UNIVERSITY OF TURKU

CHARACTERIZATION OF NOVEL FACTORS REGULATING ENDOTHELIAL BARRIER DURING METASTASIS

Institute of Biomedicine
MDP in Biomedical Sciences
Drug Discovery and Development
Master's thesis

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25.3.2024
Turku

Master's thesis

Subject: Institute of Biomedicine, MDP in Biomedical Sciences, Drug Discovery and Development
Author: Pihla Kaipainen
Title: Characterization of novel factors regulating endothelial barrier during metastasis
Supervisor(s): Professor Pipsa Saharinen, Docent, Ph.D Niina Santio
Number of pages: 49
Date: 25.3.2024

ABSTRACT

Progressive, metastatic cancer is a leading cause of cancer-related deaths worldwide. One of the critical steps of the metastatic cascade is the colonization of circulating tumor cells in the distant tissues, where cancer cells cross the blood vessel wall to initiate metastatic growth in the distant organs. Vascular endothelium acts as a gatekeeper between circulating tumor cells and metastatic organs. However, the role of endothelial cells in the regulation of cancer cell fate in the metastatic niche is poorly understood.

Here, we show that endothelial Pim-3 kinase regulates the endothelial barrier integrity during early steps of cancer cell colonization of the lungs. Single cell mRNA sequencing of the metastatic B16-F10 murine melanoma model revealed that Pim3 was upregulated in the lung endothelial cells six hours after arrival of the melanoma cells in the lungs. Upregulation of *Pim3* was further validated in spontaneous metastasis models. Further studies showed that administration of a pan-Pim kinase inhibitor AZD-1208 in mice increased melanoma metastasis and leakage in the murine lungs. In cultured human endothelial cells AZD1208 decreased Cadherin 5 based cell-cell junctions and increased endothelial cell permeability in an electrical cell impedance sensing assay. Similar Cadherin 5 reduction was seen in *PIM3* silenced endothelial cells.

Pim3 is a well-known oncogene and Pim inhibitors have been investigated as cancer therapies in recent years. However, the inhibitors did not provide the expected outcomes, and currently clinical trials have been attired. Therefore, whereas targeting Pim kinases is known to decrease tumor cell survival, results from this work suggest that Pim inhibitors may simultaneously weaken the vascular barrier. Thus, the results presented in this thesis reveal a potential mechanism behind unsuccessful Pim inhibitor cancer trials. Notably, the results also call for a better understanding of the effects of cancer therapies on the host cells of the tumor microenvironment.

Key words: Pim3, metastasis, endothelial cells, Cadherin 5

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1 Introduction

Cancer is a leading cause of mortality globally, and advanced metastatic cancer is responsible for most of the cancer-related deaths. Despite recent progress in new therapies, including immuno-oncology-based approaches and targeted therapies, metastatic cancer remains a clinical challenge. However, progress in understanding the basic biology of metastatic cancer is expected to provide the basis for improving therapies to achieve long-term outcomes in patients with advanced cancer. Metastasis is understood to be initiated by cancer cells, sometimes called as metastasis initiating cells (MICs), having stem-like and immune-evasive properties. To understand the metastatic process, it is important to learn about the characteristics, sensitivities, and origins of MICs in the primary tumour that have the capacity to initiate metastasis in distant organs. However, to develop strategies to improve the prevention and treatment of advanced cancer, it is equally important to understand the host microenvironments that support the MICs to escape immune surveillance and regenerate the tumor in the secondary sites (Hanahan & Weinberg, 2011; Massagué & Ganesh, 2021)

In this thesis, I have focused to investigate the interactions of tumor cells with the vascular endothelium, with the aim to understand, how endothelial cells in the vascular metastatic niche regulate tumor cell fate during the process of metastatic dissemination and organ colonization.

1.1 Cancer metastasis

1.1.1 Hallmarks of cancer

Tumorigenesis is a multistep process characterized by genetic alterations that drive the transformation of normal cells into malignant ones. It has been found that cancers are often diagnosed with an age-dependent incidence, related to four to seven rate-limiting, stochastic events. Moreover, lesions representing these intermediate or pre-malignant steps towards malignant transformation have been discovered through pathological analysis of tissues. It is considered that through genomic mutations and epigenetic changes cancer cells acquire capabilities that enable the essential alterations in cell physiology that collectively influence the malignant growth. These include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis or access to vasculature, and tissue invasion and metastasis, which are shared by most types of human tumors (Hanahan & Weinberg, 2011). These and additional features are collectively termed as the hallmarks of cancer. In the subsequent sections, I will focus on one of the hallmarks: metastasis.

1.1.2 Steps of tumor metastasis

Metastasis can be explained as a multistep process. For example, it can be divided into two major phases, including the physical dissemination of cancer cells from the primary tumor to distant tissues, and the subsequent adaptation of these cells to tissue microenvironments. This process enables the colonization, i.e., the growth of micrometastases into macroscopic tumours in distant organs. However, physical dissemination is not sufficient for metastatic growth, as it has been shown that many patients have micrometastases, which never progress to macroscopic metastatic tumors (Aguirre-Ghiso, 2007). In certain cancer types, micrometastases may remain dormant due to suppressing factors released by the primary tumour (Demicheli et al., 2008). Additionally, in cases like melanoma, macroscopic metastasis may appear years later, indicating that dormant micrometastases have overcome colonization barriers (Aguirre-Ghiso, 2007; Ganesh & Massagué, 2021; Jakab et al., 2022).

Initially, most circulating cancer cells are poorly adapted to the microenvironment in which they land, thus they need to develop mechanisms to survive and stay alive in the tissues. These adaptation mechanisms vary depending on colonised tissue and on cancer cell-specific intrinsic factors and may require numerous different colonisation programmes (Hanahan & Weinberg, 2011). As our understanding has improved over time, some of the tissues might have a more welcoming environment for cancer colonization than others. It is still unclear how and when the ability of cancer cells to colonise new tissue environments develops; it may arise prior to dissemination, during primary tumour formation, or as a response to selective pressure that disseminated cancer cells face as they adapt to a new microenvironment (Hanahan & Weinberg, 2011).

Additionally, supportive stroma in a primary site contributes to the ability of cancer cells to acquire metastatic properties by providing a favourable environment for cancer cells to disseminate. There is also increasing evidence that both primary tumor and circulating cancer cells induce and shape the microenvironment of a distant organ, even before disseminating cancer cells arrive at these sites. An important agenda for future research is to understand the processes and regulatory pathways involved in metastatic processes (Peinado et al., 2017)

1.1.3 Distant metastasis

The physical dissemination of cancer cells from the primary tumor to distant tissues can further be divided into several steps. First, the disseminating cancer cells needs to physically reach their target organs, after which the cancer cells are required to cross the vascular barrier to enter the tissue parenchyma and initiate metastatic growth for colonization the host organ microenvironment.

The most common way for cancer cells to spread is haematogenous, i.e. through the blood circulation, but other possible routes include the lymphatic system and extracellular spread (Ganesh & Massagué, 2021). Common metastatic sites like lungs receive venous circulation from most organs, whereas the liver receives the blood supply from the gut. This anatomic structure of the vasculature contributes to the high accumulation of disseminated cancer cells in these organs. In addition to blood flow, organ-specific differences in the anatomy of blood vessels also contribute to distant colonisation (Augustin & Koh, 2017). However, the vascular phenotypes of organotypic vessels do not entirely explain the metastatic patterns seen in many cancers, as some cancers metastasise to multiple sites, while others have a highly organ-specific metastatic pattern. For example, prostate cancer disseminates primarily into the lungs and colorectal cancer to the liver and later to the lungs (Budczies et al., 2015). Accordingly, metastatic sites are not homogeneous, and for example the liver, lung and brain parenchyma provide very different tissue microenvironments. This process of distribution of distant metastases to specific organs is known as metastatic organotropism. It is the result of a combination of a cancer cell's access to a particular organ through the bloodstream and the likelihood of cancer cells growing in the host environment of a distant organ, based on the intrinsic and acquired ability of the cancer cell to cross the barrier and exploit the supportive niche at that site (Ganesh & Massagué, 2021).

There is increasing evidence of that tumour cells interact with the organ-specific metastatic niches. Cell-cell interactions and metabolic changes within the niche help to create a supportive microenvironment (Ganesh & Massagué, 2021). In addition, the primary tumour microenvironment could already select tumor cell clones that have compatible characteristics to survive within the microenvironment of a metastatic organ, thus determining the selection of metastatic tropism of cancer cells. As an example, a breast cancer tumor with a high transforming growth factor-β (TGF-β) activity is associated with an increased lung relapse, which may be due to the ability of TGF-β to induce secretion of cytokines like ANGPTL4 in the seeding cancer cells, which enhances their accumulation to the lungs and subsequent endothelial dysfunction leading to increased cancer cell trans-endothelial passage (Padua et al., 2008).

1.1.4 Premetastatic and metastatic niche

Circulating cancer cells along with stromal components like endothelial cells, immune cells, as well as factors secreted by the primary tumor, collectively contribute to the formation of metastasis by creating favourable environment for cancer cells to seed and grow in a distant site. The principle in the (pre-metastatic) niche relies on that the disseminating cancer cells search the compatible living environment to support their growth and survival and the niche continues to evolve while the colonization progresses. The niche is formed by the arrival of disseminated cancer cells in the host tissue or modulated by primary tumor secreted factors and/or exosomes prior the seeding of disseminated cancer cells. Different tissue microenvironments, the niches, have been reported in distinct organs such as in the lungs, liver, lymph

nodes and bone. Identifying and characterization of these organ specific niches is important to better understand the metastatic process (Y. Liu & Cao, 2016).

This metastatic niche is known to be characterized by alterations in the extracellular matrix, recruitment of immune cells to create metastasis promoting immune environment, vascular permeability and/or angiogenesis and lymphangiogenesis. Important factors in the formation of the niche are the factors derived from the primary tumour and the factors secreted by the disseminated cancer cells (Y. Liu & Cao, 2016). Tumour-derived extracellular vesicles, including exosomes, microvesicles and large oncosomes, containing proteins, mRNA, etc., have the potential to travel far from the original site and educate the microenvironment of the host organ to be more hospitable to the dissemination of cancer cells (Ma et al., 2021). Tumor cells also secrete cytokines, chemokines, hormones, and other pro-inflammatory factors that help to create environment that either resists or favours metastatic colonization (Peinado et al., 2017)

Other important niche promoting factor are expressed by bone marrow -derived cells and immune cells in the niche. These cells remodel the host organ microenvironment by secreting prometastatic cytokines, growth factors and proangiogenic factors that crucially influence tumor cell colonization and proliferation (Y. Liu & Cao, 2016). Chemokines and cytokines derived from tumor cells, recruit myeloid derived stromal cells (MDCs), tumor associated macrophages (TAMs), regulatory T-cells, and tumor associated neutrophils into secondary site and promote metastasis by supporting niche formation (Zhao et al., 2012). Additionally, neutrophils have shown to have an important role during (pre-metastatic) niche formation in the lung, enhancing metastatic formation (Wieland et al., 2017). Additional stromal components, such as endothelial cells, fibroblasts as well as the extracellular matrix (ECM) can change in response to tumor-derived factors fostering the metastatic colonization and growth in secondary organ (Y. Liu & Cao, 2016).

1.2 The vascular barrier

Blood vessels form a critical anatomical border between the circulation and different organ environments, thereby reserving one of the body's largest interactive surfaces. Blood vessels maintain normal tissue homeostasis but also adapt and are involved in many pathological events. Vascular control is necessary during inflammation, haemostasis, and metabolism but also during metastasis and vascular malformations. Vascular function is mediated by an organ-specific, structurally, and functionally heterogeneous layer of endothelial cells (Augustin & Koh, 2017). A better understanding of vascular endothelial cells is crucial for understanding their involvement in many chronic and life-threatening diseases.

1.2.1 Blood vessel structure

Blood vessels are formed by endothelial cells and mural cells, including pericytes and vascular smooth muscle cells. Endothelial cells, covering the inner lining of blood vessels, form a barrier for fluids, solutes and cells between the circulation and a tissue. Large vessels, including arteries, arterioles and veins and venules carry blood from the heart to the tissues and back, respectively, connected by capillaries. There are many structural and functional differences between the different vessel types, including the distinct permeability properties (Krüger-Genge et al., 2019).

Capillaries, with a diameter ranging from 5 to 10 μm, share common functions, being responsible for nutrient and gas exchange between the tissues. However, capillaries in different vascular beds also show differences based on organ-specific capillary endothelial cells. Based on morphological difference, capillaries are classified as continuous, fenestrated, or sinusoidal. Continuous capillaries form a tight barrier between the vasculature and a host tissue and are found in most of the organs including the brain and lung. Water and small solutes diffuse through these capillaries, but larger molecules are transported by transcytosis. Sinusoidal capillaries, found in liver, allow exchange of solutes more freely. There is a gap between the endothelial cells and a discontinuous basement membrane underneath. Fenestrated capillaries, found in e.g. kidney, have intracellular pores covered with a diaphragm allowing permeability of small molecules and fluids (Augustin & Koh, 2017).

Importantly, endothelium not only forms a passive structure for the delivery of nutrients and oxygen, but rather actively controls the tissue microenvironment by expressing tissue-specific angiocrine factors that support organ development, homeostasis and regeneration (Butler et al., 2010). During tumor progression, endothelial cells create vascular niches that facilitate cancer cell growth at the primary and metastatic sites (Augustin & Koh, 2017)

1.2.2 Endothelial barrier

Endothelial cells are the main constituents of blood vessels and form a physical barrier to separate blood form underlying tissue. Structurally endothelial cells are polarized: their luminal side covered by glycocalyx is directly exposed to blood and its constituents, whereas the basolateral surface connects to the surrounding tissue via integrin mediated adhesion to the basement membrane. Despite of multiple similarities, endothelial cells have structural heterogeneity including differences in cellular morphology like size, shape and thickness of the cells, differences in a gene expression profile revealed recently through single cell RNA sequencing as well as surface properties and continuity and thickness of glycocalyx and basement membrane underneath the cell layer (Krüger-Genge et al., 2019).

In addition to acting as a gatekeeper controlling the infiltration of cells, fluids, and plasma proteins into tissues, endothelial cells play a pivotal role in detecting hemodynamic alterations in blood flow and adapting to maintaining vascular homeostasis. Endothelial cells are capable of sensing variations in blood pressure, shear stress, and other mechanical forces acting on vessel walls. In response, they can adjust vascular tone by promoting either vasodilation or vasoconstriction. This conversion of mechanical stimuli into biochemical signals involves mechanoreceptors. In a normal physiological condition, endothelial cells are in a quiescent state, meaning that there is low permeability, almost absence of endothelial cell proliferation and migration and a low expression of cell adhesion molecules (Augustin & Koh, 2017).

To ensure that tissues are adequately supplied with oxygen and nutrients, a variety of passive and active mechanisms enable delivery of substances, including ion channels and transcellular delivery systems. Additionally, cells and larger macromolecules need to cross the endothelium. For this purpose, there are two routes, transcellular or paracellular routes. During transcellular passage, cells and solutes are taken up by endothelial cells and transported from luminal side to apical side of the cell by using vesicle-like structures including caveolae and vesiculo-vacuolar organelles (VVOs) (Wettschureck et al., 2019). However, most of the transport occurs paracellularly through endothelial cell-cell contacts mediated by the opening and closing of interendothelial junctions. In basal conditions, cell-cell junctions have low permeability and barrier stability is actively maintained, but after exposure to toxins, trauma, pathogen, or other stimuli leading to tissue injury, endothelial barrier function changes. Also, pathological conditions like metastatic colonization disturb the normal homeostasis of endothelium. Mechanisms that regulate endothelial permeability have many common features, including altered endothelial junction disorganisation and gap formation, thus the vascular remodelling is disease and organ specific. For instance, the exact mechanism by which extravasation is orchestrated during metastasis, remains still incompletely known (Wettschureck et al., 2019).

1.2.3 Endothelial cell-cell junctions

Endothelial cells are connected to each other by complexes of adhesive proteins that form two junction types: adherens junctions and tight junctions. The adherens junction protein vascular endothelial cell-specific transmembrane protein Cadherin 5 (CDH5) consists of an extracellular motif, a transmembrane domain and an intracellular domain, which interacts with cytoplasmic adapter proteins plakoglobin (γ-catenin), p120 (δ-catenin) and β-catenin. α-catenin connects this complex to actin cytoskeleton (*figure 1*). The association with the actin cytoskeleton is necessary for stabilization of the junction but also for the regulation of junction opening and closure as well as maintaining the cell shape and polarity (Schulte et al., 2011). Additionally, CDH5 interacts with receptors that mediate vascular permeability, like Vascular endothelial growth factor receptor-2 (VEGFR2) and TEK receptor tyrosine kinase (Tie2).

There is also increased evidence of interaction between the different junction types, at least partially mediated by CDH5 (Taddei et al., 2008).

Tight junctions, located near the luminal side of the endothelial cells, are formed by claudin family transmembrane proteins, MARVEL domain proteins, junctional adhesion molecules together with adaptor proteins and cytoskeletal linker proteins (Zihni et al., 2016).

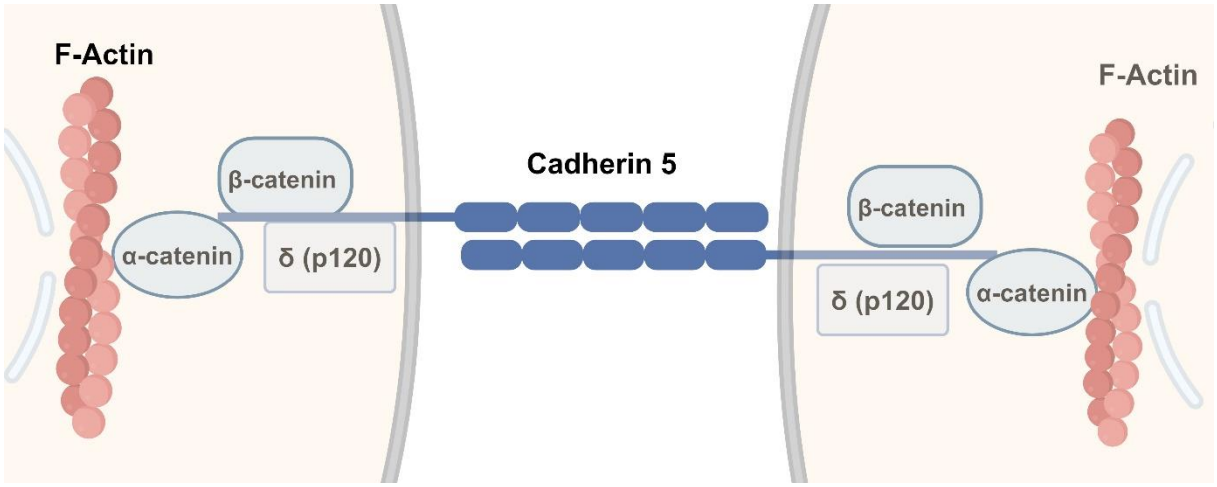

Figure 1. **Schematic picture of Cadherin 5-catenin complex in endothelial cells.** Endothelial specific, transmembrane protein Cadherin 5 forms a complex with cytoplasmic adaptor proteins p120 (δ-catenin) and β-catenin. α-Catenin connects this complex to actin cytoskeleton. Created with BioRender.com.

The opening, closure and integrity of the endothelial barrier is tightly controlled by a variety of ligand-receptor pathways that either directly or indirectly modulate barrier function. Well-known directly acting modulators are vascular endothelial growth factor (VEGF), angiopoietins and the inflammatory cytokines, indirectly acting components change the barrier function by acting in the cells that release factors to modulate barrier (Claesson-Welsh et al., 2021). The opening of the endothelial cell layer and gap formation requires detachment of the CDH5 -catenin complex (Schulte et al., 2011). In response to modulators that regulate vascular permeability or blood flow, CDH5 phosphorylation in cell-cell contacts increases, followed by ubiquitination and the disassembly of junctional complexes, internalisation and degradation or recycling of CDH5 back to the plasma membrane. This changes the distribution of CDH5 in cell junctions from a continuous band along cell borders to a zigzag pattern associated with filopodia that extend during gap formation (Claesson-Welsh et al., 2021).

Permeability factors mediate their action through intracellular pathways that involve Src family kinases (SFKs), focal adhesion kinases (FAK), Rho GTPases and eNOS/NO signalling. CDH5 has been shown to be phosphorylated at several different sites by multiple protein kinases. However, the phosphorylation of CDH5 alone is not sufficient to form gaps in the endothelium. The interplay of Rho GTPases like

Rac1 and Cdc42 as well as RhoA regulate the actin cytoskeleton and endothelial cell gap formation, especially in vitro. Less is known of their roles in vivo; thus, the cortical actin cytoskeleton is likely to coordinate gap formation by facilitating the filopodia formation during gap opening. Endothelial junctions could be also opened without losing their ability to adhere to neighbouring cells, histamine-induced changes in the actin cytoskeleton have been shown to lead to the reorganization of endothelial junctions, promoting increased permeability while still maintaining some level of cell-cell adhesion (Mikelis et al., 2015; Oldenburg & De Rooij, 2014; Wettschureck et al., 2019)

1.2.4 Regulating of endothelial barrier during metastasis

Although crossing the endothelial cell layer is often considered necessary for circulating tumor cells to initiate distant metastasis, tumor cells have also been found to proliferate within the circulation, especially in the lungs (Ehdi et al., 2000). Nevertheless, primary tumor secreted factors including exosomes and other soluble factors like VEGF, TGF-β, tumor necrosis factor a (TNF-α) modulate endothelial cells prior to or during disseminating cancer cell arrival in distant organs, and may decrease the endothelial barrier, facilitating metastasis formation. Next, I will go through some of the known soluble factors and their effects on the endothelial barrier during metastasis formation.

VEGF, secreted by the primary tumor has been reported to induce matrix metalloproteinase 9 (MMP9) expression in the premetastatic lung endothelial cells via VEGFR1/ Flt-1 tyrosine kinase, hence, enhancing cancer cell invasion (Hiratsuka et al., 2002). Additionally, VEGF has been shown to induce the hyperpermeability of endothelial cell-cell junctions by disrupting the Cadherin 5 complex, thereby opening the endothelial barrier when cancer cells cross the endothelium (Reymond et al., 2013). This may be partly mediated via VEGF-induced FAK/Scr signaling acting on adherens junction complexes. VEGF increases lung vascular leakage via phosphorylation of its receptor VEGFR2 resulting activation of c-Scr in wild type (WT) mice, but not in knock-in mice that have PY949 substitution in their VEGFR2 receptor. This way VEGF also increases CDH5 phosphorylation. WT mice also have an increased incidence of spontaneous lung metastases after melanoma cell inoculation into mice ears, when compared to the knock-in mice (X. Li et al., 2016). Vascular hyperpermeability may also be regulated through tight junctions, as VEGF induces phosphorylation of the tight junction proteins zonula occludens-1 (ZO-1) and occluding (R. Li et al., 2019).

Additionally, to tumor-secreted soluble factors, exosomes have been under intensive research during recent years. In the context of endothelial cells, exosomes containing mRNA and RNA of proangiogenic factors enhance VEGF, MMP2 and VEGFR1 expression in lung endothelial cells. In the context of ovarian cancer, primary tumor -secreted exosomes released soluble E-cadherin which then was shown to heterodimerize with endothelial cell CDH 5, thus activating β-catenin and NF-kb signaling (Tang et

al., 2018). To conclude, multiple primary tumor -secreted soluble angiogenic factors or exosomes can activate the stromal cells, including endothelial cells in the distant site, to induce endothelial cell permeability or secretion of angiocrine factors that facilitate extravasation, and therefore enhance metastatic colonization.

1.2.5 Endothelial factors that regulate the initial attachment of tumor cells

The exact cellular and molecular mechanisms enabling cancer cell extravasation into tissues are incompletely understood, but in vivo and in vitro results have shown that cancer cells mainly use transedothelial migration and squeeze through endothelial cell-cell junctions. However, there is also evidence that during distant metastasis cancer cells might cause endothelial cell death by inducing apoptosis or necroptosis (Strilic et al., 2016). To cross the endothelial barrier, cancer cells need to physically entrap within the capillaries. Besides of this passive mechanism, surpassing the endothelial cell layer requires also active processes, where the circulating cancer cells attach to the endothelium, adhere and finally extravasate. The ability of cancer cells to cross the endothelium is affected by the interaction of cancer cells/cell clusters with circulating immune cells like platelets and leucocytes, but also vascular endothelial cells and the host tissue microenvironment. Endothelial cells, as first obstacle between the circulation and stroma, express adhesion molecules, integrins, selectins and cadherins in response to cancer cell and immune cell derived factors. Additionally, cancer cells and local immune cells also secrete factors like cytokines and chemokines, that increase permeability of the endothelial barrier as well as enhance proinflammatory environment. Many of these ligand-receptor interactions between circulating tumor cells/cell clusters and endothelium promote cancer cell adhesion to the vessel wall, cancer cell migration and extravasation (Hiratsuka et al., 2006; Reymond et al., 2013; Tremblay et al., 2006)

Multiple factors that regulate cancer cell attachment and adherence to the endothelium have been identified. These include vascular adhesion molecule (VCAM) 1, intracellular adhesion molecule (ICAM) 1 and different selectins such E-selectin and P-selectin. VCAM 1 and E-selectin are not expressed by quiescent endothelial cells, thus, they require signals from cancer cells or cancer cells related inflammatory cells to be upregulated. Endothelial E-selectin is well known for its role in leukocyte migration but has also been shown to promote cancer cell transedothelial migration by binding to cancer cells and facilitating the homing of cancer cells in the liver. In vitro, tumor cells bind to E-selectin, followed by activation of the intracellular CDH5 signaling pathways Src, ERK and p38, leading to disruption of the CDH5/β-catenin complex, stress fiber formation and increased transendothelial migration in endothelial cells (Wettschureck et al., 2019). Besides their role in inducing endothelial cell permeability, primary tumor -secreted angiogenic factors may also lead to enhanced expression of endothelial adhesion molecules. In the lungs, VEGF induced upregulation of endothelial E-selectin and

focal adhesion kinases led to increased lung permeability and cancer cell homing (Hiratsuka et al., 2011). Additionally, endothelial expressed VCAM1 has been shown to interact with a α4β1 integrin expressed in cancer cells and thus, mediate cancer cell adhesion (Taichman et al., 1991). Activation of these adhesion molecules can be affected by contact with disseminating cancer cells or by soluble factors secreted by tumor cells. For example, melanoma cells secrete SPARC, which binds to endothelial VCAM1 increasing vascular permeability through actin remodeling and by activating downstream signaling through MAPK p38 leading to increased lung metastasis (Tichet et al., 2015). In lung metastasis increased endothelial Notch activation led to increased endothelial cell senescence and vascular adhesion molecule VCAM1 expression (Wieland et al., 2017).

Additionally, to cancer cell-derived factors, endothelium has been shown to actively signal with cancer cells and hence promote metastatic colonization. In vitro, in response to cancer cells, endothelial cells express chemokines such as IL8 and GroB, which act through the chemokine receptor type 2 (CXCR2) on tumor cells and enhance the transedothelial migration and invasion of cancer cells (Mierke et al., 2008). Also, other CXCL chemokine family members and their receptors are involved in cancer cell extravasation. In vitro, CXCL12 secreted from stromal cells has been found to attract cancer cells expressing its receptors, CXR4 and CRX7, thus leading to enhanced cancer cell extravasation and adhesion. In vivo, liver endothelial cells express CXCL12, and blocking the interaction between the CXCR4 and CXL12 led to decreased extravasation but did not affect cancer cell adhesion in vivo (Wettschureck et al., 2019).

1.2.6 Cancer cell-endothelial cell-immune cell communication during metastasis

In the bloodstream cancer cells interact with multiple cell types, including other cancer cells but also the myeloid cells and platelets. These interactions increase cancer cell survival in the vasculature by preventing the attack of immune cells, improve resistance to shear stress or increase the metastatic potential of cancer cells by enhancing their invasive properties. Platelets and circulating cancer cells interact immediately after the cancer cells enter the bloodstream. The formation of platelet-cancer cell aggregates prevents cancer cell lysis by natural killer cells and enhances the entrapment of cancer cells before they cross the endothelial cell layer and promote adhesion through expression of adhesion molecules such as P-selectin. P-selectin has been shown to interact with circulating cancer cells to promote lung metastasis (Gong et al., 2012). The mechanism of action of platelets is partially through the secretion of biologically active compounds such as ATP and TGF-β, which facilitate metastatic seeding, extravasation, and colonization of cancer cells (Kopp et al., 2009). Notably, platelets might have organ specific functions since they have been involved especially in the lung, but not liver metastasis (Reymond et al., 2013).

Cancer cell -activated platelets also recruit leukocytes like neutrophils, leading to enhanced metastasis promoting environment. Neutrophils, when attracted to the metastatic site and contacted with cancer cells, secrete cytokines like interleukin-8 (IL-8), to promote the pro-inflammatory environment and facilitate cancer cell transendothelial migration (Dong et al., 2005). Additionally, neutrophils can enhance the interaction between cancer cells and endothelial cells by physically connecting the cancer cells to the endothelial cells by expressing molecules such as beta-2-integrin, which further interacts with endothelial adhesion molecules such as ICAM 1 (Huh et al., 2010). Circulating cancer cells also secrete chemokines that activate endothelial cells to express adhesion molecules such as VCAM1, followed by increased recruitment of macrophages and monocytes, which further secrete VEGF to promote the opening of endothelial cell-cell junctions (Reymond et al., 2013; Wieland et al., 2017).

1.3 Pim kinases in health and disease

PIM (Proviral Integration sites for Moloney murine leukemia virus) kinases are a family of serine/threonine kinases including three different kinase family members, PIM1, PIM2 and PIM3. There is approximately 60% sequence similarity between all three isoforms at the amino acid level. The expression of PIM kinases varies in their tissue distribution, and they are expressed in many different cell types including haematopoietic, vascular smooth muscle, neuronal and endothelial cells (Eichmann et al., 2000; Zhang et al., 2009). Since PIM-kinases do not have regulatory domain, their cellular levels are mainly regulated by transcriptional and translational levels, while the constitutively active protein turnover is mediated trough ubiquitination and proteasomal degradation e.g. by phosphatase PP2A (Losman et al., 2003)(K. C. Qian et al., 2005). The main factors promoting PIM expression are various interleukins and cytokines, which signal through the JAK/STAT pathway (Shirogane et al., 1999). JAK activation leads to the phosphorylation of STATs, dimerization, and nuclear translocation. Once in the nucleus, STATs bind to the appropriate target gene promoter region and regulate gene expression (Hammarén et al., 2019).

PIM kinases are known to act as oncogenes in different malignancies and dysregulation and overexpression of PIM kinases have been connected to both hematopoietic and solid cancer progression (Bellon & Nicot, 2023). Notably, PIM overexpression alone is not enough to induce tumorigenesis and requires other activated oncogenes such as MYC family member to exert their function as an oncogene (Shirogane et al., 1999). PIM kinases promote cell survival and cancer cell motility (Santio & Koskinen, 2017), while they also regulate cell metabolism and cancer cell immune evasion, additionally they are involved during epithelial to mesenchymal transition (ECM) and cell adhesion (Santio & Koskinen, 2017; Toth & Warfel, 2021). Overexpression of PIM1 and PIM3 are related to cancer progression in different cancer types and inhibition of PIM pathway is shown to lead to a decreased level of cancer cell migration in multiple cancer cell lines (Santio & Koskinen, 2017).

Pim kinases mediate their activity by activating or inactivating several downstream effectors. For instance, all PIM kinases have been shown to phosphorylate Notch 1 in serine 2152, leading to increased migration in prostate cancer cells (Santio et al., 2016). Additionally, PIM kinases phosphorylate the pro-apoptotic BAD protein, leading to inhibition of its pro-apoptotic function and inability to bind to the pro-survival protein Bcl-XL (Aho et al., 2004; Macdonald et al., 2006), thereby increasing cell survival and inhibiting apoptosis. The activation of PIM-dependent transcription factors can regulate PIM expression by forming an indirect or direct feedback loops. Positive feedback loops have detected between PIM kinases and a family of transcription factor protein complexes like NF-κB and negative loops between PIM1 and its substrate SOCS. SOCS suppress cytokine signalling leading to attenuated Jak-Stat response and stabilize PIM-dependent phosphorylation (Santio & Koskinen, 2017). Both PIM1 and PIM2 also regulate tumorigenesis by promoting epithelial-mesenchymal transition (EMT). Cytokine induced activation of STAT3 leads to PIM1 and PIM2 upregulation, whereas PIM inhibition led to downregulation of EMT and stemness transcription factors ZEB1, snail, N-cadherin, oct4 and sox2 (Gao et al., 2019; Uddin et al., 2015).

1.4 Preliminary results as starting point of the study

1.4.1 Identification of cancer responding endothelial cells

Preliminary experiments using single cell mRNA sequencing (scRNASeq) of lung endothelial cells identified a cancer responding endothelial cell (crEC) cluster, present in healthy mouse lung but increased after tumor cell colonization of the lungs. For this purpose, B16-F10 cells were intravenously injected into C57BL7/6 mice, and after 6 hours lung endothelial cells were isolated and sorted by flow cytometry based on fluorescently labelled endothelial cell marker Pecam1 (Cd31). Thereafter, scRNASeq was performed to identify endothelial subtypes which were annotated as capillaries, arteries, veins, and lymphatic endothelial cells. In addition, a capillary endothelial cluster expressing interferon signaling genes was identified as hIFN cluster. Moreover, clustering the cells revealed a capillary EC cluster of previously unknown identity enriched in metastatic lung. These cells were named as cancer responding endothelial cells (crEC).

1.4.2 Upregulated endothelial cell pathways at the metastatic niche

The top 25 crEC specific cluster markers were identified. To identify the endothelial cell pathways activated in the lungs 6 hours after cancer cell injection, differential gene (DE) expression analysis and gene set enrichment analysis using Kyoto Encyclopaedia of Genes and Genomes (KEGG) were performed. crECs revealed several significantly upregulated pathways including the Jak-Stat pathway. The Cell Chat (Jin et al., 2021) tool showed upregulation of Vegf and Notch, as well as the Notch

pathway ligands Dll1 and Dll4 in crECs, suggesting that crECs were responding to infiltrating tumor cells by upregulating angiogenic pathways. In my thesis, I focused on three crEC cluster markers including *Pim3* and *Bcl3,* which are targets of the JAK-STAT pathway as well as *Inhbb* (*figure 2*).

1.5 Aims

The overall aim of this thesis was to identify novel endothelial cell markers of metastatic lungs and to investigate the functions of the newly discovered marker genes. Based on scRNASeq of endothelial cells from pre-metastatic mouse lungs several crEC marker genes and upregulated pathways were identified. The most promising of these was the JAK-STAT-Pim3 (Bcl3) pathway. Although Pim3 has been previously studied in cancer, its role in endothelial cells is poorly known. Therefore, I hypothesized that Pim3 plays a role in regulating the vascular metastatic niche and the vascular barrier in the metastatic niche of the lungs. The specific aims included: 1) to investigate the role of Pim3 in endothelial cells and 2) further investigate its involvement in the vascular niche during metastasis.

2 Results

2.1 crEC marker genes are upregulated in spontaneous mouse metastasis models

The upregulation of crEC marker genes in isolated mouse lung endothelial cells was confirmed by using RT-qPCR (*figure 2a*). As a result, crEC marker genes *Pim3, Inhbb and Bcl3* were upregulated after six hours of i.v injection of B16-F10 melanoma cells (200 000 cells/100µl PBS). Since i.v injection of cancer cells models cancer cell homing into the lungs, but not the entire metastatic cascade, we investigated whether the crEC marker genes were upregulated also in spontaneous metastasis models. For this purpose, B16-F10 (200 000 cells/100µl PBS) cells were subcutaneously inoculated into the neck of C57BL7/6 mice. After inoculation, tumours were allowed to progress and form the neovasculature by day 14 (*figure 2a*). Despite the absence of lung metastasis at that time point, *Mlna*, a marker for circulating melanoma cells, was upregulated in blood samples at day 14 post-inoculation when measured using RT-qPCR (*figure 2d*). Interestingly, crEC marker genes *Pim3, Inhbb and Bcl3* were upregulated in the mouse lung endothelial cells isolated by a flow cytometry (*figure 2e)* at this time point. These results indicate that crEC markers were upregulated in the mouse lungs coinciding with the time of appearance of circulating tumor cells from the primary tumor, although no melanoma metastasis was observed in the lungs.

The phenomenon of early metastasis was further understood by utilizing the orthotopic 4T1 fat pad mammary tumor model. Either mammary gland carcinoma (4T1) cells or PBS only (200 000 cells/ 100µl PBS) were inoculated into the fourth mammary fat pad of Balb/c mice. As a result, *Pim3, Inhbb* and *Bcl3* were upregulated in isolated endothelial cells from the lungs 14 days after cancer cell inoculation (*figure 2 g*). Notably, the first visible lung metastases occurred three weeks after cancer cell inoculation, whereas the first liver nodules were found at day six (*figure 2f*). These results suggest that circulating cancer cells (as indicated by the formation of liver metastasis) could upregulate crEC marker genes, even before visible lung metastasis is detected. Altogether, the upregulation of three top crEC marker genes was confirmed in pulmonary endothelial cells using two different spontaneous metastasis models.

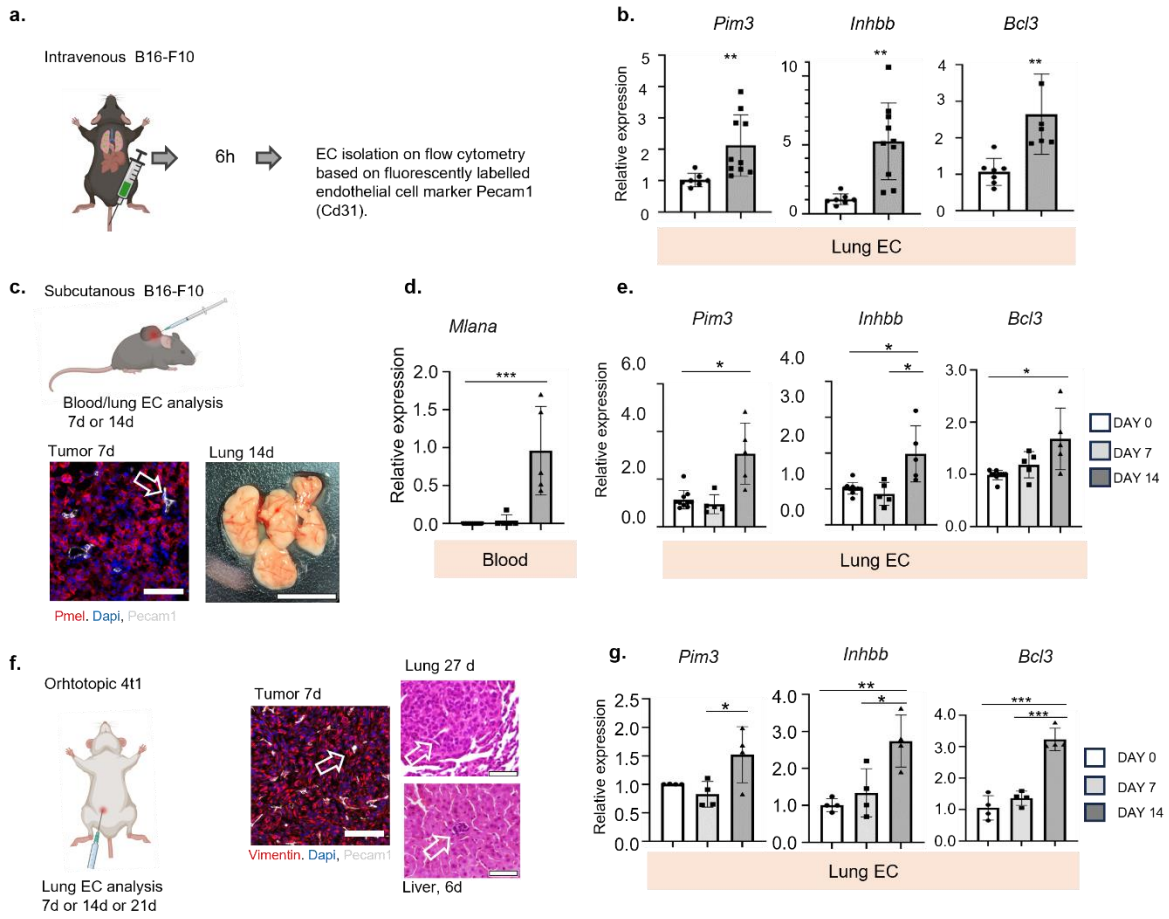

Figure 2. **crEC markers *Inhbb*, *Pim3* and *Bcl3* are upregulated in animal models. a.** B16-F10 melanoma cells or PBS (Ctrl) were intravenously (i.v.) injected (200 000 cells in PBS) into C57BL/6 mice and after 6h, lung endothelial cells (ECs) were isolated based on Pecam1/Cd31 antibody by flow cytometry (preliminary results). **b** RT-qPCR analysis of *Pim3*, *Inhbb* and *Bcl3* of ECs isolated from mouse lungs 6 h after B16-F10 or PBS i.v. injection, n=7 **c.** B16-F10 cells or PBS (200 000 cells in PBS) were injected subcutaneously (s.c.) into C57BL/ mice for 7 d or 14 d. Immunofluorescence staining for Pecam1 of the tumor at 7d to visualize the tumor vasculature as well as representative image of the lungs at day 14 post inoculation. **d.** Relative *Mlna* expression, as a marker for circulating cancer cells, measured by RT-qPCR from the blood samples. **e.** Expression of *Pim3*, *Inhbb* and *Bcl3* 7- and 14-days post implantation from isolated lung endothelial cells. **f**. 4t1 mammary carcinoma cells orthotopically injected into the BALB7s mice, resulting in vascularized tumor at day 7 post inoculation visualized by Pecam1 staining as well as representative images of lung (27d) and liver (6d) metastasis stained by Hematoxylin & Eosin (HE) staining. Arrows indicate the tumor vasculature and metastasis formation. RT-qPCR of isolated lung endothelial cells and expression of crEC marker genes at day 7 and 14. Anova variance analysis with multiple comparisons $P < 0.05$* and < 0.01** Two-tailed paired t-test $P < 0.05$*. Scale bars 50 mm. Shown are relative expression levels as compared to Ctrl, normalized to Hprt.

2.2 crEC marker genes are upregulated by cancer cell secreted factors in endothelial cell cultures

To investigate in vitro dynamics between the cancer cells and endothelial cells, human umbilical vein endothelial cells (HUVEC) were cocultured with GFP expressing B16-F10 melanoma cells. After 16h

of co-culture cells were sorted based on GFP-positive tumor cells by flow cytometry. Using RT-qPCR, the crEC marker genes *PIM3*, *INHBB* and *BCL3* were upregulated in the coculture in comparison to HUVEC monoculture (*Figure 3a,b*). This result indicates that melanoma cells are sufficient for crEC marker gene expression, and the crEC marker gene upregulation is not mediated by other cell types in the lungs.

Based on the literature, there is evidence that melanoma cells secrete soluble proteins and factors to facilitate metastasis (Anchan et al., 2020). To investigate whether crECs marker genes could also be upregulated by soluble cancer cell secreted factors, HUVEC were treated with B16-F10 conditioned medium or as a control, HUVEC conditioned media or media incubated on a plate without cells (no-cell media). Interestingly, B16-F10 conditioned media alone led to increased expression of the three crEC marker genes at different time points, indicating that melanoma cell secreted factors were responsible for crEC marker upregulation. Notably, similar increase in crEC gene transcription was observed when conditioned media from two additional cancer cell lines, 4T1 and Lewis lung carcinoma (LLC), were used (*figure 3c, d*).

To understand the pathways activated in endothelial cells by cancer cells and inducing the crEC marker genes, bulkRNA sequencing were conducted. Total RNA was isolated from HUVEC's treated with cancer cell conditioned medium or no-cell conditioned medium for 16h and analysed using the Agilent 2100 for RNA sequencing and Illumina NovaSeq Platform and Novogene (UK) Bioinformatic. Gene set enrichment analysis revealed that culture in cancer cell -conditioned media upregulates multiple pathways, of which the Jak-Stat pathway was significantly upregulated using both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Hallmark pathway analysis (*figure 3c*). Notably, the Jak/Stat pathway was upregulated in crECs 6h after i.v injection of B16-F10 melanoma cells by using single cell RNA sequencing and gene set enrichment analysis (unpublished result from a host group). Other upregulated pathways in the bulk sequencing associated with hypoxia, tumor protein p53 (TP53), mammalian target of rapamycin complex 1 (mTORC1), angiogenesis, and downregulated pathways were related to cell growth and cell cycle progression. These cell culture results therefore support the in vivo findings that activation of the Jak-Stat pathway, induced by factors secreted by tumor cells, is a hallmark of crECs.

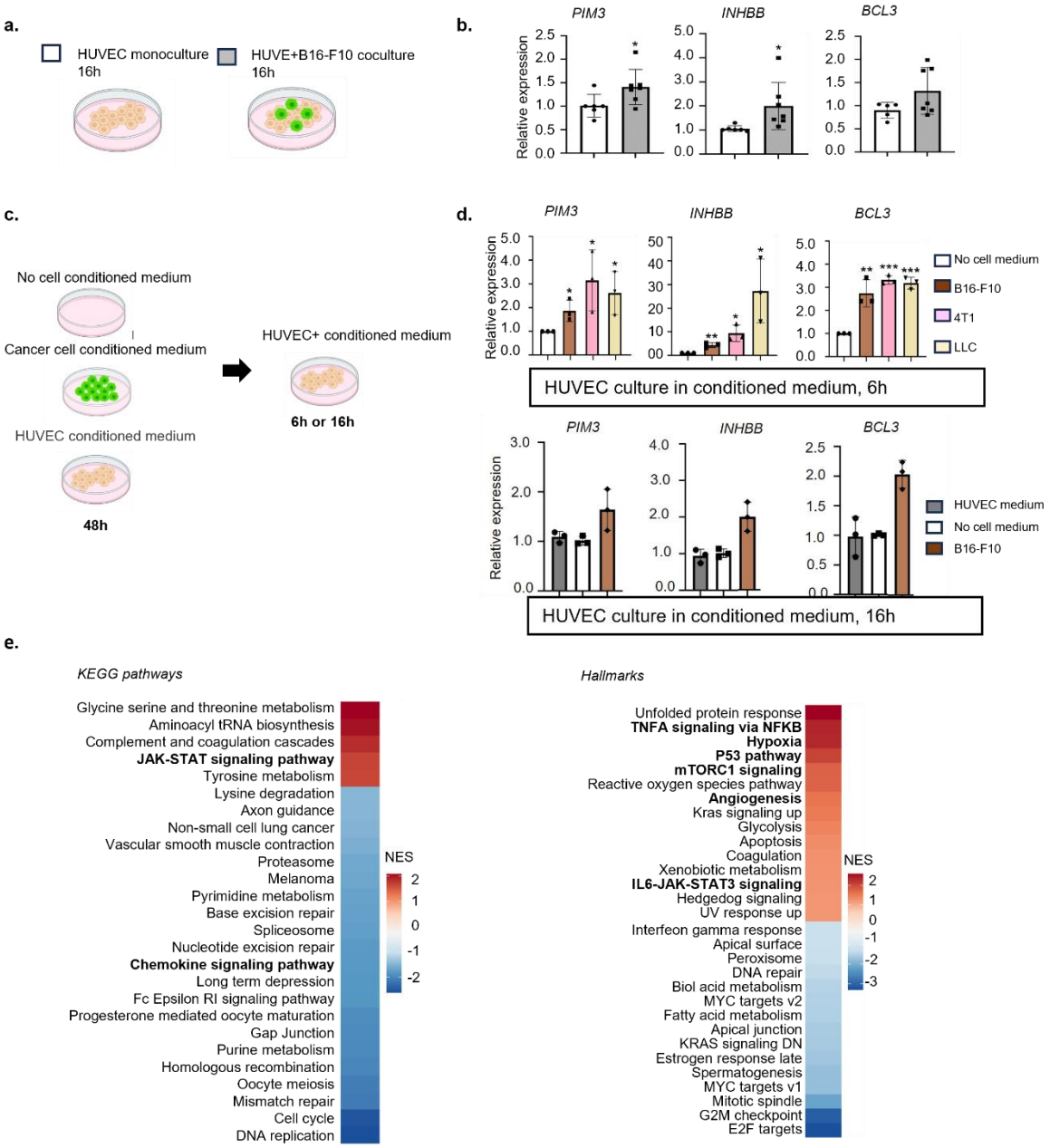

Figure 3. **CREC marker genes are upregulated by cancer cell -secreted factors in endothelial cell cultures a**. Mono- (n=6) and cocultures (n=7) of human umbilical vein endothelial cells (HUVEC) with or without GFP expressing B16-F10 melanoma cells. **b.** RT-qPCR of sorted HUVECs after 16 hours of co-culture. **c-d.** HUVEC treated for 6h and 16h with conditioned medium derived from 48 h cultures of cancer cells, no cell conditioned medium and HUVEC cell conditioned medium as a control. **d.** RT-qPCR of HUVECs treated with conditioned media derived from cancer cell cultures (n=3, each as a triplicate). Anova variance analysis with multiple comparisons P < 0.05*, < 0.01** and < 0.001*** Two-tailed paired t-test P < 0.05*. Shown are relative expression levels as compared to Ctrl, normalized to Hprt **f.** Pathway analysis of HUVEC DE genes in response to melanoma secretome by bulkRNA-Seq. HUVEC treated with B16-F10 cultured medium, as stated above, for 16h (n=4). Shown are activated and suppressed pathways after HUVEc culture in melanoma cell conditioned media. Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Hallmarks pathways of the DE genes

2.3 PIM3 regulates endothelial cell junction integrity

The functional role of PIM3 in endothelial cells was understood by first inhibiting PIM kinases using the Pan-PIM kinase inhibitor AZD-1208 (1 mM) in human dermal blood endothelial cells (BEC) and human umbilical vein endothelial cells (HUVECs) cultured on cover glasses. Inhibition of PIM kinases for 24h in the 2.5% serum containing starvation media led to a reduction of CDH5 in cell-cell junctions measured in immunofluorescence staining as compared to DMSO (0.1 %) treatment (*figure 4a, b*).

To further understand the role of PIM3 in endothelial cell barrier function, electrical cell impedance sensing assay (ECIS) were utilized for both endothelial cell types. As a result, AZD-1208 increased cell-cell contact permeability after formation of a confluent cell monolayer compared to vehicle-treated cells. This phenomenon seemed not to be related to cytotoxic effect of the compound, since AZD-1208 did not cause loss of HUVEC confluency or cell death in 2.5% serum starved conditions in an incucyte assay during 48h period (*figure 4d*).

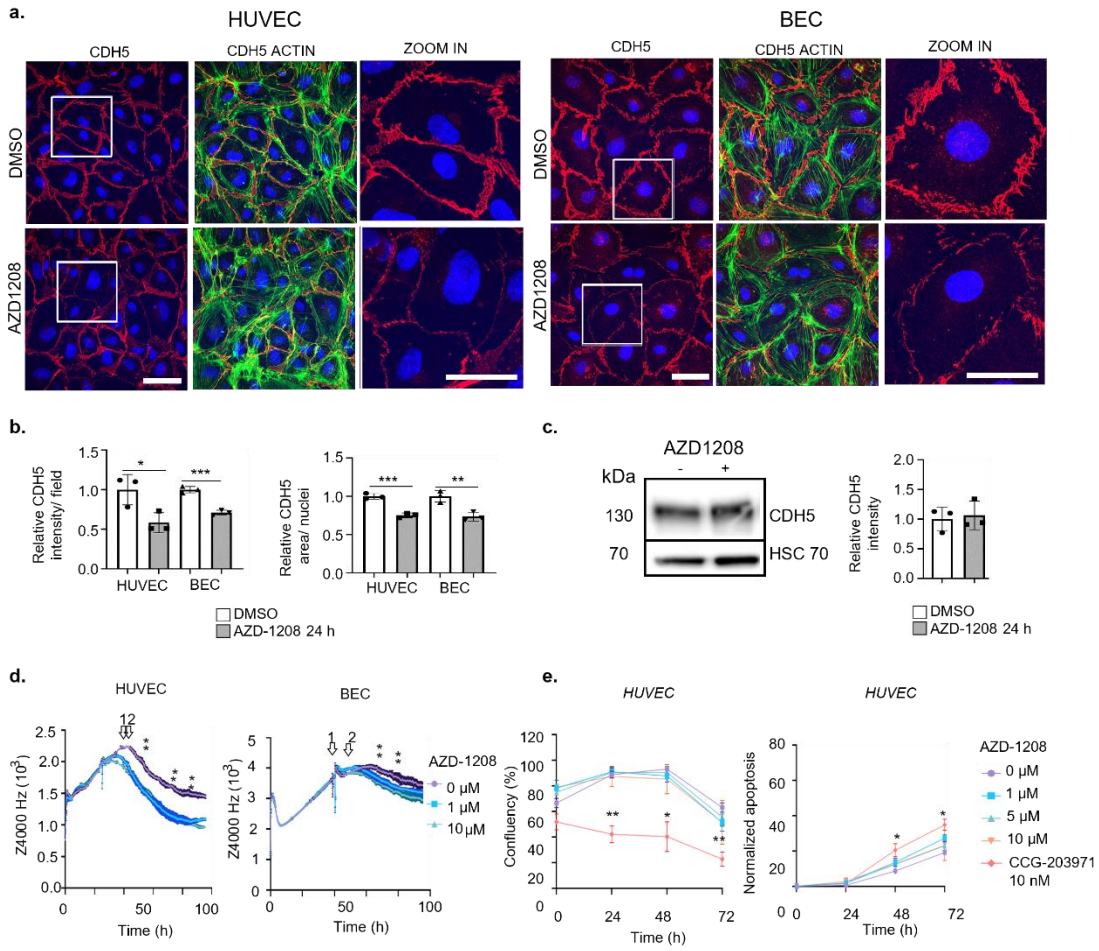

Figure 4. **PIM inhibition decreases endothelial cell barrier integrity via cell junctions a**. Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular blood endothelial cells (BEC) were treated with the PIM inhibitor AZD-1208 (1 µM) or 0.1 % DMSO as a control for 24h in 2.5% serum containing starvation media. After fixation, the cells were stained

for cadherin 5 (CDH5), F-actin and nuclei (DAPI), shown are representative immunofluorescence images (n=3) **b**. Relative CDH5 signal intensity (per field) and relative area (normalized to number of nuclei) (n=3). **c**. Western blot for CDH5 using HUVEC treated accordingly**. d**. HUVEC or BEC treated with 1 μM or 10 μM AZD1208 or 0.1% DMSO as a control and analyzed for electrical cell impedance using ECIS. Arrows indicate initiation of serum starvation (arrow 1) and start of treatment (arrow 2) (n=3) **e**. HUVEC confluency and apoptosis were analysed using IncuCyte® in the presence of the PIM inhibitor AZD-1208 as indicated or 0.1% DMSO. Soluble Rho GTPase inhibitor (CCG-203971) was used as a negative ctrl, significant effect*. Apoptosis was calculated based on Caspase Green Dye counts normalized to confluency. Two-tailed paired t-test, $P < 0.05$*, < 0.01** and < 0.001***. Scale bars 50 μm; 25 μm in close-up images.

AZD-1208 is a selective PIM kinase inhibitor with a slightly different selectivity between the different PIM kinase family members (Dakin et al., 2012). However, by using RT-qPCR we found that HUVECs and BECs prominently express *PIM3* mRNA, in comparison to other members of the PIM kinase family including *PIM1* and *PIM2* (*figure 4e*). Nevertheless, to avoid possible off target effects of AZD-1208, PIM*3* was silenced in both cell types by using shRNA lentiviral vectors. After 48 h of silencing, there was a loss of CDH5 in endothelial cell-cell junction of *PIM3* silenced but not shScramble (sh*SCR*) control silenced endothelial cells, visualized by immunofluorescence staining (*figure 5a, b*). To investigate the mechanism of CDH5 reduction, the total protein amount of CDH5 in the whole cell lysates was measured by using Western blot. As a result, the total CDH5 protein amount was unchanged in both AZD-1208 treated and *PIM3* silenced ECs (*figure 4c, 5c*).

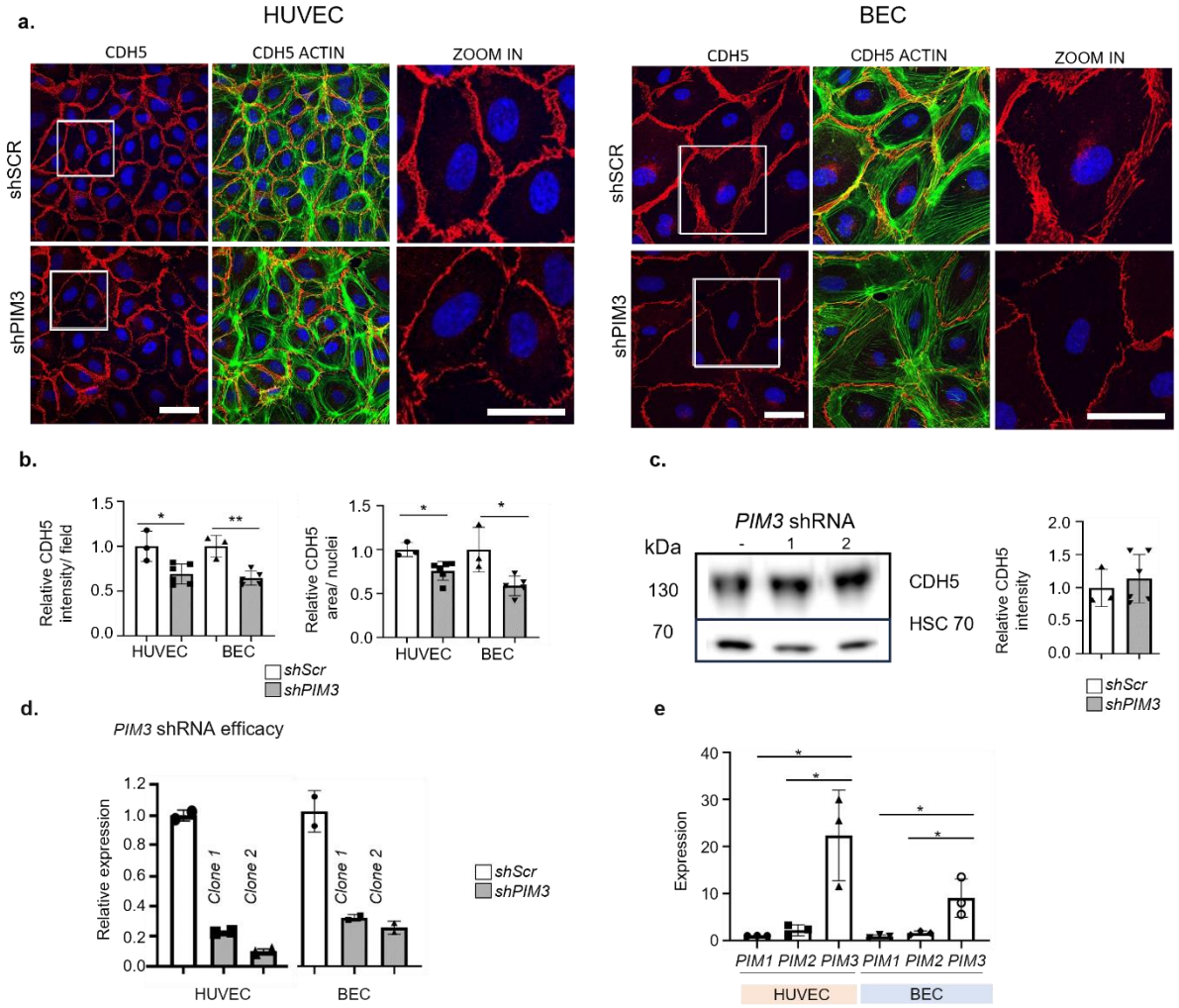

Figure 5. **PIM3 silencing decreases cadherin 5 in cell junction without affecting total protein levels.** *PIM3* was silenced for 72h using *PIM3* (sh*PIM3*) or scrambled shRNAs (shScr, as ctrl) in human umbilical endothelial cells (HUVECs) and human dermal microvascular blood endothelial cells (BECs). Shown are representative immunofluorescence images stained for cadherin 5 (CDH5), F-actin and DAPI **a**. captured with a Zeiss LSM 880 confocal microscope. **b.** Relative CDH5 signal intensity and area (normalized to number of nuclei; n = 3 for shSCR and n= 6 for sh*PIM3*). **C.** Western blot for CDH5 using HUVEC treated accordingly **d**. Efficiency of *PIM3* silencing after transfection of HUVECs and BECs using two different lentiviral vectors (sh*PIM3*) measured by RT-qPCR and compared to scrambled control (shSCR) **e** *PIM* expression levels of HUVEC and BEC analyzed by RT-qPCR. Values are normalized to *HPRT* and shown is relative expression to HUVEC *PIM1*. Two-tailed paired t-test, P < 0.05* and < 0.01**. Scale bars 50 µm; 25 µm in close-up images

In endothelial cells, β -catenin (CTNNB) and δ -catenin (CTNND) form a complex with CDH5 that is connected to actin cytoskeleton by tension sensitive α -catenin (CTNNA). Therefore, the next objective was to investigate whether *PIM3* silencing also affected the catenin's essential for junction integrity. All these adapter proteins were significantly decreased in EC-EC junctions after *PIM3* silencing (*figure 6*). Altogether, these results indicate that PIM3 inhibition affects the barrier integrity of endothelial cells

without inducing significant cellular cytotoxicity, indicating that PIM3 kinase has a role in the maintenance of endothelial cell barrier function.

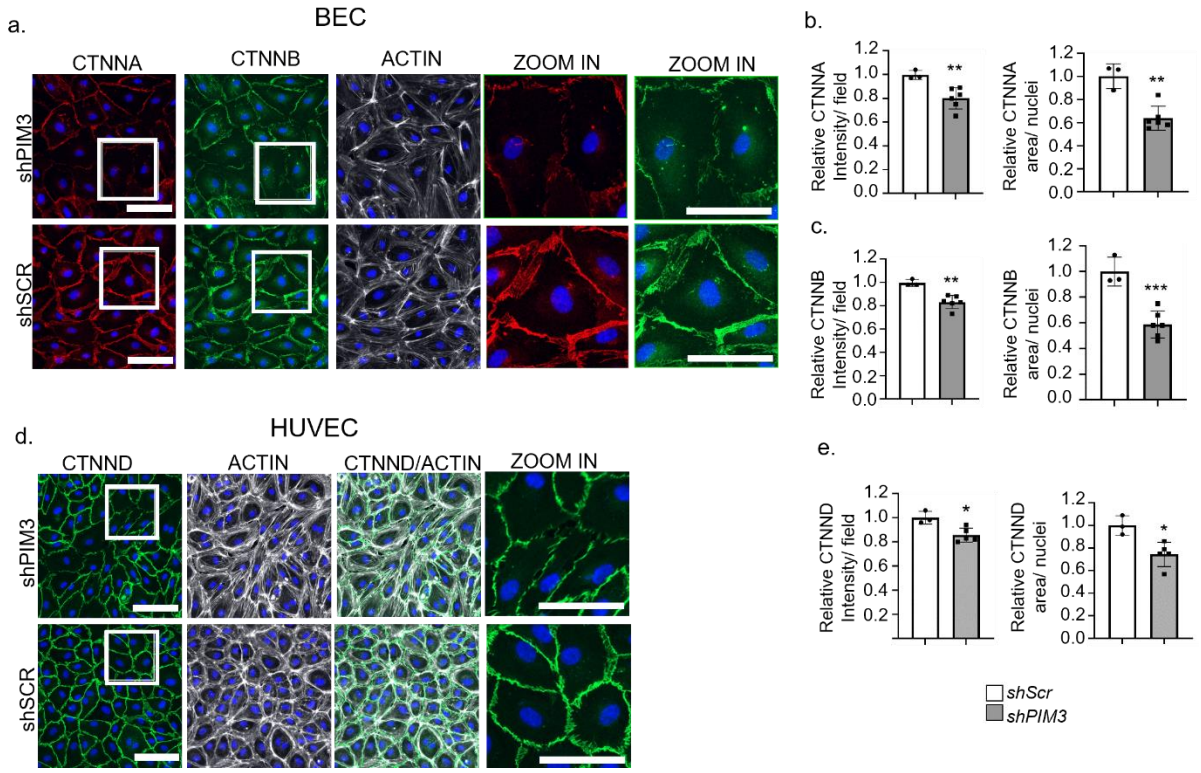

Figure 6. **PIM3 silencing decreases catenin signal in endothelial cell junctions. a** sh*PIM3* or shSCR silenced human blood dermal endothelial cells (BECs) were cultured on cover glasses and stained for α- and β-catenin (CTNNA and CTNNB) and F-actin. **d** human umbilical endothelial cells (HUVECs) were grown as in and stained for δ-catenin (CTNND) and F-actin. Nuclei were stained using DAPI. Relative **b**. α-catenin, **c**. β-catenin and **e**. δ-catenin intensities and area (normalized to number of nuclei; n =3 independent replicates). Two-tailed paired t-test, P < 0.05*, < 0.01** Scale bars 50 µm; 25 µm in close-up images.

2.4 PIM inhibition increases leakage and metastasis in mouse lungs

Since a reduction in cell junction integrity was observed after PIM3 inhibition in vitro, the next goal was to investigate whether this effect translates to in vivo. First, it was confirmed that AZD-1208 did not affect the viability of the B16-F10 cells by using Incucyte (*figure 7d*). Next, B16-F10 cell or PBS (200 000 cells/100 µl PBS) were injected i.v into C57BL7/6 mice. After allowing the B16-F10 cells to colonise the lungs for three days, the mice received AZD-1208 (30 mg.kg-1 in 10% DMSO, 40% PEG300, 5% Tween-80, in PBS, 100 µl per mouse) or vehicle (diluted accordingly), orally for five more days. Interestingly, AZD1208 did not affect the growth of the melanoma between the treatment groups at day 8 (*figure 7 a, b, c*). Thus, AZD1208 did not affect B16-F10 viability or growth in vitro or in vivo.

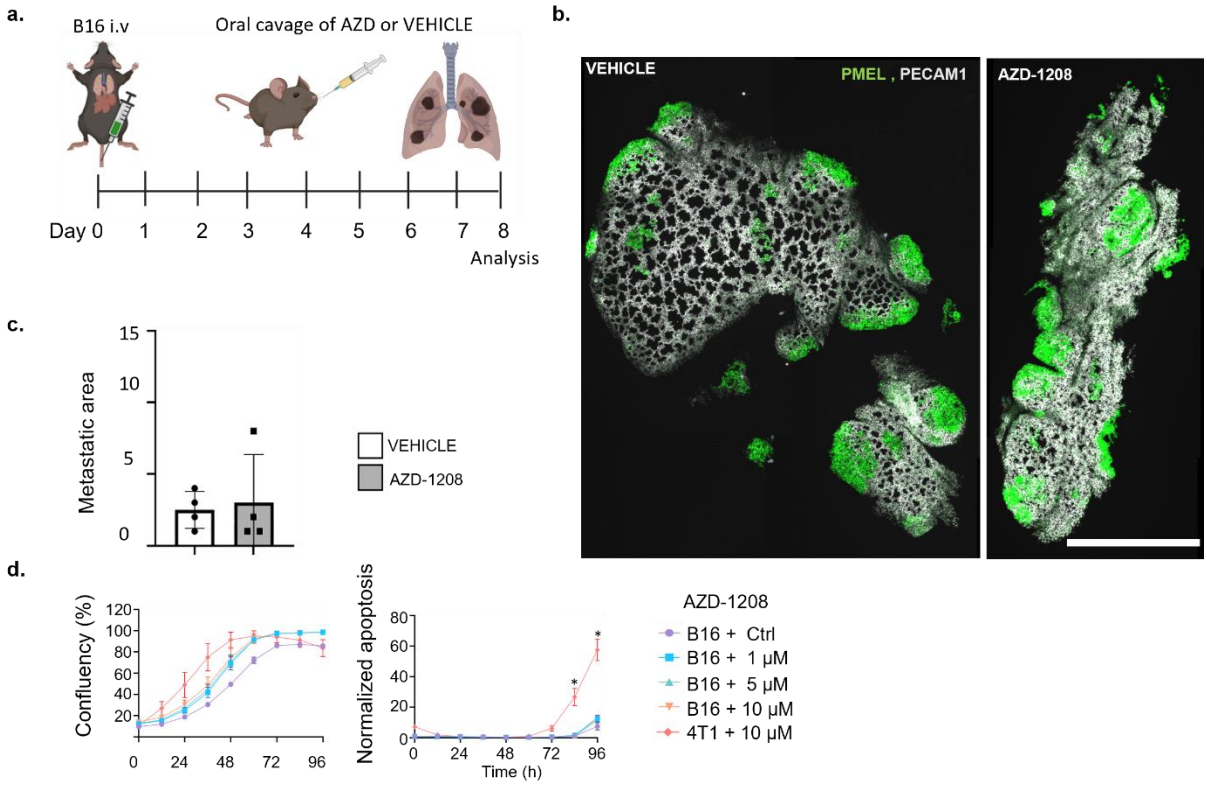

Figure 7. **PIM inhibition does not affect melanoma cell growth. a**. C57BL/6 mice were intravenously (i.v) injected with B16-F10 cells or PBS. From day three mice were orally administered either with AZD1208 or DMSO until day 8 **b**. Melanoma cells were stained by using Pmel, PECAM1 was used to stain the lung vasculature. Representative immunofluorescence images from the whole lung lobes **c.** quantification of metastatic area/lung lobe (n=4) **d.** Cultured B16-F10 melanoma cells treated with AZD-1208 or 0.1% DMSO and imaged by IncuCyte® for cell confluency and apoptosis (Caspase Green Dye counts normalized to confluency). Two-tailed paired t-test P < 0.01*, <0.001***. Scale bars 500 µm.

After finding that AZD-1208 had no effect on melanoma viability, the inhibition of PIM kinases on vascular leakage and metastasis formation were further investigated. For that purpose, C57BL7/6 mice received by oral gavage AZD-1208 or vehicle (30 mg.kg-1 in 10% DMSO, 40% PEG300, 5% Tween-80, in PBS, 100 µl per mouse) for five days. On day three, B16-F10 or PBS (200 000 cells/100 µl PBS) were intravenously injected via tail vein, and cancer cells were let to colonize and form metastatic nodules in the lungs until day seven. Leakage was measured by i.v injection of 70 kd fluorescent dextran for 10 min prior sacrificing the mice. Interestingly, analysis from the immunofluorescence staining revealed that AZD-1208 led to increased leakage as well as increased numbers of metastatic nodules in the mouse lungs (*figure 8a, b, c*).

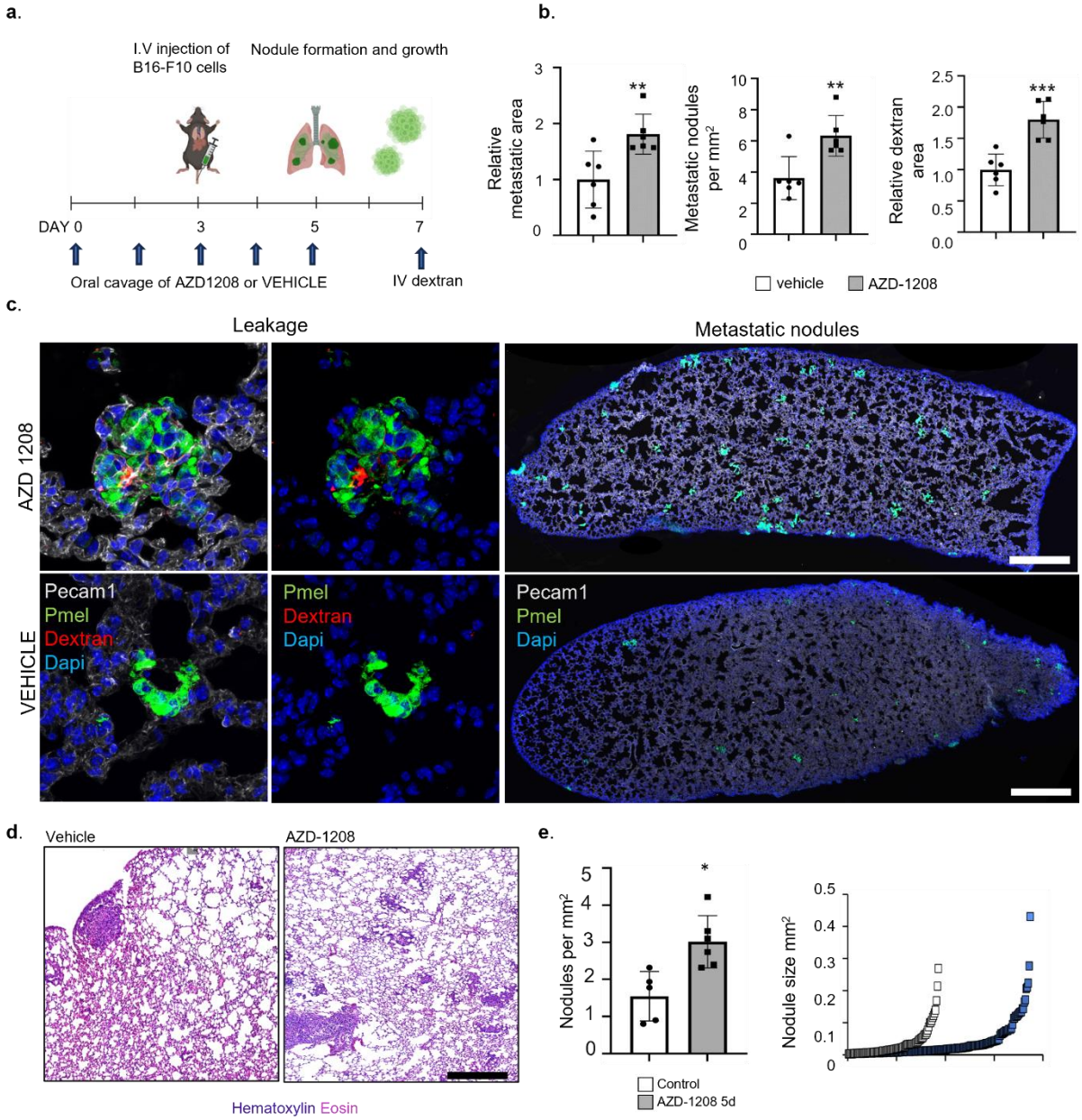

Figure 8. **Inhibition of PIM activity leads to increased vascular leakage and lung metastasis. a.** Pim inhibitor AZD-1208 (30 mg kg^{-1}) or vehicle (10% DMSO, 40% PEG300, 5% Tween-80 in PBS; 100 µl per mouse) was administered orally for five days. On day 2, B16-F10 melanoma cells were intravenously (i.v) injected via tail vein. At day 7, 70 kDa fluorescent dextran was injected i.v. for 10 minutes prior sacrification. **b.** Average metastatic area and metastatic nodules and dextran area were quantified from lung sections (n=6). **c** Representative confocal images using LSM 880, 63 x oil objective. **d.** AZD-1208 or DMSO (diluted as above) was administered orally to C57BL/6 mice for four days, followed by LLC injection on day 3 and metastasis colonization and formation until day 14, shown are representative images of Hematoxylin & Eosin (HE) stained lung sections, imaged by 3D Histec slide scanner. Scale bars 500 µm, metastatic nodules; 25 µm, leakage. Two-tailed paired t-test, P < 0.05*, < 0.01** and < 0.001***.

To further study the effect of Pim inhibition in mice, orthotopic 4T1 model and i.v LLC models were used. Similarly to the B16-F10 model, Pim inhibition by using AZD-1208, increased vascular leakage

in the orthotropic Balb/c mouse model 20 days after cancer cell inoculation. At that time lung metastasis was not visible, but probably due to the apoptotic effect of AZD-1208 in 4T1 cells (*figure 9b*), the liver metastasis were decreased in AZD-1208 treated mice (*figure 9e*). Additionally, C57BL7/6 mice received AZD-1208 four days before LLC i.v injection and cancer cells were led to grow until day 14. As a result, AZD-1208 administration prior to cancer cell injection, led to increased number of metastatic nodules as well as total metastatic area without affecting the size of the cancer nodules (*figure 9 a, c, d*). These results indicate that AZD-1208 enhanced the leakage and increased metastatic formation in the mouse lung by PIM inhibitor resistant cancer cells. However, treatment of PIM inhibitor sensitive cell lines during metastasis formation decreased metastasis in the liver. Altogether, the results show that the effect of PIM inhibition crucially depends on the addiction of tumor cells on PIM-mediated cell survival. Therefore, the use of PIM inhibitor in cancer therapies might cause unwanted and even opposite outcomes depending on the presence of PIM inhibitor resistant tumor cell clones, which might arise during therapy.

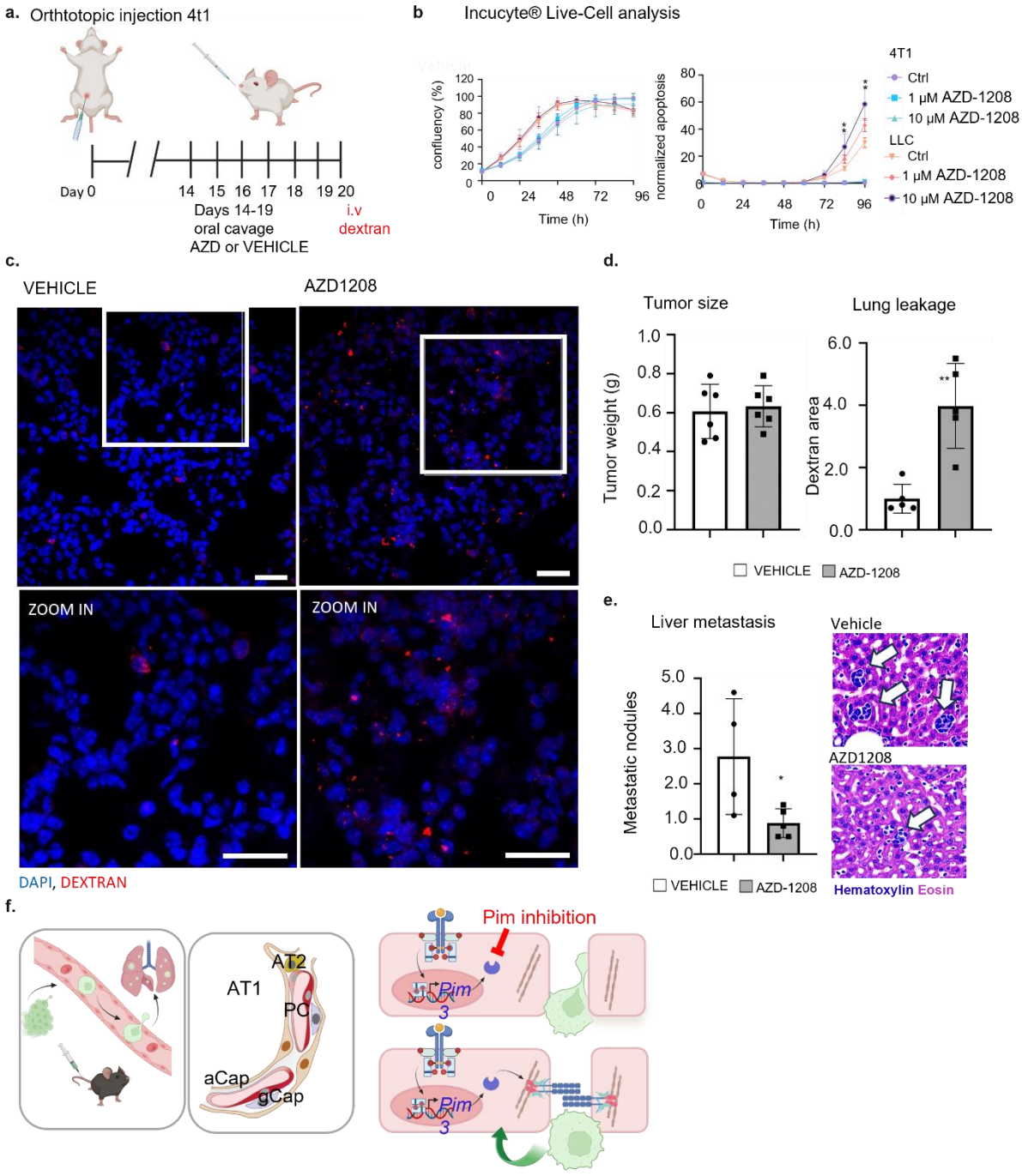

Figure 9. **PIM inhibition increases leakage in the lungs of 4T1 tumor bearing female Balb/c mice. a.** 4T1 cells were orthotopically injected into the 4th mammary fat bad of Balb/c female mice. AZD-1208 was administered daily during days 14-19 post implantation. 70 kDa fluorescent dextran was injected intravenously (i.v.) 10 minutes prior to scarification. **b**. Representative images of leakage from the lung sections stained for dextran and DAPI (nuclei), imaged by Zeiss LSM880 confocal microscope, 40x oil objective. **c.** Tumor weight at the endpoint and relative leakage quantified from lung sections. **e.** Analysis of liver metastasis (nodules with > 5 cells were counted). Representative images of Hematoxylin & Eosin (HE) stained liver sections, imaged using 3D Histech slide scanner. Arrows pointing the metastatic colonies. **f.** Schematic representation of cancer cell-induced reprogramming of endothelial cells in the metastatic niche of the lung. Two-tailed paired t-test P< 0.05*, < 0.01**. Scale bar 50 μm.

3 Discussion

Cancer is a leading cause of mortality worldwide, and advanced metastatic cancer is responsible for the majority of cancer-related deaths, causing an enormous burden to society and healthcare systems. The endothelium serves as a barrier between the circulation and the host tissue, but also actively responds and remodels the vascular environment during cancer cell colonisation and metastasis. A better understanding of the endothelial pathways activated during (pre-metastatic) niche formation will improve our understanding of metastatic colonisation and thus give a basis to improve therapies to achieve the long-term outcomes in patients with advanced cancer.

Here it was found that the endothelial Pim3 kinase regulates endothelial barrier integrity during early steps of cancer cell colonization of the lungs. Experiments using highly invasive B16-F10 murine melanoma model and single cell sequencing had revealed a new endothelial cell identity, the crECs that were enriched in metastatic lungs 6h after intravenous injection of cancer cells. In the pathway analysis, the Jak-Stat pathway emerged to be as one of the prominently upregulated pathways in the crECs. Among the top crEC marker genes and top differentially expressed gene were *Pim3, Bcl3* and *Inhbb*. Therefore, the upregulation of these marker genes was first validated using RT-qPCR in response to homing melanoma cells and using spontaneous metastasis models. Since, Pim3 has shown to be one of the downstream targets of the JAK-STAT pathway (Matikainen et al., 1999), Pim3 was chosen to be further analysed during thesis project. In cultured human endothelial cells, the selective pan-Pim kinase inhibitor AZD1208 reduced CDH5 in the cell-cell junctions and increased endothelial monolayer permeability in an electrical cell impedance assay, without affecting endothelial cell viability as measured using the Incucyte assay. Similar CDH5 reduction was seen in *PIM3* silenced cells, conforming the specificity of the pan-PIM inhibitor. Additionally, *PIM3* silencing decreased the expression of the CDH5 binding partners α-catenin, β-catenin and δ -catenin. In vivo, the administration of the pan-PIM inhibitor AZD-1208 prior to melanoma cell injection, increased melanoma metastasis and leakage in the murine lungs. This result was validated using additional mouse tumor models, including lung and breast cancer models.

3.1 Novelty and significance of the results relation to current knowledge

PIM kinases are well-known oncogenes. They have been intensively studied in cancer cells and their role in tumorigenesis by regulating pathways that enhance cancer cell survival and motility, metabolism, proliferation, and metastasis is well-established (Santio & Koskinen, 2017). The role of PIM kinases as oncogenes in tumorigenesis, but also their unique structure with a proline in the hinge region of the ATP-binding site (Dakin et al., 2012) not found in other kinases, has made PIM kinases an attractive

target for drug discovery in recent years. Unfortunately, the success has been less than expected, and all the clinical trials have been attired.

As already mentioned, the PIM kinase family includes three family members, PIM1, PIM2 and PIM3. All PIM kinases show similarities at the amino acid level, thus there have also been studies suggesting that they share some functional similarities (Mikkers et al., 2004). Despite these, PIM kinases have been shown to have differential tissue distribution as well as substrate specificity. Also, PIM kinases are expressed differently in malignancies, PIM1 and PIM2 being abundant in leukemias and lymphomas, whereas PIM3 is expressed at higher level in solid tumors and prostate cancer (Mumenthaler et al., 2009; Zheng et al., 2008). Some cancer cell lines, as our results indicated, are more sensitive to PIM inhibition induced cell death (*figure 9 b*). This is also evident from the literature where PIM3 inhibition has led to BAD-associated apoptosis in human pancreatic cell lines (Y. Y. Li et al., 2006) and PIM inhibitors have shown antitumor activity in cell-based assays or in animal models (N. Kirschner et al., 2015).

Despite these results using preclinical models, the results from clinical trials have been disappointing. Compound SG1-1776 from Astex Pharmaceuticals indicated for relapsed/refracted leukemia was withdrawn during phase 1 due to the cardiac QTc prolongation (NCT01239108). Also, studies with AZD1208, indicated for Acute Myelogenous Leukemia (ALM), was terminated after phase 1 due lack of efficacy (NCT01489722). Additionally, to their oncogenic role, PIM kinases have multiple physiological roles throughout the body. For example, PIM1 has been shown to have a cardioprotective role in the heart (Eichmann et al., 2000; Samse et al., 2016), thus some of the early phase clinical trials have been discontinued due to cardiotoxicity, as mentioned above. As observed for many kinase inhibitors, also PIM inhibitors inhibit all the PIM family members. Therefore, some of the unwanted adverse effects could be due to their polymorphic role in the body.

PIM kinases have been shown to be expressed in multiple cell types, including hematopoietic, vascular smooth muscle and neuronal cells, but also in endothelial cells. Pim1, but not Pim2 or Pim3 expression was upregulated in the lungs of endotoxin-induced mice, and the effect was mediated through ELK3 in pulmonary microvascular endothelial cells (Cao et al., 2021). In contrast, the role of PIM3 in endothelial cells is not known. Only few studies so far have been reported. It has been shown that cultured endothelial cells predominantly express PIM3 and that it plays a role in endothelial cell migration and the formation of endothelial tube-like structures (Zhang et al., 2009). In endothelial cells Akt positively regulates PIM1 but not PIM3 or PIM2. (Min et al., 2012). These results, together with a differential expression of PIM kinases in LPS -induced endotoxemia in the lungs, suggest that there are some PIM isoform specific as well as cell type specific differences in the PIM regulatory pathways. My results

show that Pim-3 has a role in maintaining endothelial cell integrity and inhibition of vascular permeability.

Additionally, it was found that PIM3 is upregulated by cancer cell -secreted factors in endothelial cells (*figure 3c, d*). Pathway analysis of DE genes in HUVECs in response to melanoma secretome after treatment with cancer cell conditioned medium, supports the role of the JAK-STAT pathway in crECs, specifically induced by tumor cell secreted factors. These results therefore reveal a novel role for endothelial Pim3 in maintaining the core adherens junction complexes in endothelial cell-cell junctions, and in maintaining junction integrity. Moreover, the results indicate that PIM3-mediated endothelial cell barrier enforcement is critical for forming the EC barrier, hindering tumor cell metastasis. Previously, the Jak-Stat pathway has been indicated in endothelial cells in lung metastasis (Transl et al., 2021), however, the downstream signals have not been characterized, and importantly endothelial Pim kinases have not been previously linked to tumor metastasis.

The results of this work suggest that although PIM inhibitors reduce tumour cell survival, they may also weaken the vascular barrier. This endothelial cell barrier impairment may result in increased tumor cell metastasis, as in our preclinical models. Thus, the results presented in this thesis may reveal a mechanism behind lack of efficacy of PIM inhibitors in cancer trials. The results emphasize the need to better understand the effects of cancer therapies on the host cells of the tumor microenvironment and metastatic niches in distant tissues. Although PIM sensitive tumors might be inhibited by the PIM inhibitor, resistant clones, potentially selected during therapy, might be more prone to initiate distant metastasis due to increased ability to cross the endothelial barrier.

3.2 Suitability of the models controlling method reliability

Experimental syngeneic metastasis models are commonly used in metastasis research. They give rapid and site-specific information of metastasis without the necessary use of immunocompromised mice, thereby considering the important inflammatory and immune system. For example, tail vein injection leads to the rapid formation of lung metastases, reflecting the trapping of cancer cells in the lung microvasculature. Despite the fast formation of metastasis, intravenous injection of cancer cells does not reflect the initial stages of cancer metastasis but rather the homing/colonization of cancer cells in the lungs, because of the large number of injected cancer cells (Gomez-Cuadrado et al., 2017). Spontaneous metastatic dissemination is an inefficient process and only a few cancer cells of the primary tumor, which have the metastatic potential, eventually form distant metastasis, due to their eradication in the vasculature. To recapitulate the entire metastatic process, the upregulation of crEC marker genes were validated, by using spontaneous models, where the orthotopically and subcutaneously injected cancer cells grew and formed tumors.

Since preclinical vertebrate animal models share many anatomical similarities with human and rodents and are easily genetically modified, they provide models that can be used to investigate pathological conditions seen in humans and are therefore a valuable tool for basic research. The use of laboratory animals also raises some ethical and moral issues, and their use is carefully considered and limited to research questions when the benefits outweigh the harm and suffering caused, as evaluated, and authorized by the Project Authorisation Board. Therefore, to follow the three R's principle to reduce, replace and refine the animals used during research, cultured endothelial cells as well as a tumor cell-endothelial cell co-culture model to further investigate the role of Pim3 were used.

The PIM inhibitor AZD-1208 inhibits selectively all the PIM kinases (Dakin et al., 2012). Although cultured endothelial cells predominantly express PIM3 (*figure 5e*) and therefore AZD-1208 inhibition would mainly occur via PIM3 in endothelial cells, the PIM3 were specifically targeted by using two distinct clones for shRNA silencing to control potential off-target effects. Accordingly, the shRNA efficiency using both *PIM3* targeting shRNA clones was effective (*figure 5d*). Additionally, silencing PIM3 allowed for controlling the effect of the 2.5% serum starvation used during AZD treatment in endothelial cells. Thus, it is known that prolonged exposure to serum starvation reduces basal cellular activity and affects intracellular pathways, in a cell line-dependent manner. Despite this, no increase in apoptosis was observed in the assays prior to 24 hours of serum starvation during AZD-1208 treatment (*figure 4e*), indicating that cell viability in endothelial cells is not dramatically affected by such conditions.

In vitro cell cultures have some limitations, such as culture of cells as a 2-dimensional (2D) monolayer or co-culture, which cannot fully recapitulate the complex interactions between cells living naturally in tissues. There is no structural support from the basement membrane and tissues underneath, also artificial substrates like polymers are foreign to endothelial cells which inevitably affects the morphology and function of the cells (Kapałczyńska et al., 2018). Thus, cell cultures have been shown to affect events like cell signaling and changes in cell internal structures. The use of two different endothelial cell lines gives more reliable and reproducible results of the role of PIM3, even though none of the cell lines used were of lung origin. Consequently, due to disadvantages that come from using cell culture, the loss of CDH5 in cell-cell junctions after PIM-inhibition was also reproduced in mouse lungs (results not shown here). To overcome the problems that arise from using a planar cell model, the use of for example trans well assay, would have given more insight how the cancer cells cross the endothelial cell layer during Pim inhibition.

3.3 Future perspective

The JAK-STAT pathway was among the significantly upregulated pathways in KEGG and Hallmark pathway analysis of transcriptome-wide scRNAseq of ECs from metastatic lungs and bulk RNAseq analysis of melanoma cell conditioned HUVECs. PIM kinases are recognized as downstream targets of signalling pathways initiated by cytokines, which activate Janus kinases (JAKs). This activation subsequently leads to the phosphorylation of a transcription activators (STATs), which directly regulate the transcription of PIM kinases (Hammarén et al., 2019).The activation of Jak/Stat pathway has been reported in endothelial cells, where the cytokines, like IL6, downregulate the junctional localization of CDH5 resulting in increased endothelial permeability, by phosphorylating the STAT3 by JAKS (Alsaffar et al., 2020). Mechanistically, STAT3 activation has been shown to lead to upregulation of endothelial cell adhesion molecules (Kim et al., 2017).

During the CellChat analysis, VEFG and DLL4- were found to be upregulated in crECs (unpublished results from the host lab). VEGF and Notch-pathway are common regulators of tumor angiogenesis, vascular permeability, tube formation and vascular sprouting (Z. L. Liu et al., 2023; Saharinen et al., 2011). Previously, PIM3 has been shown to phosphorylate NOTCH1, leading its increased activity and nuclear localisation (Santio et al., 2016). Additionally, the activation of DLL4-Notch pathway supports the integrity of endothelial cell-cell contacts and is therefore essential for endothelial barrier function. Thus, activation of DLL4-Notch leads to increased lung metastasis possibly due an increased endothelial VCAM1 expression (Wieland et al., 2017). Notch is also a known oncogene that is upregulated in many cancers and the pathway also plays a role during metastatic colonization (Zhou et al., 2022). Notch signaling is also involved during cancer cell extravasation across the endothelial cell layer. The suppression of Notch activity resulted in reduced colon cancer cell transendothelial migration by decreasing the Dll4-Notch interaction between endothelial cells and colon cancer cells. Additionally, Notch inactivation in cancer cells led to decreased lung metastasis (Sonoshita et al., 2011). Increased Notch activation enhances tumor cell invasion and migration by upregulating endothelial DLL4, leading to elevated expression of MMP9 in tumor cells (Huang et al., 2014). Therefore, endothelial Notch-DLL4 pathway has been connected to events related to metastasis. Consequently, Notch may be an endothelial downstream target of PIM3 or an important co-regulator of PIM3 in maintaining barrier integrity.

VEGF has been shown to induce Dll4 and activate Notch signaling in a paracrine fashion during angiogenesis (Hellström, 2007). Additionally, VEGFA increases lung vascular leakage via phosphorylation of its receptor VEGFR2, by activating c-Scr in a WT mice compared to knock in mice that have PY949 substitution in their VEGFR2 receptor. WT mice also have an increased incidence of lung metastases (X. Li et al., 2016). VEGF-induced vascular leakage has been shown to be related on opening of adherens junctions in the endothelial cell-cell junctions. The opening of an endothelial barrier

is regulated by increasing the phosphorylation of CDH5 via the activation of several protein kinases, of which the protein kinase Scr is a known regulator (Konstantoulaki et al. 2003; Chenetal. 2012). VEGF-mediated increased lung permeability has also been related to upregulation of endothelial cell focal adhesion kinases and E-selectin in endothelial cells, which would lead to increased cancer cell homing in the lungs (Hiratsuka et al., 2011). VEGFA has also role in activating (pre-metastatic) niche promoting chemokines; CCL2 was upregulated both in the stromal and tumor cells leading to recruitment of tumor associated macrophages and increased extravasation via activation of VEGF pathway in macrophages (B. Z. Qian et al., 2011). Therefore, also the VEGF-pathway has been shown to enhance vascular permeability through endothelial cell junctions. However, whether the VEGF signaling pathway is related to PIM3 is still unknown. To summarize the results, our findings suggest that the JAK-STAT-PIM3 pathway could provide the negative feedback loop, to control endothelial cell permeability. However, the exact mechanism and pathways through which PIM3 inhibition leads to loss of barrier integrity, remains to be determined.

Interestingly, in addition to the upregulation of crEC marker genes by the HUVEC + B16-F10 co-culture, the results from endothelial cells cultured with cancer cell-conditioned medium suggested that *PIM3* is upregulated by soluble factors derived from cancer cells, without the need for a direct contact between the cancer and the endothelial cells. This result is interesting in the context of metastatic niche formation, where many primary tumor secreted as well as disseminating cancer cell secreted factors have been reported to be involved. There is evidence that cancer cells secrete angiogenic factors that reprogram endothelial cells, which might have metastasis promoting or even inhibitory effects (Preuss et al., 2024). Previously, it has been shown that melanoma cells secrete factors that disturb brain endothelial cell function measured by ECIS (Anchan et al., 2020). Using proteomic approaches, they identified 15 soluble proteins secreted by melanoma cells that disrupted the function of brain endothelial cells. When administered individually, only two of the proteins, TGFβ and ANGPTL-4 c-terminal fragment caused a loss of barrier function. Thus, this study highlighted that the cancer cells could secrete multiple cytokines and factors that might have possible synergistic or combinatorial effects on the vascular barrier function. Additionally, barrier disruption could also be mediated via a myriad of other factors including proteases or lipid mediators (Anchan et al., 2020). However, identifying the cancer cell related factors that regulate PIM expression might give more insight how PIM3 upregulation is mediated, and thus, more knowledge of the metastatic niche formation.

3.4 Final conclusion

In 1889 Steven Paget published his seed and soil hypothesis, which was based on as idea that metastasis would be a result of a primary tumor secreting cancer cell with metastatic properties that land onto a congenial niche referring to any tissue that provides suitable environment for cancer cells to grow.

Extensive studies in recent decades have provided increasing understanding of the metastatic process. Metastasis is understood to be initiated by cancer cells (MIC) with a stem-like and immune evasion properties that disseminate to structurally available tissue with a proper tissue morphology. However, there is increasing evidence of factors that during distant metastasis dynamically interact with the host microenvironment to create the niche that supports the metastatic colonization and outgrowth. As the metastatic cancer is one of the leading causes of death worldwide, understanding the pathways that are activated during metastatic dissemination and niche formation, is crucial to be able to improve cancer therapies. My result reveals the role of PIM3 in the vascular niche, maintaining vascular integrity, with an anti-metastatic function, while also highlighting the importance of adherens junction integrity to prevent metastasis.

4 Materials and methods

4.1 Reagents and cell culture

The Mus musculus skin melanoma cell line B16-F10, Lewis Lung Carcinoma LLC1, mammary gland carcinoma cell line 4T1 (American Type Culture Collection, ATCC®CRL-6475TM, CRL-2539TM and CRL-1642TM Manassas, VA, USA) and B16-F10-eGFP-Puro (CL053, Imanis Life Sciences, Rochester, MN, USA) were cultured in Dulbecco's modified Eagle medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum, 50 µg/ml of streptomycin, 50 U/ml of penicillin and 2 mM L-glutamin. Human umbilical vein endothelial cells (HUVEC) (200P-05N, Merck, Kenilworth, NJ, USA) and HMVEC-dBlAd -Neonatal Human Dermal Microvascular Endothelial Cells (blood endothelial cells, BEC) (#CC-2543, Lonza) were cultured in EBM™-2 Endothelial Cell Growth Basal Medium-2 with EGM™-2 MV Microvascular Endothelial SingleQuots™ Kit (#00190860 and #CC-4147, Lonza). For coating, either gelatin 0.1% (HUVECs) or fibronectin 1 µg mL-1 in PBS (BECs) was used. Cells were detached by using Trypsin-EDTA (BE17-161E, Lonza). For cell experiments Pan-PIM inhibitor AZD-1208 (#SML2595-5MG, Sigma-Aldrich, St.Louis, MO, USA) was diluted in DMSO as a final DMSO concentration of 0.1%.

4.2 Immunofluorescence staining

Endothelial cells (180 000-220 000) were plated in a 6-well plate with cover glasses on the bottom and let to grow for 24h. The medium was then changed to 2.5% starvation media, followed by treatments 2 hours later. Cells were stimulated for 24h and fixed in a 4% PFA in RT 10-15 min, washed in PBS 5-10 min x3, permeabilized in 0.1% Tx100-PBS in 5 min, blocked in 1% BSA-PBS 10 min, followed by staining in primary antibody in 1% BSA-PBS 40-60 min, washed in PBS x3, blocked again with a 1% BSA-PBS and stained with a secondary antibodies and phalloidin, washed in PBS three times and mounted with DAPI-mounting medium (ab104139, ThermoFisher Scientific). Primary antibodies were used in 1:100 to 1:300. The following antibodies were used: mouse anti-human VE-cadherin (BD Biosciences, 555289), α-catenin (a kind gift from Akira Nagafuchi, Nara Medical University), b-catenin (D10A8) (Cell signaling #8480), d-catenin (D752M) (Cell Signaling Technology, 59854), Vimentin (V9) (Santa Cruz Biotechnology, Sc6260), Pecam1 (Millipore, MAB1398), Pmel (Abcam, Ab137078), PIM3 (Cell signaling, #4165). For secondary antibodies, Donkey anti-mouse AlexaFluor® 594, Donkey anti-rabbit AlexaFluor® 488, Goat anti-armenian hamster Alexa Fluore 647 (Jackson Immunoresearch, 127-605-160), Goat anti-rabbit IgG HRP (GE Healthcare 1:4000) were used 1:300, if not stated otherwise.

4.3 Electrical cell impedance assay (ECIS)

Cells were plated on E-plates (Applied BioPhysics Inc, San Diego, CA, USA), coated with an ECM protein (10ug/ml fibronectin or 0.1% gelatin in PBS). Followed by incubation at 37 °C for 1 h, rinsed with a sterile ddH2O three times, incubated with a sterile 10mM cysteine solution in RT for 30 min and rinsed with ddH2O three times. Cells were allowed to settle for 10 min after plating and let to attach around 48h after changing complete medium for 2.5% FBS medium. The treatments were added after the frequency was balanced. Frequencies 500 Hz, 1000 Hz, 2000 Hz, 4000 Hz, 8000 Hz, 16000 Hz, 32000 Hz and 64000 Hz were used.

4.4 shRNA silencing of cells

HEK293FT cells were transfected with two different *shPIM3* clones; TRCN0000037414: CGCCTGTCAGAAGATGAACAT and TRCN0000037416: CGTGCTTCTCTACGATATGGT (from Sigma Aldrich MISSION shRNA library, distributed by Genome Biology Unit core facility supported by HiLIFE and the Faculty of Medicine, University of Helsinki, and Biocenter Finland) in the presence of a packaging plasmid pCMVg and pCMV delta8.9. As a control, HEK293FT cells were transfected with a MISSION® pLKO.1-puro Empty Vector Control Plasmid DNA (SHC001). Endothelial cells were transduced for 5 h with shRNA lentiviral vectors and 0.1% polybren (Sigma-Aldrich), followed by addition of 2x complete medium, change of media the next day and analysis after 72h.

4.5 Fluorescence microscopy and image quantification

For cell and tissue imaging, Axio Imager.Z2 upright epifluorescence wide-field microscope with Zen 3.1 (blue edition) software (Zeiss, Oberkochen, Germany), Zeiss LSM880, and Pannoramic 250 FLASH II Digital Slide Scanner (3D Histech, Budapest, Hungary) at the Biomedicum Imaging unit and Genome Biology Unit, University of Helsinki, were used. 40x or 60x oil objectives were used for confocal imaging. 10x, 20x and 40x air objectives were used for whole tissue and cell imaging. Image analysis was performed using ImageJ/Fiji (1.48 s, Fiji, Wayne Rashband, National Institutes of Health, Bethesda, MD, USA) image processing software. In vitro CDH5 average signal intensity was analysed/ field and was calculated from five maximum intensity projections / sample after thresholding and results were normalized to the number of nuclei. All intensity analyses were performed on original images, either with similar brightness and contrast settings or on original images taken with the same exposure and intensity settings using ImageJ.

4.6 RNA isolation and quantitative real time PCR

Blood was collected from the heart of mice using a MiniCollect® TUBEs (1 ml K3E K3EDTA, Greiner Bio-One, Kremsmünster, Austria). For RNA isolation, NucleoSpin® RNA Blood (Macherey-Nagel, Düren, Germany) were used.
RNA from cultured cells was isolated by using NucleoSpin RNA kit (Macherey-Nagel). For cDNA synthesis, SensiFAST cDNA Synthesis Kit (Bioline, London, UK) was used. For real time quantitative PCR the DyNAmo HS SYBR Green master mix (Thermo Scientific) and the BIO-RAD C1000 Theram cycler (Bio-Rad Laboratories) was used.

Table 1. Primers for RT-qPCR.

List of human and mouse primers.

Target	Forward (5'>3')	Reverse (5'>3')
Pim3	AAGCTCATCGACTTCGGCTC	CAGGGAGAGACACCACTCAATAAG
Bcl3	AGGGACCTTTGATGCCCATTTA	CTGAGTATTCGGTAGACAGCGG
Inhbb	CAGGTGGAATGTGGTGGAGAA	CGATGAGCCGAAAGTCGATGA
PIM3	AAAATCTGCTTGTGGACCTGC	GATCTCCTCGTCCTGCTCGAA
INHBB	ACTCAACCTAGACGTGCAGTG	GGGTGCTATGATCCAGTCGTT
Mlna	TCCTGGGGATTGCTCTGCTT	CGGGCTGATGGGATTTCTCT

4.7 Bulk RNA sequencing and analysis

HUVECs were cultured for 16 h in conditioned medium from 48 h B16-F10 melanoma cell cultures or empty plates (n=4). After RNA isolation samples were sent to Novogene (London, UK) for sequencing. Data analysis for DE genes and pathways were performed by Chipster and DESeq2 (v3.18) and GSEA software (v4.3.2). Reads were aligned to GRCh38.p12 using HISAT (v2.2.1), default setting with soft clipping turned on. Mapped reads were counted using HTSeq (v2.0), reference genome GRCH38.109, default settings. Principal component analysis (PCA) was performed for sample counts. Control samples and treated samples arranged spatially in to two different groups, indicating no intra condition variability. Differentially expressed genes were analysed using DESeq2 (v3.18), default settings with cutoff value 0.05 for Benjamini-Hochberg adjusted p-values. Gene set enrichment analysis (GSEA) was performed using GSEA software (v4.3.2). For GSEA analysis, The Molecular Signatures Database (MSigDB) hallmark gene set collection and Kyoto Encyclopedia of genes and genomes (KEGG) pathway database were used. Enriched pathways with FDR < 25% were considered significant.

4.8 Endothelial cell culture in cancer cell conditioned medium

50 000 HUVECs were plated on 24-well plate and let to form monolayer for 24 h. Subsequently, cancer cell conditioned medium or no cell conditioned medium were added on top of the cells for different time points. For preparing the conditioned medium either B16-F10 melanoma cells, 4T1 or LLCs were plated on 5 cm plates in 2 ml of endothelial cell medium for 48 h. Medium was centrifuged for 10 min prior to adding it to endothelial cells. Endothelial cell medium was plated on an empty 5 cm plate (no cell control) for 48h prior adding it onto ECs.

4.9 Endothelial cell-cancer cell co-culture

180 000-200 000 HUVECs were plated on a 6-well plate well and let to form a monolayer. Subsequently, 50 000 of GFP-expressing B16-F10 cancer cell were added on top of the monolayer for 16 h. Cells were detached by using Trypsin-EDTA followed by sorting using BD InfluxTM (BD Biosciences, Franklin Lakes, NJ, USA).

4.10 Cell confluency and apoptosis assay

5000-8000 endothelial cells were plated in 96-well plate with IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent 1:1000 (Essen Biosciences) and results analysed with Incucyte S3 (Essen Bioscience, Ann Arbor, MI, USA).

4.11 Western blotting

Endothelial cells were lysed in IP lysis buffer (50mM Tris, pH 7,4, 150mM NaCl, 1% NP40) with protease and phosphatase inhibitors Na_3VO_4 and NaF. BCA protein assay was used to measure protein concentration. 10 µg of protein was loaded/ well in SDS-PAGE and transferred on PVDV membrane. Western blotting signal was imaged using the Odyssey CLx near-infrared fluorescence imaging system (LI-COR Biosciences, Lincoln, NE, USA). Signal intensity was analyzed using Fiji (1.48 s, Fiji, Wayne Rashband, National Institutes of Health, Bethesda, MD, USA).

4.12 Tissue handling

For cryo sections, tissues were fixed in 4% PFA o/n at +4°C, washed in PBS 5 x 15 min and treated with 30% sucrose o/n at +4°C. Samples were casted into Tissue-Tek Cryomolds (Sakura Finetek, Torrance, CA, USA). Prior to staining, 7 µm sections were rehydrated in 0.3% Triton-PBS for 5 min and blocked in 5% BSA, 5% Donkey serum in 0.1% Triton - PBS for 1 h at RT. Incubations using primary and secondary antibodies were performed in the blocking buffer. Primary antibodies incubated o/n at +4°C and secondary antibodies at RT for 1 h. After incubation sections were washed 4 x 10 min

with 0.3% Triton-PBS. After secondary antibody incubation, sections were fixed with 4% PFA for 10 min, washed 2x5 min in PBS and mounted with DAPI-mounting medium (Thermo Fisher Scientific).

For Paraffin sections, 5 µm sections were cut form paraffin embedded blocks for Hematxylin & Eosin (H&E) staining. Basic protocols for paraffin embedding and H&E staining were followed.

4.13 Animal experiments and licenses

All animal experimental procedures were approved by the Project Authorization Board, Regional State Administrative Agency for Southern Finland, under lisence ESAVI/4975/2019 - osahanke 2. Mice were housed in individually ventilated cages according to the guidelines of the Federation of European Laboratory Animal Science Associations. B16-F10, LLC1 or 4T1 cell suspension (200 000 cells per 100 µl) in PBS were injected via the tail vein to the C57BL/6JRj or BALB/c female mouse (Janvier labs, Le Genest-Saint-Isle, France). PBS in similar volume were injected as a control. For subcutaneous injection, 200 000 B16-F10 cells/mice were injected in the neck of 8-10-week-old C57BL/6JRj male mice. For orthotopic inoculations, 200 000 4T1 cells per 100 µl were injected into the fourth mammary fat pad per each BALB/c female mouse. Tumor width and length was measured by caliper and the tumor volume estimated by the formula: Volume = (width)2 x length/2.

The pan-PIM inhibitor AZD-1208 30 mg.kg-1 in 10% DMSO, 40% PEG300, 5% Tween-80, PBS (100 µl per mouse) or the DMSO (diluted respectively) were orally administered. For analysis of leakage, 1 mg of Dextran (Texas RedTM, 70 000 MW, Lysine Fixable, Thermo Fischer Scientific) in 100 ul of PBS per mouse was i.v. injected 10 min prior to sacrifice as previously described (Hakanpaa et al., 2018). Photographs of organs and tumors were taken by mobile phone.

4.14 Statistical analysis and illustrations

GraphPad Prism software (version 9.2.0, GraphPad Software, LLC, San Diego, CA, USA) was used for statistical analysis of other than RNAseq data. Two sample comparison was performed by two-tailed Student´s t-test. For larger data sets, one-way Anova variance analysis was performed to utilize multiple comparison by comparing the mean of each column with the mean of a control column. Sample number can be visualized from the individually shown dots in the graph bars or is stated in the figure legends. In vitro experiments were performed a minimum of three times, each dots represents one sample. In in vivo experiments one dot represents one animal. Illustrations were created by BioRender.com. Image analysis was performed as described above using Image J.

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