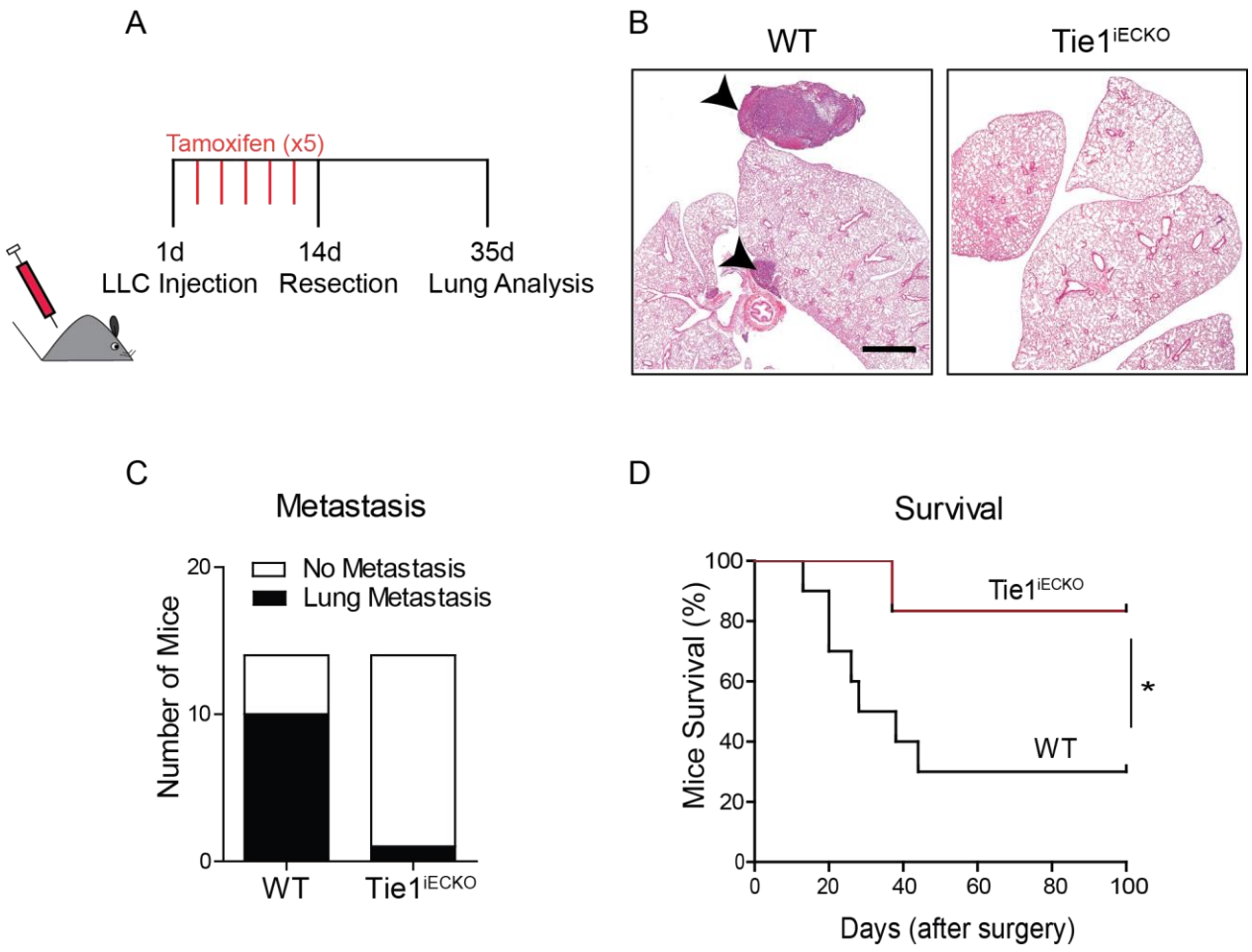

Figure 21: Tie1 deletion during primary tumor growth decreases lung metastasis and prolongs survival

A) Schematic representation of the experimental model: LLC were inoculated subcutaneously and Tie1 deletion was induced via tamoxifen administration. Tumors were surgically removed after 14 days. Mice were sacrificed and lungs were analyzed 3 weeks post-tumor resection. **B)** Representative H&E lung staining from WT and Tie1^{iECKO} mice. Arrowheads indicate metastases. Scale bar: 3 mm. **C)** Number of mice that developed lung metastases (black) in WT and Tie1^{iECKO} mice after primary tumor removal (n=14). **D)** Kaplan-Meier survival curve of WT and Tie1^{iECKO} mice after primary tumor removal (n=10 (WT) and 6 (Tie1^{iECKO}); Gehan-Breslow-Wilcoxon Test; *, $p<0.05$. Mice with less than 50% Tie1 deletion were excluded from the analysis.

3.6 Tie1 promotes tumor cell extravasation and seeding

The prominent primary tumor and metastasis phenotypes (Fig. 12, 13, 17 and 21) position Tie1 function in the early steps of the metastatic cascade (primary tumor angiogenesis, tumor cell invasion and intravasation in the circulation). To investigate its contribution to subsequent stages of metastatic dissemination (tumor extravasation, cell seeding and colonization of secondary sites), a second metastasis model was employed. B16F10 melanoma cells were injected directly into the circulation, allowing the study of extravasation and seeding of the tumor cells into the lungs (Overwijk and Restifo, 2001). Mice were pre-treated with 4 doses of tamoxifen, and after one week from the last injection,

$1x10^5$ B16F10 cells were injected into the tail vein (Fig. 22A). Fourteen days post-inoculation, lung metastatic foci were counted under a stereomicroscope and further confirmed by histology (Fig. 22C, D). Metastatic colonies were detectable in both experimental groups (Fig. 22B). Yet, $Tie1^{iECKO}$ mice showed a significant decrease in the number of metastatic foci (Fig. 22D), suggesting that the orphan receptor affected the ability of tumor cells to extravasate and seed.

Taken together, the data support an important involvement of Tie1 in tumor cell transmigration through the endothelium both at primary tumor site (intravasation) and at secondary sites (extravasation).

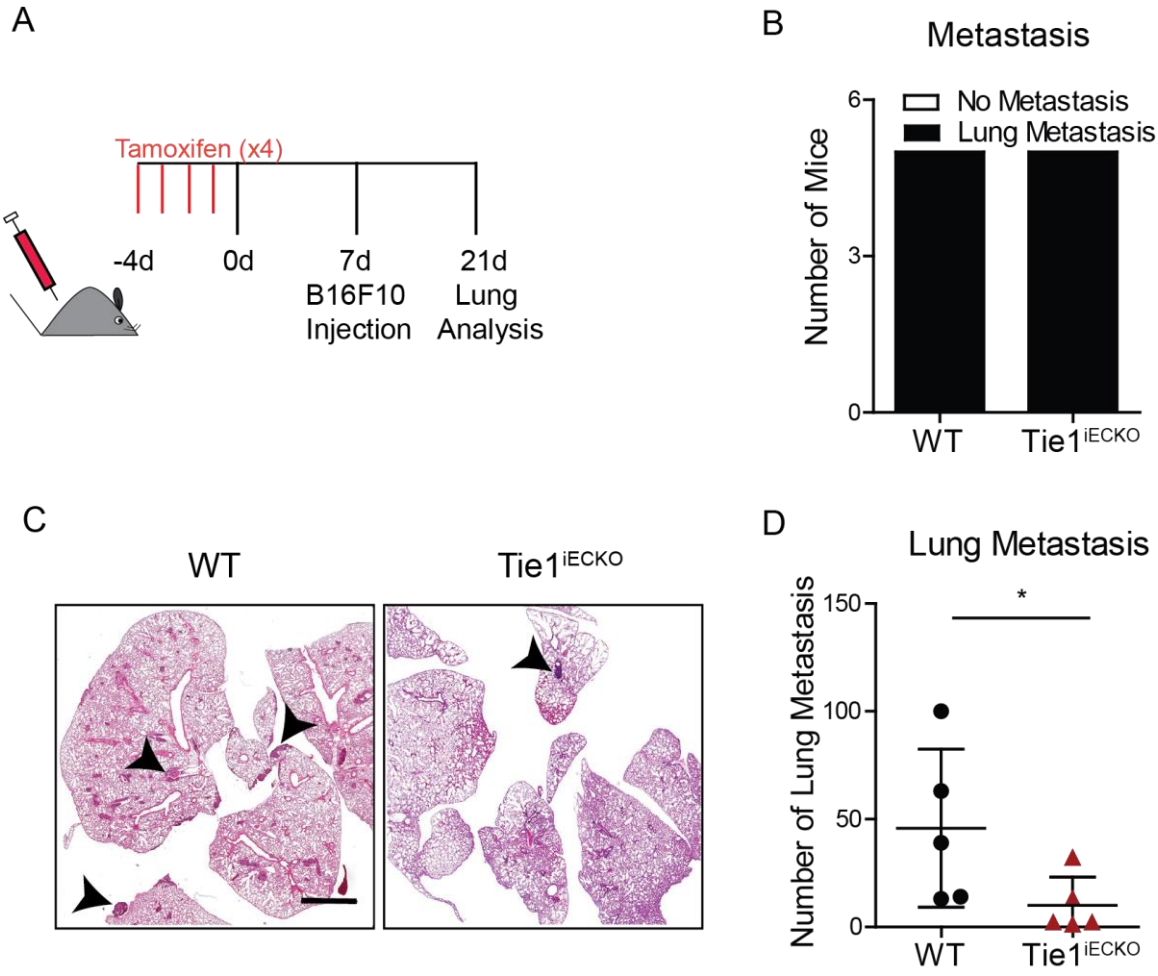

Figure 22: Tie1 deletion affects tumor cell extravasation and seeding

A) Schematic representation of the experimental metastasis model. Mice were pre-injected with 4 doses of tamoxifen and after one week from the last administration, B16F10 cells were injected intravenously. Mice were sacrificed and lungs were analyzed 2 weeks post-tumor cell injection. **B**) Number of WT and $Tie1^{iECKO}$ mice that developed lung metastasis (black) (n=5). **C)** H&E lung staining from WT and $Tie1^{iECKO}$ mice. Arrowheads indicate metastases. Scale bar: 3 mm. **D)** Quantification of the number of melanoma metastases per mouse lung (n=5; Mann-Whitney test, *, $p<0.05$).

3.7 Endothelial Tie1 deletion does not affect the growth of seeded micrometastasis

The previous experiments suggest that Tie1 affects several steps of the metastatic cascade, therefore the next investigation was aimed at investigating whether Tie1 deletion in EC affects the growth of seeded micrometastases. For this purpose, the same post-surgical metastasis model was used, but the tamoxifen administration was postponed after the primary tumor removal, when tumor cell seeding into the lungs had already occurred (Fig 23A). No difference was observed in terms of metastatic occurrence, as 6 out of 8 (75%) WT mice and 7 out of 8 (87.5%) Tie1^{iECKO} mice developed post-surgical lung metastases (Fig. 23B). In addition, the lung metastatic area, quantified microscopically by H&E staining, was also similar in both experimental groups (Fig. 23C, D).

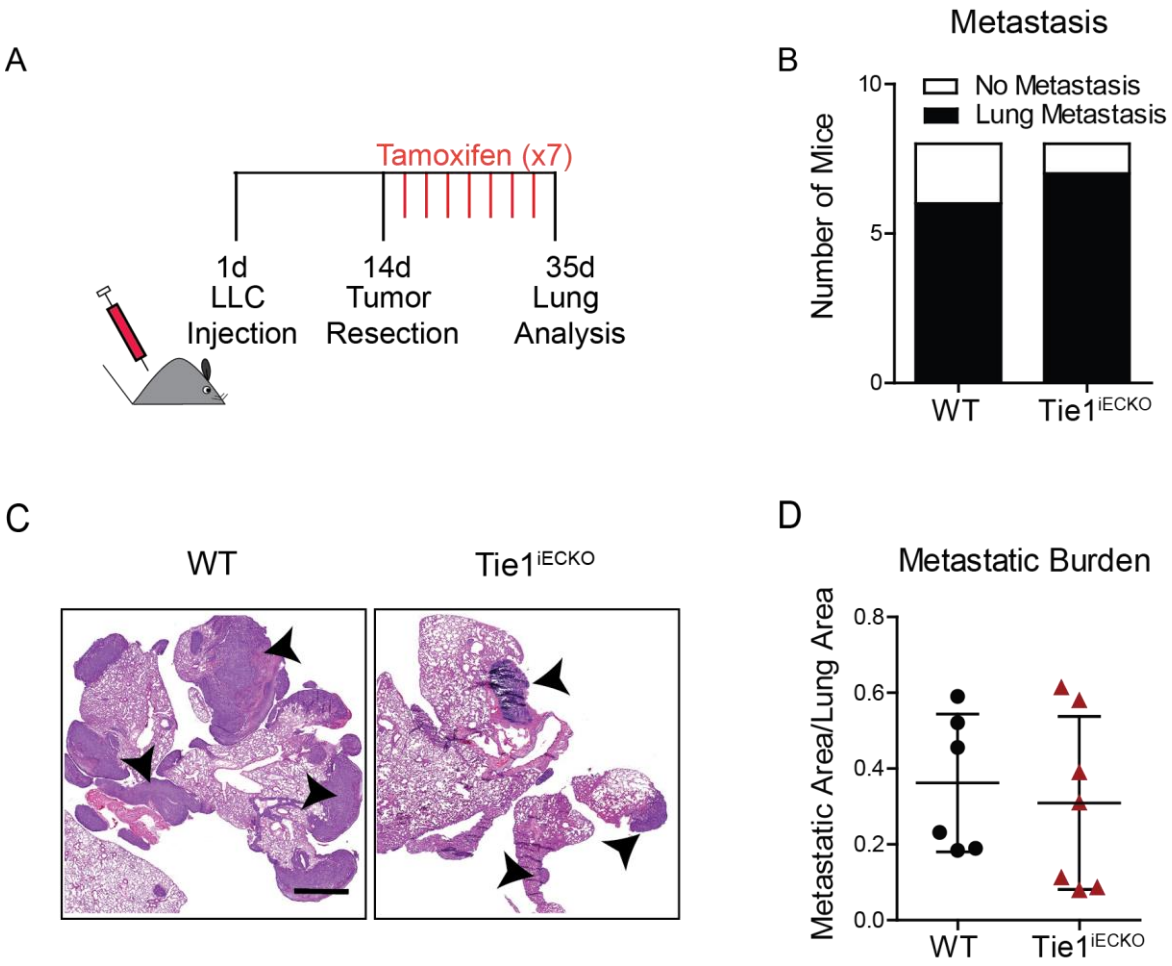

Figure 23: Endothelial Tie1 deletion does not affect the growth of seeded micrometastases

A) Schematic representation of the experimental model: LLC were injected subcutaneously and tumors were resected on day 14. Tie1 was deleted seven days after surgery, upon tamoxifen administration. Mice were sacrificed and lungs were analyzed 3 weeks post-tumor resection. **B)** Number of WT and Tie1iECKO mice that developed lung metastases (black) (n=8). **C)** H&E lung staining from WT and Tie1iECKO mice. Arrowheads indicate metastases. Scale bar: 3 mm. **D)** Quantification of the metastatic area in lungs from WT and Tie1iECKO mice (n=6 (WT) and 7 (Tie1^{iECKO}); Mann-Whitney test).

Since Tie1 deletion profoundly affected primary tumor vasculature, blood vessel area and density as well as vessel coverage were analysed also in the lung metastases, 3 weeks post-tumor removal. Consistently to the findings in the primary tumors, Tie1 genetic targeting had anti-angiogenic effect on metastatic vasculature, inducing a decrease in microvessel area as well as vessel density (Fig. 24A-C), although no significant difference in mural cell coverage was observed (Fig. 24D).

In conclusion, similar to the primary tumor growth, Tie1 endothelial deficiency induces anti-angiogenic effects on the metastatic vasculature, but does not affect the growth of seeded micrometastases.

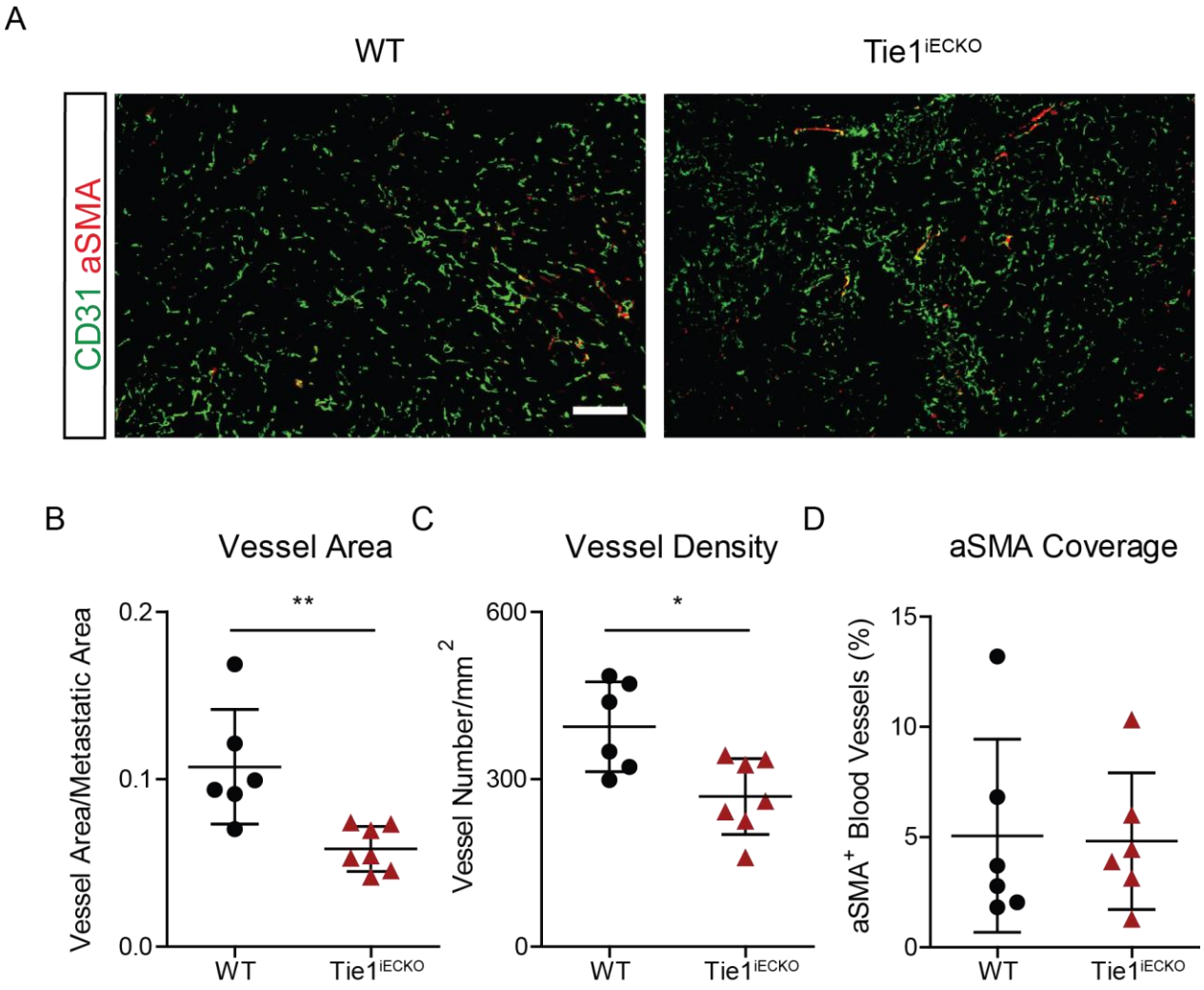

Figure 24: Tie1 deletion has anti-angiogenic effect also on metastatic vasculature

A) Representative microscope images of WT and Tie1^{iECKO} metastasis vasculature. Scale bar: 200 µm. Quantification of **B)** microvessel area, **C)** microvessel density and **D)** αSMA coverage of lung metastasis vasculature (n=6 (WT) and 6-7 (Tie1^{iECKO}); Mann-Whitney test, **, p<0.01, *, p<0.05).

3.8 Endothelial Tie1 deletion favors Tie2-expressing endothelium and promotes stabilization through Ang1 increase

The orphan receptor Tie1 exerts its effects on angiogenesis and vascular maturation by contextually affecting Ang1/Tie2 signaling (Kim et al., 2016; Korhonen et al., 2016; Savant et al., 2015). We therefore performed cytokine array experiments on whole tumor lysates 14 days post-tumor cell inoculation (Fig. 25A) in order to assess the expression of Ang1, Ang2 and other molecules, in tumors grown in WT and Tie1^{iECKO} mice. The expression of both Ang1 and Ang2 was increased in tumors lysates from Tie1^{iECKO} mice compared to WT mice (Fig. 25A, B). However, the ratio of Ang1 to Ang2 expression shifted towards Ang1 (Fig. 25C), involved in endothelial maturation and quiescence maintenance.

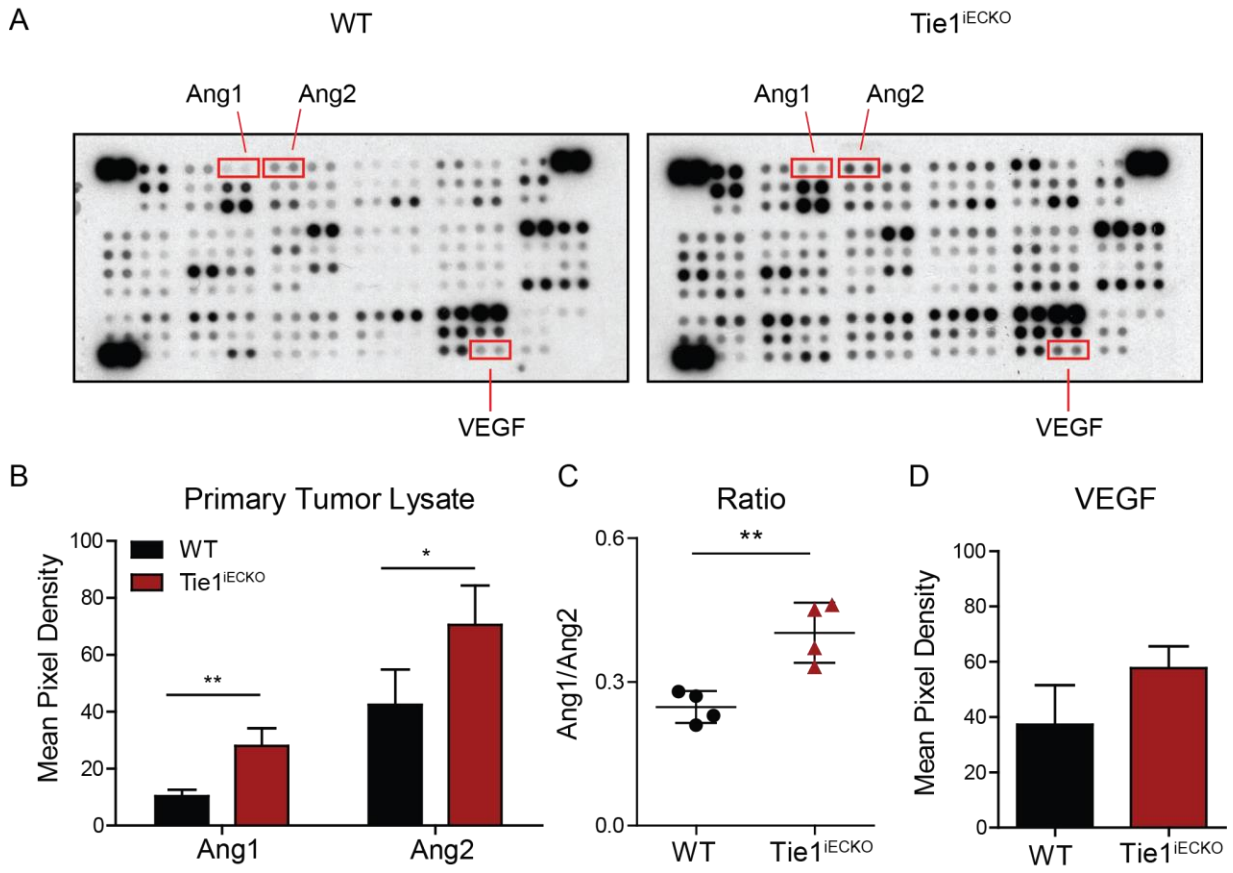

Figure 25: Tie1 deletion increases Ang1/Ang2 ratio

A) Representative immunoblotting images of the proteome profiler. Mice were inoculated subcutaneously with LLC and after 14 days, whole tumors lysates were used for the cytokine array and quantified. **B)** Densitometric quantification of Ang1 and Ang2 in WT and Tie1^{iECKO} primary tumor (n=4; Mann-Whitney test, **, $p<0.01$ and *, $p<0.05$). **C)** Intensities resulting from the cytokine array in A) were analyzed and the ratio Ang1/Ang2 was quantified (n=4; Mann-Whitney test, **, $p<0.01$). **D)** Densitometric quantification of VEGF ligand in WT and Tie1^{iECKO} tumors (n=4; Mann-Whitney test).

In cooperation with VEGF, Ang2 promotes endothelial cell migration, proliferation and sprouting angiogenesis. In order to see if VEGF/VEGFR pathway was synergistically acting with Ang2/Tie2 signalling,

VEGF protein level and VEGFR-2 mRNA were analysed. Interestingly, intratumoral expression of VEGF was not significantly affected by EC Tie1 deletion (Fig 25A, D). Yet, there was a strong reduction of *Vegfr-2* mRNA expression in FACS-sorted isolated tumor EC from Tie1^{iECKO} mice (Fig. 26A), showing that EC VEGFR-2 expression is strongly regulated by Ang/Tie signaling, as has been similarly observed by Hu and colleagues in the liver after partial hepatectomy (Hu, Srivastava et al. 2014). Unlike *Vegfr-2*, *Tie2* mRNA levels in FACS-sorted isolated tumor EC was not altered (Fig. 26B) and, similarly, Tie2 protein level in whole tumor lysates (Fig. 26C, D) was not changed in Tie1^{iECKO} mice. Yet, the microvessel density in Tie1^{iECKO} tumors was strongly reduced (Fig. 12A-C) with pronounced reduction of angiogenic sprouts (Fig. 13E). Given that Tie2 is negatively regulated during angiogenesis (del Toro et al., 2010; Felcht et al., 2012), we therefore hypothesized that EC Tie1 may not directly regulate EC Tie2 expression, but rather affect the fraction of Tie2-negative angiogenic EC, as has been suggested during postnatal mouse retinal angiogenesis (Savant et al., 2015). Indeed, the colocalization index of Tie2 and the endothelial marker CD31 in tumor sections identified a significant increase in the percentage of Tie2-positive endothelial cells (stalk cells) (Fig. 26E, F), and therefore, a reduction of Tie2-negative endothelium (tip cells). In conclusion, the vascular normalization caused by Tie1 deletion in the primary tumor is a result of increased Ang1 in the tumor microenvironment that might act on the increased fraction of Tie2-positive endothelial cells.

Together, these data indicate that Tie1 deletion promotes a more quiescent and mature Tie2-expressing endothelium that limits tumor cell intravasation and hence metastasis.

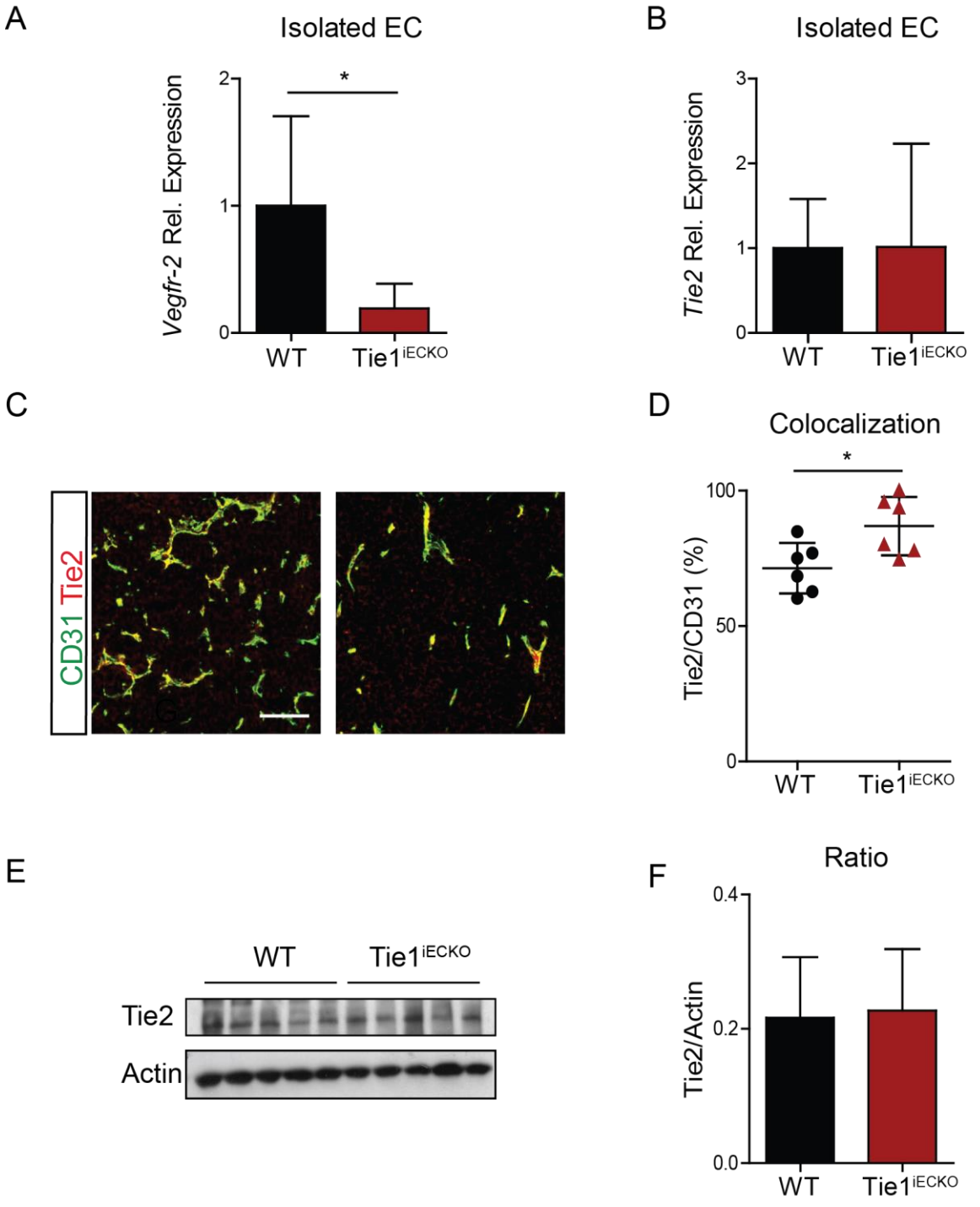

Figure 26: Tie1 deletion promotes vascular maturation

A) Relative *Vegfr-2* expression in isolated tumor EC (n=4 (WT) and 5 ($Tie1^{iECKO}$); Mann-Whitney test, *, p<0.05). **B)** Relative *Tie2* expression in isolated tumor EC (n=4 (WT) and 5 ($Tie1^{iECKO}$)). **C)** Microscope images of CD31 positive blood vessels stained for Tie2. Scale bar: 100 µm. **D)** Quantification of CD31 and Tie2 colocalization (n=6; Mann-Whitney test, *, p<0.05). **E)** Tie2 Western Blot analysis on whole tumor lysates from WT and $Tie1^{iECKO}$ mice with antibodies against Tie2 and the loading control actin. Representative picture of two independent experiments. **F)** Western Blot quantification of Tie2 relative to actin control (n=5; Mann-Whitney test).

3.9 Tie1 targeting does not affect immune cell population

The cytokine array performed on WT and Tie1^{iECKO} tumor lysates clearly showed a notable upregulation of several cytokines following Tie1 targeting (Fig. 25A). A more detailed analysis revealed that the most significant changes affected either cytokines involved in the Th1 (T helper lymphocyte type 1) anti-cancer immune response and cytokines involved in Th2 (T helper lymphocyte type 2) immune-suppression response (Walsh and Mills, 2013). To better clarify which immune cell population was affected by Tie1 deletion, a FACS analysis for the different immune populations within tumors grown for 14 days in WT and Tie1^{iECKO} mice was performed. The LLC tumors did not yield any significant differences in the proportion of total CD45$^+$ immune cells in both groups (Fig. 27A). Analyses of the lymphoid compartment showed no changes in the percentage of B cells (CD45$^+$CD19$^+$CD45R$^+$) involved in the humoral immunity (Kurosaki et al., 2015), and T cells (CD45$^+$CD3$^+$) involved in the adaptive immune response (Walsh and Mills, 2013), that populate the primary tumors (Fig. 27B, C). Amongst the T cell population, similar levels of T helper cells (CD3$^+$CD4$^+$), cytotoxic T cells (CD3$^+$ CD8$^+$) and T helper 17 cells (CD3$^+$CD4$^+$RORγt$^+$) were found (Fig. 27D). Similarly, no significant changes were detected in the myeloid compartment (CD45$^+$CD11b$^+$), important for the normal function of innate and adaptive immune response (Gabrilovich et al., 2012) (Fig. 27E). Indeed, the percentage of granulocytes (CD45$^+$CD11b$^+$Ly6G$^+$), monocytes (CD45$^+$CD11b$^+$Ly6C$^+$) and macrophages (CD45$^+$CD11b$^+$F4/80$^+$) was not altered between WT and Tie1^{iECKO} groups (Fig. 27E). Further immunofluorescent staining confirmed no differences in terms of F4/80-positive macrophages between WT and Tie1^{iECKO} tumors (Fig. 27F, G).

Macrophages were also classified according to the M1-like phenotype (CD45$^+$CD11b$^+$F4/80$^+$CD11c$^+$MMR$^-$) or M2-like phenotype (CD45$^+$CD11b$^+$F4/80$^+$CD11c$^+$MMR$^+$) (Fig. 28A, B). M1-like macrophages have been described to be pro-inflammatory and to exhibit anti-tumor activity, while M2-like macrophages have been reported to be anti-inflammatory and pro-tumorigenic (Sica and Mantovani, 2012). There were no differences in M1 and M2-like macrophages between the two experimental groups.

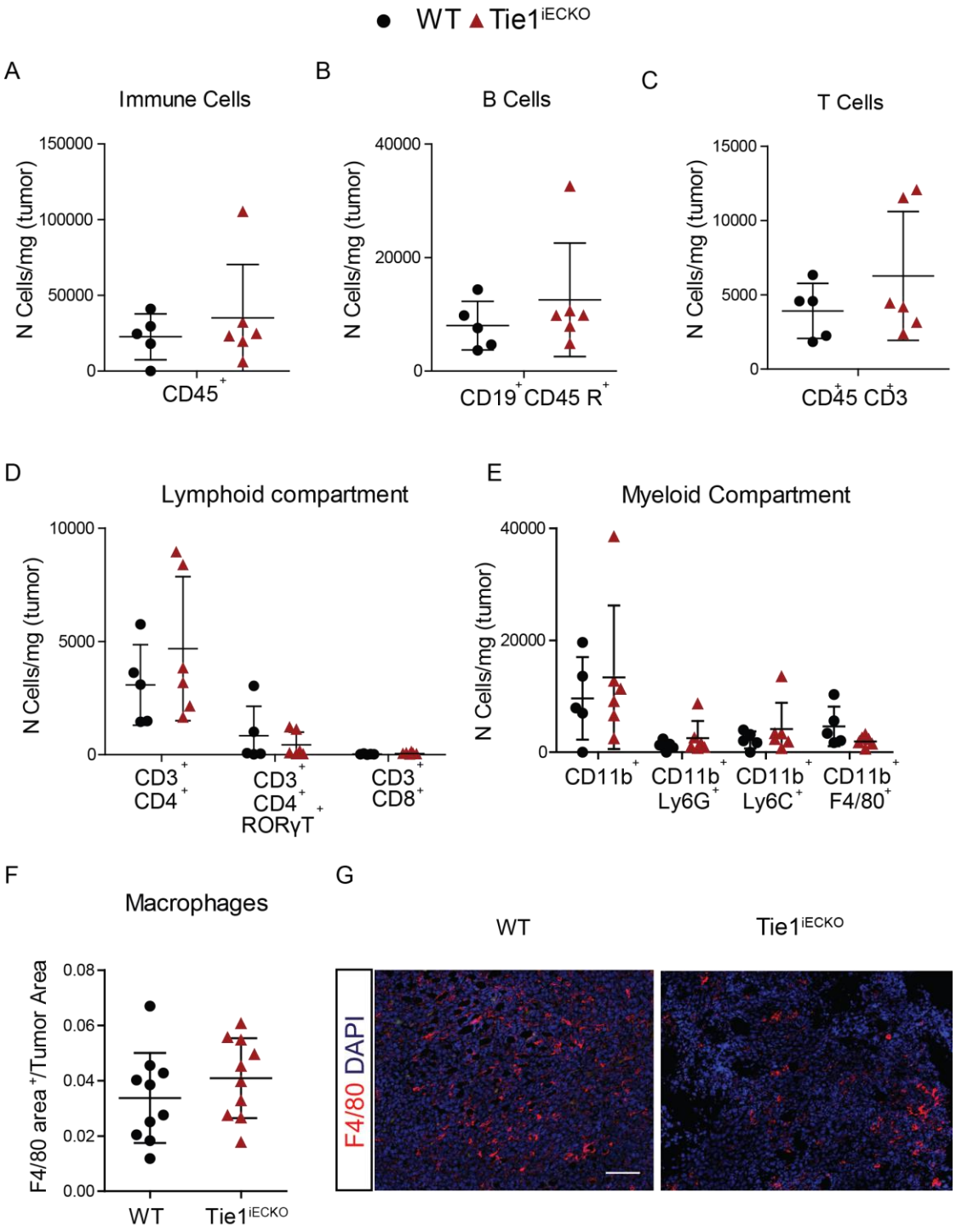

Figure 27: Tie1 targeting does not affect immune phenotype

LLC were inoculated subcutaneously and the tumors were used for immune FACS analysis 14 days post-inoculation. **A)** Quantification of total immune cell population infiltrated in the primary tumor. Values are expressed as number of cells per mg of tumor. Analysis of **B)** B cell and **C)** T cell populations. **D)** Characterizations of lymphoid and **E)** myeloid compartment (n=5 (WT) and 6 (Tie1^{iECKO}); Mann-Whitney test). Percentage of **F)** Quantification of macrophages (anti-F4/80) in the tumor area (DAPI) of WT and Tie1^{iECKO} mice (n=10; Mann-Whitney Test). **G)** Representative microscope images of macrophages (stained with anti-F4/80) in WT and Tie1^{iECKO} primary tumors. Scale bar: 100 μm.

Overall, the analyses of lymphoid and myeloid immune cell compartment in LLC tumors revealed no changes between WT and Tie1^{iECKO} mice. These findings exclude any involvement of immune cell populations in the anti-metastatic phenotype that characterize Tie1^{iECKO} mice, substantiating the unique role of Tie1 to the metastatic process.

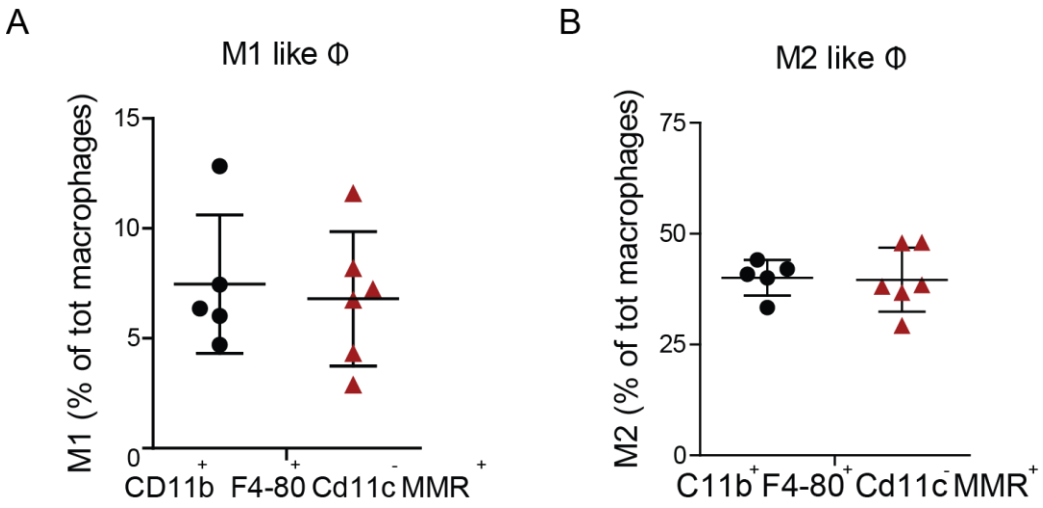

Figure 28: Tie1 endothelial deletion does not affect macrophage polarization

Percentage of **A)** M1-like and **B)** M2-like macrophages within WT and Tie1^{iECKO} primary tumors (n=5 (WT) and 6 (Tie1^{iECKO}); Mann-Whitney test).

4. Discussion

The genetic loss of Tie1 receptor in mice results in embryonic lethality between E13.5 and birth with defects in blood vessel integrity and haemorrhages as well as compromised capillary remodelling and EC survival, without overt cardiac defects (Puri et al., 1995). Savant, La Porta and colleagues have reported that EC-specific Tie1 deletion causes deficient retinal angiogenesis in postnatal mice (Savant et al., 2015), demonstrating a fundamental role of Tie1 also during developmental angiogenesis. Interestingly, Tie1 is not required under basal conditions in adult mice, and therefore its expression is downregulated in the adult vasculature.

4.1 Function of Tie1 during primary tumor growth

Although dispensable in the mature adult vasculature, the orphan receptor Tie1 has been found to be prominently overexpressed in several solid tumors such as breast cancer (Salven et al., 1996), colorectal adenocarcinoma (Ito et al., 2004), metastatic melanoma (Kaipainen et al., 1994), gastric carcinoma (Lin et al., 1999) and thyroid neoplasm (Ito et al., 2004). Tie1 receptor positively orchestrates tumor angiogenesis and its functional contribution to tumor progression has only recently begun to be explored. Conditional EC-specific Tie1 deletion in adult mice has been shown to result in decreased survival of EC in the tumor vasculature, leading to angiogenesis inhibition and reduced tumor growth (D'Amico et al., 2014). The present study was aimed at mechanistically dissecting the role of Tie1 during primary tumor growth and investigating its contribution to the process of metastasis, which is the main cause of cancer-related deaths.

To investigate these aims, Tie1 was conditionally deleted in EC ($Tie1^{iECKO}$ mice) after tumor cell inoculation. Tumor growth and vasculature were analysed at different time points (day 9, 12, 14 and 18 post-tumor cell inoculation) in order to detect at which stage Tie1 receptor influences tumor vasculature. Tumor growth in WT and $Tie1^{iECKO}$ mice did not differ until tumors had grown to almost 1 cm^3 in size. Thereafter, curves diverged leading to a significant inhibition of tumor growth and reduction of tumor weight upon Tie1 loss. This has also been reported by D'Amico and colleagues (D'Amico et al., 2014). These results suggest that Tie1 is not required for the vascularization of the primary tumor during early phases of tumor growth, but rather crucial for later stages. Indeed, until day 9 post-inoculation, Tie1 deletion induced only subtle changes in the $Tie1^{iECKO}$ tumor vasculature. In contrast, a significant anti-angiogenic effect was observed 12 days after tumor cell injection that became even more pronounced on day 14. The progressive and drastic decrease in microvessel area and density in $Tie1^{iECKO}$ tumors led to a notable increase in primary tumor hypoxia and subsequently to an increment in tumor necrosis. The anti-angiogenic effect induced by Tie1 deletion resulted from the abrogation of the immature tip cell vasculature and the regression of non-functional blood vessels. These findings correlate with those observed during physiological post-natal retina angiogenesis in $Tie1^{iECKO}$ mice. Particularly, Tie1 deletion in the developing retina induced reduction of tip cell sprouts, promoted endothelial cell apoptosis and subsequently regression of the immature vasculature without any

influence on the mature, well perfused vessels (Savant et al., 2015). Consistently, Tie1 deletion has been shown to upregulate the Notch pathway: Tie1 endothelial loss induced upregulation of Notch ligands, Dll4 and Hey1, mainly expressed in the angiogenic tip cells, and activator of Notch signaling in the neighboring stalk cells in order to suppress their migration and sprouting (D'Amico et al., 2014).

Similar to Tie1, Ang2 has been found to be upregulated in tumor blood vessels (Helfrich et al., 2009; Kuboki et al., 2008; Park et al., 2007; Scholz et al., 2007; Zhou et al., 2007). Its expression is closely associated with tumor growth, progression and metastasis (Holopainen et al., 2012; Imanishi et al., 2007; Nasarre et al., 2009), and has therefore been pursued as anti-angiogenic (AA) target. In particular, growth curve of tumors grown in Ang2-KO mice dissociated from the WT curve during early stages of tumor development, whereas tumor growth rates during later stages of primary tumor progression were similar (Nasarre et al., 2009). Furthermore, Ang2 overexpression promoted lymph node and lung metastasis in tumor xenograft (Holopainen et al., 2012). Although Ang2 global deletion and Tie1 endothelial deletion have the same vessel stabilization effect on primary tumor vasculature with higher rates of perivascular coverage (Nasarre et al., 2009), the underlying cellular mechanisms appear to be different: Whereas Ang2 deletion affects mural cell coverage with a limited effect on sprouting angiogenesis (Nasarre et al., 2009), the increase in vessel normalization in Tie1^{iECKO} mice results from the abrogation of the immature tip cell vasculature leaving behind a more covered and better perfused mature endothelium (this study). As such, differences in the target cell population of Ang2 and Tie1 could mechanistically explain the success of the combinatorial treatment of Tie1 genetic deletion and anti-Ang2 blocking antibody in sprouting-retina angiogenesis and tumor growth inhibition (D'Amico et al., 2014); Oppositely, the targeting of tip cell population promoted by both anti-VEGF antibody and Tie1 genetic deletion makes their mechanism of action redundant and explains the inefficacy of the combinatorial treatment during tumor growth (D'Amico et al., 2014). Ang2 inhibition in association with VEGF therapy has been shown to have pronounced effects on overall survival in preclinical tumor models (Kloepper et al., 2016; Peterson et al., 2016). Yet, these preclinical findings did not, so far, translate in an improved therapeutic effect of VEGF/Ang2 co-targeting in human clinical trials (Reuters, 2016). The findings of the present study provide experimental evidence suggesting that Tie1 targeting is mechanistically and temporally different from Ang2 blockade and support a strong rationale to exploit Tie1 as a therapeutic target.

Tumor vessels are characteristically chaotic, tortuous and poorly covered by perivascular cells with loose junctions and excess sprouting. These features make the blood vessels leaky and inefficient for the delivery of oxygen and nutrients to the tumor microenvironment (Jain, 2014). During the early phases of tumor growth, Tie1^{iECKO} blood vessels were phenotypically similar to the WT tumor vessels; progressively they acquired more mature characteristics similar to physiological vasculature, with higher perivascular coverage, reduced vessel sprouts, decreased vascular leakiness and improved vessel perfusion. This phenotype resembles vessel normalization, whereby disorganized and abnormal tumor blood vessels remodel into an organization similar to the physiological vessel architecture (Jain, 2013). Normalized

vasculature is more efficient in oxygen and nutrient delivery than abnormal tumor vasculature, and consists of a tighter endothelial barrier, which cannot be easily crossed by tumor cells. As tumors become less prone to tumor cells escape, vessel normalization is generally associated with decreased metastases (Jain, 2005). Despite the vascular normalization effect, LLC and B16F10 primary tumors showed a notably high hypoxic and necrotic area after Tie1 targeting. This apparent discrepancy between normalization and hypoxia/necrosis fits well to the concept of dose-dependent normalization window that occurs under AA therapy (Jain, 2005; Jain, 2014). An optimal AA strategy passively prunes away immature, dysfunctional vessels and actively fortifies those remaining. Importantly, this short-lived window improves oxygenation of the primary tumor microenvironment. Higher drug doses inhibit angiogenesis more potently but induce also an excessive pruning of the vasculature, leading to decreased oxygenation and increased necrosis. In $Tie1^{iECKO}$ tumors, the strong anti-angiogenic effect similarly relates to an extremely high deletion levels of Tie1, causing hypoxia and necrosis in the tumor microenvironment. An ideal dose-effect balance of Tie1 targeting could avoid the side effects related to an excessive AA therapy and could help to achieve the highest benefits of normalization therapy.

4.2 Function of Tie1 during tumor progression and metastasis

The strong normalization effect induced by Tie1 endothelial deletion on the primary tumor vasculature led to the investigation of its contribution to metastatic progression. To understand at which step of metastasis Tie1 plays an important role, single steps of the metastatic cascade were analyzed (tumor cell intravasation, survival in the circulation, seeding, extravasation to secondary site and colonization). Thus, *in vitro* assays, different metastasis models and temporal variation of the tamoxifen administration in these experimental protocols were adopted.

Surgery is often the first line of treatment adopted against primary tumors in the clinic. Nevertheless, patients succumb to metastatic disease that develops after operation. Similar to the clinical setting, surgical removal of the primary tumor in a mouse model enables the study of metastatic disease with spontaneous metastatic lesions establishment. The specific endothelial targeting of Tie1 during primary tumor growth led to the inhibition of post-surgical lung metastasis in 90% of $Tie1^{iECKO}$ mice and resulted in a successful improvement of the overall survival in $Tie1^{iECKO}$ mice compared to WT. Specific administration of tamoxifen during primary tumor growth allows the manipulation of tumor angiogenesis and of tumor cells intravasation into the blood stream. This last process results from the bi-directional communication between tumor cells and the tumor-associated vasculature. To isolate and better examine the process of tumor cell escape into the circulation, an *in vitro* transwell migration assay was applied, mimicking the vascular crosstalk between tumor cells and EC. These *in vitro* reconstruction experiments corroborated the vessel normalization phenotype and demonstrated that Tie1-deficient endothelium was less prone to tumor cells transmigration. In line with this, *ex vivo* permeability experiments showed that the number of tumor cells transmigrated from the primary tumor into the circulation and capable of forming tumor cell colonies, were reduced upon Tie1 loss. Although Tie1 and Tie2 are homologous receptor tyrosine kinases, they do play opposite roles in certain conditions. In

contrast to Tie1 deficiency, loss-of-function *in vitro* experiments have shown that Tie2 silencing in the EC weakens endothelial adherens junctions, rendering the endothelium monolayer more permissive to tumor cells transmigration (Hakanpaa et al., 2015). Correspondingly, gain of function *in vivo* experiments have demonstrated that the simultaneous activation of Tie2 and blockade of Ang2 ligand by the ABTAA antibody, increased VE-Cadherin distribution on tumor blood vessels and limited spontaneous lung metastases (Park et al., 2017). Similarly, in this study, the *in vivo* knock-out of Tie1 in growing tumor blood vessels promoted strengthening of endothelial adherens junctions via increased VE-Cadherin mRNA and protein, and inhibited post-surgical lung metastases. Hence, Tie1 deficiency resembles Tie2 endothelial activation phenotypes, with a robust vascular barrier and preserved vessel integrity, that limits the formation of spontaneous metastases.

Once in the circulation, tumor cells can travel within the blood stream to distant sites, attach to capillary vessel walls and extravasate into other tissues. In order to survive the new microenvironment of distant organs, tumor cells either become dormant or proliferate forming metastatic lesions (Ghajar et al., 2013; Karrison et al., 1999). All these processes can be reassumed in a rate-limiting step, known as “seeding and colonization of tumor cells”. To bypass the early steps of metastatic cascade and to relate Tie1 to the later steps of metastasis, an experimental model was performed by directly injecting B16F10 melanoma cells in the circulation of $Tie1^{iECKO}$ and WT mice. Also in this set up, endothelial Tie1 deleted mice showed a reduction in the number of metastatic melanoma foci per lung, indicating an important yet potential role of Tie1 in driving extravasation, seeding and colonization of tumor cells during metastatic spread. Similar to Tie1, Ang2 ligand also interferes with the later steps of metastatic dissemination: In the same *in vivo* experimental model, Ang2 overexpression disrupted endothelial integrity and promoted lung metastases, while its blockade improved endothelial cell-cell junctions and limited distant metastases (Holopainen et al., 2012; Park et al., 2017). These results suggest that Tie1 and Ang2 drive similar effects not only in the primary tumor vasculature but also at distant sites, during metastatic spread and metastasis formation. Further studies are needed to clarify if the two molecules have a similar mechanism-of-action, or if they act differently, as observed in normalization of the primary tumor vasculature in this study.

As mentioned previously, cancer-related deaths are generally not caused by the primary tumor, rather by the occurrence of mortal metastasis (Steeg, 2016). Indeed, while primary tumor growth is controlled to a great extent with a combination of surgery, chemotherapeutic and AA drugs, metastatic spread still poses an imminent clinical challenge with limited treatment options (Brezden-Masley and Polenz, 2014). Adjuvant AA therapy is still in its infancy and has so far not shown beneficial effects. For example, the BEATRICE trial showed that adjuvant bevacizumab failed to improve disease free survival in triple negative breast cancer patients after 3 years (Cameron et al., 2013). Similar disappointing results were obtained from the use of adjuvant Vanucizumab, that combine the effect of bevacizumab and anti-Ang2 blockade in metastatic colorectal cancer (Reuters, 2016). Limited clinical success of AA drugs calls for

better understanding of their mode-of-action and for identifying additional new inhibitors that are able to overcome the current limitations of AA therapy.

Recently, it has been shown that Ang2 targeting in a post-surgical adjuvant setting reduced the incidence of post-surgical bone metastasis as well as the growth of lung metastasis in mice (Srivastava et al., 2014). This study also demonstrated that combination of Ang2 blockade in association with low dose metronomic chemotherapy had additive effects on the metastatic inhibition and improved overall survival of mice (Srivastava et al., 2014). The present work adopted the post-surgical metastasis model to explore possible benefits of Tie1 targeting in a post-surgical adjuvant setting, when the seeding of tumor cells has already occurred and metastases have started to grow. Consequently, tamoxifen administration protocol was shifted after the primary tumor removal. Tie1 deletion in this adjuvant setting did not show any efficacy in terms of metastatic incidence and burden. Indeed, the same number of WT and Tie1^{iECKO} mice developed spontaneous lung metastasis. Interestingly, as in the primary tumor, Tie1 deletion affected the metastatic vasculature, inducing reduction of both microvessel area and microvessel density. Despite the anti-angiogenic effect on the metastatic vasculature, a difference in lung metastatic area was not evident, as instead observed during the late phases of primary tumor growth. This divergence between primary tumor and metastasis might be due to the fact that the death or sacrifice of the mice, caused by multiple and diffuse lung metastases, occurred before a potential difference in metastatic burden was evident between the two experimental groups. In conclusion, unlike Ang2 targeting, Tie1 deletion does not affect the growth of established metastatic foci in the lungs. Future experiments will show whether Tie1 inhibition in combination with high dose or metronomic chemotherapy lead to a beneficial effect as has been observed with anti-Ang2 therapy.

4.3 Tie1 targeting favors Tie2-expressing endothelium and promotes stabilization through Ang1 increase

The primary tumor milieu is characterized by dynamic networks involving cytokines, chemokines and growth factors that regulate the growth and maintenance of blood vessels (Jain, 2013). Tie1 endothelial deletion induced profound changes in the cytokine profiles within the microenvironment of Tie1^{iECKO} tumors, including the expression of growth factors Ang1, Ang2 and to a smaller extent VEGF. It has already been reported that LLC tumors are refractory to VEGF (Shojaei et al., 2009); Yet, in Tie1^{iECKO} primary tumors, a strong decrease in *Vegfr-2* mRNA levels was observed in FACS isolated tumor EC. These results are in accordance with a recent study demonstrating that EC VEGFR-2 expression is strongly regulated by Ang/Tie signaling in liver regeneration (Hu et al., 2014) and also suggest that the tumor endothelium is less responsive to VEGF/VEGFR-2 axis compared to the Ang/Tie axis.

Interestingly, in the primary tumor, Tie1 deletion led to an increase of Ang1 protein, therefore shifting the Ang1/Ang2 ratio towards Ang1. Ang1 is known to increase Tie2 activation and to maintain a quiescent EC phenotype while sustaining vessel integrity (Thurston et al., 2000; Thurston et al., 1999). Yet, it has been recently reported that sepsis-induced acute inflammation causes Tie1 ectodomain

shedding, which in turn leads to the downregulation of *Tie2* mRNA, reduction of Tie2 phosphorylation and upregulation of *Ang2* expression. In this context, Tie1 shedding prevents the agonistic action of Ang2 and favors its antagonistic role on Tie2, therefore leading to vascular remodeling (Kim et al., 2016; Korhonen et al., 2016). Similar effects were observed in the endothelial conditional Tie1 knock-out mouse, where the deletion of the orphan receptor in the endothelial compartment was favoring Ang2 antagonistic activity on Tie2 (Korhonen et al., 2016). In the current thesis, Tie1 deletion did not induce changes in *Tie2* mRNA but promoted an increase in Tie2 protein in the tumor endothelial cells as well as favored Ang1 production in the tumor microenvironment, indicating an increased activation of Ang1/Tie2 signaling upon Tie1 endothelial deletion in tumors. In other words, Tie1 does not directly regulate EC Tie2 expression, but rather affects the fraction of Tie2-negative tip cells, as has been demonstrated during postnatal mouse retinal angiogenesis. Indeed, the colocalization of Tie2 and CD31 in tumor blood vessels showed a higher proportion of Tie2-positive endothelium (stalk cells), and reduced proportion of Tie2-negative endothelium (tip cells), suggesting a less angiogenic and more quiescent tumor vasculature. Further studies are needed to clarify the mechanism driving Tie1 shedding and to decipher the context-dependent influence of Tie1 on Ang1-Ang2/Tie2 signaling.

In summary, the current work proposes that vascular normalization caused by Tie1 deletion in the primary tumor is a result of both enhanced Ang1/Tie2 signaling and a vasculature that is less responsive to VEGF and Ang2 stimuli.

4.4 Tie1 does not affect the immune system

An additional important role of blood vessels is to provide gateways for the patrolling immune cells. The excessive production of pro-angiogenic factors creates a local tumor microenvironment that leads to the preferential accumulation of immune suppressor cells in the tumor (Huang et al., 2013). The abnormal tumor microenvironment helps tumors evading the immune response through multiple mechanisms, including impairment of lymphocyte infiltration, upregulation of immune checkpoint protein expression via hypoxia, recruitment of regulatory T cells (Treg), and establishment of an immunosuppressive tumor microenvironment that impairs the function of the resident and transiting immune effector cells (Huang et al., 2013) (Motz et al., 2014). In contrast, tumor vessel normalization has been shown to correlate with immunostimulatory pathways, involved in T lymphocyte infiltration and activity (Tian et al., 2017).

A growing body of evidence indicates that AA agents normalize the abnormal tumor vasculature and potentially re-educate the tumor immune microenvironment towards a more immunosupportive profile. Along these lines, Kloepper et al. have very recently demonstrated how Ang2/VEGF bi-specific antibody reprograms macrophages to anti-tumor phenotype, via promoting tumor vessel normalization (Kloepper et al., 2016) and Schmittnaegel et al. have shown how dual inhibition of Ang2 and VEGFA elicits anti-tumor immunity via perivascular accumulation of IFN-γ expressing $CD8^+$ cytotoxic T lymphocytes (Schmittnaegel et al., 2017).

In order to understand whether the suppression of spontaneous metastasis following Tie1 targeting was not only due to limited tumor cell intravasation/extravasation at secondary sites, but also due to the reprogramming of the immune compartment, immune cell populations infiltrated in WT and Tie1^{iECKO} primary tumors were analyzed. The percentage of cells involved in either humoral response (B cells) or adaptive immune response (T cells) was not significantly altered upon Tie1 deletion. The amount of T effector cells such as CD4^{+} and CD8^{+}, normally associated to vessel normalization, was unchanged. Similarly, the number of granulocytes, monocytes and macrophages was not different between the two experimental groups and the same proportion of M1-like (anti-tumorigenic) and M2-like (pro-tumorigenic) tumor-associated macrophages was found.

These findings indicate that Tie1 genetic deletion does not improve anti-tumor immunity. Yet, it is important to note that the immune profiling was performed on day 14, when the late full-grown tumor was well established. Further studies, conducted at earlier stages, are needed to see whether Tie1 blockade promotes reconditioning of the tumor immune microenvironment towards an immunosupportive anti-tumor profile in the developing tumor.

4.5 Model: Tie1 expressing endothelium promotes tumor angiogenesis and metastatic dissemination

Taken together, the data leads me to propose Tie1 as a key-player of tumor angiogenesis, vascular abnormalization and, most importantly, metastatic dissemination. During pathological tumor angiogenesis, Tie1 is upregulated and promotes the formation of abnormal blood vessels in their feature and function. Tie1 expression induces vascular sprouting, counter-regulates Tie2 expression in tip cells, and reduces Ang1/Ang2 ratio in the tumor microenvironment promoting vessel destabilization. Therefore, Tie1-positive blood vessels present impaired mural cell coverage, loose endothelial cell-cell junctions, increased vessel leakiness and reduced vessel perfusion. The vasculature is more prone to tumor cell escape from the primary tumor into the vasculature and is more susceptible to tumor cell extravasation into secondary sites and, therefore, metastasis formation (Fig. 29).

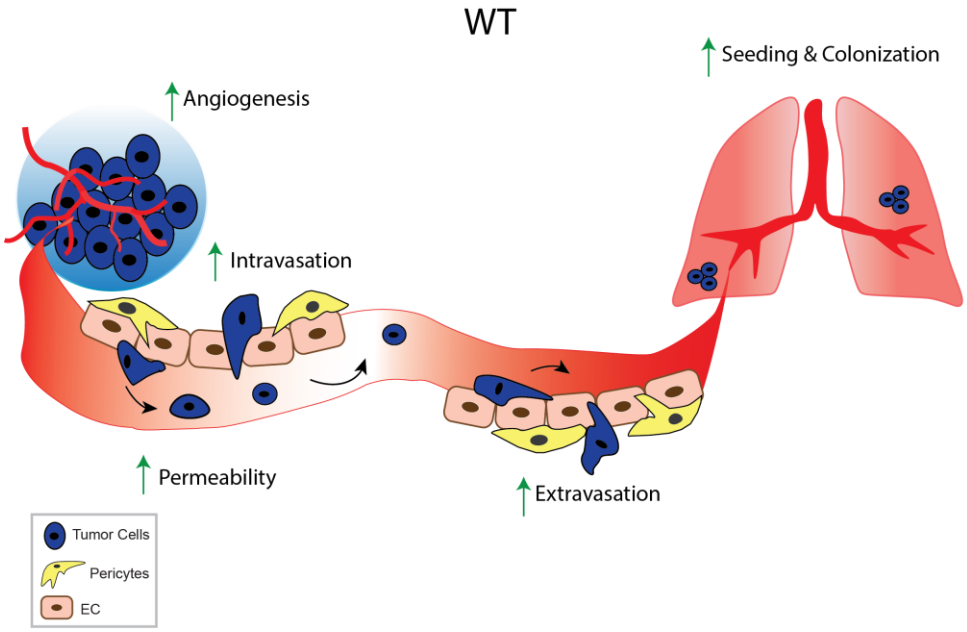

Figure 29: Tumor vasculature is abnormalized and promotes distant metastasis

Tumors are characterized by dense and irregular vasculature. Tumor blood vessels present weak endothelial cell-cell junctions, impaired perivascular coverage and increased vessel leakiness, which favor metastatic dissemination.

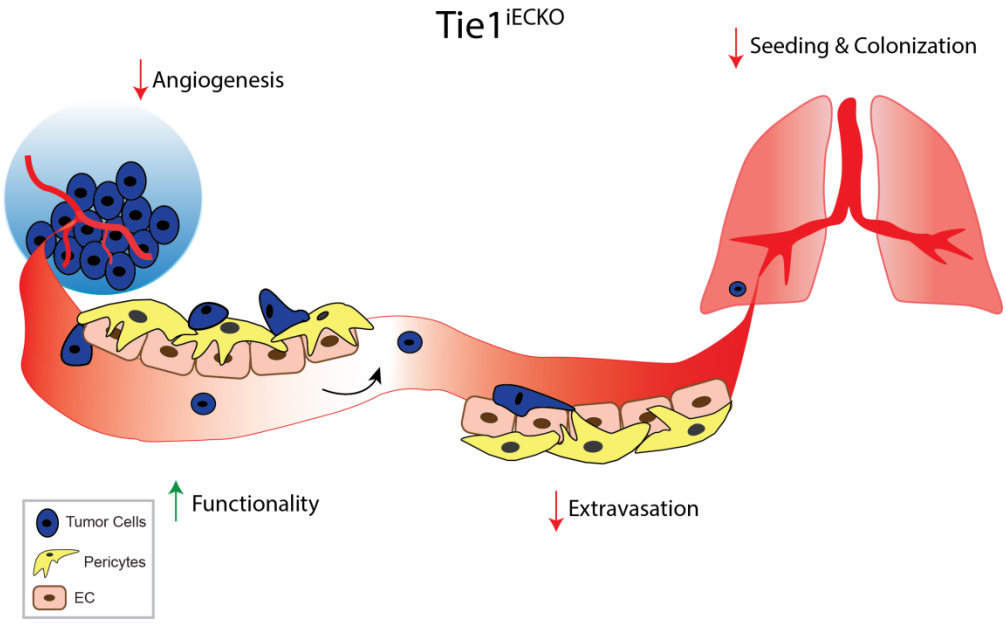

Figure 30: Tie1 targeting during primary tumor growth induces vascular normalization and blocks metastatic dissemination

Tie1 deficient tumors revealed reduced vessel density, increased mural cell coverage and improved vessel perfusion. This physical barrier limits tumor cell intravasation and extravasation, and reduces the formation of spontaneous lung metastasis.

Tie1 targeting therapy prior to primary tumor removal would limit angiogenesis and induce regression of non-functional blood vessels, while promoting normalization of the remaining vasculature, via the Ang1/Tie2 signaling axis (Fig. 30).

4.6 Tie1 as a therapeutic target and future direction

The therapeutic effect of Tie1 targeting during primary tumor growth has recently been reported by D'Amico et al (D'Amico et al., 2014). While the combination of Tie1 genetic deletion and VEGF/VEGFR blocking antibodies did not provide any additive inhibition of tumor angiogenesis and tumor growth, the combinatorial targeting of Tie1 and a soluble form of Tie2, blocking angiopoietins, resulted in improved tumor angiogenesis and tumor growth inhibition.

In the present thesis, the use of sophisticated *in vivo* metastasis models complemented with *in vitro* experiments, enabled to investigate the role of Tie1 in individual steps of tumor progression and metastatic dissemination. The gained insight into Tie1 biology uncovers new AA therapeutic opportunities focusing on Tie1 inhibition. Normalization of the primary tumor vasculature that occurs upon Tie1 deletion, and the strong inhibition of post-surgical lung metastasis offers Tie1 as an appealing target for new AA approaches. Further studies based on the combination of Tie1 inhibition and the administration of chemotherapeutic agents need to be performed to show the potential of Tie1-targeted blood vessels and improved delivery of chemotherapeutics inside the tumor microenvironment.

Furthermore, AA therapies have severe side effects because the pathways they target are essential not only for the tumor growth but also for the homeostasis of normal tissue (Carmeliet and Jain, 2011). Since Tie1 is predominantly expressed in activated blood vessels and it is downregulated in adult quiescent vasculature (Savant et al., 2015), the blockade of this receptor would have the advantage of selectively targeting the tumor vasculature without affecting other tissues. Finally, Tie1 blockade might provide a new strategy to overcome the refractoriness of tumors to the standard AA-therapy. Thus, the development of a Tie1 blocking antibody would be a good tool for translating pre-clinical evidence of Tie1 targeting into the clinics.

5. Methods

5.1 Mouse experimentation

5.1.1 Animal welfare

Mice of the C57Bl/6 background with deletion of Tie1 gene was kindly provided by Dr. Scott Baldwin. To generate $Tie1^{loxP/loxP}$ mice, a Tie1 floxed targeting vector was constructed based on the 129-Sv mouse genomic fragment used by Puri et al. (Puri et al., 1995). A 940 bp HpaI-SacI fragment containing a Tie1 minimal promoter (Iljin et al., 2002; Korhonen et al., 1995) and exon 1 (containing the initial ATG codon) was inserted into the floxed KpnI-ClaI sites of the pDELBOY plasmid (pDELBOY-3X), which contains an Frt-site-flanked neomycin gene. The targeting vector was electroporated into 129 R1 embryonic stem (ES) cells (Nagy et al., 1993) and targeting was confirmed by Southern blotting with both 5 and 3 external probes and PCR with the primers 5-ATGCCTGTTCTATTTATTTTTCCAG-3 and 5-TCGGGCGCGTTCAGAGTGGTAT-3. Correctly targeted cells were then injected into C57/BL6 blastocysts and two separate clones were found to transmit the targeted allele through the germline. Both lines were maintained on a 129-Sv and C57/BL6 background and demonstrated identical phenotypes.

$Tie1^{flox/flox}$ mice were crossed with *Tg(Cdh5-cre/ERT2)1Rha* (Cdh5CreERT2) mice (Wang et al., 2010b) to specifically delete endothelial Tie1 expression. Cre recombination was induced by injecting 2 mg tamoxifen according the protocol indicated in each experiment.

$Tie1^{flox/flox}$ mice and *Tg(Cdh5-cre/ERT2)1Rha* (Cdh5CreERT2) were bred in barriers. For tumor experiments, WT and $Tie1^{iECKO}$ littermate mice were transferred to a mouse room with an isolated ventilated cage system. All animals were monitored on a daily basis for signs of ill health and had *ab libitum* access to food and water.

For sacrificing the mice, rapid cervical dislocation of the cervical spine was performed. A part of the tail was taken for re-genotyping.

All animal experiments were performed according to the guidelines of the local Animal Use and Care Committees and were approved by the local regulatory committee Bezirksregierung Karlsruhe, Germany (G61/13 and G171/15).

5.1.2 Tumor cell inoculation

LLC ($1x10^6$) or B16F10 ($1x10^6$) tumor cells suspended in 100 µl PBS were injected subcutaneously in the flank region of 7-9 weeks old male-female $Tie1^{flox/flox}$ and $Tie1^{flox/flox}$ x VE-CadherinCreERT2 mice, using a 27G needle. Tumors were grown for a period of 2 weeks. Tumor size was monitored by caliper measurement and tumor volume was calculated as height x length x width. To induce Tie1 deletion after tumor cell implantation, both control and mutant animals were intraperitoneally treated with five doses of 2 mg tamoxifen (Sigma) dissolved in ethanol/peanut oil. LLC and B16F10 tumors were harvested 14-18 days after cell injection (volume from 600 mm^3 to 1 cm^3). Mice were sacrificed at the indicated time

points by cervical dislocation. Resected tumors were cleaned of skin and processed for fixation and staining (see 4.3 hystochemical method).

5.1.3 Spontaneous metastasis assay

Lewis Lung Carcinoma (LLC) model: LLC tumor cells ($1x10^6$) resuspendend in 100 µl PBS were injected subcutaneously in the flank region of 7-9 weeks old male-female C57BL/6 $Tie1^{flox/flox}$ (WT) and $Tie1^{flox/flox}$ x VE-CadherinCreERT2 mice, using a 27G needle. Tumors were grown for a period of 14 days and were surgically resected. To induce Tie1 deletion after tumor cell implantation, both control and mutant animals were intraperitoneally treated with five to seven doses of 2 mg tamoxifen (Sigma) dissolved in ethanol/peanut oil, according to the different schedules indicated in the figures. Three weeks post-surgery, the lungs were then harvested and processed for histological analysis. Survival studies were performed until natural death of the mice or until the mice needed to be sacrificed for ethical reasons. One hundred days post-surgery, the surviving mice were sacrificed.

Surgical resection of tumors: Surgeries were performed under systemic anesthesia induced by intraperitoneal injections of a mixture of Ketamine (100 mg/kg) and Xylazine (10 mg/kg) diluted in isotonic 0.9% NaCl. Completely anesthetized mice were placed on a heat mat and eyes were creamed with bepanthen cream to avoid drying. Electrocauterization was performed to achieve hemostasis during the surgery. Resected tumors were cleaned of skin and processed for fixation and staining (see 4.3). Where indicated primary tumors were weighed. Surgical wounds of C57BL/6 mice were sutured using number 5 suture for the peritoneum and number 4 sutures for the skin. Mice with tumors not effectively removed or with subsequent tumor recurrence were removed from analyses. Mice were sacrificed at the indicated time points or when they showed signs of ill health. Organs were collected and processed for fixation and staining (see 4.2).

5.1.3.1 Primary tumor and metastases analyses

To quantify tumor vessel density and area, the vessel number and CD31-positive area were measured and normalized to the tumor area. For analysis of mural cell coverage and vessel functionality, the αSMA- and desmin-positive area, and the lectin-positive area, respectively, were measured, and normalized to the corresponding vessel area. Vessel permeability was analysed counting the number of extravasated beads and normalizing them to the vessel number. Analyses were performed on acquired tile scan of entire tumor sections. For immunohistochemistry staining, zinc-fixed lungs and primary tumors were paraffin-embedded, sectioned and subsequently stained with Hematoxylin and Eosin (H&E) in the Institute of Pathology, Heidelberg University. Sections were analyzed by a board-certified pathologist (C.M.) and the percentage of necrotic area/slide in each tumor was assessed. To quantify the metastatic burden into the lungs, H&E images were taken using Zeiss Axio Scan and subsequently analysed with Fiji software. The metastatic area was measured and normalized to the total lung area. One lung section per mouse was analyzed. In order to quantify hypoxic area, the HIF1α-positive area was measured and normalized to the tumor area with Fiji software.

5.1.4 Experimental metastasis assay

WT and Tie1$^{flox/flox}$ x VE-CadherinCreERT2 mice were pre-injected with four tamoxifen doses. After 7 days from last injection, B16F10 cells (1x10^5), suspended in 100 µl PBS, were injected intravenously into the tail vein of 7-9 weeks old mice. Lungs were collected after 2 weeks from the inoculation and the metastatic melanoma foci counted under a stereomicroscope. Lungs were further processed for histological analysis.

5.1.5 *In vivo* vascular leakage

WT and Tie1$^{flox/flox}$ x VE-CadherinCreERT2 tumor bearing mice were injected intravenously with 100 µl of fluorescent beads (100 nm, LumaFluor Corp.) 4h before sacrifice. Mice were sacrificed and tumors were harvested, embedded in OCT and sectioned. 7 and 50 µm slides were thawed, rehydrated, fixed in acethon/methanol and stained for anti-mouse CD31. Slides were mounted with ProLong GoldTM with Antifade (Invitrogen) and fluorescent images were collected on Zeiss LSM 700 confocal microscope and Zeiss Cell Observer. Images were analysed using ImageJ.

5.1.6 *Ex vivo* culture of circulating tumor cells

Arterial blood was isolated by cardiac puncture and kept in lithium heparin Microtainer tubes (BD Biosciences) on ice. 100 µL blood was plated in 10 mL DMEM plus 10% FCS, 1% Penicillin/Streptomycin in a 10 cm tissue culture plate and 14 days later tumor cell colonies were counted under a fluorescent microscope (Olympus IX71). The total number of tumor cell colonies per plate was quantified manually in ≥2 plates for each animal on an Olympus IX71 microscope.

5.2 Histochemical methods

5.2.1 Preparation of zinc-fixed paraffin-embedded tissues and sections

Following mouse dissection, lungs and tumor tissues were fixed in zinc-fixative overnight. The next day, samples were washed once with VE-water. Samples were further processed automatically with the spin tissue processor STP120 including incubations in graded ethanol series (70-85-96%), twice in isopropanol, twice in xylol, and stored in paraffin. Samples were manually embedded in paraffin blocks. 7-8 µm thick tissue sections were prepared using the rotary microtome HM355S. The sectioning was performed from at least three different levels of the tissue sample.

5.2.1.1 Paraffin section preparation

Paraffin sections (7-8 µm) were dewaxed, dehydrated and rehydrated by consecutive incubations in a graded series of ethanol dilutions.

Table 1: Paraffin section preparation

Procedure	Reagent	Time
1	xylol	2 min
2	xylol	2 min
3	99 % ethanol	1 min
4	80 % ethanol	10 sec
5	70 % ethanol	10 sec
6	VE-water	1 min

Sections were rehydrated with VE-water.

5.2.1.1.1 H&E-Staining

Tumor sections and lungs sections were hematoxylin and eosin (H&E) stained to visualize respectively necrosis and metastasis. Therefore, sections were stained with filtered hemalaun for 4 min, washed with running tap water for up to 10 min, and then stained with 1% ethanoic eosin for 2 min. Subsequently, sections were washed three times with VE-water followed by dipping into graded ethanol series (70-80-99%) and isopropanol. Before mounting with histomount, sections were treated with histoclear for 90 sec. In addition, H&E stainings were performed at the Institute of Pathology (Heidelberg) according to standard protocols using Mayer´s hemalaun (Carl Roth, Karlsruhe, Germany). Bright field images were obtained with Zeiss Cell Observer (20x objectives) or the Zeiss Axio Scan (10x objective) and evaluated manually by an experienced pathologist (Dr. Carolin Mogler, Institute of Pathology, Heidelberg) scoring the positively stained area.

5.2.1.1.2 Immunohistochemistry (IHC)

After dewaxing, dehydratation and rehydration (see above), antigen-retrieval was achieved by boiling the sections for 20 minutes in 0.01 M pH 6.0 citrate target retrieval buffer followed by cooling in VE-water for 20 min. To block background activity of endogenous peroxidases, slides were treated with 3% H_2O_2 for 15 min and washed 2x 5 min with PBS-T or TBS-T. Unspecific binding sites were blocked with 10% rabbit serum 30 min at room temperature and further overnight slides were incubated at 4°C with primary antibody in the appropriate buffer (see table 18 for detailed primary antibody information). The next day, slides were washed 3x 5 min with TBS-T and incubated with the respective secondary antibody in the primary antibody buffer for 30 min at room temperature (see table 19 for detailed secondary antibody information). Slides were washed 3x 5 min with TBS-T. Detection was performed via a biotin-peroxidase complex, in form of Vectastain ABC solution according to manufacturers' protocol. To summarize, 1 drop of solution A was mixed into 2.5 ml PBS, followed by adding 1 drop of solution B, further mixing and incubation in the dark for 20 min at room temperature, added to sections for 30-40 min at RT. Sections were rinsed with TBS-T for 3x 5 min, followed by diaminobenzidine substrate (DAB) treatment for 2-5 min. Sections, briefly washed with tap water, were counterstained with Hematoxylin for 2 min, rinsed with running tap water for 10 min, dehydrated through graded ethanol series (2x 70- 2x

80- 2x 96%), treated with Histoclear for 5 min and mounted with Histomount. Bright field images were taken at the Zeiss Axio Scan (10x objectives) and the pictures analyzed with Fiji software.

5.2.2 Preparation of cryoblocks and sections

Immediately after mouse dissection tumor tissues were placed in cryomold immersed in OTC Cryo-medium on dry ice and stored at -80 °C. 7-50 µm sections were cut using the cryo microtome Hyrax C50 followed by drying for few minutes. Sections and frozen tumors were stored at -80 °C.

5.2.2.1 Immunofluorescence (IF) stainings

Cryosections were dried for 20 min at room temperature and subsequently fixed for 10 min using ice-cold methanol. Fixed samples were washed three times with PBS for 5 min. Samples were treated with blocking solution for 1 h at room temperature. Primary antibodies were applied to sections in appropriate dilutions in blocking serum at 4 °C overnight in the dark (see table 18 for detailed primary antibody information). Samples were washed three times with TBS-T. Afterwards, secondary antibodies were added in appropriate dilutions in TBS-T for 45 min at room temperature in the dark (see table 19 for detailed secondary antibody information). Samples were further washed three times in TBS-T to remove excess secondary antibodies. Nuclei were stained with the DNA intercalating agent Hoechst (DAPI) in a 1:3000 dilution with the secondary antibody solution. Stained sections were mounted with fluoromount. Samples were stored protected from light at 4 °C until analysis.

5.3 Image acquisition

Fluorescent images were obtained with the Zeiss Cell Observer (10x or 40x objective) or the Zeiss Axio Scan microscope (10x objective). Bright field images were obtained at the Zeiss Axio Scan (10x objective) at the imaging core facility of the DKFZ. Fiji/Image J and Imaris softwares were used for image analysis.

5.4 Cell culture methods

5.4.1 Cell maintenance

All cell types were cultured under sterile conditions in a humidified (85 %) incubator with 5 % CO2 at 37°C. Human umbilical vein endothelial cells (HUVEC, Nov. 2015, tested by Promocell via flow cytometry and PCR) were cultured in Endopan3 medium with 3% fetal calf serum (FCS) and supplements according to the manufacturer's protocol (PAN Biotech GmbH). HUVEC were used between passages one and six. Adherent cells were passaged when they reached 80-90% confluency. For passaging, the medium was discarded and cells washed once with PBS. Trypsin/EDTA solution (0.25% trypsin) was added and the cells incubated at 37 °C until they detached from the plate. Cells were spin down at 1,000 rpm for 5 min, medium was discarded and cells were resuspended in fresh media. Cells were re-seeded in a 1:3 ratio.

Lewis lung carcinoma cells (LLC; obtained from ATCC, 2006) and B16F10 (obtained from ATCC, 2015) were cultured in DMEM supplemented with 10% FCS, 1% PS. For passaging, the medium was discarded and cells washed once with PBS. Trypsin/EDTA solution (0.25% trypsin) was added and the cells

incubated at 37 °C until they detached from the plate. Trypsin-dependent digestion was stopped with 10% serum containing medium and cells were re-seeded in a 1:10 ratio.

Cell lines were routinely tested for mycoplasma by PCR and tested negative.

5.4.2 Cryopreservation and thawing of cells

Cells were resuspended in cell-type specific medium, containing 10% DMSO and 50% FCS. Cell suspension was transferred into cryostatic vials (1 ml/vial). The vials were slowly frozen in an isopropanol containing container at -80°C overnight and then stored in a liquid nitrogen tank. For thawing, cells were placed in a water bath for 2 min at 37°C. Slightly thawed cells were immediately mixed with pre-heated medium and centrifuged at 1,000 rpm for 3 min. Pellet was resuspended in fresh media and transferred into tissue culture plates. The medium was replaced with fresh medium the next day.

5.4.3 PKH staining of tumor cells

The PKH26 Fluorescent Cell linker kit from Sigma was used for PKH labeling of tumor cells. According to manufacturer´s protocol LLC cells (1 x 10^7) were washed with PBS and resuspended in 1 mL of Diluent C. 4 µl of PKH26 ethanolic dye solution was added to 1 mL of Diluent C. The cell mixture was added and incubated for 5 min while periodic mixing. Staining was stopped by addition of equal volumes of DMEM containing 10% FCS and 1% PS. Following three rounds of washing with DMEM plus 10% FCS and 1% PS and centrifugation at 1000 rpm for 5-10 min in between, cells were resuspended in DMEM plus 2% FCS and 1% PS and counted before further use in the transmigration assay.

5.4.4 Transfection with small interfering RNA (siRNA)

HUVEC were seeded in 6-well plates at a density of 120,000 cells per well. After 24 hours pre-designed annealed siRNA (100 nM) was mixed with 100 µL Opti-MEM (solution A) and 5 µL Oligofectamine transfection agent was mixed with 100 µL Opti- MEM (solution B). Both solutions were incubated for 10 min at RT. Solution A and B were then mixed and incubated for another 30 min and RT. HUVEC were washed twice with Opti-MEM and covered with 800 µL Opti- MEM after the last wash. Thereafter, the 200 µL of siRNA-Oligofectamine transfection mix was added dropwise on the cells and incubated for 4 hours. The transfection mix was then aspirated off and endothelial cell growth medium was added. Knockdown efficiency was assessed by quantitative PCR (see 4.6.4 for qPCR method).

5.5 Cellular assays

5.5.1 Transmigration assay

HUVEC were transfected with siRNAs targeting Tie1 and non-targeting siRNA as control (procedure described above in 4.4.4). HUVEC (1 x 10^5) were plated in Endopan3 medium onto the top chamber of 6.5-mm/8.0-µm 0.2% gelatin-coated Transwells (Corning) overnight. The upper and lower wells contained Endopan3 with 3% FCS and supplements according to the manufacturer's protocol. Twenty-four hours later, LLC PKH67 red fluorescent dye (Sigma) labelled (1 x 10^5) were added to the top chamber in DMEM 10% FCS, with DMEM 10% FCS in the bottom chamber. Eight hours later, Transwells were

washed three times in PBS, fixed with Roti-Histofix 4% for 10 min. Transwells were left at 4 °C in PBS until they were imaged on the fluorescent microscope Olympus IX 71 microscope (x10 magnification, 5 fields) per filter. Analysis was performed using Fiji/ImageJ software.

5.6 Molecular biology methods

5.6.1 DNA isolation and analysis from tail

Genotyping of C57BL/6 Tie1^{iECKO} mice was performed on genomic DNA isolated from mouse tails. Tails (~0.5 cm) were lysed in 200 µl Direct PCR Lysis Reagent with 10 µg Proteinase K at 55 °C overnight. To inactivate Proteinase K, tails were heated to 95 °C for 15 min the next day. The lysate was centrifuged for 10 sec, and supernatant was used for subsequent PCRs. Isolated DNA was stored at -20 °C.

5.6.1.1 PCR – Polymerase chain reaction

PCR was performed to analyze the genotype of Tie1 wildtype and knockout mice. Tie1 genotyping generated a wild-type band of 150bp and a mutant band of 200bp. The following pipetting scheme was used for the PCR mix:

Table 2: PCR reaction mix

Reagent	Amount
Q-solution	4.0 µl
10x buffer	2.0 µl
MgCl2	1.0 µl
dNTPs (5 mM)	0.8 µl
Primer Tie1flox-F (10 µM)	0.3 µl
Primer Tie1flox-R (10 µM)	0.3 µl
Primer CdhCreErt2-F (10 µM)	1.2 µl
Primer CdhCreErt2-R (10 µM)	1.2 µl
Taq polymerase	0.5 µl
Template (1 µg/µl)	2.0 µl
H_2O (nuclease-free)	6.7 µl
Total	**20 µl**

The PCR was performed with an Applied Biosystems thermocycler according to the PCR program depicted here:

Table 3: PCR program

Temperature	Time	
95°C	2 min	
95°C	30 sec	
60°C	1 min	40x
72°C	1 min	
72°C	10 min	
4°C	forever	

The amplified DNA was analyzed directly by 1% agarose gel electrophoresis or stored at 4°C until analysis.

5.6.1.2 Agarose gel electrophoresis

1% (w/v) agarose was dissolved in 0.5x TBE buffer by heating. Ethidium bromide (5 µl/100 ml) was added and solution was poured into a cast tray for solidification. Samples were loaded onto the gel, which run at 140V for 45min. The 100 bp Generuler Plus DNA-Ladder (7 µl/well) was used as a size reference. DNA was visualized under UV-light and the band size was determined relative to the DNA ladder.

5.6.2 RNA preparation from tumors

RNA was purified from mouse tumor and lung pieces. Mouse tissue pieces were lysed with 300 µl of TRIzol and completely homogenized by fragmentizing with a plastic pistil. 400 µl of TRIzol were added to the homogenized samples. In order to separate proteins from RNA and DNA, 300 µl of chloroform was added and vortexed for 20 sec. After 5 min incubation at room temperature samples were centrifuged at 10,000 rpm for 15 min at 4 °C. The aqueous phase was transferred to a new RNase- free eppendorf tube. RNA was precipitated after adding 1 volume isopropanol, tube inverting, and incubation for 10 min at room temperature. Sample was loaded onto an RNeasy spin column. RNA was prepared using the RNeasy Mini Kit from Qiagen according to the manifacturer's protocol. The RNeasy spin column placed in a 2 ml collection tube was loaded with 700 µl of the sample, centrifuged at 10,000 rpm for 15 sec and flow through was discarded. The column was washed with 700 µl of RW1 wash buffer, followed by centrifugation at 10,000 rpm for 15 sec (flow- through was discarded). Further washing was performed by adding 500 µl of RPE and centrifugation at 10,000 rpm for 15 sec (flow-through was discarded). This step was repeated and sample was centrifuged at 10,000 rpm for 2 min (flow-through was discarded). The column was dried by centrifugation at full speed for 1 min. RNA was eluted from the column after adding 30 µl of RNase- free H_2O, incubation for 1 min, and centrifugation at 10,000 rpm for 1 min. Concentration and purity was measured by analyzing 1 µl of sample with the RNA program of the Nanodrop. A 260/280 ratio of ≥2 represented protein-free RNA. Purified RNA was stored at -80°C or used directly for cDNA preparation.

RNA of FACS-sorted mouse EC was isolated with the Arcturus PicoPure RNA Isolation Kit. Cells were centrifuged at 500 g and 4°C for 5 min and the pellet was resuspended in 50 µl Arcturus PicoPure extraction buffer. RNA was isolated according to manufacturer´s instructions. RNA was eluted in 11 µl RNase free H_2O and RNA concentrations were measured using NanoPhotometer® N60.

RNA of cell culture cells was isolated using RNeasy Mini Kit from Qiagen according to the manifacturer's protocol instructions. Cells were lysed with 200 µl lysis buffer and RNA was eluted in 30 µl RNase free H_2O and stored at -80°C.

5.6.3 cDNA generation

cDNA generation was performed with the Quantitect® Reverse Transcription Kit from Qiagen according to manufacturer's instructions. Template RNA was thawed on ice and 1 µg was mixed with 2 µl of gDNA Wipeout buffer. To reach a total volume of 14 µl, RNase-free water was added. This reaction was incubated for 2 min at 42°C and immediately placed on ice afterwards.

Table 4:cDNA reaction mix

Component	Volume	Final concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcriptase	1 µl	
Quantiscript RT Buffer, 5x	4 µl	1x
RT Primer Mix	1 µl	
Template RNA		
Entire genomic DNA elimination reaction	14 µl	
Total volume	**20 µl**	

To start the transcription reaction from RNA into cDNA, the reaction was incubated for 30 min at 42°C. In order to achieve inactivation of the Quantiscript Reverse Transcriptase the reaction tube was then incubated for 3 min at 95°C. cDNA was stored at -20 °C.

cDNA of FACS-sorted EC was amplified with QuantiTect Whole Transcriptome Kit (Qiagen) according to to manufacturer's instructions and cDNA was diluted in RNAse-free water 1:250. cDNA was stored at -20 °C.

5.6.4 Quantitative realtime-PCR (qRT-PCR)

Relative gene expression analysis was performed using qRT-PCR. The Taqman mono-color hydrolysis probe method (Applied Biosystems) was used to detect differences in the amount of mRNA transcription levels. This method is based on probes that are labeled with a fluorophore (6- carboxyfluorescein, FAM) at the 5´end and a fluorescence quencher at the 3´end. The exonuclease activity of the Taq polymerase cleaves the probe and thereby allows detection of the FAM fluorescence. Reactions were performed in a 96 or 384-well plate. One reaction contained the following components:

Table 5: Taqman qRT-PCR reaction mix

Reagent	Volume
Taqman FAST Universal Master Mix (ABI)	5 µl
Taqman Probe/Primer	0.5 µl
H_2O (nuclease-free)	1.5 µl
Total	**7 µl**
+ cDNA (1:10 diluted)	3 µl
Total	**10 µl**

Each reaction was performed in technical triplicates. The qRT-PCR was performed using the Lightcycler® 480 System (for the 384-well plates) or the StepOnePlus Real-Time PCR System (for the 96-well plates) with the following temperature profile:

Table 6: Taqman qRT-PCR program

TaqMan qRT-PCR program			
Step	**Temperature**	**Time**	
Pre-denaturation	95°C	30 sec	
Denaturation	95°C	2 sec	} 45x
Amplification	60°C	20 sec	

For analysis, the ΔΔCt method was applied as described previously (Livak and Schmittgen, 2001). This was done by comparing the Ct values of the samples of interest with a control. The Ct values of both the control sample and the samples of interest were normalized to a housekeeping gene ($\Delta Ct_{\text{gene of interest}} = Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$). Here b-actin was used as housekeeping genes. Secondly, internally normalized Ct values were further normalized to the mean value of all controls (WT animal, or siNS samples) resulting in ΔΔCt values. Respective fold changes (FC) were calculated as the following: $FC = 2^{-\Delta\Delta Ctgene\ of\ interest}$.

5.7 Protein chemical methods

5.7.1 Preparation of protein lysates

Protein lysates from LLC tumors were prepared from frozen tumors. Tumor material was lysed by grinding with plastic pistils in 300-500 µl modified RIPA lysis buffer with phosphatase inhibitor orthovanadat at a concentration of 2mM (1:100). Samples were incubated on ice for 20 min. To sediment cellular debris, cell suspension was centrifuged for 5 min at 14,000 rpm at 4°C. The cleared lysate was stored at -80°C.

5.7.2 Protein concentration measurements

Exact protein concentrations were determined by using the BCA-assay (Pierce).

5.7.2.1 BCA-assay

The BCA-assay from Pierce® with a working range from 20-200 µg/ml was performed according to manufacturer's instructions for microplate formats. For the preparation of the working reagent (WR), 50 parts of reagent A were mixed with 1 part of BCA reagent B, resulting in a mixture of 50:1. The reaction in the microplate was prepared as follows: 25 µl of standard or sample were mixed with 200 µl WR and shaken for 30 sec. The covered microplate was incubated for 30 min at 37°C. After that time, the microplate was measured at λ = 562 nm using a Photometer. Standard values with known concentrations were plotted in a graph and a standard curve was generated by linear extrapolation of the plotted standard values. The unknown concentration of the samples was then calculated as the following:

$x = (y-b)/a$.

5.7.3 SDS-PAGE and western blot analysis

Proteins were separated using 8% or 10% SDS-PAGE gels depending on the protein size, and subsequently transferred to a nitrocellulose membrane. After blocking the membrane with 3% BSA in TBS-T, the respective primary antibody diluted in blocking solution was added and blots were incubated overnight at 4°C (see Table 18 for primary antibody information). Unbound antibody was removed by three washing steps with TBS-T, and the membrane was incubated with the corresponding horseradish peroxidase-conjugated (HRP) secondary antibody (1:5000 in TBS-T) for 45 min at RT. Following incubation with secondary antibody (see Table 19 for secondary antibody information), the membranes were washed extensively with TBS-T and incubated with enhanced chemiluminescence substrate (ECL). Signals were detected by exposing the membrane to a Fuji X-ray film. Actin was used as loading control.

5.7.4 Fluorescence activated cell sorting (FACS)

5.7.4.1 Isolation of tumor EC

FACS sorting was performed with freshly isolated LLC tumors using a BD Biosciences FACS Aria Cell Sorter. Mice bearing tumors were sacrificed and tumors were minced into small pieces. Single-cell suspensions were prepared by digesting the tissue in Dulbecco's Modified Eagle's medium (DMEM) containing 200 U/ml Collagenase I (Sigma, C9891) and 10 µg/ml DnaseI (Sigma, D4527) at 37°C for 30 min and subsequently sieved through a 100 µm cell strainer using the plunger of a 19 G cannula syringe. For isolating EC by FACS sorting, cell suspensions were stained with antibodies against CD45, Ter119, Lyve1 and podoplanin (Pdpn) (Table 18) to deplete blood cells and lymphatic EC for 30 min at 4°C in PBS/5% fetal calf serum (FCS). Cells were depleted by incubating with 500 µl magnetic Dynabeads (Invitrogen, 114.15D) in 750 µl PBS/5% FCS for 30 min at 4°C on the rotator. The remaining cells were positively stained with CD31 and CD34 antibodies (Table 18) in PBS/5% FCS for 30 min at 4°C. Dead cells were excluded by phosphatidylinositol (PI) staining (1:3000). $CD45^-Ter119^-Lyve1^-Pdpn^-CD31^+CD34^+$ cells were sorted with a BD Biosciences FACS Aria Cell Sorter.

5.7.4.2 Immune cell analysis

FACS analysis was performed with freshly isolated LLC tumors using the BD Biosciences FACS Aria Cell Sorter. Tumors were processed as described before (4.7.4.1). For analysis of the myeloid compartment, cell suspensions were stained with antibody against Ly6C, Ly6G, CD45 (PacOrange), MMR, CCR2, F4/80, CD11c, CD11b and CD31 (APC) (Table 18) in PBS/5% FCS for 20 min at 4°C, and finally washed twice with PBS/5% FCS. For analysis of the T-cell compartment, cell suspensions were stained first for extracellular markers: anti-CD45 (PacOrange), anti-CD4, anti-CD19, anti-CD8a, anti-CD45R, anti-NK 1.1 and anti-CD3e in PBS/5% FCS for 20 min at 4°C. Subsequently cell suspensions were fixed and permeabilized in Cyto Perm/fix for 15 min at 4°C, and washed twice in 1x wash buffer. Finally, samples were stained for intracellular markers with antibodies against Foxp3 and RORγt (Table 18). Dead cells were excluded by phosphatidylinositol (PI) staining (1:3000).

5.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data are expressed as mean ± SD or SEM (where indicated). Comparison between the two groups were made using two-tailed Student's t test, Mann-Whitney test or Gehan-Breslow-Wilcoxon-Test, as indicated in the figure legends. Differences ≤ 0.05 were considered as statistically significant. *=P<0.05, **=P<0.01 and ***=P<0.001.

6. Materials

6.1 Chemicals

Bulk chemicals were purchased from the following companies:

- AppliChem (www.applichem.com)
- Carl Roth (www.carl-roth.de)
- Gerbu (www.gerbu.de)
- Merck (www.merk.de)
- Roche (www.roche-apllied-science.com)
- Serva (www.serva.de)
- Sigma-Aldrich (www.simaaldrich.com)

6.2 Cells

Table 7: List of cells used in this study

Cell type	Company or organization
Human umbilical vein endothelial cells (HUVEC)	PromoCell
Murine lewis lung carcinoma cell line (LLC)	ATCC
Murine lewis lung carcinoma cell line (LLC)-RFP	Dr. Andreas Fischer (DKFZ)
Murin melanoma cell line (B16F10)	ATCC

6.3 Cell culture and reagents

Table 8: List of cell culture media

Medium	Cell type	Company
Endopan3	HUVEC	Pan Biotech
Dulbecco´s modified eagle medium (DMEM) Glutamax I	LLC, LLC-RFP, B16F10	Gibco
Optimem+ Glutamax	HUVEC	Life technology

Table 9: List of reagents used in cell culture

Reagent	Company
Accutase, 10x	PAA Laboratories
Dimethylsufoxide (DMSO)	Sigma-Alderich
Dulbecco's phosphate buffered saline (PBS)	PAA Laboratories
Fetal calf serum (FCS), heat inactivated	PAA Laboratories
Penicillin/streptomycin (PS), 100x	PAA Laboratories

Reagent	Company
Puromycin	AppliChem
Trypan blue	Gibco
Trypsin-EDTA solution, 10x	PAA Laboratories

6.4 PCR and qRT-PCR reagents

Table 10: PCR and qRT-PCR reagents

Component	Company
100 bp DNA Ladder plus	Fermentas
10 x Coral Load PCR buffer	Qiagen
Direct PCR Lysis Reagent	PeqLab
DNase/RNase free H2O	Gibco
dNTP mix (10mM each)	Fermentas
Ethidium bromide	Roth
MgCl2 (25mM)	Qiagen
6x Orange DNA Loading Dye Solution	Fermentas
Taq DNA polymerase (5U/µl)	Qiagen
TaqMan Fast Advanced PCR Master Mix	Applied Biosystems
Trizol	Sigma-Aldrich

6.5 Primers

All TaqMan probes were purchased from Applied Biosystems.

Table 11: Taqman probes for qRT-PCR

Target gene (ms)	Assay ID
Actb (b-actin)	Mm00607939_s1
Cadherin-5	Mm03053719-s1
Pecam	Mm01242584_m1
Tie1	Mm00441786_m1
Tek	Mm00443254_m1
Vegfr2	Mm01222421_m1

Primers for genotyping of $Tie1^{iECKO}$ mice were purchased from MWG Biotech and used as described in 4.6.1.1 (www.mwg-biotech.com).

Table 12: Primers for genotyping of Tie1^{iECKO} mice

Target	Sequence
Tie1 flox/flox-F	ATGCCTGTTCTATTTATTTTTCCAG
Tie1 flox/flox-R	TCGGGCGCGTTCAGAGTGGTAT
Cdh5CreErt2-F	GCCTGCATTACCGGTCGATGCAACGA
Cdh5CreErt2-R	GTGGCAGATGGCGCGGCAACACCATT

6.6 Enzymes

Table 13: Enzymes

Enzyme	Company
Collagenase I	Sigma Aldrich
DNase I	Roche
Proteinase K	Gerbu
RNase free DNAse	Qiagen

6.7 Kits

Table 14: Kits

Reagent	Company
Arcturus PicoPure RNA Isolation Kit	Life Technologies
Pierce Bicinchoninic acid (BCA) Protein Assay Kit	Thermo Fischer
Quantitect Reverse Transcription Kit for cDNA Synthesis	Qiagen
RNeasy Mini Kit	Qiagen

6.8 Miscellaneous

Table 15: Miscellaneous

Reagent	Company
Bepanthen eye cream	Roche
Betadine	MundiPharma
Ketavet	Pfizer
Paraffin (low melting 56°C)	Merck
Rompun	Bayer
Tissue freezing medium (Tissue TEK)	Sakura

6.9 Consumables

Table 16: Consumables

Consumables	Company
6-well plates	Beckton Dickinson
Cannula (18G, 19G, 27G)	BD
Cell culture dishes (10cm, 15cm)	TPP
Cryotubes	Carl-Roth
Embedding cassettes	Medim Histotechnologie
FACS tubes	BD Falcon
Filter containing pipette tips	Biozym
Freezing box	Thermo Scientific
Insulin syringe	BD
Microscope cover glasses	VWR international
Microscope glass slides	Menzel-Gläser
Pipette tips	Nerbe
PDVF filter (0.22 µm, 0.45 µm)	Millipore
Polyamid suture (4.0)	ETHICON/ETHILON
Polyamid suture (5.0)	ETHICON/ETHILON
qPCR plates (96-well)	Biozyme
qPCR plates (384-well)	Roche
Reaction tubes (0.5ml, 1.5ml, 2ml)	Eppendorf
Reaction tubes (15ml, 50 ml)	Greiner
Round bottom 96 well plate	Greiner
Sealing foil	Applied Biosystems
Sterile pipettes	Corning
Sterile filters	Renner
Suture clip	Braun
Syringes	Dispomed
Tissue cultures 6-well plates	Greiner
Tissue freezing medium (Tissue TEK)	Sakura
Transwell permeable supports 6.5mm, 8.0µm	Costar

6.10 Equipment

Table 17: Equipment

Product	Company
Agarose gel documentation system	Peqlab
Axio Scan	Zeiss
Canto FACS Analyzer	BD
Cell culture hood	Thermo Scientific
Cell culture incubator	Thermo Scientific
Cell Observer	Zeiss
Centrifuge	Beckman Coulter
Countess Automated Cell Counter	Invitrogen
Cryotome	Zeiss
Elisa reader (Multiskan)	Thermo Scientific
Freezing box	Thermo Scientific
Heating block	Eppendorf
Heating mat	ThemoLux
Inverted fluorescence microscope IX71	Olympus
Light cycler 480	Roche
Microm HM3555	Thermo Scientific
Multistep pipette	Eppendorf
Nanodrop 1000 spectrophotometer	Thermo Scientific
Neubauer Cell Counting Chamber	Marienfeld
Pipettes	ErgoOne
Power supply	BioRad
Cauter set	F-con
Table centrifuge (5417R)	Eppendorf
Thermocycler	Applied Biosystems
UV transluminator	Intas
Vortex	Neolab
Water bath	Julabo
Thermocycler	Applied Biosystems

6.11 Antibodies

6.11.1 Primary antibodies

Table 18: List of primary antibodies, their dilution and their applications

Antigen	Host	Clone	Conjugate	Source (Cat. no.)	Dilution	Application
ms αSMA	mouse	-	Cy3	Sigma (C6198)	1:100	IF
ms CCR2	rat	-	FITC	R&D 475301	10µL/1^6 Cells	FACS
ms CD3e	rat	-	APC-e780	eBioscience 17A2	1:150	FACS
ms CD4	rat	-	FITC	Biolegend 129.19	1:400	FACS
ms CD8a	rat	-	PE-Cy5	eBioscience 53-6.7	1:400	FACS
ms CD11b	rat	-	PE-Cy7	eBioscience M1/70	1: 200	FACS
ms CD11c	hamster	-	PerCP	Biolegend N418	1:400	FACS
ms CD19	rat	-	PerCP-Cy5.5	Biolegend 6D5	1:150	FACS
ms CD31	rat	MEC13.3	-	BD Bioscience (553370/ 557355)	1:100	IF
ms CD31	rat	-	APC	BD Pharmigen	1: 100	FACS
ms CD34	rat	-	PCB	eBioscience	1: 100	FACS
ms CD45	rat	-	APC-Cy7	BD Pharmingen (567659)	1:400	FACS
Ms CD45	rat	-	PE	BD Pharmigen	1:200	FACS
ms CD45	rat	-	PacOrange	Invitrogen 30-F11	1:200	FACS
ms Cdh5	rat	-	-	BD Biosciences (550548)	1:300	IF
ms Desmin	rabbit	-		Abcam (Ab15200-1)	1:200	IF
ms Foxp3	rat	-	V450	eBioscience FJK-16s	1:100	FACS
ms F4/80	rat		PE	Invitrogen BM8	1:100	FACS
ms HIF1α	mouse	-	-	Abcam ab8366	1:100	IHC
ms Lyve1	rat	-	APC-Cy7	eBioscence	1: 200	FACS
Ms Lyve 1	rat	-	PE	eBioscence	1: 200	FACS
ms Ly6C	rat	-	Apc-Cy7	Biolegend HK1.4	1:400	FACS
ms Ly6G	rat	-	PacBlue	Biolegend	1:400	FACS

Antigen	Host	Clone	Conjugate	Source (Cat. no.)	Dilution	Application
ms MMR	rat	-	BV650	Biolegend C068C2	1:200	FACS
ms NK 1.1	rat	-	APC	Biolegend PK-136	1:200	FACS
Podoplanin	hamster	-	488	eBioscence	1: 100	FACS
ms RORγt	rat	-	PE	eBioscience B2D	1:200	FACS
ms Ter119	rat	-	APC-Cy7	BD Pharmingen	1:100	FACS
ms Ter119	rat	-	PE	BD Pharmingen	1:100	FACS
ms Tie2	rabbit	-	-	Santa Cruz (sc-324)	1:500	WB
ms Tie2	rat	-	-	eBioscience 14-5987-81	1:100	IF

IHC=Immunohistochemistry; WB= Western Blot; IF=Immunofluorescence; FACS=Fluorescence activated cell sorting; ms=mouse.

6.11.2 Secondary antibodies

Table 19: List of secondary antibodies, their dilutions and applications

Reactivity	Host	Conjugate	Source (Cat. no.)	Dilution	Application
Rat IgG	goat	AF488	Life Technologies (A11006)	1:500	IF
Rat IgG	goat	AF546	Life Technologies (A11081)	1:500	IF
Rabbit IgG	goat	AF546	Life Technologies (A11071)	1:500	IF
Mouse IgG	goat	HRP	Jackson/Dianova 115-035-166	1:250	IHC
anti-rabbit	goat	HRP	Dako Cytomation	1:1000	WB

IHC=Immunohistochemistry; WB= Western Blot; IF=Immunofluorescence;

6.12 Additional staining reagents

Table 20: Staining Reagents

Staining	Company
Avidin/Biotin blocking solution	DAKO
BSA	Gerbu
Eosin Y solution	Sigma-Aldrich
Fluorescent mounting medium	DAKO
Histomount	Invitrogen
Hoechst Dye 33258, 1mg/ml	Sigma-Aldrich
Liquid DAB Substrate Chromogen System	DAKO
Mayer's Hematoxylin solution	Sigma-Aldrich
Normal goat serum	DAKO
Normal goat serum ready-to-use (10%)	Zymed
Roti-Histofix 4% (pH 7)	Carl Roth

6.13 Solutions and buffers

Solutions and buffers for agarose-gels, FACS and Western blotting were prepared according to standard methods.

Table 21: Solutions and buffers

Solutions	Composition	
Blotting buffer (1x)	192mM	Glycine
	25mM	Trizma Base
Digestion mix	150mg	Collagenase 1A
	0.20%	DNaseI
	50mL	serum free medium
FACS	5%	FCS in PBS
Modified RIPA lysis buffer	50mM T	Tris-HCl pH 7.5
	150mM	NaCl
	1mM	EDTA
	1%	NP-40
	0.25%	Na-deoxycholate
	100mM	Na-orthovanadate
	1x	Protease inhibitor Mix G
PBS-T	1% [v/v]	Tween-20 in 1x PBS
Running buffer (1x)	192mM	Glycine
	25mM	Trizma Base
	0.10%	SDS
Tris-Borate-EDTA buffer (TBE)	89mM	Tris/HCl, pH 8.0
	89mM	H_3BO_3
	1mM	EDTA
Tris-Buffered Saline Tween-20 (TBS-T)	10mM	Tris/HCl, pH 7.5
	100mM	NaCl
	0.10%	Tween-20
Zinc fixative	3mM	$Ca(C_2H_3O_2)_2$
	2.2mM	$Zn(C_2H_3O_2)_2$
	3.6mM	$ZnCl_2$
	0.1M	Tris-HCl (pH 7.4)

6.14 Software

Table 22: Software

Software	Company
Fiji	ImageJ
FlowJo	Miltenyi Biotec
Light Cycler 480 software	Roche
Living Image software 4.0	PerkinElmer
ZEN blue	Zeiss

7. Abbreviation

3D	3-dimensions
GRB2	Growth factor receptor-bound protein2
ACTA2	Alpha-smooth muscle actin
ADAM	a disintegrin and metalloproteases
AF	Alexa-Fluor
Ang	Angiopoietin
Ang2-KO	Ang2 global knock out
Angptl4	Angiopoietin like 4
SMA	Alpha-smooth muscle actin
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BM	Basement membrane
BRAF	B-Raf-proto-oncogene Serine/Threonine Kinase
Bv8	Bombina Variagata 8
C	Celsius
C57BL/6J	C57 Black 6
CCL	C-C motif chemokine ligand
CD31	Cluster of differentiation 31
Cdh5	Cadherin 5
cDNA	Complementary DNA
COMP-Ang1	cartilage oligomeric matrix protein-Angiopoietin 1
CT	Threshold Cycle
CTC	Circulating Tumor Cells
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Desoxy nucleotide triphosphate
E	Embrionic day
EC	Endothelial Cells
ECM	Extrcellular Matrix
EGF	Epidermal Growth factor
EMT	Epithelial-Mesenchymal-Transition
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular signal regulated kinase
FACS	Fluorescent activated cell sorting
FAK	Focal Adhesion kinase
Fc	Fusion Construct
FCS	Fetal Calf Serum
FDA	Food and Drugs Administration
FGF	Fibroblast growth factor
FITC	Fluorescein thioisocyanate

7. Abbreviation

FOXO-1	Forkhead Box protein O1
FSP	Fibroblast-Specific Protein
g	Gram
h	Hour(s)
H&E	Hematoxylin/Eosin
H_2O	Water
HB-EGF	Heparin binding-epidermal growth factor
HGF	Hepatocyte growth factor
HIF1	Hypoxia Inducible Factor 1a
HRP	Horseradish peroxidase
HUVEC	Human Umbelical Vein Endothelial Cells
i.p.	Intraperitoneally
i.v.	Intraveneously
ICAM	Intercellular adhesion molecule
IGF	Insulin growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
Il	Interleukin
ILK	Integrin Linked Kinase
kDa	Kilo Dalton
KLF2	Kruppel-like-Facotor
KO	Knock Out
l	Liter
LEC	Lymphatic Endothelial Cells
LLC	Lewis Lung Carcinoma Cells
LOX	Lysyl Oxidase
LPS	Lipopolysaccharide
Ly6d	Lymphocyte antigen 6D G6D
Ly6g	Lymphocyte antigen 6D
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-Epithelial Transition
min	Minute(s)
mm	Millimeter
MMP	Matrix metalloproteinase
ms	Mouse
n	Nano
NFKB	Nuclear Factor kappa-light chain enhancer of activated B cells
NK	Natural Killer
nm	Nanometer

PAR1	Protease activated receptor 1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PFA	Paraformaldehyde
PFS	Progression Free Survival
pH	Power of hydrogen
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PMA	para-Methoxyanfethamine
PTEN	Phosphatase and tensin homologue
qRT-PCR	Quantitative real time polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
Rho A	Ras homolog gene family, member A
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen Species
rpm	Rounds per minute
RT	Room temperature
RTK	Receptor Tyrosine Kinase
RTKI	Inhibitor
s.c.	Subcutaneous
SD	Standard Deviation
SDS	Sodium Dodecylsulfate
SEM	Standard error of the mean
siNS	Short interfering RNS Control
siRNA	Short interfering RNA
siTie1	Short interfering target
SMAD4	Small Mother against decapentaplegic
SMC	Smooth muscle cells
Src	Proto-oncogene tyrosine-protein kinase Src
TAM	Tumor Associated Macrophages
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween
TGF	Transforming growth factor
Tie	Tyrosin kinase with immunoglobulin-like and EGF-like domain
$Tie1^{iECKO}$	Tie1 inducible endothelial cells knock out
Tie1-KO	Tie1 global Knock out
Tie2-KO	Tie2 global knock out
TSP1	Thrombospondin 1

7. Abbreviation

U	Unit
v/v	Volume/Volume
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth fctor receptor
w/v	Mass Volume (mass concentration)
WB	Western Blot
WHO	World health organization
WT	Wildtype

8. Publications

Savant S, **La Porta S**, Budnik A, Busch K, Hu J, Tisch N, Korn C, Valls AF, Benest AV, Terhardt D, Qu x, Adams RH, Baldwin HS, Ruiz de Almódovar C, Rodewald HR, Augustin HG. *The orphan receptor Tie1 controls angiogenesis and vascular remodeling by differentially regulating Tie2 in Tip and stalk cells*. Cell Rep. 2015; 12, 1761-1773; 2015.

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