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Lymphatic endothelial cells as a pro-metastatic component of the melanoma microenvironment

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To my loved ones

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Abstract

The tumor microenvironment (TME) has a tremendous impact on cancer progression, and studies of cancer communication with the TME can provide new avenues for combinatorial cancer treatments. Tumor associated lymphatic vasculature is well acknowledged for its role in mediating cancer cell dissemination to distant sites. However, lymphatic vasculature does not only act as a passive route for cancer metastasis. For example, although melanoma cell dissemination to the draining lymph nodes is used as a prognostic factor, lymph node removal does not improve the outcome of the patients, suggesting that lymphatic vasculature actively drives melanoma aggressive characteristics already in the primary tumor. The purpose of this thesis was to characterize the active, cancer-promoting crosstalk between melanoma and lymphatic endothelial cells (LECs), the crucial component of the lymphatic vasculature.

We found that the melanoma cell-LEC crosstalk changes the biological properties in both cell types. In vitro, melanoma cells originating from the melanoma metastases became more invasive upon LEC co-culture, whereas LECs started sprouting when cultured as spheroids in a 3D fibrin matrix and showed impaired tube formation and barrier function. An in vivo mouse xenograft assay indicated that melanoma cells originating from metastasis formed significantly more metastases to liver and lung when pre-cultured with LECs compared to the melanoma cells from a parental monotypic culture. Mechanistically, we found that the LEC co-culture induced a change in the MMP14 localization in melanoma cells from the cytosol to the plasma membrane, and that MMP14 mediated Notch3 induction and beta 1 integrin activation in melanoma cells. We showed that all three proteins were required for the more invasive phenotype of melanoma cells, and MMP14 and Notch3 were required for the increased metastasis of melanoma cells as shown using a zebrafish embryo xenograft model. We further showed that Notch3 on the melanoma cell surface is activated by the Notch ligand DLL4, a ligand abundantly expressed by LECs, and that the activated Notch3 induces WNT5B transcription in melanoma cells. WNT5B, in turn, contributes to the functional changes that melanoma cells cause in LECs. In a publicly available melanoma patient dataset, high Notch3 expression in melanoma samples was found to correlate with worse prognosis, and Notch3 and WNT5B expression coincided in these samples. When primary and metastasis samples in a cohort of 31 melanoma patients were analyzed by IHC, the co-localization of Notch3 and WNT5B was found to be significantly higher in the metastasis samples if compared to the primary tumor, further supporting that the co-expression of Notch3 and WNT5B is characteristic for the aggressive melanoma type. In a mouse ear xenograft model, WNT5B expression in melanoma cells enhanced their metastasis to the draining lymph nodes. These results provide evidence for the bi-directional, active melanoma cell-LEC communication and represent possible leads to targets of new combinatorial treatment options.

Tiivistelmä

Syöpää ympäröivällä kudoksella, niin kutsutulla syövän mikroympäristöllä, on merkittävä vaikutus syövän kehittymiselle ja sairauden etenemiselle, minkä vuoksi syövän mikroympäristön tutkiminen voi avata ovia uusille, tehokkaille yhdistelmähoidoille. Syövän mikroympäristöön kuuluvan imusuoniston tiedetään olevan tärkeä väylä syövän leviämiselle ja etäpesäkkeiden muodostumiselle, mutta imusuonisto ei toimi ainoastaan passiivisena kulkureittinä syöpäsoluille. Esimerkiksi, vaikka melanooman diagnostiikassa syöpäsolujen löytyminen vartijaimusolmukkeista korreloi huonomman ennusteen kanssa, metastaattisten vartijaimusolmukkeiden poisto ei kuitenkaan paranna potilaiden ennustetta. Tämä viittaa siihen, että imusuonisto saa aikaan aggressiivisia muutoksia melanoomasoluissa jo primaarikasvaimessa. Tämän työn tarkoituksena oli tutkia aktiivista, melanooman leviämiseen johtavaa vuorovaikutusta melanoomasolujen ja imusuoniston endoteelisolujen, eli imusuoniston seinämän muodostavien solujen, välillä.

Työni osoitti, että melanoomasolujen ja imusuoniston endoteelisolujen vuorovaikutus aiheuttaa molekulaarisia ja toiminnallisia muutoksia molemmissa. Työssäni näytin, että metastaaseista lähtöisin olevat melanoomasolulinjat tulevat invasiivisemmiksi ja metastasoivat herkemmin, kun niitä kasvatetaan yhdessä imusuoniston endoteelisolujen kanssa. Vastavuoroisesti melanoomasolut aiheuttavat muutoksia endoteelisolujen kasvutavassa 3-ulotteisissa soluviljelmissä sekä häiriöitä niiden muodostaman solukerroksen rakenteessa ja integriteetissä. Näiden muutosten seurauksena imusuonistosta voi tulla läpäisevämpi, mikä edesauttaa syöpäsolujen siirtymistä primaarikasvaimesta imusuonistoon ja sieltä kauempana sijaitseviin elimiin.

Väitöskirjatyössäni osoitin myös, että imusuoniston endoteelisolut saavat aikaan MMP14:n siirtymisen melanoomasolun sytoplasmasta solun plasmamembraanille, ja että tämä saa aikaan Notch3:n indusoitumisen ja beta 1 integriinin aktivoitumisen melanoomasoluissa. Näytin työssäni, että kaikki kolme proteiinia ovat tärkeitä melanooman lisääntyneelle invaasiolle, ja seeprakalan alkiota hyödyntävässä kokeessa osoitin, että MMP14 ja Notch3 tarvitaan melanooman aggressiivisempaan metastaasiaktiivisuuteen. Notch-ligandi DLL4, jota on runsaasti imusuoniston endoteelisolujen pinnalla, saa aikaan Notch3-reseptorin aktivaation melanoomasolussa. Aktivoitu Notch3 lisäsi WNT5B:n transkriptiota melanoomasoluissa, ja WNT5B puolestaan oli osallisena melanooman aikaansaamiin biologisiin muutoksiin imusuoniston endoteelisoluissa. Julkisesti saatavilla olevan melanoomakohortin analyysi osoitti, että korkea Notch3:n ilmentyminen melanoomakasvaimissa korreloi huonon ennusteen kanssa, ja lisäksi Notch3:n ja WNT5B:n ilmentyminen korreloi keskenään. Näytteet sellaisesta melanoomakohortista, johon oli saatavilla näytteet sekä primaarikasvaimesta että metastaaseista, osoitti, että Notch3 ja WNT5B lokalisoituvat yhdessä erityisesti metastaasinäytteissä, viitaten siihen, että molemmat proteiinit ilmentyvät yhdessä erityisesti aggressiivisissa melanoomasolupopulaatioissa. Lopuksi osoitin, että WNT5B edistää melanooman metastasointia vartijaimusolmukkeisiin hiirimallissa. Työni tulokset selvittivät mekanismeja melanooman ja imusuoniston kaksisuuntaisesta, aktiivisesta vuorovaikutuksesta, ja voivat auttaa löytämään potentiaalisia kohteita uusille melanooman yhdistelmähoidoille.

Original publications

This thesis is based on the following original publications, which are referred to in the text by the following Roman numerals:

I. Pekkonen, P.*, **Alve, S.*,** Balistreri, G., Gramolelli, S., Tatti-Bugaeva, O., Paatero, I., Niiranen, O., Tuohinto, K., Perala, N., Taiwo, A., Zinovkina, N., Repo, P., Icay, K., Ivaska, J., Saharinen, P., Hautaniemi, S., Lehti, K. and Ojala, P. M. Lymphatic endothelium stimulates melanoma metastasisand invasion via MMP14-dependent Notch3 and beta1-integrin activation. eLife 7. 2018. *equal contribution.

II. **Alve, S.**, Gramolelli, S., Jukonen, J., Juteau, S., Pink, A., Manninen, A.A., Hänninen, S., Monto, E., Lackman, M.H., Carpén, O., Saharinen, P., Karaman, S., Vaahtomeri, K., Ojala, P.M. DLL4-Notch3-WNT5B axis mediates bi-directional pro-metastatic crosstalk between melanoma and lymphatic endothelial cells. JCI Insight 2023.

Abbreviations

3D	three-dimensional
ADAM	a disintegrin and metalloproteinase
AKT	protein kinase B
BEC	blood endothelial cell
CAF	cancer associated fibroblast
CBF1	C-promote binding factor 1
CCL/R	CC chemokine ligand/receptor
CD	cluster of differentiation
COUP-TFII	chicken ovalbumin upstream promoter transcription factor II
CT	computed tomography
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DKK	Dickkopf
DLL	delta like ligand
DVL	Dishevelled
E-cadherin	epithelial cadherin
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal-transition
EndMT	endothelial-to-mesenchymal-transition
ERK	extracellular signal-regulated kinase
FZD	frizzled
GFP	green fluorescent protein
HES	hairy enhancer of split
HEY	hairy/enhancer-of-split related with YRPW motif protein
HIF-1	hypoxia inducible factor 1
HUVEC	human umbilical endothelial cell
ICAM	intercellular adhesion molecule
JAG	Jagged
JNK	c-Jun N-terminal kinase
LAG3	Lymphocyte-activation gene 3
LEC	lymphatic endothelial cell
LPS	lipopolysaccharide
LRP	lipoprotein receptor-related protein
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
MAML	mammalian mastermind-like
MAPK	mitogen-activated protein kinase
MITF	Microphthalmia-associated transcription factor

MMP	matrix metalloproteinase
mTORC	mammalian target of rapamycin complex
N-cadherin	neural cadherin
NECD	Notch extracellular domain
NGF	neural growth factor
NICD	Notch intracellular domain
NICD	Notch intracellular domain
NLS	nuclear localization sequence
NRP	neuropilin
PD-1	programmed cell death protein 1
PDGF-B	platelet-derived growth factor B
PEST	proline/glutamic acid/serine/threonine-rich sequence
PET-CT	Positron emission tomography–computed tomography
PI3K	Phosphoinositide 3-kinase
PIK3	Phosphoinositide 3-kinases
PROX1	prospero homeobox protein 1
PTEN	Phosphatase and tensin homolog
RAM	RBPjk association domain
RBPjk	recombination binding protein for immunoglobulin kappa J
RNA	ribonucleic acid
ROS	reactive oxygen species
sFZD	secreted Frizzled-related protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIRT1	sirtuin 1
SLUG	snail family transcriptional repressor 2
SNAIL	snail family transcriptional repressor 1
SOX18	sex determining region Y box 18
Sp1	specificity protein 1
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF-β	transforming growth factor β
Tks	tyrosine kinase substrate
TLR	Toll-like receptor
TME	tumor microenvironment
UV	ultraviolet
VCAM	vascular adhesion molecule
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VLA	very late antigen
WHO	World Health Organization

WIF1	WNT inhibitory factor 1
WNT/PCP	planar cell polarity/WNT pathway
ZEB	zinc finger E-box binding homeobox
ZO-1	Zona occludens, tight junction protein 1

Introduction

Lymphatic vasculature plays an essential role in maintaining tissue fluid balance and residing and trafficking immune cells. When compared to the blood vasculature, the lymphatic capillary walls are more permeable, and in addition, the fluid pressure is lower in the lymphatic vasculature and the environment is less oxidizing, making lymphatic vessels an efficient route for cancer cell dissemination. The role of the lymphatic vasculature in metastasis of solid cancers is supported by observations that high density of the lymphatic vasculature and cancer cell metastasis to the tumor draining lymph node correlate with worse prognosis (Dieterich et al., 2022). However, lymphatic vasculature does not only act as a passive route for cancer cell dissemination but there is active, cancer promoting molecular crosstalk between the two. Although the significance of the lymphatic system for cancer cell spreading is well acknowledged, the molecular events mediating the cancer cell interaction with the lymphatic vasculature and their contribution to cancer progression are not yet completely understood.

Melanoma, like many other solid cancers, often disseminates via the lymphatic vasculature. In this study I have characterized molecular events mediating the cancer promoting crosstalk with the lymphatic endothelial cells (LECs) using melanoma cell-LEC co-cultures as a model. I identified functional changes in both cell types and molecular events mediating these changes. The results presented in this thesis will help to understand the mechanisms how melanoma cells gain more aggressive properties through their communication with LECs and how melanoma cells modify the LEC functional properties for more efficient cancer cell metastasis.

Review of the literature

1 Cancer – a disease of dysregulated cell-environment communication

All cells of the multicellular organisms are strictly controlled regarding their metabolic pathways, proliferation, growth, apoptosis and state of differentiation or stemness. In cancer cells, these processes are no more controlled in normal ways, but instead they are unbalanced to maximize the cancer cell survival at the expense of the homeostasis of the tissue. Traditionally, cancer was viewed as a disease of overly active cell proliferation, initially caused by mutations in the genome. Although mutations causing overly active cell division is still a hallmark of cancer, there are multiple biological aspects to consider in cancer biology reviewed in (Hanahan, 2022; Hanahan & Weinberg, 2000, 2011), which do not only involve the cancer cells but the tissue surrounding the cancer as well. Instead of being only a clump of proliferating cells, cancer can be viewed as a disease of malfunctional cell and tissue regulation, with remarkable heterogeneity regarding the molecular and functional aspects observed in both the cancer cells and the cancer associated tissue (Dagogo-Jack & Shaw, 2018). The surrounding tissue associated with cancer, also called tumor microenvironment, displays aberrant biological and molecular functions as well, which contribute in multiple ways to cancer progression.

1.1. Tumor microenvironment (TME)

The tumor microenvironment (TME) consists of similar components that make up normal tissues; various tissue type specific cells, supporting cells such as fibroblasts, blood and lymphatic vasculature, immune cells, extracellular matrix (ECM, further discussed in section 1.2.1.) and soluble factors such as growth factors, cytokines, nutrients and oxygen. These can be roughly divided into cellular and non-cellular components of the TME.

While cancer very often modifies and educates the stromal cells to adopt tumor promoting roles, the role of immune cells can vary widely from tumor promoting to tumor suppressing. In many cancers, chronic inflammation is a common finding and considered as one of the cancer hallmarks. Inflammation induces accumulation and regulation of both adaptive and innate immune cells, which both have important functions in cancer progression. Innate immune cells such as macrophages, neutrophils, natural killer cells and dendritic cells can in their tumor promoting role, for instance, promote angiogenesis and lymphangiogenesis, modify extracellular matrix, stimulate cancer cell epithelial-to-mesenchymal-transition (EMT) switch (further introduced in section 1.2.) and suppress antitumor activity of other immune cells. In contrast, in their tumor suppressing roles, they can for example create an inflammatory environment that supports the antitumor activity of other immune cells, secrete toxic substances which kill cancer cells and present tumor specific antigens to boost adaptive immune cell cancer cell killing. Adaptive immune cells consist of T cells, which can be further classified as regulatory (CD4+) cytotoxic (CD8+) T cells and B cells, which all recognize specific antigens as their targets. Depending on the cell class, these cells can produce and release proteins that promote other cells in target killing or suppress the tissue damage caused by pathogens or directly kill the target cell (Gonzalez et al., 2018). During the recent years, remarkable progress has been made by utilizing either naturally occurring or genetically engineered patient derived T cells in cancer therapy, especially in hematogenous cancers (Waldman et al., 2020).

Stromal cell composition typically varies according to the cancer type and the site of the primary tumor but typically it consists of fibroblasts, adipocytes and endothelial cells. Moreover, there may be other tissue specific cell types. Just like immune cells, stromal cells can elicit either tumor promoting or tumor suppressing effects. Stromal cells are an important source of growth factors and cytokines that may promote cancer cell invasion and proliferation, angiogenesis, immunosuppression, and therapy resistance. For instance, cancer associated fibroblasts (CAFs) are a common component of the TME, but they show remarkable intratumoral heterogeneity (Ping et al., 2021). Tumor associated vasculature and endothelial cells in turn have a crucial role in mediating cancer cell dissemination, and my thesis will especially focus on the role of lymphatic vasculature and lymphatic endothelial cells upon cancer cell crosstalk.

1.1.1 Tumor vasculature and endothelial cells

Cancer cells rely on support and cues provided by the environment. Like normal cells, they need access to nutrients, oxygen, and maintenance of fluidic balance around the cells. Blood and lymphatic vasculature are involved in these events in

the TME. Research during the last decades has clearly indicated, that there is active communication between cancer cells and the blood and lymphatic vascular systems (Dieterich et al., 2022; Farnsworth et al., 2014). This communication leads to many changes in the vasculature, which gains multiple abnormal characteristics. Ultimately, the cancer cell communication with vascular systems allows cancer cell invasion into tumor draining lymphatic and blood vessels, dissemination of the cells from the primary tumor, and establishment of distant metastases.

The integral part of both the blood and lymphatic vasculature are the endothelial cells. These are cells that form the inner lining of the vascular wall facing the lumen. They have multiple important functions, which depend on the anatomical location and temporal changes in the endothelial cell environment. Endothelial cells typically control fluid balance between vascular lumen and underneath tissue, including regulation of passage of substances such as metabolites and peptides. Blood endothelial cells (BECs) in addition have a crucial role in mediating passage of glucose and oxygen to be used in underneath tissues. Both blood and lymphatic endothelial cells (LECs) respond to various stimuli in their environment. For instance, inflammatory factors induce changes in their surface protein expression, leading to alterations to endothelial cell contacts with each other and immune cells (Dieterich et al., 2022; Rajendran et al., 2013).

1.1.2 Lymphatic vasculature and system

Lymphatic vasculature functions in maintaining fluid balance, lipid absorption and immune cell trafficking. It collects interstitial fluid along with metabolites produced by cells from the body and returns it to the blood stream. The structure of lymphatic vasculature varies in various organs to best fit the function; for instance, in small intestine the lymphatic vasculature is arranged in each villus with blood capillaries for efficient uptake and transport of dietary lipids in the form of chylomicrons (Petrova & Koh, 2018). In contrast to blood vasculature, lymphatic vasculature does not form a circulatory network through which the lymph fluid flows, but instead, typically starts with blind-end capillaries which connect to larger vessels (Breslin et al., 2018). The smallest ends of the lymphatic vessels, referred to as lymphatic capillaries, are only formed of a layer of lymphatic endothelial cells, LECs, which are a characteristic component of all lymphatic structures. Lymphatic capillaries differ from blood capillaries in that LECs are loosely connected with each other by discontinuous junctions, allowing easy passage of fluids, macromolecules, and immune cells into the lymphatic vascular system. In addition, lymphatic capillaries have discontinuous basement membrane, and they lack pericytes and vascular smooth muscle cells (Breslin et al., 2018). The LECs in the lymphatic capillaries typically have an oak leaf like

shape and they are connected to each other with button-like junctions (Figure 1 A-B) (Norden & Kume, 2020). Lymphatic capillaries are connected to pre-collecting lymphatic vessels, in which the junctions between LECs are more abundant and there is a smooth muscle layer around the vessels to aid fluid flow. These pre-collecting vessels connect to larger connecting vessels. In addition of having relatively continuous basement membrane, covered by smooth muscle cells, collecting vessel lumens have valves to prevent fluid flow to wrong direction (Breslin et al., 2018). LECs in the collecting vessels have continuous zipper-like cell-cell junctions (Figure 1 A). Collecting lymph vessels eventually connect to the thoracic duct and the right lymphatic duct which allow discharging of lymphatic fluid into the veins.

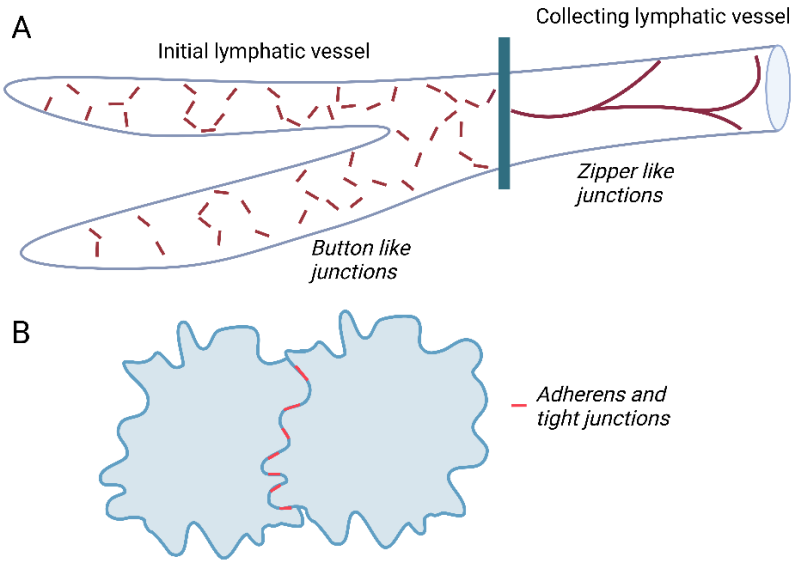

Figure 1. Arrangement of junctions between lymphatic endothelial cells in lymphatic capillaries into button like junctions (A-B) and continuous zipper like junctions in collecting lymphatic vessels (A). Figure created using BioRender.com.

In addition to the lymphatic vasculature, lymphatic system also consists of lymph nodes, which have a crucial role in the initiation and amplification of immune responses and filtering the stimuli that comes from the interstitial fluid and returns to blood circulation (Oliver et al., 2020). In addition to LECs, lymph nodes also consist of and harbor multiple other cell types, such as lymphocytes, myeloid cells and supporting stromal cells (Dieterich et al., 2022). Single cell RNA sequencing analyses have shown that lymph nodes harbor multiple different types of LECs and that these have distinct gene expression patterns from the peripheral lymphatic vessels LECs (Fujimoto et al., 2020; Takeda et al., 2019).

Since different parts of the lymphatic vasculature have distinct roles in collecting and transporting the lymphatic fluid, it is not surprising that the establishment of variable cell-cell connections is essential for the function of lymphatic system. Both the button and zipper-like junctions are mainly formed of adherens junctions, consisting of VE-cadherin, beta-catenin and p120-catenin, as well as tight junctions consisting of occluding, claudin-5 and ZO-1 (Zona Occludens-1) (Zhang et al., 2020). Both adherens and tight junctions are the best studied in the context of blood endothelial cells, and it remains to be determined whether similar functional and regulatory mechanisms apply in the context of LECs. In the adherens junctions, the transmembrane protein VE-cadherin (Vascular endothelial cadherin) mediates cell binding to the neighboring cell. In its cytoplasmic tail VE-cadherin harbors binding sites for different catenin proteins, which form a link to the actin cytoskeleton (Giannotta et al., 2013). In the context of BECs, tight junctions have been especially reported to mediate important barrier function role. For example, BECs in the blood brain barrier have an extraordinarily tightly regulated and relatively impermeable vascular wall that is characterized with abundant tight junctions (Stamatovic et al., 2008). Mouse studies have shown that LEC junctional proteins have an important role during the development of the lymphatic vasculature: for example, conditional deletion of VE-cadherin (Hagerling et al., 2018) or constitutive deletion of beta-catenin (Cha et al., 2016) in LECs results in severe defects in the lymphatic vasculature development, edema and prenatal death. Although the role of several proteins involved in these junctions are well characterized in the context of developing lymphatic vasculature, less is known how these junctions are maintained and regulated in adult tissues.

In addition to maintaining the fluid balance and trafficking of the immune cells, lymphatic vasculature is also an important source of signaling factors, referred to as lymphangiocrine signals, which mediate the development and maintenance of the surrounding tissues (Liu & Li, 2023). For instance, LEC derived reelin mediates the heart muscle cell growth during the development and heart repair upon myocardial infarction (Liu et al., 2020), and in intestine, R-Spondin 3(RSPO3) produced by LECs controls the intestinal stem cell population maintenance, proliferation and intestinal epithelium repair (Palikuqi et al., 2022).

1.1.3 Development of the lymphatic vasculature

The past work has revealed that there are several distinct cellular mechanisms during the lymphatic vasculature development, and these are widely accepted to complement each other.

The venous specification model implies that lymphatic vasculature arises from the early veins shortly after specification of arterial and venous endothelial linages. This was initially observed in studies with mammalian embryos in the early 20th century, in which ink injections to the superficial lymphatic vessels were traced to the ducts budding from the venous endothelium (Jafree et al., 2021). Later studies have indicated that the first lymphatic vessels arise from the cells of the anterior wall of the cardinal vein and the adjacent intersomitic veins that express prospero homeobox protein 1 (PROX1) (Wigle et al., 2002; Wigle & Oliver, 1999). Later on, also transcription factors chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (Lee et al., 2009) and sex determining region Y box 18 (SOX18) (Francois et al., 2008) have been shown to be expressed in this subpopulation of the cells, all of which in coordination with each other drive the lymphatic identity of these cells. This subset of cells also begins to express lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), which despite being a useful lymphatic marker does not seem to have any obvious function in the development of the lymphatic vasculature (Gale et al., 2007). Once the cell populations adopting the lymphatic vasculature fate have been established, the cells exit the cardinal vein and unite to form the first lymphatic vessels close to the cardinal vein (Francois et al., 2012).

During the next steps, new lymphatic vessels sprout from the already existing primary vessels in a process called lymphangiogenesis. Lymphangiogenesis is driven by a secreted vascular endothelial growth factor C (VEGF-C), expressed by the nearby mesenchymal cells, which binds to its cognate receptor VEGFR3 (vascular endothelial growth factor receptor 3) on the LEC surface (Karkkainen et al., 2004), promoting LEC survival, migration and proliferation (Makinen et al., 2001). In addition to VEGF-C, VEGF-D has been shown to bind to VEGFR3 to activate lymphagiogenesis but it seems to be mainly dispensable during development (Baldwin et al., 2005), and adult VEGF-D deficient mice show smaller dermal lymphatic capillaries with less efficient adsorbtion properties (Paquet-Fifield et al., 2013). The lymphangiogenic signaling is further supported by PROX1, that maintains VEGFR3 expression in LECs (Srinivasan et al., 2014), and by COUP-TFII, that induces the expression of VEGFR3 co-receptor Neuropilin 2 (NRP2) (Lin et al., 2010; Xu et al., 2010).

In addition to venous specification and lymphangiogenesis, lymphvasculogenesis is a complementary lymphatic vasculature formation mechanism in which the tissue residing LEC clusters proliferate and extend protrusions towards other LEC clusters or existing lymphatic vessels. During the developmental progression, the number of these clusters declines, suggesting that these clusters actively fuse to expand the tissue lymphatic network (Jafree et al., 2021). The molecular mechanisms mediating lymphvasculogenesis still require further investigation. In addition, the contribution of each of the lymphatic vessel developmental

mechanisms varies from one tissue to other. For instance, lineage tracing experiment showed that large parts of the dorsal and lumbar midline skin lymphatic vasculature form independently of the venous cell origin through lymphvasculogenesis of the tissue residing LEC clusters (Martinez-Corral et al., 2015). This study along with other lineage tracing studies suggest that in addition to veins there are other contributing sources of LECs, such as the non-venous endothelium (Pichol-Thievend et al., 2018; Stanczuk et al., 2015).

1.1.4 Lymphangiogenesis

Lymphangiogenesis, the expansion of the lymphatic network, normally occurs only during the development and is largely inactive in adults. In adults, lymphangiogenesis typically is associated with pathogenic conditions such as inflammatory responses, compromised wound healing, and cancer (Maby-El Hajjami & Petrova, 2008). There are several different secreted factors that mediate lymphangiogenesis, of which VEGF-C is the most potent and studied. Upon activation by ligand binding VEGFR3, the receptor for VEGF-C, dimerizes and its cytoplasmic tail becomes phosphorylated, which in turn activates downstream signaling. Upon VEGFR3 phosphorylation, adaptor proteins bind to the cytoplasmic tail of the receptor and this leads to activation of downstream signaling, including AKT, ERK and JNK pathways, which promote LEC survival, growth and migration (Makinen et al., 2001; Salameh et al., 2005). Although VEGF-C – VEGFR3 system has been classically characterized in promotion of lymphangiogenesis, this pathway has important roles also in maintaining lymphatic vasculature. In mouse models, VEGF-C is required for maintenance of the intestine lymphatic vessels (Nurmi et al., 2015), and VEGFR3 deletion in endothelial cells decreases the lymphatic vessel area in the intestine and trachea (Karaman et al., 2022). VEGF-C - VEGFR-3 signaling is also required for the maintenance of the meningeal lymphatic vessels in adult mice (Antila et al., 2017). In addition to VEGF-C there are other supporting lymphangiogenic factors, which either promote directly LEC proliferation or which are involved in LEC survival, or both (Dieterich et al., 2022).

1.1.5 Lymphangiogenesis in cancer

In many solid cancers, lymphatic remodeling is a common phenomenon (Figure 2). Both intratumoral and especially high peritumoral lymphatic vessel density, correlate with the occurrence of lymph node metastasis and therefore with poor prognosis in many solid cancers (Zhang et al., 2017). In melanoma, lymphatic

vessel density in primary tumor as well as melanoma cell lymphatic invasion correlate with poor outcome in melanoma patients, suggesting that the lymphatic system is especially important for melanoma metastatic dissemination (Pastushenko et al., 2014). Tumor cells, but also other cell types in the TME, produce lymphangiogenic factors to stimulate the existing lymphatic vessels to sprout and enlarge. In addition to VEGF-C, other lymphangiogenic factors also contribute to lymphangiogenesis in cancer. For instance, melanoma sheds extracellular vesicles, which contain neural growth factor (NGF) which induces lymphangiogenesis in LECs (Garcia-Silva et al., 2021). Many inflammatory signals in the TME promote lymphangiogenesis in an indirect manner (Dieterich et al., 2022). For instance, a proinflammatory cytokine interleukin-1 beta induces VEGF-C production in human lung fibroblasts (Ristimaki et al., 1998), and this cytokine is also expressed by melanoma associated macrophages, which promotes VEGF-C production in an autocrine manner in them (Peppicelli et al., 2014).

Despite the presence of increased lymphatic vessels in the tumor cell mass, the vessels are often found collapsed and non-functional due to pressure caused by the pressing tumor cells and high interstitial fluid pressure (Padera et al., 2004). If the intratumoral lymphatic vessels remain open, they can enhance the tumor cell lymphatic metastasis by having increased velocity of fluid flow into the lymphatic capillaries (Hompland et al., 2012). The peritumoral lymphatic vessels in turn are often found to be dilated and can sometimes be filled with cells; these vessels are thought to be primarily responsible for the tumor lymphatic metastasis (Dieterich et al., 2022).

Lymphangiogenesis is not limited to the primary tumor sites but occurs also elsewhere. It has been shown in mouse models, that tumor draining lymph nodes become larger due to expansion of the lymphatic network by LEC sprouting and proliferation within the lymph node (Commerford et al., 2018; Harrell et al., 2007) and similar findings have been shown in cancer patients (Van den Eynden et al., 2007). Lymphangiogenesis additionally occurs at the sites of distant metastasis, as shown in the metastatic lungs of melanoma patients (Ma et al., 2018).

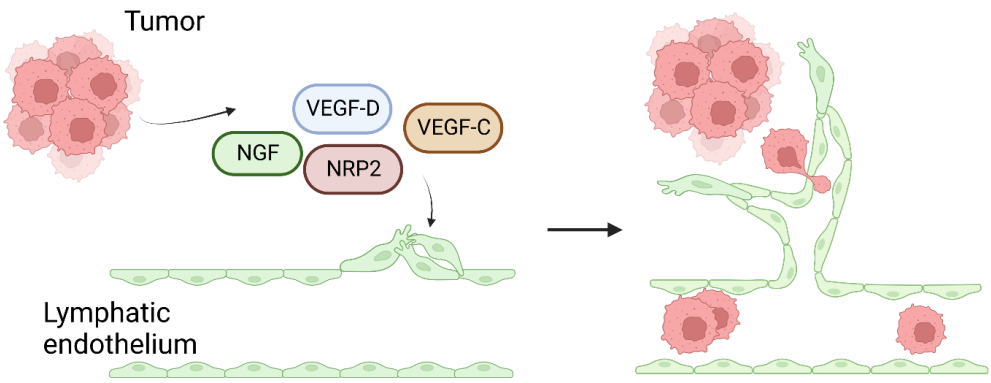

Figure 2. Cancer cells together with other TME cells secrete lymphagiogenic factors (left) to stimulate lymphangiogenic sprouting (right). Figure created using BioRender.com.

1.1.6 Tumor angiogenesis

Due to cancer cell dependency on energy resources, nutrients and oxygen, it has been estimated that the expanding tumor cell mass can reach around 1 mm diameter without connection to the blood vasculature before reaching the limits of resources needed for its growth (Folkman et al., 1963). Therefore, reorganization and expansion of the blood vascular network within and around the tumor are essential steps for cancer progression. There are several different ways by which cancer can promote these steps. The most common way is angiogenesis, in which the existing blood vessels form new sprouts to expand the vessel network. In the intussusceptive angiogenesis, the existing blood vessel is split into two by creating capillary wall in the middle of the vessel lumen (Saravanan et al., 2020). Cancer cells may also gain properties of endothelial cells and become parts of the vascular walls in a process called vascular mimicry. In addition, endothelial cell progenitor cells can be recruited to the newly forming tumor vasculature (Armani et al., 2021). Cancer cells and cells from the surrounding tissue can release an array of factors to initiate angiogenic signaling. In normal tissue, there are always present anti- and pro-angiogenic factors; upon the onset of the angiogenesis, the balance of these has shifted to favor angiogenesis initiation, in a step referred as “angiogenic switch” (Bergers & Benjamin, 2003). The most common trigger for their production is hypoxia, which induces the expression of HIF-1 in cells that in turn upregulates multiple pro-angiogenic factors (Al-Ostoot et al., 2021). Among the downstream targets are for example VEGF-A, TGF-β and PDGF-B, all inducers of angiogenesis in blood endothelial cells (Viallard & Larrivee, 2017). Especially VEGF-A signaling has a crucial role in the induction of angiogenic sprouting of the existing blood vessels. High VEGF concentration stimulates blood endothelial cells (BECs) by binding to receptor VERGFR-2 , which in turn leads to upregulation of

Notch ligand DLL4 in cells (Phng & Gerhardt, 2009). DLL4 binds to Notch1 receptor on the neighboring cell, leading to its activation and release of the Notch intracellular domain NICD. NICD acts as a transcriptional regulator which limits the expression of multiple genes involved in the response to VEGF signaling (Bray, 2016). DLL4 expressing cells adopt a role of so-called tip cells, and they gain highly motile, invasive properties and can respond to numerous growth factors and properties of the surrounding ECM. Tip cells express multiple proteolytic enzymes, such as matrix metalloproteinases (MMPs, described further in section 1.2.2.) which are essential in the process. Upon angiogenesis, MMPs remodel the basement membrane and degrade extracellular matrix components to make room for the newly forming vessels. In addition, MMPs can detach pericytes from the vessels and release pro-angiogenic factors from the degraded extracellular matrix (Wang & Khalil, 2018). The cells following the tip cells expressing Notch1 adopt "stalk cell" properties and become proliferative and maintain the integrity of the newly forming vessels (Viallard & Larrivee, 2017). Upon formation of the new vessels, they need to mature by forming the basement membrane, recruit pericytes and establish proper cell-cell contacts for fully functional vascular performance. However, tumor associated vasculature typically shows defects in these maturation processes. The vasculature found within tumors can be heterogenic with either hypo- or hypervascular sites. The vessels are frequently found to be excessively permeable, leading to poor perfusion, increased interstitial pressure in tumor and hypoxic sites (Al-Ostoot et al., 2021). These promote tumor cells to gain stem-like characteristics and allow easy intravasation of the tumor cells. In addition, poor blood flow can hinder accumulation of anti-tumorigenic immune cells into the tumor and the entrance of antitumor drugs (Al-Ostoot et al., 2021; Hida et al., 2022; Viallard & Larrivee, 2017). Therefore, normalization of the tumor vasculature as a combination therapy is under active investigation (Zheng et al., 2021).

1.2 Cancer cell invasion and extravasation

In addition to sustained cell proliferation, one key biological aspect of cancer is the local invasion into the surrounding tissue. The local invasion involves cancer cells to lose their connections to the surrounding tumor cell mass, gain mobility as single cells or as small clusters of tumor cells, and to be able to attach and remodel the surrounding extracellular matrix.

Cancer cells lose connections to their neighbors very often by decreasing their epithelial characteristics and gaining mesenchymal ones in their place, a process called epithelial-to-mesenchymal transition (EMT). During this process, cells with epithelial characteristics change towards more mesenchymal ones, including remodeling of cell-cell and cell-matrix interactions, leading to cell detachment

from each other and the basement membrane. During EMT, cells downregulate the proteins of cell-cell contacts, especially those of tight and adherens junctions, including for example E-cadherin, occludins and claudins. Cells lose their cobblestone-like shape typical for epithelial cells and become spindle shaped, and gain expression of proteins associated with mesenchymal cells, such as N-cadherin, vimentin and fibronectin (Dongre & Weinberg, 2019). EMT does not necessarily occur as a full epithelial or mesenchymal switch but instead there are multiple gradual stages between the two, which have been reviewed in (Pastushenko & Blanpain, 2019). Since cells may exhibit and switch between various states of epithelial-mesenchymal phenotypes, a term epithelial-to-mesenchymal plasticity has been adopted to describe this flexibility (Haerinck et al., 2023). The EMT changes are orchestrated in cells by transcription factors, such as ZEB1, ZEB2, SNAIL, SLUG and TWIST, which all have slightly different repression or activation pattern of hundreds of different downstream genes.
To invade locally, detachment of the cancer cells from each other and ability to survive as single cells or cell clusters is not sufficient but in addition, cancer cells need to be able to modify and degrade the surroundings and attach to various tissues. Invasion locally can be a very difficult task for the tumor cell, considering that the cell must be able to squeeze through complex matrixes or invade through basement membranes (Cox, 2021). ECM components have a dramatic effect on cancer cell invasion as they regulate cancer invasive processes (Mierke, 2020). Therefore, the changes induced by EMT are also linked to cancer cell ability to modify the surrounding ECM. For example, SNAIL1 has been shown to upregulate several MMP proteins involved in ECM degradation (Miyoshi et al., 2004), a protein family further discussed in section 1.2.2.

1.2.1 Extracellular matrix in cancer

Extracellular matrix (ECM) refers to all supportive non-cellular tissues found all around the body and organs. Extracellular matrix components are synthesized and secreted by various cells, which form three-dimensional, often insoluble structures. The secreted components can form different types of networks with similar or different types of constituents, forming multiple types of extracellular environments which differ in density, stiffness, biochemical and mechanical properties. These different environments offer cues for the residing cells, and therefore ECM affects the cells' biology and behavior. Consequently, the cells within ECM and ECM components are in a dynamic relationship where both continuously affect each other; the cells deposit, break down and modulate the ECM, and these changes in turn affect the cell behavior. The two main components of ECM are collagens and glycoproteins. Collagens are the most abundant ECM

constituent of human body, providing mechanical strength and sites for cell adhesion and migration. Collagens are also a major factor of specialized ECM structure, basement membrane, which forms an interface between the endothelial or epithelial cell layers and the surrounding tissue. Glycoproteins are characterized by carbohydrate chains attached to the protein core. Glycoproteins are structurally a very diverse group, and therefore they function in multiple processes, including but not limited to, offering resistance to compressive forces, regulation of cell adhesion, migration and proliferation and binding of other biologically active molecules, such as cytokines and growth factors (Cox, 2021).

In solid tumors, ECM deposition, modification and degradation are usually dysregulated. In many solid cancers, desmoplasia, meaning deposition of excessive and aberrant ECM components around and within the tumor, is very common. In desmoplasia, both cancer cells and cancer associated cells, such as fibroblasts, contribute and respond to the abnormal ECM composition. Tumor associated ECM does not only differ in the amount but also in the properties of the deposited matrix; cancer cells as well as cancer associated cells express different types of enzymes capable of modulating the existing ECM. For example, crosslinking of the collagen fibers increases the stiffness of the ECM (Egeblad et al., 2010), and the more stiff ECM in the TME has been shown to promote tumorigenesis in multiple cancer types (Levental et al., 2009). Additionally, tumors modulate the orientation of ECM fibers; in normal tissues, the fibers are usually found non-oriented, but in tumors they are often arranged linearly to promote and direct cancer cell local invasion (Provenzano et al., 2006). Solid cancers are often found to aberrantly express ECM modulating enzymes, such as LOX proteins which mediate collagen crosslinking (Yuan et al., 2023), or WISP1 which promotes collagen fiber linearization (Jia et al., 2019).

Solid tumors not only show abnormal deposition and modulation of tumor associated ECM, but also degradation of the ECM is an important aspect of cancer progression. ECM degradation releases bound cytokines and growth factors, which promote tumorigenic transformation of cancer cells or modulate the behavior of the TME cells (Cox, 2021). It is well known that to invade and start metastasis, cancer cells need to degrade the basement membrane. For example, breast cancer cells activate matrix-degrading pathways that are normally active during mammary epithelial development to facilitate cancer cell invasion in the tumor periphery (Feinberg et al., 2018). There are several different classes of matrix degrading enzymes expressed by cancer cells, such as matrix metalloproteases discussed next.

1.2.2 Matrix metalloproteinases as mediators of cancer cell invasion

To invade into the surrounding tissue, cancer cells must be able to actively modify their surroundings. Matrix metalloproteinases (MMPs) are a large family of zinc and calcium-dependent proteinases capable of cleaving various extracellular matrix proteins, including fibrin, collagens, gelatins and proteoglycans, to name a few (Verma & Hansch, 2007). MMP protein family in humans consists of 24 proteins with variable structures and substrate specificities. Depending on the specific MMP, they are either secreted or tethered on the plasma membrane. In normal homeostatic conditions, most of the MMP transcription is maintained at relatively low level but may increase dramatically when cells are stimulated with cytokines, chemokines or growth factors. In addition, various changes in cellular signaling by protein phosphorylation may induce MMP transcription and translation (de Almeida et al., 2022). Regulation of MMP transcription is overall a rather complex process, and their expression is increased usually in pathological conditions, such as during inflammation or tumorigenesis.

MMPs have variable structures but most of the MMPs contain a signaling peptide, a pro-domain, a catalytic site, a linker region and a hemopexin domain. In addition, membrane-bound MMPs have a transmembrane region (de Almeida et al., 2022). The signaling peptide targets the protein for the secretory pathway (Sternlicht & Werb, 2001). The pro-domain is a negative regulator of the enzymatic activities of the protein, and upon cleavages of the pro-domain MMPs can maturate into fully active peptidases (Maskos, 2005; Visse & Nagase, 2003). The pro-domain of most of the MMPs, depending on the given MMP, can be cleaved and activated by trypsin, other MMPs, furin-like convertases or plasmin (Niland et al., 2021). The catalytic site harbors chelated zinc ion which mediates catalytic activity, along with additional two zinc ions and three calcium ions which stabilize the tertiary structure of the catalytic domain (Cui et al., 2017). The hemopexin domain mainly facilitates the substrate identification and target specificity (Dufour et al., 2008).

The TME contains various substrates for MMPs, most importantly ECM components, such as fibrin, collagens, gelatins and laminins. Sometimes, these are abundantly deposited to the tumor surroundings, forming a capsule around the tumor (Cox, 2021). Upon their degradation, they may form biologically active components, matrikines, which regulate tumor progression and metastasis (Niland et al., 2021). In addition, ECM binds soluble factors, such as growth factors, which can be released to the TME upon degradation by MMPs (Winkler et al., 2020). Therefore, by degrading the ECM components, MMPs are contributing to many cancer promoting activities including cancer invasion, neovascularization, and metastasis. The substrates of the MMPs are not limited, however, to ECM components; some MMPs are able to cleave and release cell

surface bound proteins such as cytokines and growth factors and their receptors (Niland et al., 2021), which may have tumor promoting effects. The proteolytic activity of MMPs links them also to the cancer-related EMT; their activity enables cancer cells to migrate and invade locally, for example, by degrading the basement membrane. There are several different MMPs which are linked to EMT phenotype in cancer cells, such as MMP14, also known as MT1-MMP (Gonzalez-Molina et al., 2019; Liu et al., 2018). MMP14 is of particular interest in context of cancer since it has been found to be highly expressed in multiple cancer types and it also correlates with poor prognosis (Gifford & Itoh, 2019). MMP14 is described as a major collagenase, although its substrates also include various other ECM components, such as basement membrane laminins, fibronectin, vitronectin and fibrinogen, to name a few (Barbolina & Stack, 2008; Pahwa et al., 2014). In addition to ECM components and other MMPs, MMP14 can also cleave various other proteins; for example, MMP14 is able to cleave TGF-beta-1 binding protein, resulting to release of latent TGF-beta1 (Tatti et al., 2008). In addition, MMP14 can cleave several other MMPs, such as MMP2, MMP9 and MMP13, leading to their activation (Niland et al., 2021). In a mouse knockout model, it has been found that MMP14 is indispensable for normal development and that other MMPs are not able to compensate for the MMP14 loss (Zhou et al., 2000).

MMP14 expression and activity has multiple points of regulation. Epigenetic regulation likely plays a role in the regulation of MMP14 gene expression; in highly migratory glioblastoma cells, MMP14 promoter was hypomethylated when compared to the poorly migratory breast cancer cells (Chernov et al., 2009). MMP14 expression in cells can be induced by different stimuli, such as several growth factors, cytokines and extracellular signaling ligands (Turunen et al., 2017). For example, in renal cell carcinoma, MMP14 is positively regulated by hypoxia-inducible factor 2-alpha (Petrella et al., 2005), and in melanoma, MMP14 expression is increased upon Akt-dependent Sp1 activation (Hong et al., 2014). EMT can also induce MMP14 transcription and for instance, in breast cancer cells MMP14 gene expression is upregulated upon Snail1 induction (Ota et al., 2009). MMP14 promoter harbors binding sites for PROX1, which acts as a repressor for MMP14 transcription (Gramolelli et al., 2018). Newly synthesized MMP14 protein requires proteolytic conversion by furin to become an active enzyme (Yana & Weiss, 2000). After MMP14 protein is synthesized and cleaved, cancer cells can still spatially and temporally regulate the MMP14 enzymatic activity. In U87MG glioblastoma cells, kinesin superfamily protein 1B is required for efficient MMP14 plasma membrane translocation from the cell cytoplasm (Chen et al., 2016). Cancer cells may also maintain a pool of MMP14 in intracellular vesicles and translocate and release MMP14 on the cell surface on demand (Infante et al., 2018). The activity of the cell surface MMP14 can be inhibited by several inhibitory proteins, such as tissue inhibitor of matrix metalloproteinases, TIMPs (Niland et

al., 2021). Finally, MMP14 presence on the cell surface is also under regulation. Either through autocatalytic or non-autocatalytic activity, MMP14 can be shed from the cell surface, releasing a soluble fragment. The soluble fragment can still have some proteolytic activity; MMP14 released from the breast cancer cells can cleave endoglin on the epithelial cell surface (Tobar et al., 2014). However, cell membrane bound MMP14 likely is the main contributor of extracellular matrix degradation, since soluble MMP14 cannot mediate cell invasion (Hotary et al., 2000).
In addition to degrading the surrounding extracellular matrix and other proteins, cancer cells need to also attach to the surrounding tissue. The efficient invasion usually requires coordinated function of MMPs mediating tissue degradation and integrins (described in the next section) which are responsible for cell attachment on various surfaces.

1.2.3 Role of integrins in cancer cell local invasion

Integrins are an essential family of adhesion proteins, mediating cell-cell, cell-ECM and cell-pathogen interactions. Functional integrins on the cell surface are formed of α and β heterodimers. In humans, there are at least 18 α and 8 β subunits, known to form 24 different types of heterodimers (Takada et al., 2007). The extracellular part of the heterodimer binds to the ligands outside of the cell. The intracellular parts of the two subunits are relatively short, but they have important functions mediating the regulation of the integrin binding, and they link integrins to a variety of other intracellular structures, such as cell cytoskeleton linker proteins. Integrins are present on the plasma membrane in different conformations, reflecting varying affinities to the ligands. In a low affinity conformation, the integrin extracellular domains appears to be folded, “closed”, and the transmembrane and cytosolic parts are located close to each other. In a high affinity conformation, the extracellular part becomes extended and the cytosolic domains of the α and β subunits become dissociated, allowing binding of cytosolic linker proteins to the integrin cytosolic domains (Barczyk et al., 2010; Takada et al., 2007).
Integrins act as mediators of signaling both outside-in and inside-out (Figure 3). The outside-in signaling means that integrin receptor binding provides cues to the cells about the abundance, stiffness and directions of the ligand ECM components. Upon ligand binding on the cell surface, integrins undergo conformational changes, including the separation of transmembrane and cytoplasmic domains. These changes allow interaction of the intracellular signaling proteins with the integrin cytoplasmic tails. In the inside-out signaling, the cell regulates the affinity of integrins to their ligands. Integrins are regulated by G-protein-coupled receptors, which phosphorylate the cytoplasmic tail of the β subunit, leading to its

dissociation from the α subunit and opening of the integrin structure to a high affinity state. In addition, cytoplasmic linker proteins such as talin are able to regulate integrin function (Takada et al., 2007). Upon activation of the complex in either way, integrins together with additional linker proteins kindlin and talin (Ye et al., 2014) form complexes called adhesomes, special cellular components mediating signal transduction in cell (Horton et al., 2015).

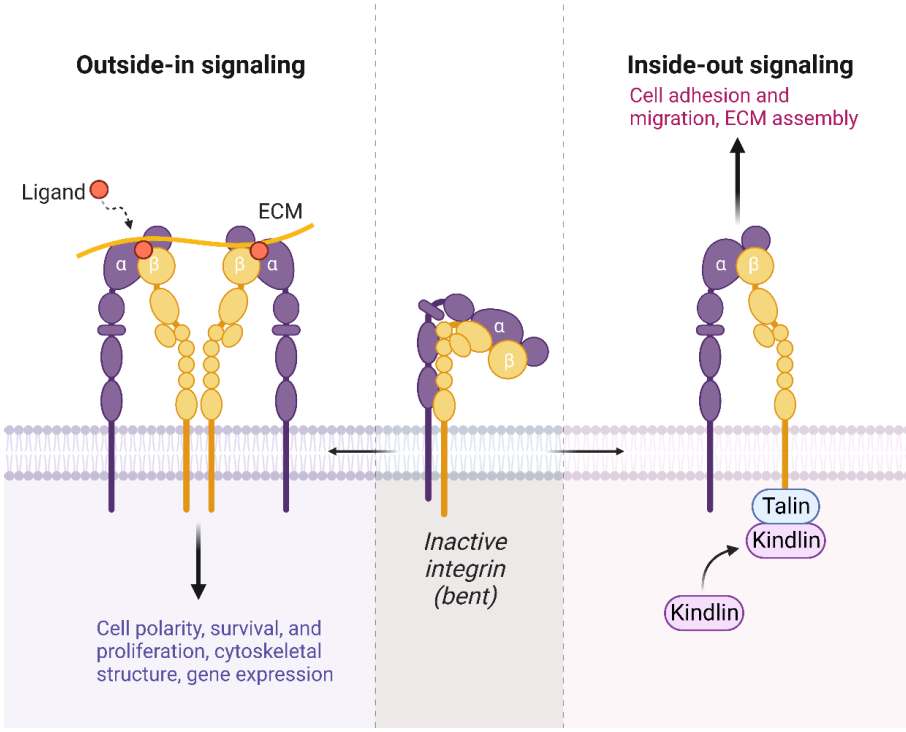

Figure 3. Integrin-mediated signaling. Figure created using BioRender.com.

Integrins are involved in basically all aspects of cancer progression, from tumor initiation to metastasis establishment. In addition to cell adhesion, integrins have roles in various processes, such as promoting oncogenic growth factor receptor signaling or supporting the survival of circulating tumor cells (Hamidi & Ivaska, 2018). Here, however, the focus is on integrin involvement in local cancer invasion. During cancer cell invasion, the cell needs to alter and adjust its adherence to the surroundings. Integrins mediate an important function during cancer cell invasion, and in many cancers it has been shown that cancer cells have atypical expression levels of integrins (Seguin et al., 2015). In addition, cancer cells may aberrantly activate integrins through linker proteins such as talin and kindlin (Valdembri & Serini, 2021), or cause changes in kinetics of normal integrin

endocytosis and recycling back to the plasma membrane (Paul et al., 2015). A common finding in many solid tumors is that cancer cells or cancer associated TME cells deposit aberrantly and excessively ECM proteins, such as collagen or fibronectin. These deposited ECM components can form stiff, thick capsular structures around the tumor. The modified ECM provides cancer with multiple pro-migratory and pro-invasive cues and specific ECM proteins in combination of defined, active integrins can control the invasion of cancer cells.

In addition to forming adhesive sites between cancer cells and ECM, integrins and their activation can regulate other pro-invasive signaling pathways in cells. As an example, β1 integrin signaling can affect E-cadherin internalization dynamics (Canel et al., 2010), and therefore contribute to the EMT switch in cancer by weakening the cell-cell contacts. Integrins are also involved in switching between different invasion strategies; for example, primary melanoma explants cultured in 3D collagen exhibited collective invasion mode dependent on β1 integrin, and when inhibited, melanoma cells switched to amoeboid like invasion (Hegerfeldt et al., 2002).

In all cells, including cancer cells, cell adhesion and matrix degradation are coordinated processes and can even occur simultaneously at the same sites. For example, angiogenic processes require that the proliferating endothelial cells can coordinately both bind to and degrade ECM to efficiently form new vessels (Spuul et al., 2016). This is achieved by coordinated deposition of adhesive and degrative proteins on defined locations on the cell plasma membrane. Such specialized cell-ECM contact sites are called podosomes or invadopodia, collectively named as invadosomes (Linder et al., 2023). Podosomes occur in normal cells and are characteristically smaller and have usually arranged into coordinated arrays. Invadopodia instead typically occur in cancer cells, are larger in size and typically do not exist in coordinated arrays. In both podosomes and invadopodia, actin filaments provide mechanical force and structural strength to form protrusions on the plasma membrane. Different matrix degrading enzymes, MMP14 being one of the most abundant, are present on the plasma membrane at the end of the actin filaments linked through various linker proteins. The area rich of degradative enzymes is surrounded by a ring of adhesive proteins, most importantly integrins. The invadosomes are highly dynamic structures, their protein composition can vary quite rapidly and overall the life-time of a single invadosome varies from few minutes to around one hour (Linder et al., 2023). Therefore, cell adhesion and ECM degradation are interconnected processes, resulting in complex regulatory mechanisms ensuring meaningful cell behavior in various environments. Importantly, this coordinated process along with lymphangiogenesis allows cancer cells reach lymphatic vasculature to further enhance cancer progression.

1.2.4 Lymphatic invasion of cancer cells

Tumor associated lymphatic vessels can often appear ruptured, with damaged vessel wall and tumor cells within the lumen; this indicates, that tumor cells may passively, by mechanical force, damage the endothelial barriers and gain access to the lymphatic vessel lumen (Niimi et al., 2001). However, there are accumulating evidence that cancer cell invasion into lymphatic vessels would be an active process.

LECs are a crucial component of the functioning immune system. Upon inflammatory conditions, LECs express several cell adhesion molecules, such as VCAMs and ICAMs. Immune cells express cognate receptors for these, and their binding facilitates immune cell attachment to LECs and immune cell intravasation. Cancer cells may express these receptors in addition to other cell adhesive molecules to mediate cell attachment to the lymphatic vessel wall. For instance, melanoma cells have been shown to express the VCAM cognate receptor VLA, also known as α4β1 integrin dimer (integrins further discussed in section 1.2.3.), which plays a crucial role in mediating melanoma cell attachment to endothelial cell layers (Garcia-Martin et al., 2019; Taichman et al., 1991). In addition to the adhesion molecules, LECs express chemokine CCL21, which forms a gradient that facilitates immune cell migration towards lymphatic vasculature. Cancer cells may express the CCL21 cognate receptor, CCR7, which may guide cancer cells towards lymphatic capillaries; in several cancer types, higher CCR7 expression correlates with increased lymph node invasion (Dieterich et al., 2022). Once the tumor cells have localized and attached to lymphatic vessels, they need to gain access to the lumen to initiate the metastatic process. Although lymphatic capillaries are already relative permeable, cancer cells may further modulate the permeability to enhance the intravasation. VEGF-C does not only act as a lymphagiogenic factor, but it can also modulate the LEC barrier function. In a mouse model, VEGF-C facilitated colorectal cancer lymphatic entry by impairing the barrier function due to decreased cell junctional VE-cadherin production (Tacconi et al., 2015). Other TME cells may also facilitate cancer cell lymphatic invasion. For example, tumor associated macrophages in a breast cancer model attached in an integrin dependent manner to the tumor associated lymphatic vessels and released transforming growth factor beta 1 to increase lymphatic vessel permeability (Evans et al., 2019). In addition, extracellular composition of the TME can modify the permeability of LECs. For instance, a low molecular weight form of hyaluronan has been shown to induce permeability of LECs by disrupting ZO1 containing tight junctions (Yu et al., 2015).

1.2.5 Cancer cell extravasation

To form metastasis, cancer cells need to escape out from the lumen of the lymphatic or blood capillaries through the vessel wall. This requires the cancer cell to attach on the vessel wall and modification of the wall so that the cancer cell may squeeze through the vascular cells and enter the tissue beneath. This process requires coordinated action of proteins involved in cell adhesion and degradation, such as integrins and matrix metalloproteinases. In addition, many secreted factors that modulate the vasculature during (lymph)angiogenesis, such as vascular growth factors and angiopoietins, are involved for the more efficient cancer cell extravasation (Shenoy & Lu, 2016).

During the first steps of extravasation, cancer cells need to become entrapped to the vessel walls. This typically occurs in the capillary networks, where the fluid pressure is smaller and therefore cancer cells can interact more efficiently with the vessel wall. The initial attachment can be reached using multiple adhesion molecules, such as selectins, integrins, immunoglobulins and cadherins (Reymond et al., 2013). For instance, melanoma cell lines expressing high N-cadherin, a classical epithelial-to-mesenchymal (EMT) marker, were shown to adhere on the HUVEC layers, whereas cell lines with very little N-cadherin expression did not (Li et al., 2001). In a zebrafish xenograft model, renal cell carcinoma and pancreatic cancer cells with high Neuropilin-2 expression extravasated more efficiently compared to control cells, and in vitro analyses showed that α5 integrin on the HUVEC surface was a binding partner for Neuropilin-2 (Cao et al., 2013). In a prostate cancer model, E-selectin mediated prostate cancer cell rolling on the bone marrow endothelium after which cancer cell attachment was mediated by several different β-integrins. E-selectin depletion caused a marked reduction in extravasation of cancer cells from bone marrow endothelium, which consequently reduced bone metastasis formation (Barthel et al., 2013).

During the next step of extravasation, cancer cells need to squeeze between the endothelial cell layers and breach through the basal lamina in order to reach the underneath tissue layers. In this process, tissue degradative and adhesive molecules are utilized coordinately. These molecules can be arranged into specific protrusions on the cancer cell plasma membrane, called invadopodia (further described in section 1.2.3.). An intravital imaging study using a chicken embryo ex ovo model showed that multiple different cancer cell lines formed protusions through the endothelial cell junctions which were particularly enriched with invadopodia markers, such as the degradative enzyme matrix metalloproteinase 14 (MMP14; described in section 1.2.2.) and tyrosine kinase substrate with four/five Scr homology 3 domains (Tks4 and Tks5), which are required for the formation of invadopodia (Leong et al., 2014). Depletion of Cortactin by shRNA, a regulatory protein that mediates invadopodia formation, inhibited bladder cancer

cell migration in a lung microvascular endothelial cell layer model and reduced lung metastasis formation in mice (Tokui et al., 2014).
It is worth to note that extravasation does not only mediate cancer cell escape directly to the new colonization site, but this may occur upon switching the route between blood and lymphatic vasculature for further metastatic dissemination. In mouse models, cancer cells in lymph nodes extravasated to the lymph node associated blood vasculature (Brown et al., 2018; Pereira et al., 2018). Therefore, metastasis of cancer cells can occur via multiple routes, reflecting further plasticity and resilience of the cancer cells in changing environments. Moreover, since the collecting lymphatic vessels eventually empty their contents into the subclavian veins, the tumor cells that circulate in the lymph fluid and which do not either attach to the lymphatic vessels or form metastasis through them will eventually be shed into the bloodstream. However, melanoma mouse models suggest that melanoma dissemination from the primary tumor preferentially occurs initially via the lymphatic vasculature; in both immunocompromised mice with patient derived xenografts as well as immunocompetent mice with mouse melanomas, more melanoma cells per microliter were found in tumor draining lymph than in tumor draining blood (Ubellacker et al., 2020). In bloodstream, the presence of reactive oxygen species (ROS) makes melanoma cells vulnerable for ferroptosis, a form of apoptosis caused by ROS exposure. The study by Ubellacker et al. further found that lymph fluid contains multiple components that can protect melanoma cells from ferroptosis, and that melanoma cells isolated from lymph nodes were more resistant to ferroptosis and formed more metastases compared to cells isolated from a subcutaneous tumor when both cell types were injected intravenously (Ubellacker et al., 2020). These studies suggest that lymphatic and blood vasculature are not necessarily alternative routes in cancer cell dissemination, but rather both systems can be utilized to maximize both cancer cell survival and efficient cell spreading in the body.

1.2.6 LECs as a source of cancer promoting factors

LEC-derived secreted factors modulate the function of the surrounding tissue (discussed in section 1.1.2.), and cancer cells are not an exception. As discussed in section 1.2.4, LEC-produced CCL gradients guide cancer cells to migrate towards lymphatic vasculature (Dieterich et al., 2022). In a breast cancer model, tumor associated LECs promoted cancer cell proliferation by secreting EGF, and enhanced tumor angiogenesis and pericyte recruitment by increased PDGF-BB production (Lee, Pandey, et al., 2014). In a human skin carcinoma model, tumor exposed LECs showed a huge upregulation of IL6, which in turn induced tumor cell growth *in vivo* (Van de Velde et al., 2021). In a head and neck cancer model, stimulation of LECs by cancer cell conditioned medium induced the LECs to

produce CXCL5, which promoted cancer cell invasion and metastasis to the lymph nodes (Lee et al., 2021). In addition to pro-tumorigenic proteins, LECs can produce tumor promoting miRNAs, as shown for instance in a breast cancer model where ELK3-regulated miRNAs promoted tumor cell dissemination (Kim et al., 2019).

In addition to affecting cancer cell proliferation, lymphatic factors induce changes in immune system function leading to the immune tolerance of the tumor cells. In a mouse melanoma model, tumor cell-derived extracellular vesicles were taken up by lymph node LECs, which resulted in tumor specific antigen cross-presentation on MHC-I, further leading to apoptosis of antigen specific CD8+ T cells (Leary et al., 2022). Lymphangiogenic factor VEGF-C similarly promotes CD8+ T cell apoptosis in the primary tumor in a melanoma mouse model (Lund et al., 2012), likely due to the upregulation of PDL1 in LECs (Dieterich et al., 2017). Tumor associated lymphatic vessels may also sequester CD8+ T cells in the tumor periphery, leading to their egress from the tumor and therefore impaired antitumoral T cell functions; mechanistically, LEC-produced CXCL12 attracts CXCR4 expressing CD8+ T cells leading to their exit from the tumor, and the blockage of this signaling axis improves the antitumoral effect of CD8+ T cells (Steele et al., 2023). Macrophages exist in the tumor microenvironment in multiple forms, many of which are strongly tumor promoting (Mantovani et al., 2022). LECs can enhance the recruitment of macrophages with chemoattractants such as CCL2, and the macrophages may secrete VEGF-C to further fuel LEC expansion (Petkova et al., 2023). Taken together, although the lymphatic vasculature is needed for the efficient leukocyte infiltration to the tumors (Lund et al., 2016), LECs exhibit multiple way to suppress the immune response against the cancer cells.

1.3 Colonization of distant organs

Upon extravasation, metastasis formation requires that cancer cells survive and establish macroscopic colonies in distant sites. Usually, the new environment is very different compared to the one in the primary tumor, exposing the disseminated cancer cells to potentially hostile factors. The number of tumor cells which circulate in the bloodstream of cancer patients typically far exceeds the number of detectable metastasis that will be found in the patients (Nagrath et al., 2007). In mouse models, such as those performed with melanoma cells, indicate that although the circulating cancer cells are rather efficient in extravasation to the metastatic site, a large portion of the cells die, and that large fraction of the cells that survive are dormant (Cameron et al., 2000; Luzzi et al., 1998), indicating that the colonization is a rate limiting factor in cancer progression. In addition to distinctively new environment to which cancer cells fail to adapt quickly, the tissue

resident immune cells may actively destroy the infiltrating tumor cells (Massague & Obenauf, 2016).

Many tumor types show typical metastasis site patterns, indicating that these locations are more favorable for the metastatic colony establishment. For instance, areas around the small blood vessels are such sites; brain metastases of lung and breast cancer and melanoma are usually found right next to the blood capillaries (Carbonell et al., 2009). However, cancer cells typically modify the environment to create their own niche. The specific factors and components required for such niches likely vary depending on the given cancer type. However, all cancers require some similar components at the distant sites which promote survival and growth of the primary tumor, such vicinity of the blood vasculature for oxygen and nutrient uptake and the suitable ECM composition in order to progress to macroscopic colonies (Massague & Obenauf, 2016). The distant site niche formation can occur even before the arrival of the cancer cells through education by the primary tumor. Melanoma for instance secretes exosomes which educate the bone marrow progenitor cells and induce tumor promoting vascular leakiness and inflammation (Peinado et al., 2012).

The establishment of the macroscale colonies does not necessary occur immediately upon arrival of the cancer cells to the distant metastasis site, but instead, cancer cells may be dormant for prolonged times. The dormancy may result from multiple factors, such as tissue residing growth inhibitory factors, lack of supportive vasculature or presence of tumor growth suppressive immune cell populations (Massague & Obenauf, 2016). However, molecular mechanisms mediating cancer cell dormancy or awakening are not yet completely understood.

2 Selected developmental pathways activated in cancer

During development, there are multiple signaling pathways activated in cells which are under tight spatiotemporal control. In adult tissue, these pathways are mainly shut down in most of the cells, or a limited selection of the pathways is coordinately, constantly active to ensure maintenance of cell identity and tissue stem cells. In many cancers, such as melanoma, many pathways that were once active during development typically become aberrantly activated again, giving the cells a more plastic nature. The developmental signaling pathways typically regulate cell proliferation, migration, apoptosis and expression patterns of cell type specific genes.

2.1 Notch pathway

The Notch pathway is highly conserved in evolution and plays a major role in cell fate determination during development. Notch gene was first characterized in *Drosophila melanogaster*, where a mutation in this gene caused notching of the wing margin, and hence the gene obtained its name (Dexter, 1914). Since then, Notch has been studied in various organisms where it has been found to be involved in various developmental processes, such as proliferation, apoptosis and maintenance of stem cell populations. Notch proteins are single-pass plasma membrane receptors, where the extracellular domain interacts with ligands on the neighboring cell surface and the intracellular domain mediates signal transduction to the nucleus. The extracellular part of Notch receptor has a signal peptide and an array consisting of multiple EGF-like repeats which are involved in Notch ligand binding. The intracellular part consist of a RBPjk association domain (RAM domain), a nuclear localization sequence (NLS), ankyrin repeats and a PEST domain (proline/glutamic acid/serine/threonine-rich sequence) (Sachan et al., 2023). In humans, there are four different Notch receptors (Notch1-4) and five different Notch ligands (Jagged1,2 and DLL1,3,4).

Notch is first synthesized as a precursor protein, which then becomes cleaved by furin-like convertases in the trans-Golgi network, resulting in an N-terminal extracellular domain and a C-terminal intramembraneous-intracellular domain, which are held together by non-covalent bonding. This mature Notch receptor is translocated to the cell plasma membrane. In the canonical Notch signaling

(Figure 4), upon binding of the ligand on the neighboring cell, Notch extracellular domain is cleaved by A disintegrin and metalloproteinase (ADAM) family proteases, resulting in a membrane tethered Notch receptor truncation. Cleavage by ADAMs results in a conformational change in the intracellular region of Notch, allowing another cleavage by a gamma-secretase complex to take place. This releases the Notch intracellular domain (NICD) from the plasma membrane. NICD then translocates to the nucleus by the aid of importin alpha 3. In the nucleus, NICD acts as a transcriptional co-factor in a complex involving CSL, in humans known also as CBF1 or RBPjk (CBF1, C-promoter binding factor 1 or RBPjk, recombination binding protein for immunoglobulin kappa J) and mammalian mastermind-like (MAML). The most common Notch targets include genes belonging to *HES* or *HEY* family of genes, which are transcription repressors and mainly inhibit transcription of genes involved in differentiation of the cells. However, there are multiple other Notch downstream targets, which are usually context dependent (Sachan et al., 2023). In the absence of NICD, CSL recruits corepressors to the genes (Oswald et al., 2005).

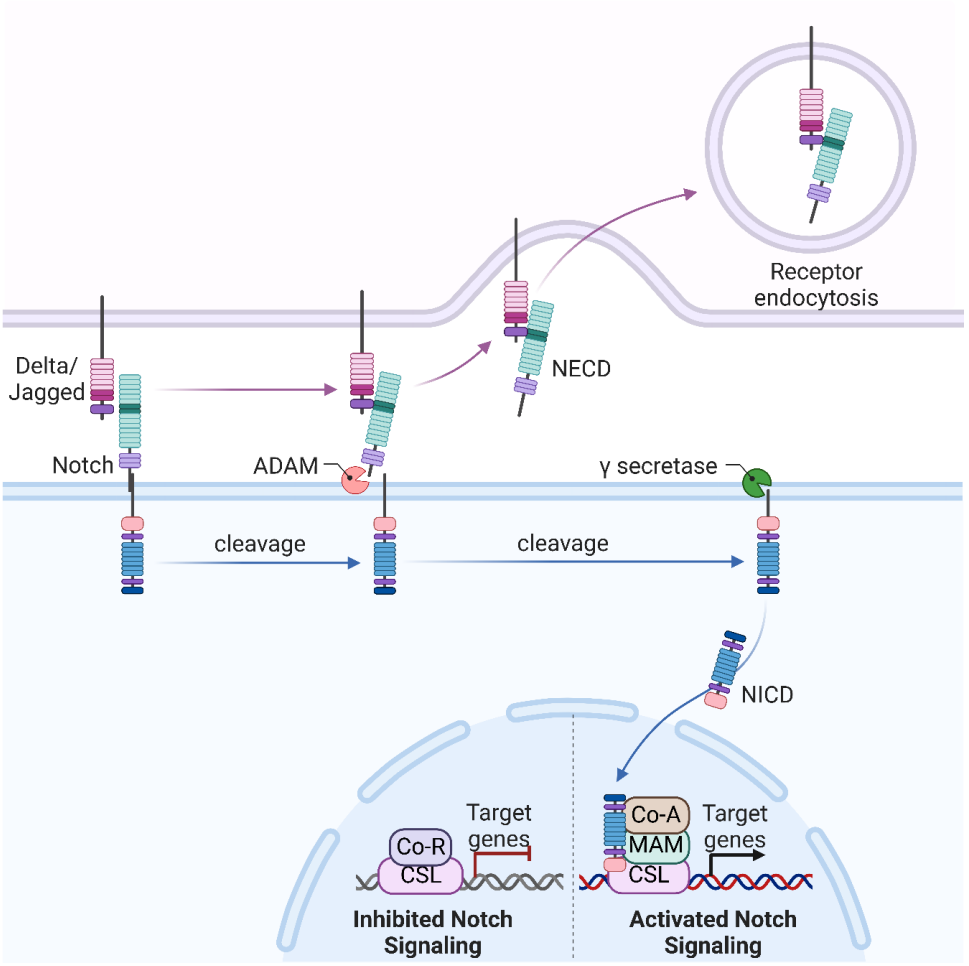

Figure 4. Canonical Notch signaling. Figure created using BioRender.com.

The non-canonical Notch signaling refers to ligand- or CSL-independent functions of Notch (Andersen et al., 2012). Some of the first evidence of non-canonical Notch signaling was obtained from a study, where increased levels of Notch1 inhibited differentiation of myoblasts into muscle cells, and this inhibition mechanism was not dependent on CSL (Shawber et al., 1996). NICD has been shown to be able to interact with other transcription factors, such as components of mTORC, PTEN, AKT and WNT signaling pathway, to modulate the transcription of target genes (Zhou et al., 2022). LPS (lipopolysaccharide) induces IL-10 and IL-2 expression in dendritic cells through Notch1 and TLR dependent signaling without the involvement of gamma-secretase (Gentle et al., 2012). In endothelial cells, full length Notch3 cleavage by caspase-9 induces apoptosis,

whereas culturing the endothelial cells with lung carcinoma cell lines with abundant JAG1 levels blocks the Notch3 induced apoptosis (Lin et al., 2017).

Notch signaling is regulated at multiple steps. The Notch signaling activation is regulated by mechanical pulling force on the Notch receptor, induced by binding of the Notch ligand to the receptor. This mechanical pulling exposes the cleavage sites of Notch receptors to the proteases. It has been suggested that most of the pulling force is induced when the ligand becomes endocytosed upon binding to the Notch receptor (Suarez Rodriguez et al., 2023). However, endocytosis of the ligand is not necessarily mandatory for Notch activation, since *in vitro*, immobilized ligands can still activate Notch receptor (Varnum-Finney et al., 2000). It may be that the pulling effect is achieved with the immobilized ligands *in vitro* through the micromovement of the Notch expressing signal receiving cells. In addition to the traditional pulling force Notch receptor activation model, other types of receptor activation can take place, possibly complementing the traditional pulling force activation and creating opportunities to fine-tune the signal initiation. For instance, soluble JAG1-Fc ligands presented on the DNA origami to the cells could induce Notch activation, suggesting that instead of creating the pulling force, Notch ligands may sequester EGF-like repeats, leading to the exposure of the ADAM-cleavage site (Smyrlaki et al., 2024). Post-translational modifications target both the extracellular and intracellular domains and mediate the ligand binding as well as the amplitude and duration of the signal. In the extracellular domain, there are multiple different glycosylation sites reported, most of which are in the EGF-like repeats. Mechanistically, different glycans can completely inhibit Notch ligand binding, or inhibit binding of specific Notch ligands (Urata & Takeuchi, 2020). In Notch1, acetylation of NICD by the p300 acetyltransferase promotes NICD dependent transcription (Oswald et al., 2001), which is reversed upon SIRT1 mediated acetyl group removal (Guarani et al., 2011). The presence of Notch receptors, as well as Notch ligands, is controlled by endocytosis, which may then either lead to protein recycling back to the cell membrane or protein degradation in lysosomes. Notch pathway activation is terminated upon ubiquitination and subsequent degradation of NICD (Sachan et al., 2023).

In normal cells Notch signaling is tightly regulated to maintain homeostasis. In adult tissues, aberrant Notch signaling regulation is observed upon pathogenic conditions, such as pathogen infection and cancer. For instance, in LECs, infection by Kaposi sarcoma herpesvirus induces MMP14-dependent invasive properties and EndMT changes through Notch pathway activation (Cheng et al., 2011). As the effect of Notch signaling in development is spatiotemporal, it is not surprising that in cancer Notch can either have tumor promoting or inhibiting effects. Notch signaling activation has been described being oncogenic in breast, ovarian, lung, colorectal and hepatocellular cancers (Aster et al., 2017). Notch signaling in cancers can be activated by different ways such as mutations resulting in structural

alterations in the Notch receptor or aberrant upstream signaling (Zhou et al., 2022). The cells in the TME may express abundantly Notch ligands which activate Notch signaling in cancer cells (Meurette & Mehlen, 2018). Notch signaling can be boosted in the cancer cells by different effectors that post-translationally modify Notch components. For instance, Pim kinases phosphorylate Notch1 and Notch3 intracellular domains, and phosphorylation of NICD1 on the NLS domain enhances its nuclear translocation and downstream target transcription, promoting the tumorigenicity of breast and prostate cancer cells (Santio et al., 2016), whereas NICD3 phosphorylation hinders NICD3 interaction with CSL, maintaining the tumorigenic potential of breast cancer cells under estrogen-containing conditions (Landor et al., 2021). The exact result of pro-tumorigenic Notch activation depends on the cancer type, and can include driving of the cell proliferation potential, enhancing local invasion, induction of EMT phenotype and driving treatment resistance, to name a few. In few cancers, such as in squamous cell carcinomas and pancreatic ductal adenocarcinoma, Notch has been described to have tumor suppressing effect. In these cancers, Notch signal activation typically induces transcription of other genes with tumor suppressing effects, leading for instance to cell cycle arrest (Zhou et al., 2022).

Notch signaling does not only act on cancer cells themselves but is an important pathway inducing various effects upon cancer crosstalk with the TME. In a mouse model of lung carcinoma, tumor myeloid cells expressed high levels of Dll4, which activated tumor promoting Notch signaling in cancer cells, while inhibition of Notch activation reduced tumor growth (Ohnuki et al., 2014). Cancer cells may activate Notch signaling in the TME cells as well; for instance, a single-cell RNA sequencing analysis of cribriform prostate cancer patients revealed that the cancer cells expressed abundantly *JAG1*. Blood endothelial cells in the TME in turn expressed *NOTCH1* and *NOTCH4*, and the expression of the Notch downstream targets *HEY1* and *HES1* were significantly increased in the tumor associated blood endothelial cells compared to the blood endothelial cells in a benign prostate (Wong et al., 2022) suggesting a possible Notch signaling activation in the blood endothelium by cancer cells, resulting to induction of angiogenesis in the cancer microenvironment.

2.2 WNT pathway

Like the Notch signaling pathway, WNT pathway is a highly conserved, essential pathway regulating multiple aspects from development to tissue and stem cell maintenance. WNT pathway mediates intercellular signaling where the signal sending cell produces and secretes lipid modified WNT ligands. These bind to the surface receptor of the signal receiving cells, which leads to intracellular signaling cascade mediating various responses in the signal receiving cell. In humans, there

are 19 genes encoding different WNT ligands (Nusse & Varmus, 1992), and multiple receptor families are responsible for the binding of WNT ligands and signal transduction in the WNT signaling; therefore, WNT signaling is one of the most versatile and complex signaling systems under normal and pathogenic conditions.

WNT signaling can be divided into two different classes depending on the signal receiving receptor and downstream effectors of the signal. In the canonical signaling, the best characterized WNT pathway, beta-catenin is a central signal mediating factor in the cytoplasm of the signal receiving cell (Figure 5). When WNT signaling is inactive, beta-catenin becomes phosphorylated by a protein complex, referred as a destruction complex, which consists of multiple proteins. Phosphorylated beta-catenin becomes subsequently ubiquitinated and degraded. Upon canonical WNT signal activation, WNT ligand binds to two distinct receptors on the plasma membrane: a Frizzled-family receptor (FZD) and an accessory receptor which is a member of the low-density lipoprotein receptor-related family, LRP5 or LRP6. Wnt ligand binding induces conformational changes in the receptor, creating a platform for binding of multiple Dishevelled proteins (DVL). Oligomerized DVL in turn sequesters the destruction complex, which leads to accumulation of beta-catenin in the cell cytoplasm, enabling its translocation to the cell nucleus. There, beta-catenin binds to chromatin bound TCF/LEF transcription factors which enables transcription of the downstream targets. When the cytoplasmic and nuclear beta-catenin levels are low, TCF/LEF is bound to transcriptional repressors, such as Groucho, preventing transcription of these genes (Rim et al., 2022).

In the signal sending cell, the newly synthesized WNT is first modified in the endoplasmic reticulum by Porcupine, which adds a palmitoleic acid moiety to the WNT ligand (Galli et al., 2007), a modification essential for WNT signal transduction (Willert et al., 2003). Matured WNT ligands are transported to the cell surface by Wntless (Banziger et al., 2006; Bartscherer et al., 2006). Since palmitoleic acid modification of WNT makes it rather hydrophobic, it is possible that the distance in tissues where WNT signal travels is rather limited. However, there are indications that WNT ligands may be transported via cell divisions in which FZD carries the bound ligand further away from the signal sending cell (Farin et al., 2016).

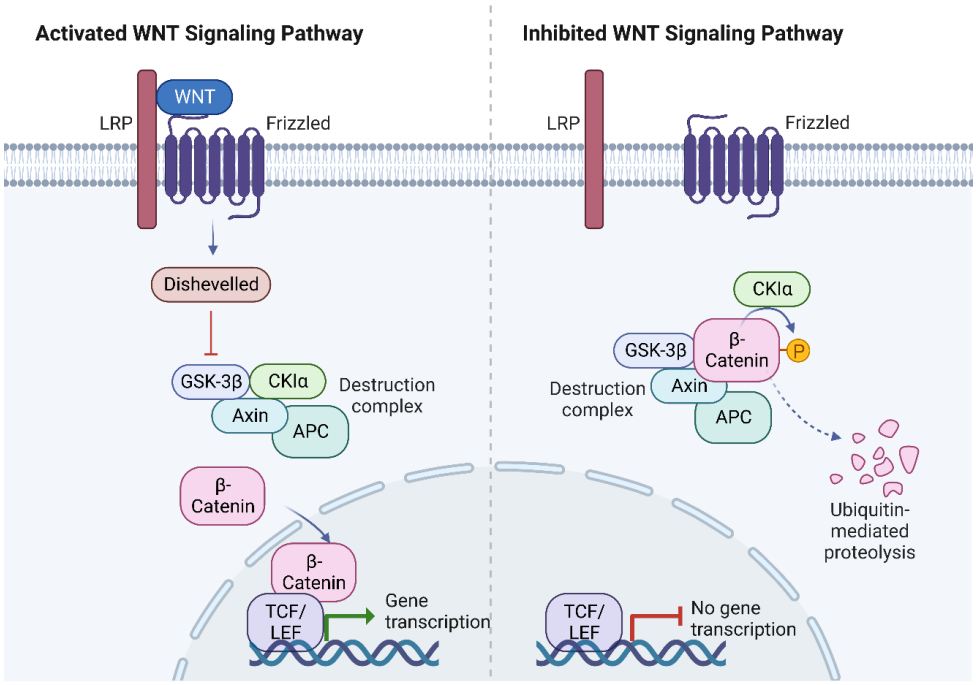

Figure 5. Canonical WNT signaling. Figure created using BioRender.com.

In the signal receiving cells, FZD receptor composition mainly defines the responsiveness of the cell to different WNT ligands, however, to date there is not yet a comprehensive mapping study available of the different WNT ligand-FZD interactions, mainly due to large number of members in both families. There are several studies which show that WNT ligands have different preferences to FZD receptors to initiate the signaling (Rim et al., 2022). In addition, some WNT ligands may bind to and induce active signaling through a broad spectrum of different FZD receptors whereas others may act through limited selection of receptors (Voloshanenko et al., 2017). An additional layer of the WNT signal regulation comes in the form of soluble factors that compete and inhibit the WNT ligand binding to the receptors, such as secreted Frizzled-related proteins (sFZDs) or WNT inhibitory factor 1 (WIF1) (Leyns et al., 1997; Zhang et al., 2023). WNT receptors are subject to ubiquitination, resulting in receptor degradation (Hao et al., 2012; Koo et al., 2012), and the ubiquitination of the receptors is inhibited by internalization of the ubiquitination enzymes by R-spondin, a potent enhancer of WNT signaling (Hao et al., 2012). Finally, in the canonical WNT signaling, beta-catenin mediated activation of gene transcription is in addition to TCF1/LEF fine-tuned by several context specific additional transcriptional factors and chromatin modifiers (Rim et al., 2022).

The non-canonical WNT signaling refers to signaling pathways where beta-catenin is not involved. These are a diverse collection of pathways with variable receptors involved in signal transduction. Of these, the best characterized are the planar cell polarity/WNT pathway and the WNT/Ca^{2+} pathway. The planar cell polarity/WNT pathway (WNT/PCP) has been characterized to be involved in cell movement, for example during neural tube formation. In WNT/PCP, WNT ligand binds to the FZD receptor on the cell surface and an accessory ROR/Ryk receptor. This induces changes in the cell cytoskeleton organization and induction of downstream gene transcription through Rho-family of GTPases and JNK proteins (Koca et al., 2022). WNT/Ca^{2+} activation in turn leads to activation of phospholipase C, which in turn mediates calcium influx in cell, leading to various calcium dependent responses in cell (De, 2011).

Just like in Notch signaling, the effect of WNT signaling in the context of cancer varies and is dependent on multiple factors, including the type of cancer. In many cancer types, WNT signaling hyperactivation is very common, but the routes to reach such a state varies a lot between different cancers (Parsons et al., 2021). Again, the canonical WNT pathway is the best characterized in the context of cancer. Mutational targets that induce hyperactivation of the canonical WNT pathway can be roughly divided into those that still involve WNT ligand binding to the receptor (such as mutations in the R-spondin gene) or those acting downstream of WNT ligand induction. Of the latter one, a classic example is the hereditary forms of colon cancers where components involved in beta-catenin destruction complex are mutated, leading to constantly active WNT signaling (Basu et al., 2016). Depending on the cancer cell type, WNT signal activation can induce various effects, such as maintenance of stem cell characteristics. In addition to the primary tumor, WNT signaling can play pro-tumorigenic role by promoting cancer cell survival in metastatic sites; for instance, interleukin 1 beta produced by the bone marrow induces autocrine Wnt-signaling in breast cancer cells, which promotes bone metastasis (Eyre et al., 2019). Wnt signaling can involve cells in the TME, including the tumor vasculature. As an example, in colorectal cancer, cancer associated fibroblasts secrete WNT2, which stimulates endothelial cell migration and angiogenesis. In a mouse xenograft model, colorectal cancer cells highly expressing WNT2 induced markedly increased tumor vascularization and tumor growth (Unterleuthner et al., 2020). Human brain microvascular endothelial cells treated with glioma-conditioned medium increased mesenchymal marker protein expression, which was mediated by the induction of canonical WNT signaling. In a mouse model, active WNT signaling mediated glioblastoma resistance to temozolomide chemotherapy, and administration of the WNT signaling inhibitor in combination with temozolomide therapy erased tumor associated endothelial cells, reduced tumor growth and improved mouse survival (Huang et al., 2020).

3 Cutaneous melanoma

Cutaneous melanoma arises from melanocytes, the melanin pigment producing cells in skin. The incidence of melanoma has risen dramatically during the last decades, especially in white populations. In Finland, the incidence rates have increased dramatically with almost 2000 new cases per year diagnosed during the last years (https://syoparekisteri.fi/tilastot/tautitilastot/). The majority of the melanoma cases, estimated 75 % in white population, arise from the damaging UV exposure to the skin, which causes highly increased mutation burden on the melanocytes (Arnold et al., 2018; Long et al., 2023). Individuals with fair skin tone, high nevus count, history of chronic sun exposure and sun burns have increased risk of developing melanoma. Although UV exposure has been widely accepted as a primary risk factor for cutaneous melanoma, its role is complex; a large proportion of primary melanomas arise on sites which usually are only modestly exposed to sun light, such as trunk or back (Stanienda-Sokol et al., 2017).

3.1 Clinical and mutational characteristics of melanoma tumors

Melanoma tumors, due to the high impact of UV radiation causing accumulation of mutations, possess characteristically high mutational burden compared to other types of cancers. Therefore, primary melanomas typically have high number of passenger mutations, and it may be sometimes problematic to distinguish them from the tumor driving mutations. However, there are several regularly mutated genes in primary melanoma tumors with distinctive disease driving potential. The aberrant activation of Mitogen-activated protein kinase pathway (MAPK) is especially common in melanoma. The MAPK pathway regulates various cell biological aspects, including proliferation, apoptosis, migration, and cell differentiation. The MAPK pathway is activated when a growth factor binds to its receptor tyrosine kinase on the cell surface. This activates RAS proteins, leading to further activation of downstream proteins RAF and ERK. The activated ERK translocates to the nucleus to activate transcription factors, leading to transcription of the genes required for the biological processes mentioned above (Teixido et al., 2021). In melanoma, components of the MAPK pathways are commonly mutated, the most common mutation being *BRAFV600E*. These mutations lead to hypersensitivity and aberrant activation of these pathways. The mutational status of several of the MAPK pathway members is routinely used in

melanoma diagnostics. In addition to MAPK, genes regulating the pathways such as PI3K-AKT, cell cycle control, MITF or NFkB can also be regularly mutated in primary melanomas (Long et al., 2023).

Melanomas can be categorized into different subtypes, and this categorial information is used in the clinic to assess prognosis and treatment options. The most recent classification system developed by WHO categorizes melanomas based on histological, clinical and genetic features (Yeh, 2023). This classification system also describes precursor lesions and intermediate forms of neoplastic lesions with second genetic mutation, collectively called as melanocytomas (Long et al., 2023), and thereby melanomas are not strictly classified as benign or malign tumors anymore. Although melanocytomas have a low risk into developing into malignant lesions, individuals with multiple such lesions have an increased risk of eventually advance to metastatic melanoma. Primary melanomas typically occur as darkly pigmented lesions, although a subset of cases occurs as non-pigmented, amelanotic lesions which hampers the early detection of the tumor. To confirm the identification of the suspected melanoma tumor, a biopsy and pathological assessment are required. Histopathology is routinely used to assess the samples, and factors such as tumor thickness and ulceration, mitotic rate, lymphovascular invasion and existence of tumor infiltrating lymphocytes are characterized. Usually, histopathologic examination is supplemented by immunohistochemistry to identify the mutational status of specific oncogenes, especially *BRAF* and *NRAS* (Long et al., 2023). To determine whether the tumor has progressed to a metastatic state, a fine needle or core biopsy of the tumor draining lymph node is taken. These are usually taken if the primary tumor shows high risk features, including high mitotic rate, lymphovascular invasion or the tumor has invaded deeply into the underneath tissue. For the patients with a positive sentinel lymph node finding or with a primary tumor possessing high risk factors, whole body imaging methods such as CT or PET-CT are recommended to assess the potential existence of further metastasis.

3.2 Melanoma treatment

For the patients with only primary tumor and no indications of high risk factors, surgical removal of the primary tumor is in many cases enough to cure the disease. In locoregional melanoma spread, melanoma has metastasized to the nearby areas of skin (called satellites), in lymphatic vessels or to tumor draining lymph nodes. For these patients, both primary tumor as well as nearby lymph node(s) are removed as a standard care. However, two randomized clinical trials have found no significant survival benefit between the sentinel lymph node removal or observation of the node (Faries et al., 2017; Leiter et al., 2019); therefore, these

patients need close clinical follow up after the surgical removal of the tumors and may benefit of administration of systemic treatments.
For patients with advanced melanoma and *BRAF* mutations, there is a well-tolerated BRAF inhibitor Dabrafenib treatment available. The response rate is high for this subset of patients, with variable tumor regression and rapid improvement of symptoms. Although the initial responses with these treatments are usually outstanding, majority of the patients develop resistance over time (Long et al., 2023). Development of immunotherapies has tremendously improved the survival of melanoma patients with metastatic disease; before, only 10% of the patients with metastatic disease survived beyond five years. Today, immunotherapies have succeeded to increase the proportion of these patients to around 50%. The immunotherapies target PD-1, CTLA-4 or LAG3, of which targeting the first one has been proven to usually show the best efficacy in melanoma patients. The immunotherapies can be administered as single or combination treatments for the patient. The selection which immunotherapies option to provide for the given patient is not straightforward though, as there are not yet good markers to select which patients benefit most from the therapies; for instance, PD-L1 status of the tumor is not helpful for excluding patients of PD-1 based therapy (Placke et al., 2023). Moreover, immunotherapies come with varying levels of side effects. Furthermore, around 50% of the patients with a metastatic disease do not either respond to these therapies or acquire resistance (Long et al., 2023). Therefore, further investigation of melanoma metastatic processes is crucial to find new therapeutic pathways and targets.

3.3 Notch and WNT pathways in melanoma

Developmental pathways, such as Notch and WNT signaling, can be found aberrantly regulated in melanoma. Both signaling pathways are required for the normal melanocyte development. For instance, canonical WNT signal mediates expression of melanoblast lineage-specific MITF (Takeda et al., 2000); high MITF expression in neural crest cells induces cell development to melanocytes, whereas low expression mediates differentiation into glial cells, neurons and cartilage (Mort et al., 2015). In hair follicles, Notch-mediated signaling maintains melanoblast and melanoblast stem cell populations (Moriyama et al., 2006) through activation of Notch1 and Notch2 (Kumano et al., 2008).
Especially the Notch1-mediated signaling has been linked to more aggressive melanoma. Notch1 levels have been shown to be higher in melanoma tumors compared to melanocytic nevi, and in a mouse xenograft model Notch1 induced melanoma cell proliferation and protected the cells from apoptosis (Bedogni et al., 2008). SOX10, a classical melanoma marker widely used in the clinic, has been

shown to induce Notch1 expression (Tang & Cao, 2021). Notch4 is highly expressed in cell lines from aggressive melanomas, where it balances cell proliferation and protects the cells from apoptosis, and induces vascular mimicry -like growth phenotype in 3D collagen matrix (Hardy et al., 2010). Involvement of active Notch signaling in the melanoma pro-tumorigenic properties is further supported by a study which showed that Notch downstream target Hey1 mediates increased invasion and metastasis through the PIK3/Akt pathway (Pu et al., 2021). Overall, the multiple studies show that Notch signaling promotes more aggressive behavior of melanoma cells, and therefore Notch pathway inhibition may represent a viable option for combinatorial treatments for patients with aggressive disease.

The role of WNT signaling in melanoma initiation and progression has been quite extensively studied, with variable conclusions and sometimes conflicting results. However, several studies suggest that the canonical WNT signaling acts as a tumor suppressive pathway in melanoma, whereas the non-canonical signaling is pro-tumorigenic. In melanomas, low levels of nuclear beta-catenin have been reported in vitro, resulting in increased invasive properties in the melanoma cells (Arozarena et al., 2011), and in freshly isolated cells from patient samples low nuclear beta-catenin was associated with TNF-alpha induced inflammatory pathway and chemoresistance (Kovacs et al., 2016). In melanoma patient samples, beta-catenin levels have been shown to be lower in melanoma metastases versus primary melanoma (Kageshita et al., 2001), and higher overall nuclear beta-catenin expression in patient samples correlates with better prognosis (Chien et al., 2009). In a melanoma mouse model, however, abundant beta-catenin levels in cancer cells promoted melanoma metastasis to lung (Gallagher et al., 2013), possibly reflecting that beta-catenin is required for reversing the EMT process to enable establishing colonies at distant metastasis sites for cell colony growth. In addition, there are several reports which indicate that WNT antagonists may act as a suppressor for melanoma. For instance, levels of DKK, a WNT antagonist, are relatively low in melanoma cell lines and were shown to be low in melanoma patient samples as well (Kuphal et al., 2006). Overall, these studies suggest that the canonical WNT signaling maintains the less migratory and invasive characteristics of the cells, and that suppression of beta-catenin mediated signaling leads to more aggressive phenotype required for melanoma initiation, possibly through the EMT changes, and that the establishment of macroscopic distant metastasis benefits from the induction of the canonical WNT signaling. However, further investigation with systematic studies of the role of different WNT ligands and their receptors upon the canonical Wnt signaling in the context of melanoma are still required.

The best characterized WNT ligand in melanoma is WNT5A, which has been linked to both the canonical and non-canonical signaling depending on the receptor

context (Gajos-Michniewicz & Czyz, 2020). In melanoma, WNT5A can increase the canonical Wnt signaling in an unique way: binding of WNT5A to FZD4/LRP6 activates ARF6, which in turn potentiates beta-catenin release from N-cadherin on the plasma membrane, thereby increasing the pool of free beta-catenin that can translocate to the nucleus (Grossmann et al., 2013). WNT5A through binding to ROR2, a non-canonincal Wnt ligand receptor, suppresses tumor growth by inhibiting the YAP/TAZ pathway (Wang et al., 2023). WNT5A induction of melanoma cells leads to phosphorylation of ATP1, which then in turn catalyzes depalmitoylation of the pro-metastatic adhesion molecules CD44 and MCAM, which promotes melanoma cell invasion (Sadeghi et al., 2018). WNT5A induction can also regulate the expression of WNT receptors: WNT5A downregulates ROR1, a marker of poorly invasive melanoma cells. In a mouse xenograft model, silencing of ROR1 by siRNA in melanoma cells inhibited primary tumor growth but enhanced metastasis (O'Connell et al., 2013). These studies suggest that WNT5A acts as one of the switch proteins mediating melanoma cell to change from a proliferative to a less proliferative, invasive cell. Altogether, WNT signaling may be an important switch between the different phenotypes in melanoma and mediator of cell plasticity. While the non-canonical WNT signaling mediates melanoma cell invasion and metastasis, the canonical signaling is important for tumor growth and the melanoma cell colony formation at the metastatic sites.

Aims of the study

Cancer cells and the TME cells exhibit remarkable plasticity during cancer progression. For instance, cancer cells as well as other cell types in the TME are known to induce lymphangiogenesis, and in melanoma, a dense cancer associated lymphatic network correlates with poor prognosis. However, in addition to lymphangiogenesis, there are other mechanisms involved in the melanoma communication with the lymphatic endothelium, which are not yet thoroughly understood. The purpose of this thesis is to characterize the tumor promoting melanoma crosstalk with lymphatic endothelial cells. Specifically, the aims are:

1. To characterize if the lymphatic endothelial cell – melanoma cell interactions contribute to cancer progression and metastasis.

2. To discover and characterize the key molecules involved in the melanoma cell – lymphatic endothelial cell communication.

Materials and methods

1. Mouse models (I, II)

To test the tumorigenicity of the melanoma cells in the study I, the control Bowes and WM852 as well as LEC co-cultured melanoma cells were injected subcutaneously into immunocompromised C.B17/IcrHanTMHSD-Prkdc Scid (SCID) mice. The melanoma cells expressed a douple eGFP-luciferase reporter, which was used to the in vivo imaging of the organs (Caliper IVIS Kinetic System). The mice were followed up to 70 days after which the mice were sacrificed and the tumors were collected for further analysis by immunohistochemistry and the metastasis was assessed by luciferase activity. In addition, human cell presence in the lungs was measured by detecting the human cell specific ALU sequences and mouse genomic DNA was used as a control.
In the study II, the siRNA treated, control or LEC-cocultured WM852 cells were injected intradermally into ear pinnae of NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (NOD-SCID). The mice were followed for 7, 8 or 14 days after which the mice were sacrificed, and the ears and superficial cervical lymph nodes were collected. The ears were split to dorsal and ventral halves and fixed with 4% PFA and imaged with a Zeiss LSM 780 confocal microscope. The relative total areas occupied by the GFP positive melanoma cells was quantified by ImageJ. The presence of melanoma cells was either addressed by FACS, to which the lymph nodes were processed to obtain single cell suspension and analyzed with Accuri C6 Plus analyzer (BD Biosciences), or by first extracting the genomic DNA followed by measuring of relative ALU sequence presence by qPCR. The mouse experiments were approved by the Committee for Animal Experiments of the District of Southern Finland (license ESAVI/10548/2019 or ESAVI/434/04.10.03/2012).

2. Zebrafish embryo model (I)

The zebrafish embryos (Danio Rerio) of casper strain (roy-/-; mitfa-/-) were collected two days after spawning and injected with siRNA treated, monotypic or LEC-cocultured WM852 melanoma cells into the pericardial space. For the injections, we used CellTramVario (Eppendorf), Injectman Ni2 (Eppendorf) micromanipulator, and borosilicate glass needles pulled from glass capillaries (TW100-4, World Precision Instruments Ltd) using micropipette puller (PB-7, Narishige). Four days after injection, embryos were imaged with Zeiss

StereoLumar V12 fluorescence microscope. The zebrafish embryo experiments were approved by the Committee for Animal Experiments of the District of Southern Finland (licence ESAVI/9339/04.10.07/2016).

3. Cell lines (I, II)

The cell lines used are listed in table 1. The culture conditions are described in the references.

Table 1. Cell lines used in this thesis.

Cell line	Origin	Identifier
Adult dermal microvascular lymphatic endothelial cell (LEC)	Lonza, Basel, Switzerland	CC-2543
WM852	Wistar Institute Philadelphia	RRID:CVCL_6804
WM165	Wistar Institute Philadelphia	RRID:CVCL_L033
Bowes	D.B. Rifkin, Rockfeller University	RRID:CVCL_3317
WM793	Wistar Institute Philadelphia	RRID:CVCL_8787

4. Cell culture models

4.1. 2D Melanoma cell-LEC co-culture (I, II)

For the co-culture assays, melanoma cells and LECs were plated on the fibronectin (Sigma) precoated plates in 1:1.5.1:3 ratio in endothelial cell media (EGM-2, Lonza, supplemented with all other supplements except VEGF). The cells were cultured for two days before cell sorting.
For the study I, co-cultures were sorted using magnetic cell separation. To this end, melanoma cells were prelabeled with 1mg/ml fluidMAG-DX (Chemicell) magnetic beads before the co-culture. After the co-culture, the melanoma cells and LECs were sorted using MidiMACS separator and LS columns (Miltenyi Biotec). For the study II, the co-cultures of LEC and GFP expressing melanoma cells were sorted using SH800Z cell sorter (Sony).

4.2. 3D fibrin embedded cells (I,II)

For the LEC spheroid assays, LECs were first plated on the agarose coated U-bottom plates. Next day, the preformed spheroids were harvested and embedded into the fibrin matrix using a mixture of plasminogen-free fibrinogen (final concentration 3 mg/ml, Calbiochem) and thrombin (final concentration 2U/ml, Sigma) in Hank's balanced salt solution supplemented with aprotinin (200µg/ml, Sigma). Melanoma cells were embedded as a single cell suspension into fibrin using a mixture described above. After four days, fibrin embedded cells were fixed, and depending on the assay, further stained. The cells were imaged either with a Nikon Eclipse TS2 phase contrast microscope or a Zeiss LSM780 confocal imaging system. The spheroid sprouting was quantified using ImageJ and the melanoma sprouts using ImageJ or Bioimage XD.

4.3. Tube formation assay (II)

LECs were plated on a 96-well plate precoated with Cultrex (R&D Systems). Next day, the wells were imaged with a Nikon Eclipse TS2 phase contrast microscope and the quantification of the relative length of the structures was performed with ImageJ.

4.4. Electrical cell-substrate impedance assay (II)

The 96-well plates by Ibidi (96W10idf) were precoated with 10mM L-cysteine and 5 µg/mL fibronectin. LECs were seeded on the plate and the resistance of the cell layers was recorded at 4000Hz with ECIS Z Theta instrument and ECIS software, version v.1.4.8 (Ibidi) over four days.

5. RNA interference (I,II)

For the RNA interference experiments, cells were transfected using siRNAs listed in table 2 using RNAiMAX according to the instructions by the manufacturer and as explained in the references. Cells were cultured in the presence of siRNAs for 24h before used for subsequent assays. The silencing efficiency was assessed by qPCR.

Table 2. siRNAs used in this thesis.

siRNA	ref. no	source	used in
FZD6	L005505-00-0005	Dharmacon	II
FZD8	L-003962-00-0005	Dharmacon	II
MMP14	SI03648841	Qiagen	I
MMP14	SI00071176	Qiagen	I
non-target control siRNA	MA4390843	Ambion	I, II
non-target control siRNA	D-001810-10-05	Dharmacon	I, II
non-target control siRNA	1022076	Qiagen	I
NOTCH3	L-011093-00-0005	Dharmacon	I, II
NOTCH3	4392420	Ambion	I, II
WNT5B	L-009761-00-0005	Dharmacon	II

6. Transient transfection (I,II)

Melanoma cells were seeded on cell culture dishes one day prior transfection to reach 80%–90% confluence the next day. Cells were then transfected with either NICD3-pCLE (Addgene), MMP14 Sport6 (GenomeBiology Unit, University of Helsinki) or PcDNA3 (Invitrogen) using Fugene HD (Promega) according to the manufacturers' instructions.

7. Inhibitor and ligand stimulation assays (I, II)

For the inhibitor assays, Gamma-secretase inhibitor DAPT (Sigma) and pan-MMP inhibitor GM6001 (Tocris Biosciences) at 10 μM concentrations, MMP14 hemopexin domain inhibitor NSC 405020 (Selleckchem) at 50 μM and the β1-integrin blocking antibody AIIB2 were applied to the growth medium during the 48 h cell cultures or 96 h in 3D fibrin assays. Recombinant WNT5B protein (7347, R&D Systems) at 1000 ng/mL was applied was applied on LECs for 16 h.
For the Notch receptor induction, cell culture plates were coated with the Fc domain, or Notch ligand fused with the Fc domain at concentration of 10 μg/mL for 6 hours at room temperature (DLL1-Fc, 10184-DL-050 Biotechne; DLL3-Fc, DL3-H5255 ACRO Biosystems; DLL4-Fc, 158- 10171-H02H-100 Sino Biological;

JAG1-Fc, 158-11648-H02H Sino Biological, and JAG2-Fc, 1726-JG-050 R&D Systems). Melanoma cells were cultured for 48 h on the coated plates hours and then harvested and used for subsequent assays.

8. Immunofluorescence of 2D and 3D cultures (I,II)

Cells were fixed with 4% PFA in PBS for 15-60 min at room temperature followed by permeabilization with 0.1-0.3% Triton X. The 3D cultures were in addition fixed with 1:1 acetone-methanol for 1 min. The fixed samples were stained with primary antibodies listed in table 3. The secondary antibodies were labelled with fluorochromes AlexaFluor 488, 594 or 647. Hoechst 33342 was used for nuclear staining. The samples were imaged with Zeiss epifluorescent microscope or Zeiss LSM780 confocal imaging system.

Taulukko 1 **Table 3.** Antibodies used for immunofluorescence staining.

antibody	description	source	used in
active β1-integrin	30394	Abcam	I
active β1-integrin	553715	BD Pharmingen	I
MMP14	51074	Abcam	I
Notch3	sc-5593	Santa Cruz	I
PECAM	M0823	Dako	I
total β1-integrin	P5D2, DSHB hybridoma	Johanna Ivaska	I
VE-cadherin	555661	BD Biosciences	I,II
WNT5B	ab94914	Abcam	II
ZO-1	402200	Invitrogen	II
β-catenin	610153	BD Biosciences	II

9. Real time quantitative PCR (qRT-PCR) (I,II)

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey Nagel). RNA was reverse transcribed using the TaqMan reverse transcription kit (Applied Biosystems). The qPCR reactions were performed using SYBR Green PCR mix (Applied Biosystems) and Lightcycler 480 (Roche). The primers used are listed in table 4.

Table 4. Primers used for qRT-PCR

primer target	sequence/reference	source	used in
ACT	TCACCCACACTGTGCCATCTACGA, CAGCGGAACCGCTCATTGCCAATGG	Oligomer	I,II
CD105	AGC CCC ACA AGT CTT GCA G GCT AGT GGT ATA TGT CAC CTC GC	Oligomer	II
CD31	AACAGTGTTGACATGAAGAGCC, TGTAAAACAGCACGTCATCCTT	Oligomer	I
CD34	TGGGCATCACTGGCTATTTC, CCACGTGTTGTCTTGCTGAA	Oligomer	I
DLL1	GAT GTG ATG AGC AGC ATG GA CCA TGG AGA CAG CCT GGA TA	Oligomer	II
DLL3	AAT CGC CCT GAA GAT GTA GAC C GCA CCA CCG AGC AAA TAC AA	Oligomer	II
DLL4	CTG GAG CTC AGC GAG TGT GAC CCT GGT CCT TAC AGC TGC CTC	Oligomer	II
EDN1	GAC ATC ATT TGG GTC AAC ACT C GGC ATC TAT TTT CAC GGT CTG T	Oligomer	II
FLT4	GACAGCTACAAATACGAGCATCTG, CTGTCTTGCAGTCGAGCAGAA	Oligomer	I
FZD1	TCG ACT TCC TGA AGC TGG AT AAG GTG GGA GAA GGG AGT GT	Oligomer	II
FZD10	CCT CCA AGA CTC TGC AGT CC GAC TGG GCA GGG ATC TCA TA	Oligomer	II
FZD2	CCC GAC TTC ACG GTC TAC AT CTG TTG GTG AGG CGA GTG TA	Oligomer	II
FZD3	TCT CTT TGG CCC TTG ACT G ACA AAG AAA AGG CCG GAA AT	Oligomer	II
FZD4	CCA GGA TTC CTT CCA AGT CA CCA TGT CCT TGT GGC CTA CT	Oligomer	II
FZD5	AGC TAA AAT GGC CAG AGC AA AAT TCC CCC TGG GAA CTA TG	Oligomer	II
FZD6	TTG TTG GCA TCT CTG CTG TC	Oligomer	II

	CCA TGG ATT TGG AAA TGA CC		
FZD7	CGA CGC TCT TTA CCG TTC TC GCC ATG CCG AAG AAG TAG AG	Oligomer	II
FZD8	GAC ACT TGA TGG GCT GAG GT CAA ATC TCG GGT TCT GGA AA	Oligomer	II
FZD9	AGA CCA TCG TCA TCC TGA CC CCA TGA GCT TCT CCA GCT TC	Oligomer	II
GAPDH	TCACCACCATGGAGAAGGCT, GCCATCCACAGTCTTCTGGG	Oligomer	I
HES1	TCAACACGACACCGGATAAA, TCAGCTGGCTCAGACTTTCA	Oligomer	I,II
HEY1	GTTCGGCTCTAGGTTCCATGT, CGTCGGCGCTTCTCAATTATTC	Oligomer	I,II
HEY2	TTGAGAAGACTTGTGCCAACTG, GTGCGTCAAAGTAGCCTTTACC	Oligomer	I
IL32	CTG TGA AGA CAG CCT TGG TG GAG TGA GCT CTG GGT GCT G	Oligomer	II
JAG1	TGC CAA GTG CCA GGA AGT GCC CCA TCT GGT ATC ACA CT	Oligomer	II
JAG2	TGG CAC TCG CTG TAT GAA AG AGG GCC ACA TCA ATA ACC AG	Oligomer	II
MMP14	GCAGAAGTTTTACGGCTTGCAA, CCTTCGAACATTGGCCTTGAT	Oligomer	I
NOTCH1	GAGGCGTGGCAGACTATCATGC, CTTGTACTCCGTCAGCGTGA	Oligomer	I
NOTCH2	CCTGGGCTATACTGGGAGCTACTG ,ACACCCTGATAGCCTGGGACAC	Oligomer	I
NOTCH3	QT00003374	Qiagen	I,II
NOTCH4	AATCCCACTGCCTCCAGACT, TTGTGGCAAAGGGAAGAGAC	Oligomer	I
WNT5B	CGT GGA GTA CGG CTA CCG CT CAG GCT ACG TCT GCC ATC TTA T	Oligomer	II

10. ChIP-PCR (II)

WM852 cells were transfected with the expression plasmid NICD3-pCLE for 48 h after which the chromatin was processed by the SimpleChIP kit according to the manufacturer's instructions (Cell Signaling Technology). The chromatin was precipitated using antibodies against Notch3 (M-134, Santa Cruz Biotechnology or 8G5, Cell Signaling Technology) or normal goat or rat IgG antibody (sc-2028 or sc-2026, Santa Cruz Biotechnology). DNA was purified using the PCR Purification kit (Macherey-Nagel) according to the manufacturer's instructions. After DNA purification, the promoter regions of *WNT5B* were amplified and analyzed by qPCR.

11. SDS-PAGE and immunoblotting (I,II)

Cells were lysed in RIPA buffer (150 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Bio-Rad). Equal amounts of lysates were loaded on 4-15% gradient SDS-PAGE gels (Bio-Rad). After SDS-PAGE runs, proteins were transferred on nitrocellulose membranes (Bio-Rad). The blots were blocked in 5% non-fat dry milk and probed with primary antibody dilutions (anti-β1-integrin; 553715, Abcam, anti-Notch3; sc-5593, Santa Cruz, anti- β-actin; A1978, Sigma) followed by incubation with HRP-conjucated secondary antibodies (Millipore or Cell Signaling). Bands were detected by chemiluminescence using ECL solution (Advansta) and Chemi-Doc (Bio-Rad).

12. Immunohistochemistry of tissue sections (I,II)

Mouse tissues and tumors were fixed with 4% PFA for 24h, embedded into paraffing and cut into sections. Before the primary antibody incubation, tissue sections were treated with antigen retrieval buffer (10nM citrate, pH 6.0). Omission of the primary antibody served as negative control. The signal amplification biotin system kit (Perkin Elmer) was used to detect the signal. The samples were imaged using Alexa594 and Alexa647 fluorochrome conjugated secondary antibodies for MMP14 and LYVE-1 and anti-rabbit HRP and DAB as a substrate for detection of Notch3. The images were acquired with 3DHistech Pannoramic 250 FLASH II digital slide scanner (3DHISTEC Ltd.) or Zeiss LSM780 confocal imaging system.

For the patient sample study, the paraffin-embedded melanoma tumor or metastasis sections were provided by the Helsinki Biobank. Tissue sections were deparaffinized and the EnVision FLEX+ kit (Dako, Agilent) was used to stain the

tissues. Epitope retrieval was performed using the EnVision FLEX Target Retrieval solution, high pH, for 15 minutes at 95°C. Endogenous peroxidase blocking was performed according to the manufacturer. Tissue was blocked using the normal horse serum blocking solution (S2000, Vector Laboratories) diluted 1:20 in antibody diluent (Dako). Sections were stained with either anti-Notch3 (HPA044392, Sigma-Aldrich) or anti-WNT5B antibodies (ab94914, Abcam) diluted in the Dako antibody diluent for 16 hours at 4°C. Secondary antibody treatment was performed according to the instructions of the kit. Chromogenic reactions were performed using the Romulin AEC Chromogen kit (Biocare Medical) and hematoxylin staining using Mayer's Hematoxylin (Dako). The slides were imaged with a Pannoramic 250 Flash II.

13. TCGA data analysis

The RSEM-quantitated mRNA-seq data and the associated clinical data from the TCGA Melanoma PanCancer Atlas data set were analyzed by Kaplan-Meier survival association analysis with the log-rank method and Pearson's correlation analysis. SPSS v29.0.1.0 (IBM) was used to perform the analyses and visualize the data.

14. Fluorescence-activated cell sorting (FACS) (I,II)

In the mouse studies, lymph nodes were processed to obtain single cell suspension for FACS as described in (Broggi et al., 2014). Isolated cells were then suspended in PBS and 30,000 cells were analyzed using an Accuri C6 Plus analyzer (BD Biosciences). The lymph nodes from mice without tumor cell injection were used as a negative control for GFP gating. For the cell culture studies, LECs and GFP expressing melanoma cells were sorted using a SH800Z cell sorter (Sony).

15. Global gene expression analysis (I,II)

In study I, total RNA from three biological replicates was extracted using a TRI reagent (Sigma) protocol supplemented with phenol-chloroform precipitation step. The RNA quality was checked by Bioanalyzer (Agilent Technologies). RNA sequencing was performed with NextSeq500 sequencer (Illumina). The data was aligned to HS GRCh38.76 reference genome and the differentially expressed genes were obtained with DESeq2 Bioconductor package. GAGE Bioconductor package was used for pathway analysis and KEGG pathway maps were generated with Pathview. The gene heatmap was generated with Morpheus.

For study II, LECs sorted from WM852 melanoma co-cultures (Sample 2) and a mix of parental LECs and WM852 (Sample 1) were analyzed by single cell RNA

sequencing. The parental melanoma cells were mixed to the sample 1 in 1:10 ratio to identify the potential residual WM852 cells from the sample 2 after the cell sorting. The sample cells were processed according to the 10x Genomics guidelines. The Single Cell 3′ Reagent kit v2 was used to process the samples and generate the cDNA library. Data postprocessing and quality control were performed with 10× Genomics Cell Ranger (v2.1.1) software. The filtered count matrices were preprocessed and clustered using the Seurat v4.3.0 tool in an RStudio v2023.03.0 environment. Upregulated genes in co-cultured versus control sample were used for gene ontology analysis using ShinyGO v0.77.

16. Statistical analysis (I,II)

All the numerical values present the mean +/- SD. Statistical significance was determined using t test or ANOVA and Microsoft Excel or GraphPad Prism.

Results and Discussion

1. Melanoma cell-LEC crosstalk leads to functional changes in both cell types.

In melanoma, the presence of a dense lymphatic network within the primary tumor correlates with poorer prognosis (Pastushenko et al., 2014), and the occurrence of lymph node metastasis is used as a standard prognostic factor for clinical outcome (Long et al., 2023). However, removal of the tumor draining lymph node(s) does not improve the outcome among the patients (Faries et al., 2017; Leiter et al., 2019). These results suggest that the lymphatic system increases aggressiveness of melanoma already before the cancer cells have reached the lymph node. This cancer promoting crosstalk likely occurs already in the skin, since the skin has a relatively extensive lymphatic network (Skobe & Detmar, 2000). In this project we hypothesized that melanoma cell-LEC interaction would lead to melanoma progression through changes in melanoma cells, LECs, or both. To investigate the potential functional changes *in vitro* in both cell types, we cultured primary human LECs together with GFP expressing melanoma cell lines for two days, after which the cell types were separated and used for further assays (Fig. 1a in II). First, the melanoma cells were subjected for invasion assays in 3D fibrin matrix (Fig. 3a in I). WM852 and WM165 cells, originating from melanoma metastases, formed round spheres when the cells were derived from the monotypic, parental control cultures, whereas the LEC-co-cultured cells formed sprouts, resembling collective cell invasion. Two cell lines originating from primary tumors, Bowes (from a horizontal growth phase tumor) and WM793 (from a vertical growth phase tumor), did not change their growth phenotype upon LEC co-culture. These results indicate that melanoma cells with metastatic, aggressive origin are capable to boost rapidly their invasive potential following contact with LECs. The role of LECs being a potent cancer cell invasion promoting driver in the TME is supported by a similar finding from a prostate cancer cell line study, where LECs enhanced the invasion of an invasive PC-3 cell line but not a noninvasive DU-145 in Matrigel (Shah et al., 2016), and from a malignant skin keratinocyte model where LECs promoted invasion of cancer cell spheroids in 3D collagen matrix (Van de Velde et al., 2021).

Next, we performed several functional assays with the monotypic and melanoma co-cultured LECs to assess whether melanoma cells impact their biology. We observed multiple functional changes in LECs upon co-culturing them with melanoma cell lines WM852, WM165 and WM793. LEC spheroids after melanoma cell co-culture with all three cell lines showed induction of sprouting when

cultured in a 3D fibrin matrix, indicating changes in the LEC motility and matrix degradation (Fig. 1b in II). Moreover, we observed impaired spontaneous tube formation induced by melanoma cells (Fig. 1c in II) and disrupted barrier function (Fig. 1d in II), suggesting weaker cell-cell junctions. Therefore, we studied the expression of few proteins involved in adherens or tight junctions in LECs. In accordance with the weaker barrier function of the LEC layers upon melanoma co-culture, we observed less ZO-1 and beta-catenin signal in the LEC cell-cell junctions (Fig. 1e in II). Although we did not see any significant reduction in the VE-cadherin signal in the cell-cell contact sites, the co-cultured LECs showed less serrated and reticular staining pattern, possibly indicating weaker adherens junctions (Henderson et al., 2021; Kakei et al., 2014). Overall, these results suggest that upon melanoma co-culture, LECs gain invasive properties including the ability to degrade and migrate in the 3D fibrin matrix, and that their cell-cell contacts get distorted, making LEC layers more permeable. It has been suggested that cancer cells may gain access to the lymphatic vasculature through mechanically damaging the vessel walls (Chen et al., 1999; Niimi et al., 2001). However, our study together with other studies using other cancer types indicate that cancer cells actively modify the LEC junctions, making the LEC layers more permeable. For instance, an orthotopic 4T1 mouse model indicates, that many inflammation associated genes are upregulated in the tumor associated LECs, including VCAM-1, which causes displacement of VE-cadherin from the LEC surface, leading to increased permeability and facilitation of cancer cell lymphatic invasion (Dieterich et al., 2019). In an *in vitro* colorectal cancer model, VEGF-C reduces VE-cadherin expression in LECs and this same study has shown with a mouse model that VEGF-C expression by an adenoviral vector induces trans-endothelial migration of colorectal cancer cells (Tacconi et al., 2015). Therefore, it is likely that different types of cancer cells gain access to the lymphatic vasculature by actively making the lymphatic endothelial cell barrier more permeable.

2. Melanoma cell-LEC crosstalk induces transcriptomic changes in both cell types.

To characterize the molecular changes in both melanoma cells and LECs, we performed total RNA sequencing for the parental monotypic and LEC-cocultured WM852 and Bowes melanoma cells lines (Fig. 2a-c in I) and single cell RNA sequencing for the parental monotypic or WM852 co-cultured LEC (Fig. 2a-b in II).

The pathway analysis (GAGE, Generally Applicable Gene-set Enrichment for Pathway Analysis) of the bulk RNA sequencing from the melanoma cells indicated, that in both WM852 and Bowes cell lines pathways involved in regulation of actin cytoskeleton, Notch and WNT signaling, and gap junctions were upregulated upon

LEC co-culture (Fig 2a in I). Interestingly, ECM-receptor signaling pathways were upregulated in Bowes as opposed to WM852. In addition, pathways related to focal adhesion, TGF-beta signaling, and tight junctions were only enriched in Bowes. When compared to a study by Chida et al, 2021, several of these findings are consistent with a mouse *in vivo* study, in which a mouse melanoma cell line with enhanced lymph node metastasizing capability was generated by subcutaneous injection of C8161 melanoma cells, after which the cancer cells in the tumor draining lymph nodes were isolated and the procedure was repeated once more. This study shows that pathways regulating cell-cell junctions are upregulated in the lymph node metastasis prone melanoma cell line compared to the parental cell line (Chida et al., 2021). Our RNAseq data indicated upregulation of the Notch pathway in melanoma cells upon LEC co-culture. This is in accordance with a study where melanoma patient cohort from the TCGA database as well as tumor microarray samples were analyzed for Notch1 mRNA and protein levels, indicating that high Notch1 expression correlates with poor overall survival (Su et al., 2021). Taken together, these studies suggest that melanoma cell contact with LECs favors induction of pathways that may facilitate melanoma cell invasion and metastasis.
Changes in the LEC transcriptome upon melanoma co-culture was analyzed by single cell RNA sequencing (scRNAseq). For this, we prepared two different samples, the control sample (Sample 1, Fig. 2a in II) consisting of parental LECs mixed with parental WM852 melanoma cells in a 9:1 ratio and sample 2 consisting of LECs originating from the WM852 co-cultures (Sample 2, Fig. 2a in II). For sample 1 the parental melanoma cells and LECs were mixed to identify the possible residual remaining melanoma cells after the cell sorting using magnetic beads. Upon sample analysis, nine different cell clusters could be distinguished. We observed that the distributions of the samples 1 and 2 varied in clusters (Fig. 2b in II), indicating melanoma-induced transcriptomic changes in LECs. We used the expression of *PROX1* (a LEC marker) and *SOX10* (a melanoma marker) to locate the LECs and the melanoma cells in the clusters (Supplemental figure 2a in II), and the clusters were subsequently named as LEC or melanoma clusters (Fig. 2a-b in II). The GO (Gene Ontology) analysis of differentially expressed genes in the LECI and LECII clusters indicated for instance, that Renin-Angiotensin pathway was upregulated in LECs upon melanoma cell co-culture (Fig. 2a and Supplemental data table 2 in II) and that Pyrimidine metabolism pathway was downregulated, suggesting suppression of cell proliferation. In the Sample 2 LECs, we could also find upregulated several genes involved in inflammatory or endothelial cell activation processes, and upregulation of these was confirmed by qPCR (Supplemental fig. 2b in II). Our results are in line with a study where LECs were conditioned by A375 melanoma cell media by culturing the two cell types in a Transwell-chamber system followed by bulk RNA sequencing and showing changes in pathways involved in cytokine response, lymphatic activation and cell

proliferation (Turati et al., 2023). In addition, a study where LECs were conditioned with the medium derived from malignant human skin keratinocyte cells shows increase of inflammatory response genes in LECs upon conditioning, such as ICAM-1 and IL6 (Van de Velde et al., 2021). A mouse study where the gene expression of afferent lymphatic vessels of the lymph nodes with gastric cancer metastasis was studied by RNA sequencing indicates that for instance cytokine-cytokine receptor signaling and tube morphogenesis pathways are altered in LECs upon induction by cancer cells (Xu et al., 2012). Overall, different transcriptomic studies, including ours, indicate that cancer cells cause wide transcriptional changes in LECs to promote cancer progression.

3. LEC co-culture promotes distant metastasis of melanoma cells *in vivo.*

To characterize the consequences of LEC interaction on melanoma cell characteristics *in vivo*, WM852 and Bowes originating from monotypic or LEC 3D (co)-cultures (Fig. 1a in I) were injected subcutaneously into immunocompromised mice. We did not observe any difference in the tumor growth rate between the monotypic and LEC co-cultured samples (Fig. 1c-d in I). However, when WM852 cells had first been cultured with LECs they showed significantly enhanced metastasis to liver and lung compared to the monotypic control WM852 cells (Fig. 1e-f in I). In line with the non-metastatic origin of Bowes, these cells did not metastasize to liver or lung (Fig. 1 – figure supplement 1g in I), however, they formed metastasis in some of the isolated lymph nodes (Fig. 1 -figure supplement 1e in I).

Lymphatic endothelium may enhance cancer cell aggressive behavior by different mechanisms, which ultimately lead to increased metastatic burden. For instance, a study with ovarian cancer cells shows that the cells cultured in LEC conditioned medium exhibit increased numbers of pseudopodia, proliferate faster and show increased invasive and migratory abilities (Xie et al., 2016). MDA-MB231 breast cancer cells induce LECs to express CCL5, which facilitates the cognate receptor CCR5 expressing cancer cell metastasis to lung (Lee, Fertig, et al., 2014). Increased invasive, migratory and adhesive properties evoked by the contact with LECs or by soluble signals received from them ultimately create more opportunities for tumor cells to get and be in contact with the blood and lymphatic vasculature, and therefore increases the probability of metastatic dissemination. However, the molecular mechanisms to achieve the more aggressive outcome are various and may vary from one cancer type to another. Therefore, we set to investigate the molecular events mediating increased melanoma cell aggressiveness upon the LEC co-culture.

4. Notch3 mediates the invasive sprouting of melanoma cells.

To characterize the factors inducing melanoma cell invasion upon the LEC co-culture, we cultured the WM852 melanoma cells in LEC or LEC-WM852 co-culture derived conditioned medium. We did not observe induction of the invasive sprouting phenotype in melanoma cells, indicating that direct cell-cell contact is needed for the invasive switch (Fig. 3d in I). Our RNA seq data indicated Notch pathway as one of the most upregulated pathways in the melanoma cells upon co-culture. Interestingly, a molecular profiling study has indicated that *NOTCH3* is one of the upregulated pathways in melanoma cells upon culturing the cells with HUVECs (Stine et al., 2011), and that NICD3 overexpression in the melanoma cells activates downstream *HEY1* expression which enhances cell migration (Howard et al., 2013). Our data indicated that *NOTCH3* mRNA is induced in the metastatic WM852, as well as the Notch downstream targets *HEY1*, *HES1* and *HEY2* (Fig. 2d in I). Moreover, we observed upregulation of Notch3 protein levels in WM852 and WM165 upon co-culture (Fig. 2f and Figure supplement 2f in I). Silencing *NOTCH3* by siRNA in the melanoma cells diminished the LEC co-culture induced invasive sprouting of the metastatic WM852 and WM165 cell lines. Finally, we observed upregulation of Notch3 protein levels also in the LEC primed WM852 mouse tumors compared to the monotypic WM852 tumors (Fig. 2e and Figure supplement 2e in I). Overall, these results indicate that Notch3 is an important key protein when the aggressive potential of the metastatic melanoma cells becomes unleashed by LECs.

5. MMP14 is involved in increased aggressiveness and activation of Notch3 in melanoma cells.

Since we observed the increase in invasive sprouting of the metastatic melanoma cells in 3D fibrin upon LEC co-culture, we hypothesized that melanoma cells boost the activity or presence of the ECM degrading enzyme(s) on the plasma membrane. We suspected the contribution of MMP14, since its expression in melanoma correlates with tumor progression (Hofmann et al., 2000), and this protease degrades fibrin (Hiraoka et al., 1998). By IFA we observed a relocalization of MMP14 to the plasma membrane in WM852 cells upon LEC co-culture (Fig. 4a and Fig.4 supplement 1b in I), a slight increase in the MMP14 levels in WM165 (Fig. 4 figure supplement 1b), but neither increase in protein level nor MMP14 relocalization was observed in Bowes or WM793 (Fig. 4 figure supplement 1b). Accordingly, MMP14 inhibition by GM6001 or silencing by siRNA dramatically reduced the LEC co-culture induced invasive sprouting of WM852 (Fig. 4 Figure supplement 1 d-e in I).

Since *NOTCH3* depletion also inhibited the invasive sprouting of the LEC primed melanoma cells, we investigated whether Notch3 and MMP14 were co-regulated in the WM852 cell line. Immunofluorescence staining indicated that Notch3 and MMP14 occasionally co-localize on the plasma membrane of the co-cultured WM852, whereas co-localization was not observed in the monotypic WM852 (Fig. 4c in I). Depletion of *MMP14* by siRNA led to decreased levels of *HEY1* and *NOTCH3* (Fig. 4d in I), and MMP14 inhibition reduced both the full length and cleaved Notch3 levels in the co-cultured WM852 cells (Fig.4 figure supplement 2d-e in I). Together, these data indicated that MMP14 positively regulates Notch3 activity in the LEC primed metastatic melanoma cells.

Notch3 regulation by MMP14 adds an additional layer on top of the conventional activating ligands and proteases known to activate it. In melanoma cells, Notch1 is shown to be activated by MMP14-mediated cleavage (Ma et al., 2014). Interestingly, MMP14 and the classical Notch activating enzymes, ADAM10 and ADAM17, have other mutual cell surface substrates as well, such as the IL-11 receptor (Sammel et al., 2019). In addition to the Notch receptors, MMP14 has been shown to be able to cleave the Notch ligand DLL1; in bone marrow stem cells, MMP14 cleaves DLL1 to inhibit Notch signaling, resulting in the maintenance of normal development of B cells (Jin et al., 2011). These studies suggest that Notch signaling regulation by MMP14 is context dependent.

6. Beta 1 integrin is involved in the enhanced invasive phenotype of melanoma cells and is regulated by MMP14.

Our RNAseq data of the LEC primed melanoma cells indicated changes in cell adhesion pathways. Adhesive molecules, such as integrins, have been described as crucial factors of cancer cell invasion and an important component of cancer cell invadosomes (Linder et al., 2023). Beta 1 integrin has been associated with melanoma progression (Danen et al., 1994; Natali et al., 1993), and therefore we characterized its role in the co-culture system.

WM852 and WM165 cell lines showed increase in active beta 1 integrin intensity in immunofluorescence staining after the LEC co-culture, whereas no change was observed in Bowes or WM793 cell lines (Fig. 5a and fig. 5 figure supplement 1a-b in I). Inhibition of beta 1 integrin activity by an extracellular domain binding, function blocking AIIB2 antibody in WM852 cells reduced the sprouting of the co-cultured cells close to the level of monotypic control cells (Fig. 5b in I), indicating the contribution of beta 1 integrin in the invasive sprouting of the LEC primed metastatic melanoma cells. Since the degrading and adhesive proteins in the invadosomes are tightly regulated (Linder et al., 2023), and in the developing mammary epithelium MMP14 associates and controls the expression of beta 1 integrin (Mori et al., 2013), we investigated the reciprocal regulation of MMP14

and beta 1 integrin in melanoma cells. We found a partial co-localization of active beta 1 integrin and MMP14 on the plasma membrane in the co-cultured WM852 cells (Fig. 5c in I). Silencing of *MMP14* by siRNA in WM852 cells significantly reduced the intensity of active beta 1 integrin immunofluorescence staining in the LEC co-cultured melanoma cells (Fig. 5d-e in I) and led to a less pronounced decrease in the total beta 1 integrin level (Fig. 5f-g in I). *NOTCH3* silencing did not alter beta 1 integrin activation in cells (Fig. 5 figure supplement 2a-b in I), and on the other hand, inhibition of beta 1 integrin did not change the MMP14 and Notch3 mRNA levels (Fig. 5 figure supplement 2C) or protein levels (Fig. 5 figure supplement 2d-e). Altogether, these results indicate that the invasive sprouting phenotype of the LEC primed metastatic melanoma cells depend on Notch3 and active beta 1 integrin, which both are positively regulated by MMP14. The exact mechanism by which MMP14 leads to the activation of beta 1 integrin in melanoma cells upon LEC co-culture requires further characterization. In a mouse model, MMP14 expressed by the skeletal stem cells mediates pericellular ECM remodeling, leading to changes in the cell shape necessary for beta 1 integrin activation (Tang et al., 2013). It is also worth to note that although we did not observe in our study settings MMP14 regulation by beta 1 integrin, this has been shown to occur in a breast cancer cell model *in vitro*, where active beta 1 integrin mediated MMP14 phosphorylation through the Src-EGFR signaling, leading to enhanced recycling of MMP14 to sites of active ECM modelling on the cell plasma membrane (Grafinger et al., 2020).

7. Expression of the intracellular domain of Notch3 induces invasive sprouting in WM793 cells.

To further validate the role of MMP14 relocalization and Notch3 and beta 1 integrin in the invasive phenotype in the LEC primed melanoma cells, we investigated whether their expression or activation would induce the invasive phenotype in WM793 melanoma cells. Ectopic expression of MMP14 induced cell death shortly after transfection. Beta 1 integrin activation by the 12G10 antibody was not sufficient to induce invasive sprouting of the cells (Fig. 6b-c in I). However, ectopic expression of the intracellular active domain of Notch3, NICD3, induced invasive sprouting of WM793, in a similar manner observed in the metastatic melanoma cell lines upon LEC co-culture (Fig. 6a in I).

To investigate whether the NICD3 induced invasion was further affected by MMP14 or beta 1 integrin activities, the NICD3 transfected cells were treated with the MMP14 inhibitor NSC405020 or the beta 1 integrin blocking antibody AIIB2 during the invasion assay. Beta 1 integrin inhibition efficiently blocked the invasive sprouting, whereas MMP14 inhibition caused a less pronounced reduction (Fig. 6d in I), supporting that Notch3 and beta 1 integrin are the downstream targets of

MMP14. This data further indicates that although both Notch3 and beta 1 integrin are essential for the increased melanoma cell invasion in 3D fibrin, Notch3 is not involved in the beta 1 integrin activation.

8. Notch3 and MMP14 are required for enhanced metastasis of the LEC co-cultured WM852 *in vivo.*

To study the involvement of Notch3 and MMP14 in melanoma invasion and metastasis *in vivo*, we treated the WM852 with siRNAs targeting *NOTCH3* or *MMP14* before the co-culture with LECs and injected the monotypic and co-cultured melanoma cells into the pericardial space of the zebrafish embryos (Fig. 7a-b in I). Like the results from the mouse xenograft experiment indicated (Fig. 1c-d in I), co-culturing with LECs did not significantly change the size of the melanoma cell xenografts in the zebrafish (Fig. 7c in I). Furthermore, silencing of *NOTCH3* or *MMP14* in the WM852 cells prior to injection into zebrafish did not affect the size of the primary tumor (Fig. 7c in I). The co-cultured, control melanoma cell xenografts exhibited decreased circularity compared to the monotypic control xenografts, reflecting increased invasiveness of the cells upon LEC co-culture (Fig. 7d in I). The circularity of the tumors was partially restored upon depletion of *NOTCH3* or *MMP14* (Fig. 7d in I). Importantly, co-culturing of WM852 with LECs increased the number of cells/cell clusters which had invaded outside of the pericardial cavity (Fig. 7e in I), and the number of cells/cell cluster was reduced upon *NOTCH3* or *MMP14* reduction.

MMP14 has been shown to be involved in the melanoma metastatic spreading in a mouse orthotopic model, where shRNA mediated reduction of *MMP14* in melanoma cells abolished metastasis after the surgical removal of the primary tumor (Shaverdashvili et al., 2014). Similar to this study, our results show that MMP14 does not provide a growth advantage for the primary tumor but is rather required for the metastatic dissemination. Our study indicated that Notch3 depletion, being positively controlled by MMP14, results in decreased invasiveness and metastasis *in vivo* akin to MMP14 depletion. A mouse study to characterize the impact of Notch3 on primary melanoma growth has indicated that Notch3 depletion by shRNA would rather reduce the growth of melanoma tumors, and in the case of WM852, completely results in the inability to establish the primary tumor (Hsu et al., 2017). However, although some reduction in the Notch3 protein levels upon *NOTCH3* targeting by sh*NOTCH3* is shown in the melanoma cells, there were still abundant levels of the protein remaining in the cells, raising an uncertainty of the contribution of possible off-target effects to the results (Rao et al., 2009).

9. Melanoma cell derived WNT5B contributes to the functional changes in LECs.

The data we had collected from the co-culture model *in vitro* had demonstrated that melanoma cells cause multiple changes in LECs (Fig. 1 in II). Therefore, we next set to investigate the molecular factors leading to these changes. To address if the melanoma cell induced functional changes in LECs require direct cell-cell contact or are mediated by soluble factors (Fig. 1 in II), we repeated the tube formation assay described in Section 1, in the presence of WM852 or WM852-LEC conditioned medias. The WM852 conditioned media, but especially the conditioned medium from co-culture caused defects in the LEC tube formation (Fig. 3a in II), suggesting the contribution of a soluble factor(s). We looked for the possible candidates from the RNA-seq data of the melanoma cells +/- LEC co-culture (in I) and chose one of the genes, *WNT5B*, found highly upregulated in the LEC co-cultured WM852. We confirmed the upregulation of WNT5B upon co-culture in WM852 and WM165 cell lines by qPCR and IF (Fig. 3b-c, Supplemental figure 3b in II). WM793 cell line did not show any significant change upon the co-culture, however, this cell line had around a 10-fold higher baseline expression level of *WNT5B* compared to the metastatic cell lines (Supplemental figure 3a in II), suggesting a distinct, intrinsic regulation mechanism of WNT5B expression in this cell line.

To characterize if WNT5B expressed by the LEC primed melanoma cells contribute to the LEC functional changes, melanoma cells were pretreated with *WNT5B* targeting siRNA before the co-culturing with LECs, and the functional cell assays were performed using FACS sorted LECs. Out results indicated that *WNT5B* depletion in melanoma cells reduced the sprouting of the LEC spheroids (Fig. 3d in II), partially restored the tube formation of the LECs (Fig. 3e in II) and the barrier function of the LEC layers (Fig. 3F in II). In addition, LECs treated with recombinant WNT5B protein showed a weaker barrier function (Supplemental figure 3d in II) and a decrease in beta catenin and ZO-1 on the cell membrane (Supplemental figure 3e in II), indicating weakened cell-cell junctions.

We also attempted to identify the receptor for WNT5B in the LECs responsible for receiving and mediating the signal. Receptors for WNT5B have not yet been extensively characterized (Suthon et al., 2021). Nevertheless, we addressed the role of FZD receptors, known to be involved both in the canonical and non-canonical WNT pathways (Zeng et al., 2018). Our scRNA-seq and qPCR data from the parental and WM852 co-cultured LECs (Supplemental figure 4a-b) showed that especially *FZD4*, *FZD6* and *FZD8* were expressed in LECs, and that there were no significant changes in any *FZD* expression levels between the parental and co-cultured LECs. We next treated the LECs with siRNAs targeting these receptors and subjected the cells to functional assays after the co-culture with WM852 cells.

We were not able to generate si*FZD4* LEC spheroids, possibly due to decreased levels of beta-catenin and VE-cadherin compared to the control cells (Supplemental figure 4c in II). Silencing the expression of *FZD6* and *FZD8* by siRNA did not significantly affect the spheroid formation (Supplemental figure 4d in II) or tube formation (Supplemental figure 4e in II) of the cells upon the co-culture, suggesting either contribution or redundant effect by other receptors.
The role of WNT5B in cancer progression has not yet been extensively studied, although several reports indicate it having tumor promoting effects. In the basal type breast cancer, high WNT5B expression correlates with a poor prognosis, and in a mouse xenograft model WNT5B depletion reduced tumor growth (Jiang et al., 2019). In oral squamous cell carcinoma cells, WNT5B enhances cancer cell migration (Takeshita et al., 2014). In LECs, recombinant WNT5B has been reported to induce expression of SNAIL and SLUG, two classical mesenchymal markers (Wang et al., 2017); therefore, it is possible that the melanoma derived WNT5B induces a partial EMT response in LECs, which is contributing to the functional changes observed after the melanoma co-culture.

10. WNT5B facilitates melanoma cell escape from the primary injection site to the lymph nodes.

To characterize the effect of WNT5B on melanoma progression *in vivo*, we treated WM852 cells with a *WNT5B* targeting siRNA before subjecting them to monotypic or LEC-co culture and subsequent tumor cell injection into the mouse ear pinna (Fig. 4a in II). We observed that the co-cultured cells exhibited a more dispersed colony growth phenotype compared to the monotypic control cell samples (Fig. 4b in II), suggesting increased motility of the cells in the tissue. Importantly, we found less cells to remain in the initial injection site in the co-cultured, control siRNA treated melanoma cell samples compared to the monotypic control samples (Fig. 4b in II), suggesting further metastatic spread of these cells from the primary site. However, if *WNT5B* was depleted in the melanoma cells before the co-culture, the cells were better retained in the primary injection site. As we did not find any difference in the melanoma cell growth rate between the monotypic and the co-cultured control samples (Supplemental figure 5a in II), these results suggest that co-cultured melanoma cells escape more efficiently from the primary injection site with the aid of WNT5B.
Next, we investigated if the melanoma cells escaped from the primary site had metastasized to the tumor draining lymph nodes. To this end, we harvested the tumor draining cervical lymph nodes, and measured the presence of WM852 by qPCR for human-specific ALU sequences and FACS for GFP (Fig 4c-d in II). The results indicated that the LEC co-cultured melanoma cells metastasized more

efficiently to the lymph nodes when compared to the monotypic control cells, and that *WNT5B* depletion before the co-culture significantly reduced the number of GFP expressing melanoma cells and the abundance of human ALU sequences, further confirming the presence of human melanoma cells in the tumor draining lymph nodes.

In an oral squamous carcinoma mouse xenograft model, depletion of *WNT5B* by shRNA in the cancer cells has been shown to reduce the number of mice that altogether developed lymph node metastasis (Wang et al., 2017). Moreover, this study showed that the size of the primary tumor did not change upon *WNT5B* depletion, supporting our findings that WNT5B indeed facilitates lymphatic dissemination. Interestingly, the same study showed that higher WNT5B expression levels are found in the oral squamous cell carcinoma cell lines which show a preference for high lymphatic metastasis, and the patients with high serum WNT5B levels have an increased risk for lymphatic metastasis. This study together with our results implies, that WNT5B expression by the cancer cells contributes to a more aggressive disease through its effects on the lymphatic system. They further indicate that WNT5B enhances cancer progression through mediating changes in the LECs thereby leading to enhanced lymphatic dissemination, and that WNT5B mediated lymphatic metastasis occurs in different cancer types.

11. Notch3 regulates WNT5B transcription in melanoma cells.

Activated Notch receptors also regulate the expression of other downstream targets besides the best known *HEY* and *HES* family members (Sachan et al., 2023). In ovarian cancer cells, characterization of the Notch3 downstream targets by ChIP-on-chip analysis suggested that Notch3 may bind to the *WNT5B* promoter area, however, this finding was not validated in the study (Chen et al., 2012). We therefore hypothesized, that Notch3 may regulate WNT5B in the LEC primed melanoma cells. *NOTCH3* depletion before the start of the LEC co-culture or treatment of co-cultures with the Notch pathway inhibitor DAPT significantly reduced the LEC induced *WNT5B* upregulation in WM852 and WM165 cells (Fig. 5a-b and Supplemental figure 6a-b in II). WM793 did not show any significant changes upon si*NOTCH3* or DAPT treatment, indicating that the pathway(s) regulating WNT5B expression is distinct from Notch3 (Fig. 5a-b and Supplemental figure 6a-b in II). ChIP-PCR performed using two different Notch3 antibodies in the WM852 melanoma cells transfected with NICD3 indicated that Notch3 binds to the *WNT5B* promoter region (Fig. 5c in II) further supporting the role of Notch3 regulating *WNT5B* in the LEC-primed metastatic melanoma cells.

We next characterized the co-expression of Notch3 and WNT5B in melanoma primary tumors from a cohort of 55 patients where the Breslow thickness of the primary tumor was less than 4 millimeters and patients showed no lymph node

metastasis at the time of diagnosis, but still manifested an aggressive disease with average survival being only 3.75 years (Supplemental table 3 and Supplemental figure 6c in II). To this end, sections of these patient samples were stained with anti-Notch3 and anti-WNT5B antibodies. In addition to the primary tumors, 44 metastasis samples from this cohort were stained, and the scoring was successfully performed for 47 primary tumor and 35 metastasis samples, resulting in a total of 31 patients from whom both the primary tumor and metastasis sample was successfully scored both for Notch3 and WNT5B expression. Co-distribution of the two proteins was observed in 35% of the primary tumors and 77% of the metastasis samples (Fig. 5d-e in II), with the metastatic samples showing significantly more co-expression compared to the primary tumors (Fig. 5f in II), indicating that the two proteins are more regularly co-expressed in the aggressive melanoma cell populations. A mRNA expression analysis of the patient cohort of 442 patients publicly available from the Cancer Genome Atlas Program (TCGA) dataset (Fig. 5f in II) further showed a positive correlation of *WNT5B* and *NOTCH3* expression in melanoma samples. Moreover, high *NOTCH3* expression in this cohort showed correlation with poor survival in the entire cohort and in those patients who did not have distant metastasis at the time of diagnosis (Fig. 5 h in II). These findings further strengthen the role of Notch3 in promoting melanoma progression, likely through increasing the melanoma cell invasiveness and changes in the function of lymphatic endothelial cells as shown in this thesis. This also suggests that Notch3 may represent a useful marker in addition to the marker panel currently used for diagnosis to determine which patients are at risk of developing an aggressive disease before the onset of distant metastasis.

12. DLL4 induces Notch3 and WNT5B in melanoma.

To better understand the molecular events of the LEC-melanoma cell crosstalk, we investigated how the Notch3 signaling is activated in melanoma upon LEC co-culture. We hypothesized that a Notch ligand expressed by LECs binds to and activates the Notch3 receptor on the melanoma plasma membrane. Quantitative PCR analysis indicated that LECs expressed all the five Notch ligands at a detectable level (Supplemental figure 7a in II). We cultured the different melanoma cell lines on cell culture dishes coated with different, recombinant Notch ligand-Fc fusion proteins. DLL4 was the strongest inducer of the expression of *NOTCH3* and *HEY1* in WM852 and WM165 cell lines (Fig. 6a in II). Less pronounced increase was also achieved with DLL1 coating in these cell lines. *HES1* levels did not show significant changes in any of the cell lines when cultured on the Notch ligands. We also assessed the levels of the full length and cleaved Notch3 by immunoblotting. DLL4 induced the accumulation of NICD3 in WM852 and WM165 cells and to smaller extent in WM793. We also observed accumulation of

the full length Notch3 by DLL4 induction, suggesting that Notch3 is induced by an autoregulative loop. DLL1 also increased the full length and cleaved Notch3 levels in WM852 and WM165 cell lines but the increase was less pronounced compared to the induction by DLL4. In the 3D fibrin invasion assay, DLL4 treatment of WM852 and WM165 induced invasive sprouting (Fig. 6c and Supplemental figure 7 d in II), supporting that DLL4 induces Notch3 dependent invasion *in vitro*. Lastly, we observed that DLL4 ligand induced approximately a 4-fold increase in the *WNT5B* mRNA levels in WM852 and WM165 (Fig. 6d in II). DLL1 in addition induced in lesser extend *WNT5B* upregulation in WM165 (Supplemental figure 7e). We did not see any significant changes in the *WNT5B* levels in WM793 cells upon induction by the different Notch ligands (Fig. 6 d and Supplemental figure 7e). Overall, these results indicate that in the metastatic melanoma cell lines DLL4 induces Notch3 activation, leading to an increase in *WNT5B* expression.

DLL4 binding and its abundant expression in the context of cancers has been generally associated with tumor promoting effects and poor prognosis in many different types of cancers (You et al., 2023). So far, studies which have specifically addressed the role of vascular DLL4 in the context of cancer have mainly focused on the blood vasculature. In an ovarian cancer mouse model, treating the mice with siRNA targeting the mouse *dll4* resulted in decreased tumor growth, which was likely due to the decreased blood vasculature density and impaired pericyte recruitment to the tumor vasculature, inducing hypoxia (Hu et al., 2011). Interestingly, a study of breast cancer patient samples found that high DLL4 expression, as evaluated both in the cancer cells and cancer associated endothelial cells, was associated with axillary lymph node metastasis and postoperative recurrence (Xiao et al., 2014), suggesting that high endothelial DLL4 may promote lymphatic invasion.

Conclusions and perspectives

Metastasis is the leading cause of cancer associated death, including melanoma. Lymphatic vasculature has a clear, active role in promoting melanoma progression in addition to providing an escape route for cancer cell dissemination. This study shows that LECs increase the invasive and metastatic ability of melanoma cells. Mechanistically, this occurs through MMP14 relocalization to the melanoma plasma membrane, which in turn induces Notch3 expression and beta 1 integrin activation on the cell surface. All three proteins are required for the increased invasiveness of the melanoma cells induced by the LEC co-culture, and MMP14 and Notch3 mediate the enhanced metastasis *in vivo*. Notch3, activated by the Notch ligand DLL4 on the LEC surface, induces transcription of *WNT5B* by binding to its promoter in the melanoma cell. The melanoma derived WNT5B then enhances metastasis by promoting the molecular and functional changes in LECs (Figure 6).

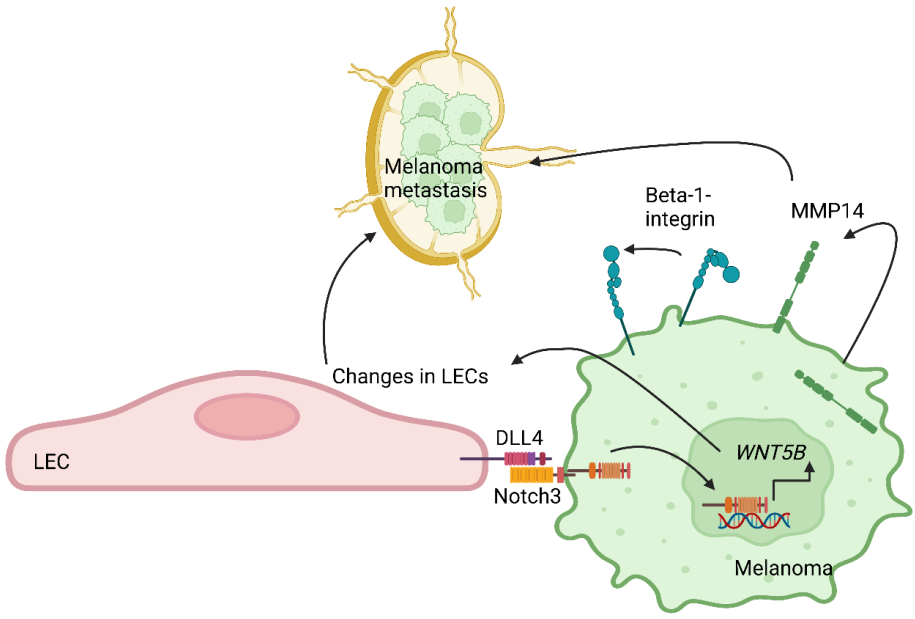

Figure 6. The molecular events of the bidirectional, prometastatic melanoma cell-LEC crosstalk revealed in this thesis. Figure created using BioRender.com.

Our study demonstrates that MMP14 has a distinct role in promoting the Notch3 mediated tumorigenesis. MMP14 might act through cleaving Notch3, leading to its activation and the autoregulatory signal amplification. Alternatively, binding of the Notch3 receptor to MMP14 might direct Notch3 accumulation to the LEC-melanoma cell contact sites, where DLL4 is available for binding, leading to the Notch3 activation. The data presented in this thesis indicates that Notch3 possesses a dual function in enhancing melanoma progression: via the increased invasiveness and metastatic potential and by induction of WNT5B, which mediates the tumor promoting changes in LECs. Since Notch3 has such a prominent role in melanoma, it might be a good target for combinatorial melanoma treatments. For instance, ligand-specific inhibitors of Notch signaling, called Notch decoys (Kangsamaksin et al., 2015) interfering with DLL4-Notch3 signaling might, through competitive inhibition, be able to limit melanoma progression.

Today, there are several small molecule inhibitors to target WNT signaling. Majority of them target either the components of the canonical WNT pathway or inhibit the function of enzymes needed for production of the WNT signaling components, thus widely affecting the WNT signaling in body. For instance, several inhibitors have been developed to inhibit porcupine, an enzyme that mediates the palmitoylation of WNT ligands. Although porcupine inhibitors have shown great potential in many preclinical cancer models, only few have this far entered the clinical trials, mainly due to challenges to balance the effectiveness and side effects upon dosing (Shah et al., 2021). The development of inhibitors targeting specific WNT ligands, and their receptors would likely help to reduce side effects. Specific inhibition of WNT5B might reduce lymphatic metastasis as shown by us for melanoma and by others for oral squamous cell carcinoma (Takeshita et al., 2014). Although WNT5B contributes to the lymphatic metastasis of melanoma, there are also other factors participating in this process; for example, a mouse melanoma *in vivo* study has shown that periostin blocking antibody reduces sentinel lymph node metastasis (Gillot et al., 2022). Combinatorial blocking of factors which mediate melanoma lymphatic metastasis may be beneficial for patients who are at risk of developing distant metastasis.

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