Thesis for the degree of Philosaphiae Doctor

Tumor – microenvironment interactions in malignant melanoma

Impact on metastatic phenotype and drug resistance

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ABBREVIATIONS

2D – Two-dimensional

3D – Three dimensional

AKT/PKB – Protein kinase B

BMDC – Bone marrow-derived cell

BRAF – Rapidly accelerated fibrosarcoma protein kinase B

CAF – Cancer associated fibroblast

CCL – C-C motif ligand

CCND1 – Cyclin D1

CCR – C-C motif receptor

CDK – Cyclin-dependent kinase

CDKN2 – Cyclin dependent kinase inhibitor 2

CDSS – Cancer drug sensitivity screening

c-MET /MET/HGFR – Hepatocyte growth factor receptor

COL5A1 – Collagen type V alpha 1

COT/MAP3K8 – Mitogen-activated protein kinase kinase kinase 8

CTL4 – Cytotoxic T-lymphocyte antigen 4

CXCL – C-X-C ligand

DKK – Dickkopf Wnt signaling pathway inhibitor

DTIC – Dacarbazine

E-cadherin – Epithelial cadherin

ECM – Extracellular matrix

EMT – Epithelial-mesenchymal transition

ERK – Extracellular regulated kinase

FACS – Fluorescence-activated cell sorting

FAK – Focal adhesion kinase

FAP – Fibroblast activation protein

FDA – Food and drug administration

FN – Fibronectin

GFP – Green fluorescence protein

GJ – Gap junction

GSK3 – Glycogen synthase kinase 3

HGF – Hepatocyte growth factor

HT – High-throughput

i.v. – Intravenously

IF – Immunofluorescence

IKK – I kappa B kinase

IL – Interleukin

ILK – Integrin-linked kinase

JNK – The c-Jun NH_2-terminal kinase

L.V. – Left ventricle

LOX – Lysyl oxidase

LUC – Luciferase

MAPK – Mitogen-activated protein kinase

MEK – Mitogen-activated protein/extracellular signal-regulated kinase kinase

MITF – Microphthalmia-associated transcription factor

MLANA – Melan-A

MMP – Matrix metalloprotease

mTOR – the mechanistic/mammalian target of rapamycin

mTORC – the mechanistic/mammalian target of rapamycin complex

N-cadherin – Neural cadherin

NF-κB – Nuclear factor kappa B

NK – Natural killer

NRAS – Neuroblastoma RAS viral oncogene homolog

PD-1 – Programmed death-1

PDGF – Platelet-derived growth factor

PDGFR – Plateled-derived growth factor receptor

PD-L1 – Programmed death ligand-1

PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase

pS6 – Phosphorylated ribosomal protein S6

PTEN – Phosphatase and tensin homolog

qPCR – quantitative/real-time Polymerase chain reaction

RAF – Rapidly Accelerated Fibrosarcoma

RAS – Rat Sarcoma

RPPA – Reverse phase protein array

RSK – p90 Ribosomal S6 kinase

RTK – Receptor tyrosine kinase

S6K – p70 Ribosomal protein S6 kinase

SA – Serum amyloid

SOX10 – SRY-related HMG-box 10

STAT – Signal transducer and activator of transcription

SWI – Simple Western immunoassay

TAM – Tumor associated macrophage

TEC – Tumor endothelial cell

TGFβ – Transforming growth factor beta

THBS1 – Thrombospondin 1

TME – Tumor microenvironment

TNC – Tenascin C

TNF – Tumor necrosis factor

TYR – Tyrosinase

UVR – Ultraviolet radiation

VEGF – Vascular endothelial growth factor

WB – Western immunoblot

Wnt –Wingless-related integration site

WNT5A – Wnt signaling Member 5A

LIST OF PUBLICATIONS

This thesis is based on the following original publications, included in the second part of the thesis. They will be reffered to in the text by their roman numerals **I-III**.

I. Metastasis-associated protein S100A4 induces a network of inflammatory cytokines that activate stromal cells to acquire pro-tumorigenic properties

Bettum IJ, Vasiliauskaite K, Nygaard V, Clancy T, Pettersen SJ, Tenstad E, Mælandsmo GM, Prasmickaite L.

Cancer Lett. 2014 Mar 1;344(1):28-39.

II. Fibroblast-induced switching to the mesenchymal-like phenotype and PI3K/mTOR signaling protects melanoma cells from BRAF inhibitors

Seip K, Fleten KG, Barkovskaya A*, Nygaard V, Haugen MH, Engesæter BØ, Mælandsmo GM, Prasmickaite L.*

*Contributed equally to the study

Oncotarget. 2016 Apr 12;7(15):19997-20015.

III. Targeting stroma-supported melanoma cells resistant to BRAF inhibitors

Seip K, Jørgensen K, Haselager MV*, Albrecht M, Haugen MH, Egeland EV, Lucarelli P, Pettersen S, Engebraaten O, Sauter T, Mælandsmo GM, Prasmickaite L.*

*Contributed equally to the study

Manuscript

1. INTRODUCTION

1.1. Cancer

Cancer is a class of diseases characterized by abnormal cell growth and invasion to other parts of the body. Due to genomic alterations, highly proliferating cells form a mass of tissue called a tumor. Eventually, tumor cells start to interfere with normal cells, leading to disruption of body homeostasis [1]. Cancer can be classified as non-malignant (non-invasive), referred to as a benign tumor, and malignant (invasive), where the latter is responsible for most of the cancer-related mortalities [2].

Cancer is a leading cause of death worldwide. 8.2 million cancer-related deaths was reported globally in 2012 [3], and this number is constantly increasing. According to the latest estimation, new cancer cases will increase by 70% over the next two decades and will reach more than 23 million cases worldwide [3]. This expected increase might be associated with overall increased population life span, since cancer is most often diagnosed in older people [4]. In Norway, almost 32 000 new cases and 11 000 deaths (i.e. 25% of all deaths) were reported in 2014. Incidence of new cases of cancer in Norway increases each year [5]. These high numbers signify the urgent need for better diagnostic and treatment options to improve cancer prevention and care.

At the cellular level, cancer can be considered as an evolutionary process, where cancer cells, due to genetic and epigenetic alterations, and support from tumor microenvironment (TME), gain a survival advantage [6]. Properties providing this advantage are known as cancer hallmarks. Hanahan and Weinberg defined 8 hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, de-regulation of cellular energetics, avoidance of immune destruction and tissue invasion and metastasis [7, 8]. In addition, tumor-promoting inflammation and genome instability are proposed to be enabling characteristics that make it possible for the cells to acquire cancer hallmarks [8].

1.1.1. Metastasis

Metastasis (from Greek meaning "displacement") is a multi-step process, where cancer cells spread from the place of origin (a primary tumor) to distant locations in the body. There are two fundamental models of metastasis. The linear-progression model describes metastasis as a late event in tumor development, where primary tumor cells migrate to other organs only after they accumulate genetic alterations necessary for the metastatic dissemination [9]. The parallel-progression model argues that tumor cells disseminate relatively early, and that metastases develop in parallel to and independently from the primary tumor [10]. Regardless of the model, the metastatic cascade includes the same steps specified in Figure 1. Lately, it was acknowledged that, in addition to the metastatic properties of tumor cells themselves, host-derived microenvironment factors participate actively in the establishment of metastases (discussed later in chapter 1.2). Even though cell motility and dissemination are relatively efficient processes, where millions of cancer cells per gram of tumor can enter the circulation daily, only 0.01% of all circulating cells will be able to survive and successfully overcome all the steps along the metastatic cascade, leading to macrometastases [11].

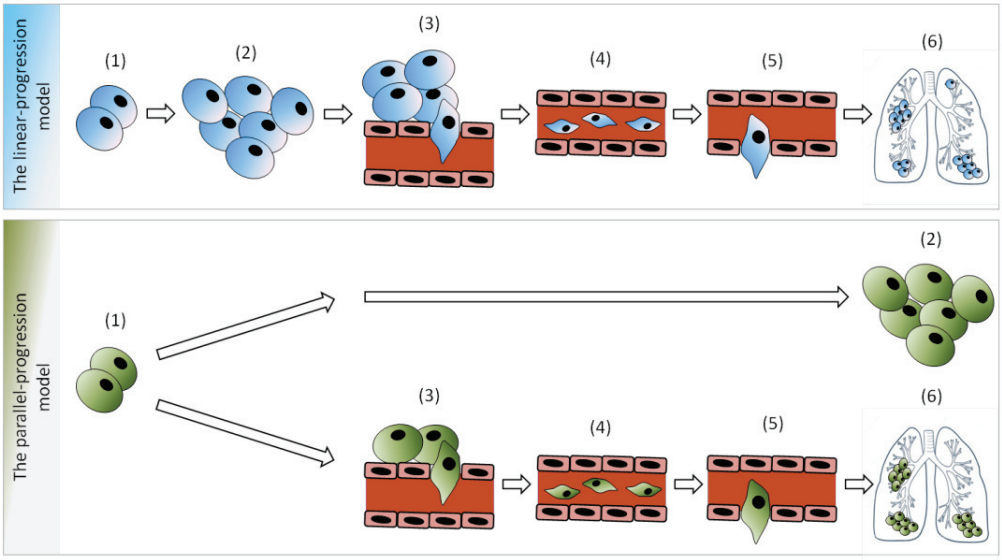

Figure 1. Development of metastases. Tumor cells (1) either proliferate and establish a primary tumor (2), followed by a metastatic cascade (the linear-progression model), or develop metastases in parallel with the development of a primary tumor (the parallel-progression model). The metastatic cascade involve the following steps: tumor cell invasion (3), intravasation into circulation (4), survival in the circulation, arrest at distant site, extravasation into distant organs (5), and initiation of growth by forming micrometastases and eventually macrometastases (6).

In recent years, metastasis has been linked to the trans-differentiation process called epithelial-mesenchymal transition (EMT). EMT is essential during development and is a reflection of phenotypic plasticity, i.e. the capacity of one genotype to change its phenotype in response to signals from the microenvironment [12]. During EMT, epithelial cells lose epithelial properties (cell – cell contacts, polarity) and gain mesenchymal features (reorganization of cytoskeleton and elongated mesenchymal morphology). Thus, cells become more motile. A number of alterations in gene expression and cell signaling are involved in EMT. Epithelial (E)-cadherin/neural (N)-cadherin switch and transforming growth factor beta (TGFβ) signaling are among the most described (reviewed in [13]).

1.1.2. Cancer associated signaling

Hallmarks of cancer, as well as changes within the TME, affect signaling in tumor cells. In the following section, signaling pathways explored in this thesis, such as the mitogen-activated protein kinase (**MAPK**), Phosphatidylinositol-4,5-bisphosphate 3-kinase (**PI3K**) – protein kinase B (PKB, also known as **AKT**), the mechanistic (previously known as mammalian) target of rapamycin (**mTOR**) and Glycogen synthase kinase 3 **(GSK3),** will be briefly introduced.

The MAPK and PI3K/AKT signaling pathways are often dysregulated in human cancers. Their activation is known to enhance cellular proliferation and survival and induce EMT (reviewed in [14]). The MAPK and PI3K/AKT pathways can be activated via both receptor tyrosine kinases (RTKs) and integrins [14]. Further signal transduction occurs through multiple intracellular effectors (specified in Figure 2) leading into the nucleus, where regulation of target gene transcription takes place.

The mTOR kinase interacts with several proteins to form two distinct complexes, named mTOR complex (mTORC) 1 and 2, which play different roles in cell biology. mTORC1, which has been explored in this thesis, is typically activated by the PI3K/AKT signaling axis [15], but MAPK-dependent activation has also been observed [16]. The mTORC1 regulates phosphorylation of p70 ribosomal S6 kinase (S6K), which further activates protein S6.

Phosphorylated S6 (pS6) participates in protein translation initiation, ribosome biogenesis and other cell growth-related events.

GSK3 is an important signaling mediator implicated in different signaling pathways, including PI3K-AKT and wingless-related integration site (Wnt)/β-catenin, a complex developmental pathway also involved in tumorigenesis (reviewed in [17]). The major role of GSK3 in Wnt signaling is the regulation of β-catenin. In the absence of active Wnt, GSK3 is active and phosphorylates β-catenin, targeting it for degradation. When Wnt is activated, GSK3 activity is suppressed and β-catenin is stabilized so it can enter the nucleus, where it interacts with transcriptional regulators [18]. GSK3 can also be inactivated by AKT, which contributes to the stabilization of β-catenin [19]. In general, suppression of GSK3 was also shown to activate mTORC1 [20].

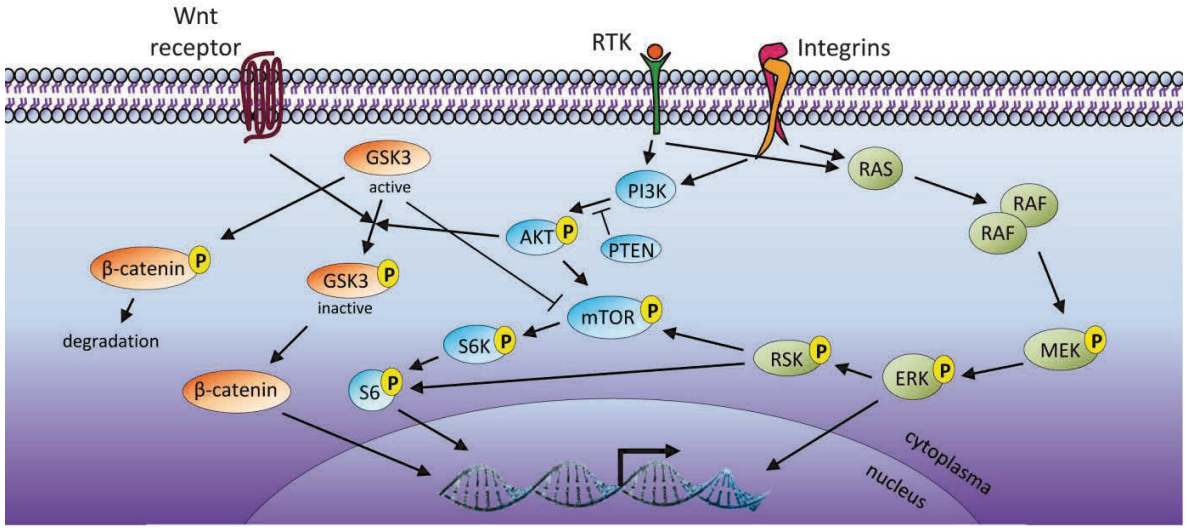

Figure 2. Cancer associated signaling pathways. Proteins involved in MAPK, PI3K/AKT/mTOR and GSK3-mediated Wnt/β-catenin signaling are colored in green, blue and orange, respectively. The MAPK pathway is triggered by extracellular signals, which leads to the activation of small GTPase-rat sarcoma (RAS). Activated RAS binds to rapidly accelerated fibrosarcoma (RAF) kinase, inducing a conformational change, which results in RAF activation and dimerization. Activated RAF then initiates a phosphorylation cascade from one kinase, mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK), to another, extracellular regulated kinase (ERK). Under normal conditions, the PI3K/AKT pathway is suppressed by phosphatase and tensin homolog (PTEN). However, due to its mutation/deletion (in addition to several other accompanying factors), PI3K/AKT signaling is activated in many cancers, leading further to mTOR signaling stimulation and subsequent phosphorylation of S6 kinase and S6 protein. In addition, mTOR can be activated by MAPK signaling through p90 ribosomal S6 kinase (RSK) protein. Activated Wnt ligand binding to the Wnt receptor inactivates GSK3, which cannot phosphorylate (and prime for degradation) β-catenin and block activation of mTOR. Finally, activated ERK, S6 and β-catenin can be translocated into the nucleus where they interact with other partners and/or transcription factors that stimulate expression of various cancer-related genes.

1.2. TME and its role in cancer progression and resistance

Until rather recently, it has been assumed that cancer is a cell-autonomous event, where only intrinsic characteristics of malignant cells play a role in tumor development and metastasis. However, now it has been acknowledged that non-malignant cells and extracellular architecture, i.e. stroma, can contribute to both primary tumor growth and metastatic colonization of distant organs [21]. Generally, the idea about the role of TME in cancer is not new. It is more than 100 years since Stephen Paget introduced the "seed and soil" hypothesis [22]. Paget suggested that a metastatic cell, "a seed", is only capable to grow at secondary sites with a permissive microenvironment, "soil". This hypothesis was based on clinical observations that different tumors have a tendency to establish metastasis at certain organs, i.e. show preferences for specific sites. In the case of e.g. breast cancer, the metastases are frequently established in lung, liver or bone, while prostate cancer often disseminates to the bone [23, 24]. The skin cancer, such as melanoma, however, can establish metastasis in many different organs (e.g. lymph node, lung, brain, liver, skin), showing less preference to a certain site [25].

The biological mechanisms responsible for how TME factors facilitate metastatic growth at distant sites are not yet fully understood. Recent studies have introduced the concepts of pre-metastatic and metastatic niches. The niches denote tumor-friendly microenvironmental conditions, generated at distant sites, before or after the arrival of metastatic cells, respectively. It has been shown that the primary tumor can secrete factors, e.g. exosomes, that can act over a long distance and initiate the formation of the pre-metastatic niche [26]. In addition, the disseminated cells settled at distant sites can change their local microenvironment, forming the metastatic niche [27, 28]. The function of both pre-metastatic and metastatic niches is to help incoming tumor cells to survive and grow at foreign sites [27]. Niche formation often involves accumulation of pro-inflammatory soluble factors, reorganization of extracellular matrix (ECM) and recruitment/activation of stromal cells, such as bone marrow-derived cells (BMDCs) or fibroblasts, that all together create an inflammatory milieu, beneficial for homing and growth of the metastasizing cells [29, 30].

In the following sections, components of the TME addressed in this thesis will be discussed, revealing how they interact with the tumor.

1.2.1. Tumor interactions with TME

A TME consists of various stromal cells (such as fibroblasts, endothelial cells and BMDCs, including monocytes, macrophages and other immune cells), ECM, a variety of secreted soluble factors (like chemokines, cytokines and growth factors) and microvesicles called exosomes [31-33]. All these components together constitute a complex system accompanying the tumor. As the tumor progresses, its microenvironment also changes, forming a permissive environment that helps tumor cells to survive, grow and invade, i.e. to become more aggressive [34]. These changes within the microenvironment are usually initiated by the tumor. Recent years of investigation have shed more light on how tumor – stromal cell cross-talk can be executed. Cells can communicate with each other by means of direct cell – cell contact or via secretion of soluble factors and exosomes. In addition, the communication can involve deposition of ECM that affects the behavior of ECM-binding cells [35-37].
Among the mechanisms enabling direct cell – cell communication are intercellular channels called gap junctions (GJs), which are composed of six connexin molecules. When connexins from neighboring cells merge, one complete GJ channel is formed. GJs enable transport of ions, short interfering RNAs, nucleic and amino acids or metabolites between two connected cells (reviewed in [38]). The appearance and permeability of these channels can change depending on cell needs and microenvironment signaling [39]. Down-regulation of connexins is associated with enhanced migratory and invasive capacities of tumor cells [40]. On the other hand, tumor cells exploit connexins to establish GJs with stromal cells to facilitate tumor development and survival [41, 42]. GJ-based communication was reported to be especially important for tumor establishment in the brain microenvironment, where tumor cells connect to astrocytes via GJs [43-45].

Another important mechanism of cell – cell communication involves integrins, which also mediate cell – ECM interactions [46]. Integrins are transmembrane cell adhesion receptors that bind counter-receptors on adjacent cells or various ECM molecules, e.g. fibronectin (FN)

or collagen. Integrins can mediate bi-directional signaling: “inside-out” or “outside-in” [47]. The “inside-out” signaling is initiated by intracellular events (e.g. reorganization of cytoskeleton), which modulates integrins’ affinity for its ligand. The “outside-in” signaling is triggered by extracellular changes (e.g. within ECM), leading to integrin-ligand binding with subsequent activation of focal adhesion kinase (FAK) and integrin-linked kinase (ILK) [48]. As a consequence, downstream signaling pathways are activated, which regulates various cell functions, such as proliferation, survival, polarity, motility or differentiation (reviewed in [49]). Integrins, depending on microenvironment stimuli, can switch “on” and “off”, where only the first state can bind the ligands [49]. Thus, integrins function as an important bridge between intracellular and extracellular protein networks and thereby tune cellular responses to microenvironment cues.

While integrins and GJs mediate interactions between adjacent entities, released small soluble molecules and exosomes can also mediate long-distance communication. Both tumor and stromal cells release a variety of cytokines and growth factors that can act in an autocrine and paracrine manner by binding to their respective receptors. This leads to activation of downstream signaling pathways affecting cell survival, growth, motility and further production of soluble factors or ECM. For example, it has been shown that through secretion of soluble factors or exosomes, tumor cells can recruit and educate BMDCs [37, 50, 51]. These BMDCs can further secrete factors that affect other stromal cells, e.g. endothelial cells [37]. Such a cascade of multi-cellular interactions is beneficial for the tumor, since it can enhance vascular permeability, facilitating extravasation [50, 52], or create a milieu stimulating drug resistance [37]. Cellular responses to soluble factors can depend on the cell’s adhesion to substrate [49], indicating that crosstalk via secreted soluble factors and direct cell – cell /ECM interactions might be tightly interconnected.

Tumor – stroma communication via ECM is another important mechanism utilized by the developing tumor. Changes within the ECM can remodulate the composition/stiffness of the ECM [53], which can be sensed by tumor cells through e.g. integrin signaling. Rigidities of the ECM maintain a directional cell movement, preferentially towards a stiffer environment [53, 54]. A recent study by Oudin et al. [55] demonstrates that tumor cells move towards a higher gradient of FN, which is typically found at the tumor border [56] and near blood

vessels [56, 57]. Modification of ECM is also observed at sites of pre-metastatic niches and metastasis [26, 27, 58, 59]. For example, it has been shown that FN is involved in pre-metastatic niche formation, where it facilitates recruitment of BMDCs and stimulates metastasis initiation [26]. Tenascin C (TNC), another factor of ECM, was shown to be produced by disseminating tumor cells in order to facilitate metastasis initiation. At later stages, stroma takes over as a source of TNC, further stimulating metastatic growth [58].

1.2.2. Components of TME

1.2.2.1. Fibroblasts

Fibroblasts are the most abundant cell type in connective tissue. One of their main functions is to synthesize the proteins of ECM. Thus, fibroblasts are a rich source of FN, collagen and TNC, and they also secrete a variety of growth factors and chemokines [60].

During tumor progression, fibroblasts are reprogrammed into cancer associated fibroblasts (CAFs). However, there is no consensus about the origin of CAFs. It has been suggested that CAFs can derive: i) from normal fibroblasts via mesenchymal – mesenchymal transition [61], which can be triggered by a variety of tumor-derived soluble factors, ii) through endothelial to mesenchymal transition [62] or iii) from the malignant epithelial cells themselves [63]. There is an agreement, though, concerning the importance of CAFs in tumor progression. CAFs are mainly localized in the tumor invasion front [33], where they secrete ECM components, matrix-remodeling enzymes and soluble factors, promoting tumor growth and invasion [64, 65]. It has been suggested that CAFs can take the leading role in tumor cell migration, where the tumor cells just follow fibroblasts-generated tracks [66, 67]. Due to production of soluble factors, CAFs also participate in recruitment of other stromal cells, such as macrophages and endothelial cells, to support tumor development [68].

1.2.2.2. Inflammatory cells: monocytes and macrophages

BMDCs, such as monocytes and macrophages, are important components of the innate immune system. They are critical during inflammation, where they are responsible for immediate short-lasting defense against infections or injury [69]. The inflammatory response is characterized by rapid accumulation of macrophages at sites of damage and production of pro-inflammatory soluble factors, with the goal to protect the organism and initiate healing. Thus, the acute inflammatory response is normally localized and has a protective function. However, if inflammation becomes chronic, it increases cancer risk. Inflammation and inflammatory cells are increasingly recognized as an essential component of tumor development. While in the beginning of tumor development inflammatory cells can challenge tumor progression [70], established tumors find a way to exploit them for their own need and enhance tumor-promoting chronic inflammation [71].

Macrophages are among the most abundant immune cells found in the TME and are commonly termed tumor associated macrophages (TAM) [72]. In many cancers, elevated numbers of TAMs are associated with a high-grade and poor prognosis [73]. It is thought that TAMs derive from tumor recruited monocytes rather than a transformation of local macrophages [74]. Due to high plasticity of macrophages, they can alter their polarization state between the classically activated pro-inflammatory M1 phenotype and the alternatively activated M2 phenotype with anti-inflammatory function [75]. In contrast to normal pro-inflammatory macrophages that display the M1 phenotype, TAMs are mainly of the M2 phenotype and are shown to actively promote tumor progression [76]. Due to various tumor and stromal cell-derived soluble factors (e.g. interleukins- (IL) 4 and 10, and chemokines C-C motif ligand (CCL) 2 and C-X-C ligand (CXCL) 12 [77-79]), TAMs accumulate in the TME. Here TAMs exhibit a lower activity of nuclear factor kappa B (NF-κB) signaling [80, 81], which normally plays an important role in regulating the immune response. Instead, TAMs increase TGFβ/PI3Kγ/AKT signaling, which suppress the cells' pro-inflammatory phenotype and T-cell-mediated anti-tumor immunity [82, 83]. It was also reported that TAMs can further recruit other myeloid cells, thereby establishing a cascade of chronic inflammation in the TME [79]. There is no consensus regarding markers of the M1/M2 phenotypes. However, M2 is often linked to increased expression of arginase 1, IL-10 and TGFβ as well as elevated

levels of the surface molecules CD206 and CD163. The M1 phenotype is often characterized by high levels of inducible nitric oxide synthase, tumor necrosis factor (TNF) and IL-12 [84].

TAMs are found in both the periphery and inside of a tumor mass. In the periphery, TAMs are reported to contribute to tumor cell invasiveness, by modulating ECM through secretion of matrix metalloproteases (MMPs), and angiogenesis, by releasing vascular endothelial growth factor (VEGF) A, IL-8 and TGFβ [85]. Inside the tumor mass, TAMs mainly induce the growth of new blood vessels [86]. There are also reports indicating that monocytes/macrophages play a role in initiation of metastases, where inflammatory monocytes are among the first to be recruited to facilitate extravasation [30]. This recruitment is initiated through the CCL2-C-C motif receptor (CCR) 2 axis, where both tumor and stromal cells secrete CCL2, which attracts CCR2-positive monocytes to the metastatic site [50].

1.2.2.3. Endothelial cells

Endothelial cells are one of the main construction blocks of blood vessels that take part in vasculogenesis and angiogenesis, i.e. new vascular network formation *de novo* and from already existing vessels, respectively [87]. Angiogenesis plays an important role in tumor progression, facilitating supply of nutrition and oxygen.

In contrast to normal endothelial cells, tumor endothelial cells (TECs) are shown to possess altered gene expression [88], where genes associated with cell proliferation, migration and tube formation are highly up-regulated [89-91]. These characteristics facilitate TECs response to pro-angiogenic growth factors, such as VEGF, epidermal growth factor and basic fibroblast growth factor [89, 92]. In addition, TECs undergo morphological changes, which negatively affect blood vessels' integrity by diminishing tight cell – cell interconnections [93]. As a consequence, new blood vessels become leaky, leading to inflammation [94] and facilitating intravasation/extravasation [95].

Although angiogenesis is the best characterized tumor-promoting phenomenon involving endothelial cells, it appears that the endothelial cells/vascular environment can also regulate tumor cell dormancy and outgrowth [96]. It has been shown that a stable microvasculature

produce thrombospondin 1 (THBS1), which keeps tumor cells dormant. However, unstable, sprouting neovasculature produce the ECM factor periostin and release TGFβ1 that sparks metastatic growth [96]. Furthermore, it has been observed that e.g. brain metastases, particularly in melanoma, are often initiated in close proximity to the existing blood vessels, a phenomenon known as vascular co-option [97, 98]. These observations indicate that endothelial cells/vascular niches create a friendly milieu for the tumor.

1.2.2.4. S100A4 – a TME factor

In humans, the S100 protein family contains more than 20 members. These are small, Ca-binding, multifunctional proteins acting intracellularly or as extracellular factors. They regulate various cellular processes and appear to be implicated in different pathological conditions. Elevated expression of several members of this family is a common feature of many cancer types and is found to be associated with metastasis. The level of S100 proteins is also elevated in inflammatory disorders, indicating their involvement in the inflammation process (reviewed in [99]). These observations fostered the idea that the pro-metastatic role of S100 proteins might be executed through its pro-inflammatory functions. S100A8/A9 are examples of two family members with a well-established role in inflammation. In metastasis, S100A8/A9 were found to be secreted by tumor cells creating a local inflammatory environment and recruiting BMDCs, thereby forming a (pre)metastatic niche [100]. It has also been revealed that BMDC-produced S100A8/A9 can mediate chemoresistance [37], indicating that S100 proteins might also influence efficacy of therapy.

Another member of the family, S100A4 (also known as metastasin or fibroblast-specific protein 1), is also associated with cancer as well as inflammatory disorders (reviewed in [101, 102]). Elevated levels of S100A4 was found in many cancers [103, 104], where the protein was detected in tumor cells, various stromal cells, and extracellular space [103, 105]. The latter defines the protein as a TME factor, and it has been explored as such in the current thesis.

It is generally accepted that S100A4 up-regulation is associated with metastatic disease (reviewed in [101]). Exactly how S100A4 promotes metastasis is not clear, although several

mechanisms of pro-metastatic activity of intracellular and extracellular S100A4 have been suggested. It has been demonstrated that S100A4 can stimulate cancer cell migration and invasion by direct S100A4 and myosin-IIA interactions [106] or S100A4-induced MMPs secretion [107]. It has also been shown that S100A4 can induce EMT, promoting a mesenchymal, more-motile phenotype in cancer cells [108]. Lately, the ability of extracellular S100A4 to act on stromal cells and participate in tumor – stroma interactions has been addressed. This revealed a pro-angiogenic activity, where extracellular S100A4 activated endothelial cells, forcing them to form capillary-like structures and invade the matrix [109]. The ability of S100A4 to recruit inflammatory cells (monocytes/macrophages) has also been reported [110], although not explored further with respect to metastasis. Finally, a recent study by Hansen et al. [111] demonstrates a link between extracellular S100A4 and inflammatory soluble factors, acute phase serum amyloid (SA) A1/A3 and S100A8/A9, which mediated the pro-invasive, pro-metastatic effects of S100A4. This data strengthens the notion that the pro-metastatic function of S100A4 might be related to its pro-inflammatory activity.

1.2.3. TME and drug resistance

The last decade of investigations has revealed TME as an important player in promoting resistance to cancer therapy. Various stromal cells, such as macrophages [112], fibroblast [113], endothelial cells [114] and astrocytes [43], were shown to be able to protect cancer cells from therapy. This ability could be mediated by: i) stromal cell-secreted soluble factors, like IL-6 [115], CXCL12 [116] or hepatocyte growth factor (HGF) [117, 118], ii) cell attachment to the ECM, stimulating e.g. integrin signaling [119], or iii) via direct cell – cell communication through GJs [43, 44]. Besides this, TME can stimulate tumor cells to undergo EMT [120-122], and the mesenchymal state generally shows lower sensitivity towards treatment [123]. Since most of the anti-cancer treatments target highly proliferative cells, invading tumor cells with lower proliferative abilities are capable of escaping from treatment [124]. Overall, disclosure of microenvironmental factors involved in drug resistance could offer novel means for therapy. In addition to all above-mentioned factors that could be explored as targets, TAM elimination has also been suggested as an alternative option [125].

So far, immune checkpoint inhibitors, leading to enhanced T cell activity towards suppressed tumor immunity, is the best example of successfully implemented TME targeting in clinics.

1.3. Melanoma

Melanoma is a cancer type derived from melanocytes, i.e. cell producing the melanin pigment, found predominantly in the skin and eyes. Malignant melanoma is one of the most aggressive, drug-resistant human cancers, which can easily adapt to the microenvironment at different anatomic sites. This might explain their high tendency to establish metastases in multiple organs [126].

Even though melanoma is not the most common cancer type, its ability to metastasize places it among the most deadly of human cancers [127]. Early primary melanoma, with thickness below 1mm (Breslow's thickness), is usually highly curable by surgery, but once the disease disseminates, it is usually fatal. Patients who progress to metastatic melanoma have a median survival of $\leq$ 1 year, and the 5 years survival rate is lower than 10% [128]. Melanoma incidence has increased rapidly during recent decades among Caucasian populations worldwide [129], and new estimates suggest a doubling of this rate every 10 to 20 years [130]. In Norway, 2 thousand cases and 322 deaths related to melanoma were reported in 2014 [5].

There are several risk factors contributing to melanoma development. One of them is ultraviolet radiation (UVR) (both from the sun and tanning beds) [131-133]. Other risk factors include family history of melanoma [134], multiple benign or atypical nevi [135] and previous melanoma [136]. Furthermore, immunosuppression (both drug [132] and age related [137]) and fair skin/red hair [138] is associated with a higher melanoma incidence. Even though some of the risk factors are unavoidable, exposure to UVR can be strictly controlled. UVR causes genetic changes in the skin, impairs immune function, increases the local production of growth factor and induces the formation of DNA-damaging reactive oxygen species that affect skin cells, melanocytes and keratinocytes (reviewed in [139]). In the skin, melanocytes reside in the basal layer of epidermis, where their function is firmly controlled by

keratinocytes [140, 141] (Figure 3). During skin exposure to UVR, damaged DNA of keratinocytes stimulates melanocytes to produce melanin, which is packed in melanosomes and transported to keratinocytes where it protects from UVR-generated free radicals, causing the appearance of tanning [133]. Paradoxically, melanocytes can also be injured and transformed by UVR. Oxidative stress can disrupt the homeostasis of melanocytes, compromising their survival or leading to malignant transformation [142]. Even though most melanomas still produce melanin, some damaged melanocytes lose this ability, resulting in a colorless appearance, which makes early diagnosis more challenging [143].

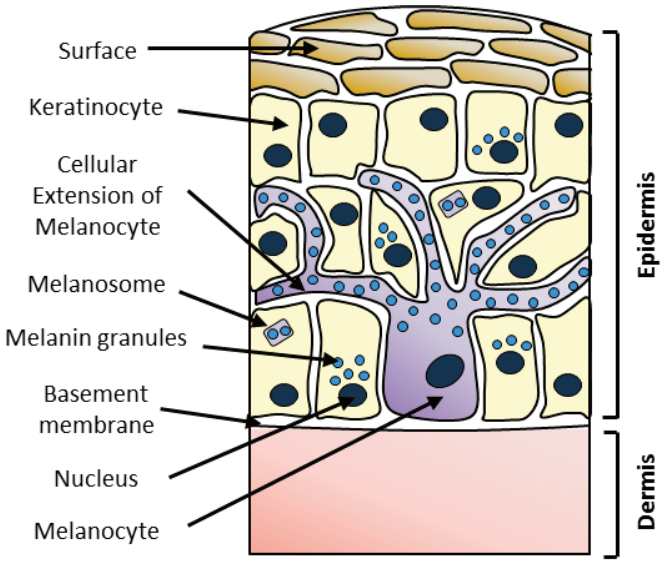

Figure 3. Schematic illustration of the cellular organization in the epidermis. The epidermal structure includes keratinocytes and melanin-producing melanocytes. The transportation of melanosomes to neighboring keratinocytes is allowed by the cellular extension of the melanocytes. The transferred melanin forms a shield against UVR.

1.3.1. Melanoma progression and interaction with the surrounding environment

The classical melanoma progression model emphasizes a stepwise transformation of normal melanocytes to malignant melanoma through several intermediate stages [139, 144] (Figure 4). The first two stages represent out of control growth of melanocytes, where benign nevi is composed of structurally normal melanocytes, while dysplastic nevi starts to gain properties of structural atypia. During a radial growth phase, non-malignant primary melanomas starts to invade intraepidermally, however, they still do not show metastatic properties. If the melanoma is detected at this stage, it can still be completely removed surgically. When the melanoma loses the expression of E-cadherin and gains the expression of N-cadherin, the progression through a vertical-growth stage occurs, and from this point, the

disease is not only capable to invade dermis, but also to establish multiple metastases at distant organs, where the final stage of melanoma progression takes place.

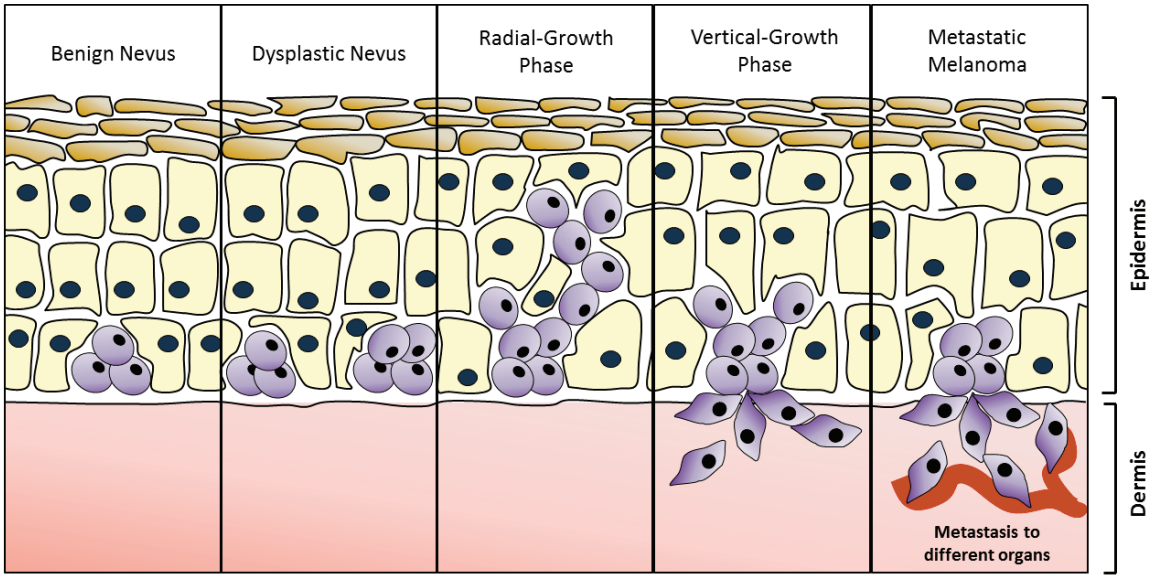

Figure 4. The traditional Clark model of melanoma progression. Due to local proliferation of melanocytes, a benign nevus appears, followed by a dysplastic nevus stage with characteristics of abnormal size, color, surface and border. The radial-growth phase is associated with cell migration to the nearby epidermis. When tumor cells undergo loss of connections with surrounding keratinocytes and establish new contacts with the stromal cells localized in the dermis, the vertical-growth phase is initiated. This progression stage is associated with increased integrin expression, leading to induced expression of MMP-2, an enzyme that degrades the collagen in the basement membrane. In addition, integrin signaling stimulates the motility of melanoma cells through reorganization of the melanoma cytoskeleton, leading to the formation of metastasis.

Cadherins are transmembrane proteins involved in Ca^{2+}-dependent cell – cell adhesion. Through E-cadherins, keratinocytes control melanocytes' growth and phenotypic state [145, 146]. When E-cadherin expression is suppressed, the melanocyte-keratinocyte connection is lost, enabling cells to move [147, 148]. The basement membrane, a layer separating epidermis and dermis, is then degraded. This happens when tumor cells, via surface adhesion molecules (integrins or cell surface proteoglycans), interact with collagen IV [149], a component in the basement membrane. As a result, MMPs are released [149]. Dermis-infiltrated tumor cells further establish new interactions with collagen I [150]. Tumor cell obtained expression of N-cadherins conveys new adhesive properties, allowing them to interact with N-cadherin expressing stromal cells, such as fibroblast and endothelial cells [151, 152]. In addition, the

tumor cells also utilize other adhesion molecules, such as integrins and GJs, to connect to stromal cells (reviewed in [153]). The established interactions increase the expression of pro-survival genes and stimulates the motility of melanoma cells [139]. Dermal invasion and later stages of metastasis are associated with increased production of various soluble factors, such as IL-6/8, CCL1/2, TGFβ, platelet-derived growth factor (PDGF), VEGF and others [154-157]. These factors modulate the melanoma microenvironment, which further contributes to tumor vascularization and growth as well as facilitates migration/invasion of both melanoma and stromal cells. Under these conditions, melanoma cells successfully survive outside their original environment and are able to establish metastasis.

1.3.2. Molecular phenotype and "phenotype switching" in metastatic melanoma

In 2006, Hoek et al. reported that melanoma is more than just a consequence of various mutations in the genome and that the ability of melanoma to progress is depending on its phenotype plasticity [158]. Melanomas can display distinct molecular phenotypes with characteristic gene expression signatures, which can switch back-and-forth as metastasis progress [159].

By gene expression profiling of 86 melanoma cell lines, Hoek et al. [158] identified two main groups of cells, distinctive in their transcriptional signatures. One group demonstrated a high proliferative rate and a weak invasive capacity, while the other group exhibited opposite features, with strong invasive capacity and low ability to proliferate. The molecular signatures of the two groups are further referred to as the proliferative and invasive phenotype, respectively [158]. The proliferative phenotype is driven by Wnt/β-catenin signaling, leading to increased expression of microphthalmia-associated transcription factor (MITF) (a master regulator for melanocytic differentiation) and its target genes, such as SRY-related HMG-box 10 (SOX10), melan-A (MLANA) and tyrosinase (TYR). The invasive phenotype, however, shows down-regulation of the above-mentioned genes and up-regulation of TGFβ signaling-driven Wnt inhibitors (like Wnt family member 5A (WNT5A), Dickkopf Wnt signaling pathway inhibitor (DKK) 1 and 3) and genes involved in modulation of ECM, including lysyl oxidase (LOX), collagen type V alpha 1 (COL5A1) and THBS1 [158] (Figure 5).

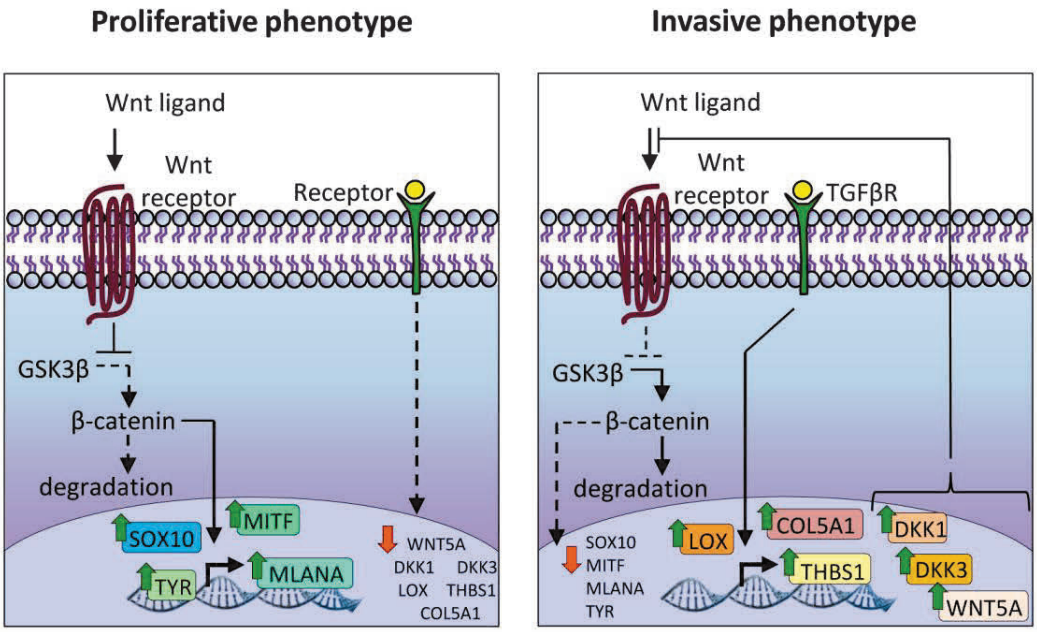

Figure 5. Schematic illustration of melanoma phenotype-associated signaling. Proliferative and invasive phenotypes are driven by Wnt and TGFβ signaling, respectively. See the text for further details. Solid and dashed lines represent activation or de-activation of the signaling, respectively. Green and red arrows indicate increased or decreased gene expression, respectively.

The notion that melanoma can switch between phenotypic states was validated in models *in vivo*. Following subcutaneous injection into mice, melanoma cells of each phenotype could initiate tumor growth. The formed tumors consisted of cells of both phenotypes regardless of the phenotype of the cells of origin [159]. Furthermore, intravital imaging of melanoma cells *in vivo* revealed transient changes in the phenotype, i.e. pigment production and expression of MITF-related genes. The non-motile cells in the primary tumors and metastases were highly pigmented and displayed a MITF-driven signature. In contrast, the motile cells and disseminating cells in the blood stream were amelanotic, with a suppressed MITF signature [160]. From our own research, we observed phenotype dynamics during development of brain metastases *in vivo* [161]. While the invasive MITF^{low} phenotype was dominant in the early-phase lesions, the late/lethal metastases were enriched with proliferative MITF^{high} phenotype cells. We suggested that a conversion to the invasive phenotype might be necessary to adapt to the foreign microenvironment and to initiate metastatic growth. Further tumor expansion, though, might benefit from the proliferative state.

What triggers the phenotype switching is not completely established, but TME factors seem to be involved [162]. For example, it has been observed that hypoxia [163] and inflammation [161, 164] are able to control melanoma cell de-differentiation and phenotype transition.

1.3.3. BRAF mutation

Melanomas harbor elevated amounts of somatic mutations compared to many other solid tumors [165, 166], and the majority of them are associated with UVR-induced cytidine to thymidine transitions [167]. The most commonly mutated genes in melanoma, rapidly accelerated fibrosarcoma protein kinase B (BRAF) and neuroblastoma RAS viral oncogene homolog (NRAS) (with mutation frequencies of 52 and 28%, respectively [168]), however, do not harbor this transition [167] and appear due to UVR-induced oxidative stress [169].

The most abundant BRAF gene mutation, accounting for more than 80%, is the substitution of valine to glutamic acid at position 600 (denoted as V600E). Other substitutions at residue 600, V600K and V600D, are less common and account for 16% and 3% of all BRAF mutations, respectively. $BRAF^{V600E}$ does not require RAS-depended BRAF dimerization and acts as a monomer instead. This can result in a 500-fold over-activation of the BRAF kinase, which is a member of the MAPK signaling cascade [170], and leads to constant activation of the downstream components of the pathway, including MEK and ERK [171, 172]. Normally, the constitutive activation of BRAF induces senescence in melanocytes, preventing their progression to malignancy. Thus, additional genetic alterations should take place in order to overcome this and drive cancer progression [173-175].

1.3.4. Melanoma treatment

Due to high therapeutic resistance of metastatic melanoma, treatment possibilities have been limited. In mid-1970s, the first drug, dacarbazine (DTIC), was introduced to the clinics, but the overall response rate to the drug was low (7%- 35%) [176] and no complete or long-lasting remission has been achieved [177]. A new era of treatment for advanced melanoma

patients began when targeted therapy against mutated BRAF was introduced. The first food and drug administration (FDA) approved BRAF inhibitor, vemurafenib (also known as zelboraf or PLX4032), was initially evaluated by Flaherty et al. [178] in a Phase I clinical trial. The results revealed an 80% response rate among patients with $BRAF^{V600E}$ mutation, and at that time, this was the highest response rate ever recorded for melanoma drug treatment [178]. To compare the efficacy of vemurafenib *versus* DTIC, a Phase III clinical trial, randomizing 675 patients with previously untreated metastatic melanoma with $BRAF^{V600E}$ mutation, was initiated. At 6 months, the overall survival and response rates were 84% and 48% in the vemurafenib group, compared to 64% and 5% in the DTIC group, respectively [179].

Vemurafenib is designed to target $BRAF^{V600E}$ and shows a 10-fold increased potency over the wild-type kinase [180]. In addition to $BRAF^{V600E}$, the drug also targets the less common $BRAF^{V600K}$ mutation [181]. Vemurafenib selectively binds BRAF monomers and inhibits their activity, thereby preventing phosphorylation of MEK and ERK, i.e. inhibiting the activation of the MAPK pathway. However, if vemurafenib is used on BRAF wild-type tumors, it binds to one of the RAF monomers, leading to the trans-activation of the second, inhibitor-free RAF monomer, promoting elevated MAPK signaling and enhanced tumor growth [182].

Even though vemurafenib is one of the most efficient drugs available for melanoma patients carrying the $BRAF^{V600E}$ mutation, its effect is short lived. Almost all melanoma patients develop resistance towards the drug within 6-12 months after treatment initiation [181]. Also other BRAF inhibitors, like dabrafenib, demonstrates a good initial response, however, resistance emerges soon after [183]. To improve treatment responses, focus has shifted towards understanding the mechanisms behind resistance to BRAF inhibition [184].

Malignant melanomas are highly immunogenic, which makes them perfectly suited for immunotherapy aiming to activate the person's immune system to fight against cancer cells [185]. The anti-cancer activity of T-cells can be hampered by immune checkpoint molecules, such as cytotoxic T-lymphocyte antigen 4 (CTL4), and programmed death-1/programmed death ligand-1 (PD-1/PD-L1). Tumor cells, as well as tumor infiltrating macrophages or fibroblasts, often over-express PD-L1, which helps evading the immune system [186-188]. These observations give a rationale for using immune checkpoint inhibitors as a way to

potentiate immune response against cancer. Today, immunotherapy with checkpoint inhibitors, such as anti-CTL4 and anti-PD-1, are at the frontline of melanoma treatment. In comparison to BRAF inhibitors, checkpoint inhibitors show lower early response and higher toxicity, but they can induce durable responses [189]. Currently, there are several immune checkpoint inhibitors approved by FDA (e.g. ipilimumab targeting CTL4, and nivolumab and pembrolizumab targeting PD-1), and many more are in clinical development.

1.3.5. Resistance towards BRAF inhibitors

Resistance to BRAF inhibitors can be classified into innate and acquired. Briefly, innate resistance is a natural property of a cell not to respond to the treatment, but only a small fraction of BRAF mutated melanomas demonstrates such resistance. The most common is acquired resistance, which develops as a consequence of drug treatment.

1.3.5.1. Acquired resistance

Acquired resistance usually involves reactivation of the MAPK signaling pathway [190] or activation of alternative signaling cascades, often PI3K/AKT [191] (Figure 6). With respect to MAPK reactivation, no secondary mutation in $BRAF^{V600E/K}$ was found [192, 193]. However, alternative splicing of $BRAF^{V600E}$ [194] and copy number amplification [195] were identified. Due to alternative splicing, dimerization of BRAF occurs, resulting in strong activation of MEK and ERK, even in the presence of a BRAF inhibitor [194]. Over-expression of BRAF was shown to be sufficient to activate ERK in the

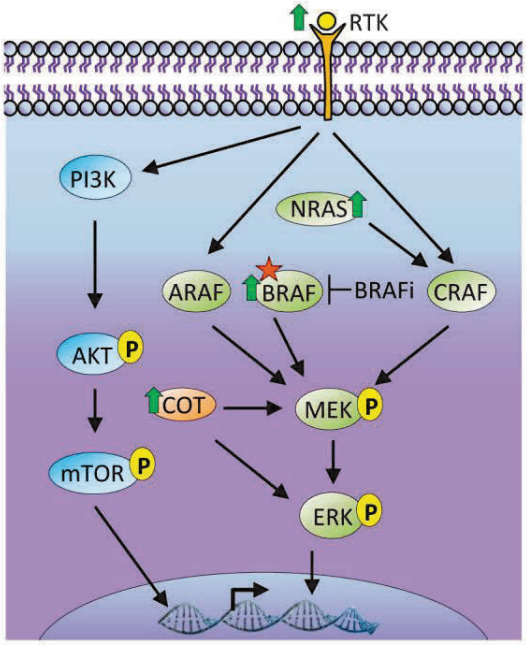

Figure 6. Schematic illustration of the mechanisms behind acquired resistance. See the text for further detailes. Green arrows represent increased expression of the protein, while star indicates additional acquired changes within the protein. BRAFi – BRAF inhibitor.

presence of BRAF inhibition in 20% of cases [195]. Up-regulation of NRAS [196] and amplification of mitogen-activated protein kinase kinase kinase 8 (COT/MAP3K8) [197] were also reported to reactivate the MAPK pathway, leading to acquired resistance to BRAF inhibitors.

RTKs were often shown to be up-regulated and activated in BRAF inhibitor treated melanoma patients. Generally, RTK can initiate signaling cascade through both the MAPK cascade and alternative pathway(s) like PI3K/AKT. Villanueva and colleagues reported that both MAPK reactivation through ARAF and CRAF, as well as an enhanced insulin like growth factor 1 receptor/PI3K signaling cascade, are involved in acquired resistance [193]. Another mechanism implicates up-regulation of the PDGF receptor (PDGFR) β, which leads to activation of PI3K/AKT/mTOR signaling [196, 198].

1.3.5.2. Innate resistance

1.3.5.2.1. Genetic factors and phenotype

Innate resistance to BRAF inhibition has been linked to mutations in a number of genes, such as cyclin-dependent kinase inhibitor 2A (CDKN2A), cyclin-dependent kinase (CDK)-4, cyclin D1 (CCND1), PTEN and AKT3. Although, it should be noted that these genetic defects can also contribute to acquired resistance [191].

Under normal physiological conditions, MAPK signaling drives the cell cycle by increasing the expression of CCND1, which then binds to CDK4 and CDK6 and initiate progression through the cell cycle (Figure 7A). Inhibition of BRAF aims to stop this process and arrest cell proliferation. However, alterations in genes regulating the cell cycle can lead to resistance to BRAF inhibition. About 17% of BRAF mutated melanomas have an amplification of CCND1, which alone, or together with mutated CDK4, can contribute to initiation of the cell cycle and thus resistance [199]. Another common genetic lesion in melanoma is the deletion/mutation in CDKN2A, which encodes $p16^{INK4}$, an inhibitor of CDK4. This genetic defect, as well as mutations in CDK4, which abolish its interaction with $p16^{INK4}$, keeps CDK4 active and thereby reduce the effect of BRAF inhibition [200, 201] (Figure 7B).

A tumor suppressor PTEN, which negatively regulates PI3K/AKT signaling, is mutated/deleted in >10% of melanomas [202]. However, PTEN loss alone is not sufficient to activate PI3K/AKT signaling and additional factors are needed [203]. One such factor is found to be FN [204], which, through α5β1 integrins, maintain PI3K/AKT signaling and thereby reduce the effect of BRAF inhibitors [204, 205]. It has also been suggested that overexpression of AKT isoform 3 (a predominant isoform in melanoma) can elevate AKT signaling, facilitating innate resistance [206] (Figure 7C).

Recently, it has been disclosed that melanoma cells with low MITF expression and high levels of AXL [207, 208] and WNT5A [209], known as the invasive phenotype, poorly respond to BRAF/MAPK inhibitors (Figure 7C). This suggests that inducers of the invasive phenotype, like factors from the TME, might be important contributors to innate resistance.

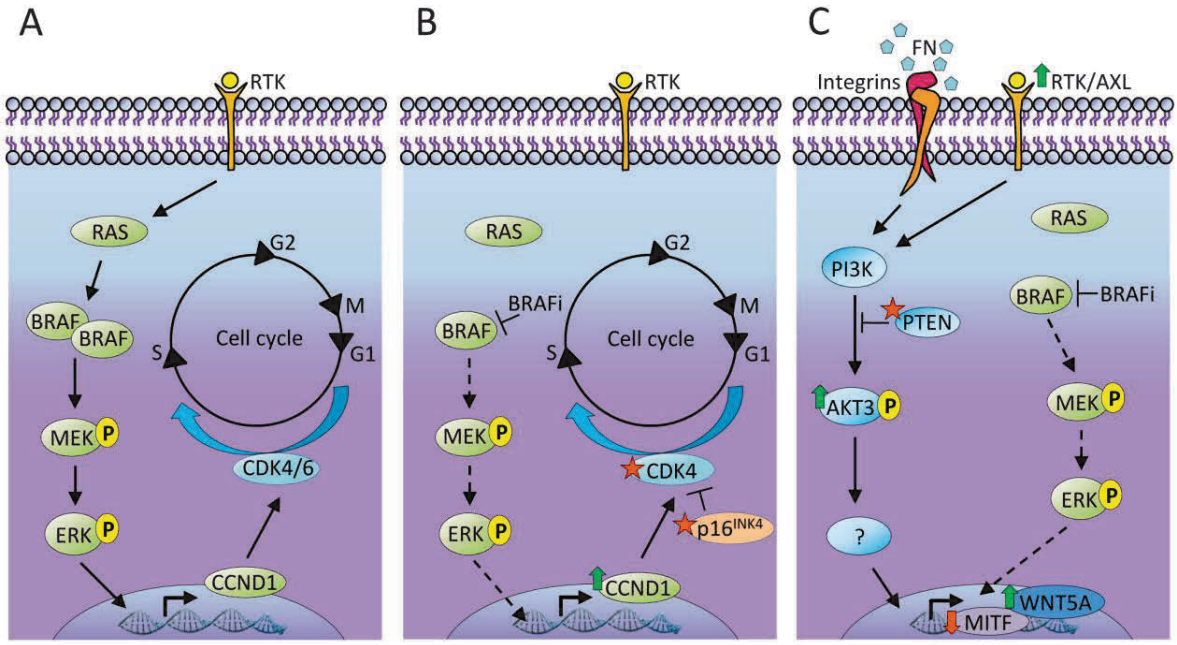

Figure 7. Schematic illustration of the mechanisms associated with tumor genetic changes and phenotypic state in innate tumor resistance. A) normal conditions, B) MAPK independent activation of the cell cycle as a result of common lesions in melanoma C) Alternative signaling involvement from activation of the PI3K/AKT pathway and phenotype switch. See the text for further detailes. G1, S, G2 and M represent different phases of the cell cycle. BRAFi – BRAF inhibitor. Green and red arrows represent increased or decreased expression of the protein, respectively. Red stars indicate different changes within the protein. Solid and dashed lines represent activation or de-activation of the signaling, respectively.

1.3.5.2.2. TME influence

Several recent studies highlight the role of TME, both soluble factors and components of ECM, in regulating melanoma response to BRAF inhibitors (Summarized in Figure 8). Straussman et al. [117] demonstrated that fibroblast-secreted HGF activates its receptor MET, which stimulates the MAPK and PI3K/AKT signaling pathways, reducing the effect of the drug.

It has also been reported that macrophages that infiltrate BRAF inhibitor-treated melanomas secrete TNF that acts via IκB kinase (IKK)/NF-κB/MITF signaling, which makes melanoma resistant to BRAF inhibition [112].

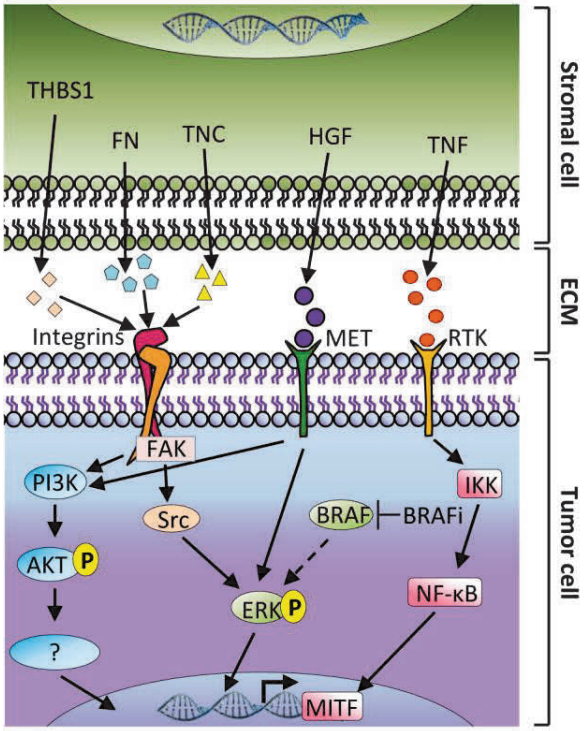

Figure 8. Schematic illustration of the influence of TME on melanoma innate resistance. See the text for a detailed explanation. Solid and dashed lines represent activation or de-activation of the signaling, respectively.

Another recent study by Seifert et al. [210] has shown that extrinsic factors, derived from different organs/sites, might have different influence on melanoma resistance. Factors from the central nervous system were shown to have the strongest influence, and this effect was mediated via the activation of PI3K signaling [210].

Hirata et al. [113] described a resistance mechanism mediated via ECM. This study showed that the BRAF inhibitor (PLX4072) also acts on melanoma-associated fibroblasts, making them produce different ECM proteins, including FN, THBS1 and TNC. Due to changed matrix composition and stiffness, the melanoma cells elevate integrin β1/FAK/Src signaling and thereby escape the drug effects [113].

1.3.6. Combinatorial treatment strategies to potentiate treatment efficacy

The resistance mechanisms discussed above (chapter 1.3.5.) suggest new treatment strategies, where a BRAF inhibitor can be combined with another drug targeting pathways/molecules associated with resistance.

In pre-clinical models, BRAF inhibitors in combination with inhibitors of CDK4 [211], COT [197], various RTKs [117], integrins [113] and IKK [112] signaling were investigated, showing an improved anti-melanoma effect. Targeting the signaling pathways regulating the tumor invasive phenotype, like TGFβ and WNT5A, has also been under investigation. Inhibition of WNT5A signaling demonstrated increased sensitivity to BRAF inhibitors, leading to tumor regression [163, 212]. Several TGFβ inhibitors were developed for cancer therapy and demonstrated efficacy in preclinical studies, but in clinical trials they have showed no success so far [213].

Clinical trials have primarily focused on combinations of BRAF inhibitors with other MAPK pathway inhibitors or inhibitors of PI3K/mTOR signaling. The efficacy of a BRAF inhibitor in combination with a MEK inhibitor was compared to a BRAF inhibitor alone in a phase III clinical trial, randomizing 423 previously untreated patients with $BRAF^{V600E/K}$ metastatic melanoma. The median progression-free survival and response rates in the combination treatment groups were 9.3 months and 67%, respectively, compared to 8.8 months and 51% in the mono-treatment group. At 3 years, the overall survival rate was 44% in the combination treatment group and 32% in the mono-treatment group (NCT01584648) [214, 215]. Even though this combination treatment reduces the risk of progression and improves the response rate and overall survival, resistance develops in the majority of the patients after approximately 1 year [216].

Promising results from inhibiting the PI3K-AKT-mTOR axis in experimental models [193, 198] encouraged the initiation of several clinical trials. Clinical trials with PI3K/mTOR inhibitors as single agents have shown limited success in melanoma [217]. A number of clinical trials where PI3K or mTOR inhibitors are combined with inhibitors of BRAF/MEK are on-going. Several trials are completed, but the results are not yet made public (NCT01820364, NCT01390818, NCT01337765) [216]. However, the latest results presented

at 2014 ASCO Annual Meeting report about high toxicity when combining both PI3K and MEK inhibitors [218].

As mentioned in chapter 1.3.4., immunotherapy with checkpoint inhibitors is at the frontline of melanoma treatment. Given that melanoma treatment with BRAF/MEK inhibitors increases the expression of immunosuppressive molecules, like PD-L1, [219], a combination of checkpoint and BRAF/MEK inhibitors is an attractive option. In pre-clinical studies such treatment significantly prolonged mice survival and retarded tumor growth [220]. Consequently, several clinical trials have been initiated (NCT01673854, NCT02224781, NCT01940809) to assess immunotherapy and targeted therapy combinations [221].

2. AIMS OF THE STUDY

Malignant melanoma is notorious for its abilities to metastasize and resist therapy, and we assumed that such aggressive behavior is favored through crosstalk with its microenvironment. The aim of this study was to investigate how TME factors promote pro-metastatic and drug resistance characteristics of melanoma, with the ultimate goal to find means for improving therapeutic response.

The specific aims for this project were to:

1) Explore how the pro-metastatic, pro-inflammatory soluble factor S100A4 influences melanoma cell interactions with stromal cells, and to reveal the consequences for aggressive behavior of tumor cells (**paper I**)
2) Investigate how different types of interactions with stromal cells influence melanoma cell sensitivity to the BRAF inhibitor vemurafenib and to reveal possible mechanisms behind stroma-mediated resistance (**paper II**)
3) Identify characteristic signaling pathways/molecular factors in the stroma-protected melanoma cells and explore them as targets in mono-therapy and in combination with the BRAF inhibitor vemurafenib (**papers II and III**)

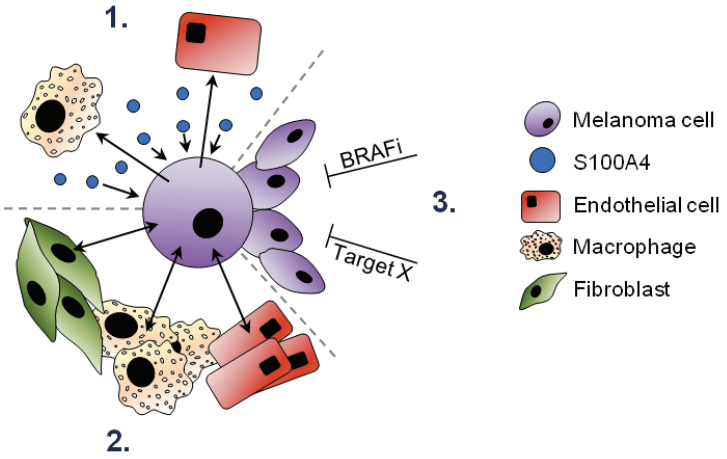

Figure 9. Overview of the project. Investigation of tumor – microenvironment crosstalk through soluble factors (1) and melanoma cell – stromal cell close proximity (2), and its influence on metastatic and resistance functions of melanoma (3). BRAFi – BRAF inhibitor; Target X - molecular features of stroma-influenced melanoma that could be evaluated as therapeutic target.

3. SUMMARY OF PUBLICATIONS

Paper I. Metastasis-associated protein S100A4 induces a network of inflammatory cytokines that activate stromal cells to acquire pro-tumorigenic properties

This paper was initiated by the following hypothesis: when present in the microenvironment, the pro-metastatic soluble factor S100A4 triggers tumor – stromal cell crosstalk, which is beneficial for the tumor and favors metastases.

By using malignant melanoma as a model, we have demonstrated that S100A4 is enriched in the tumor – stroma border in metastatic TME *in vivo*. This observation motivated further *in vitro* studies, where we analyzed how extracellular S100A4 affects melanoma cells. By analyzing the transcriptional profile and secretome of melanoma cells stimulated with S100A4, we identified enhanced production of a variety of pro-inflammatory cytokines/soluble factors, such as IL-8, CCL2, CXCL1, IL-6, IL-1b and SAA1. The tumor-conditioned media containing these proteins were further shown to activate endothelial and monocytic cells, which gained pro-angiogenic properties and the pro-tumorigenic M2 phenotype, respectively, i.e. characteristics of an inflammatory milieu. To evaluate whether such tumor-educated stromal cells can act back on tumor cells and promote their metastatic properties, melanoma cells were exposed to the activated monocytes/macrophages. We observed enhanced melanoma cell proliferation and migration, where the malignant cells left the tumor spheroid and readily spread on collagen matrix.

Overall, our data revealed how the pro-metastatic factor S100A4 stimulates melanoma cells to secrete pro-inflammatory cytokines that engage angiogenic and inflammatory stromal cells to potentiate metastatic properties. This study highlights the tumor cells' ability to create an inflammatory environment and use it to facilitate metastasis.

Paper II. Fibroblast-induced switching to the mesenchymal-like phenotype and PI3K/mTOR signaling protects melanoma cells from BRAF inhibitors

This paper was initiated by the following hypothesis: stromal cells foster a melanoma cell phenotype with impaired sensitivity to BRAF inhibition and thereby contribute to resistance.

Melanoma cells were co-cultured *in vitro* with lung fibroblasts, endothelial cells or monocytes to mimic different types of interactions during treatment with vemurafenib. We showed that the lung fibroblasts and endothelial cells, but not the monocytes, reduced significantly the melanoma cells' sensitivity to the drug. This effect was primarily dependent on close proximity/cell – cell contacts between tumor and stromal cells and could not be achieved via soluble factor-mediated communication. The reduced sensitivity to vemurafenib in the co-cultures with fibroblasts was validated by demonstrating the presence of a large fraction of melanoma cells that stayed proliferative, i.e. normally progressing through the cell cycle and positive for the proliferation marker Ki-67. Furthermore, fewer transcriptional changes were observed after vemurafenib treatment in co-cultures compared to mono-cultures. To investigate the possible biological mechanism behind fibroblast-promoted resistance, the molecular profile of melanoma cells from co-cultures and mono-cultures were compared by global gene expression. We observed that melanoma cells from co-cultures exhibited a clear switch towards the invasive, mesenchymal-like transcriptional state, characterized by down-regulation of melanocytic markers (MITF and its targets) and up-regulation of mesenchymal markers, such as AXL, PDGFR and FN. At the signaling level, a higher level of p-mTOR and its downstream target pS6 was observed in the treated melanoma cells from co-cultures compared to mono-cultures. This suggested that the co-cultured melanoma cells, i.e. the invasive phenotype, might be dependent on the active PI3K/mTOR pathway. To explore this possibility, mTOR or the upstream PI3K signaling pathways were targeted, and the effects in co-cultures and mono-cultures were compared. We demonstrated eradication of pS6-positive melanoma cells as well as an enhanced anti-proliferative effect in co-cultures when PI3K/mTOR inhibitors were used in combination with vemurafenib. In addition, the benefit of mTOR and BRAF co-inhibition was also seen in early-stage lung metastases *in vivo*.

In conclusion, our findings signify the importance of stromal cells, specifically lung fibroblasts, in regulating phenotype switching in melanoma that is associated with resistance to BRAF inhibition. In addition, this study highlights the importance of phenotype-specific targeting in order to potentiate the overall efficacy of the treatment.

Paper III. Targeting stroma-supported melanoma cells resistant to BRAF inhibitors

This work was built on our previous observation that the presence of fibroblasts significantly reduces melanoma cells' sensitivity to the BRAF inhibitor vemurafenib (BRAFi). This finding demonstrated the need to find other targets than BRAF in order to improve the anti-cancer effect in melanoma influenced by fibroblasts.

Melanoma co-cultures with lung fibroblasts and mono-cultures were utilized to mimic stroma-interacting and non-interacting tumor cells, respectively. To identify protein signatures that discriminate co-cultured and mono-cultured melanoma cells, we performed proteomic analysis by reverse phase protein array. The data indicated a clear proteome-modulating effect of fibroblasts. Thus, co-cultured melanoma had elevated levels of proteins common for the mesenchymal/inflammatory cellular state (such as AXL, PDGFR and p-c-Jun, pSTAT3) and reduced levels of proteins regulating melanocytic differentiation (MITF and β-catenin). Markers indicating cell proliferation (Ki67 and pRb) were also at lower levels in the co-cultures. Altogether, this indicates that in the presence of fibroblasts, melanoma cells acquire a mesenchymal-like, less proliferative phenotype. To target such a phenotype, we first tested inhibitors of up-regulated PDGFR and AXL. However, no significant improvement in the anti-cancer effect in the BRAFi-treated co-cultures was observed. In search of other possible targets, we performed a large-scale screening of anti-cancer drugs in combination with BRAFi. We compared the efficacy of 384 drugs from the Selleck Chemicals Cambridge cancer compound library on melanoma cells grown in co-cultures *versus* mono-cultures. Forty-one compounds reduced cancer cell proliferation equally efficient or better in the co-culture than in mono-cultures. Among them, there were 10 inhibitors of PI3K/mTOR, pointing at the PI3K-linked signaling pathway as a promising target. However, GSK3β inhibitor AR-A014418 (GSK3i) induced the strongest anti-proliferative effect in the co-cultures compared to mono-cultures. Further analysis on cell survival and levels of the proliferation marker Ki67

validated the potency of GSK3i in the co-cultures. However, in the mono-cultures GSK3i induced an adaptive response that attenuated the effect of BRAFi. Thus, while BRAFi alone pushed the mono-cultured cells into the quiescent state, defined as $pS6^{low}/Ki67^{low}$, co-treatment with GSK3i promoted the $pS6^{high}/Ki67^{low}$ cellular state. Restored high levels of pS6 might indicate GSK3i-induced re-activation of mTOR in these culture conditions, i.e. the differentiated melanoma phenotype, and such an effect might not be desirable in a clinical situation. Further, we followed up on PI3K as a target and evaluated the efficacy of a clinically relevant pan-PI3K inhibitor, buparlisib (PI3Ki) in different cell cultures. PI3Ki induced a stronger anti-proliferative effect in co-cultures than mono-cultures. Furthermore, in the co-cultures the PI3Ki+BRAFi combination was more efficient than BRAFi alone. The combined treatment of the co-cultures was able to eliminate the proliferative $pS6^{high}/Ki67^{high}$ cell fraction and push the cells into the quiescent $pS6^{low}/Ki67^{low}$ state. In contrast to GSK3i, PI3Ki did not influence the anti-cancer effect of BRAFi in the mono-cultures. Thus, co-treatment with PI3Ki was beneficial against fibroblast-interacting melanoma cells that generally show poor response to BRAFi. In melanoma cells that lack fibroblasts support, i.e. good responders to BRAFi, PI3Ki treatment did not aid in the overall anti-cancer effect.

In conclusion, our findings demonstrate stroma-influenced, phenotype-dependent responses to targeted drugs. This study also highlights the importance of understanding biological mechanisms behind each drug action on cells with different phenotype. Designing rational combinations of drugs, which induce anti-proliferative effects in cancer cells of distinct phenotypes, could potentiate the overall efficacy of the treatment.

4. METHODOLOGICAL CONSIDERATIONS

Tumor – stroma communication is a complex process where different cells interact with each other, affecting properties and behavior of the cells. To recapitulate this complexity in an experimental system and track tumor cells specifically is a challenge. In this study, malignant melanoma was chosen as a model, since it readily adapt and respond to different microenvironmental stimuli, which might be one of the important reasons why melanoma easily develops metastasis and resistance to therapy. In addition, there is a huge need to improve therapeutic options in this aggressive disease.

In order to investigate how melanoma cells interact with different stromal cells and how this affects melanoma aggressiveness, we used *in vitro* co-cultures of different composition. To discriminate tumor cells from stromal cells, melanoma cells were stable labeled with green fluorescence protein (GFP)-Luciferase (LUC) construct. The GFP tag allowed us to identify tumor cells by fluorescent microscopy, immunofluorescence (IF) and fluorescence-activated cell sorting (FACS). LUC-generated bioluminescence, on the other hand, allowed us to track tumor development and response to treatments *in vivo,* by using an *in vivo* imaging system, and measure viability/proliferation exclusively in tumor cells when they were co-cultured with stromal cells.

4.1. Model systems

4.1.1. *In vitro* cultures

A majority of the experiments in this thesis have been performed using cultured human cell lines. Generally, cell lines are widely used in preclinical research as a simple and cheap model for investigation of biological mechanisms or evaluating new therapeutic strategies. Cell lines are easy to handle in functional studies, and it is easy to harvest sufficient amounts of material (proteins or RNA) for molecular analyses. Cell line experiments are also relatively easy to control, which is reflected in high reproducibility. Finally, it is a big advantage if *in vitro*

systems can be used to substitute animal models, since use of the latter involves ethical considerations.

Growing cells as two-dimensional (2D) mono-cultures in a dish lacks most of the complexity observed in a living organism [222], i.e. three dimensional (3D) structure, ECM and neighboring stromal cells, meaning that no tumor – stroma interactions are recapitulated in such cultures. Some of the complexity/interactions can partly be re-created by forming 3D mono-culture spheroids and seeding them on a collagen/matrigel matrix [223] (as we did in **paper I**). Usage of co-cultures, where tumor cells are allowed to interact with stromal cells via cell – cell contacts or soluble factors (as we did in **papers I-III**), further brings *in vitro* systems closer to a real situation [224]. The co-cultures, however, are difficult to utilize in molecular and functional studies, since tumor cell-specific signals have to be discriminated from signals produced by the stromal cells. Thus, cell type specific gene/protein expression analysis by conventional methods (quantitative real-time polymerase chain reaction (qPCR), Western immunoblotting (WB) or Simple Western immunoassay (SWI)) requires cell sorting based on a specific marker (e.g. GFP in our case) before the analysis. An alternative is to utilize multi-parameter flow cytometry (see chapter below 4.2.3.), which can discriminate GFP^+ tumor from GFP^- stromal cells, and simultaneously measure a protein of interest (used in **papers II** and **III**). However, this technique usually shows limited sensitivity and cannot capture small differences in protein levels. In addition, tumor cell viability/proliferation in co-cultures cannot be measured by conventional assays that score the cells' mitochondrial activity (e.g. MTS or CellTiter-Glo). To overcome this challenge, we made use of the LUC tag on the tumor cells and quantified LUC-produced bioluminescence as a measure of cell viability/proliferation. A comparison of this method with conventional assays in mono-cultures showed similar results (see **paper II**), validating its relevance.

None of the difficulties mentioned above are encountered in mono-cultures exposed to conditioned mediate collected from separately-cultured cells (as done in **paper I**). However, this strategy allows investigation of cell communication only through soluble factors and not cell – cell contacts. If soluble factors are the main mediators of the crosstalk, the conditioned media-based strategy is advantageous over the co-cultures due to its simplicity.

Seeding density of 2D cell cultures is of high importance, since over-confluent cellular density can lead to underestimation of cell proliferation, signaling and drug efficacy [225, 226]. Therefore, optimization of cell densities for different culture conditions was performed for all our models. Cell cultures were carefully inspected before and during the experiments to be sure that cell density was optimal (samples were discarded if cell confluence reached > 90%).

4.1.1.1. Cell lines

A large number of melanoma cell lines have been used in this thesis, though only three of them were in focus: Melmet 1 and Melmet 5 in **paper I**, and Melmet 5 and HM8 in **papers II** and **III**. All three cell lines were established in our Department and are very well characterized *in vitro* and *in vivo* [161, 227], making them attractive models for melanoma/metastasis studies. The cell lines originate from different metastatic sites in melanoma patients: subcutaneous metastases (Melmet 1), lymph node metastases (Melmet 5) and brain metastases (HM8) (all approved by the Norwegian Research Ethics Committee 2.2007.997, S-01252, and 2011/2183). In contrast to Melmet 5 and HM8, which represent the proliferative phenotype, Melmet 1 is associated with the invasive phenotype. Thus, a Melmet 1 and Melmet 5/HM8 cellular system is a good model to study phenotype-specific responses.

Three different types of stromal cells have been used in this thesis. Monocytic cell line THP-1 and embryonic lung fibroblast cell line WI-38 are commercially available and widely used by many laboratories. Human Umbilical Vein Endothelial cells (HUVEC) were isolated from human umbilical cord and provided by our collaborators at Department of Pathology, Oslo University Hospital, Norway. In **paper I,** which focused on inflammation-related interactions, monocytes and endothelial cells were used, since they are known to be involved in inflammatory processes [228, 229]. In **paper II** and **III**, which focused on stroma-dependent drug resistance, fibroblasts were used, due to their acknowledged role in EMT and resistance [117, 230]. Furthermore, patient-derived fibroblasts were tested in co-cultures, revealing a similar effect on melanoma drug resistance as the WI-38 lung fibroblast cell line (unpublished).

4.1.2. *In vivo* experimental models

In vivo animal models have the TME complexity and can thus well reproduce interactions between tumor and host cells. In addition, these models allow us to investigate side effects of treatment *in vivo*. The mouse is a leading organism for modeling human disease and testing experimental therapies *in vivo*. In order to generate human tumors in mice, the mouse immune system has to be suppressed. The absence of an intact immune system is one of the most important limitations of these *in vivo* models, since the immune cells play an important role in tumor development and therapy. Ethical considerations regarding the use of animals in research is another important limitation for the *in vivo* models.

In this thesis, athymic nude foxn1^{nu} mice have been in use. Due to disruption of the gene FOXN1, thymus is lost, resulting in loss of adaptive immunity cells, i.e. T cells, while cells involved in innate immunity, like macophages and natural killer (NK) cells, are preserved. This means that nude mice do not allow studies on effects involving T-cells, but responses involving BMDCs, macrophages and NK cells can still be observed. In addition, the hairless appearance of these animals simplifies vital imaging when tumor formation or response to treatment is scored by measuring bioluminescence derived from LUC^{+} tumor cells (see **paper II**).

Two different experimental metastasis models have been used in this thesis: tumor cell injection into the left ventricle (L.V.) of the heart (**papers I** and **II**) and intravenously (i.v.) (**paper II**). The L.V. injection allows tumor cells to circulate within the body, followed by homing to preferred sites. By doing this, metastases at multiple sites can be initiated. The i.v. injection delivers tumor cells primarily to the lungs. Even though neither of these models reproduce the tumor cells' escape from a primary tumor and subsequent intravasation, they involve the other steps of the metastatic cascade [231] (see chapter 1.1.1.). Therefore, we can study site-specific differences with respect to metastatic growth or therapeutic response, as we did in **paper II**. A spontaneous metastasis model, where tumor cells are injected subcutaneously or orthotopically, form a "primary" tumor and eventually disseminate to distant sites, recapitulate all the steps of the metastatic cascade [231]. However, this model is difficult to establish, and our group has no such a model yet in place.

4.2. Experimental tools

4.2.1. High-throughput (HT) techniques

Multiple HT approaches were utilized in this thesis to explore biological mechanisms involved in tumor – stroma crosstalk and/or pinpoint potential targets for therapy: i) cytokine arrays to map soluble factors released by the tumor cells (**paper I**); ii) Illumina BeadChip array (**papers I** and **II**) and reverse phase protein arrays (RPPA) (**papers II** and **III**) to map gene expression and levels of (phospho)proteins, respectively, in tumor cells when stimulated with microenvironmental factors and/or therapy; iii) cancer drug sensitivity screening (CDSS) to explore tumor cell (with/without stroma) responses to compounds from the Selleck Chemicals Cambridge Cancer Compound Library (**paper III**). Even though such HT techniques supply a vast amount of data, their further analysis and integration into a coherent biological picture requires bioinformatics expertise. This aspect was only partially addressed in this thesis, and work is ongoing in collaboration with systems biology groups. HT-identified specific proteins/genes need to be validated. We used enzyme-linked immunosorbent assay (ELISA) and WB/SWI for protein validation, and qPCR for gene expression validation.

The hits identified through CDSS also need to be validated through dose-response cell survival studies. CDSS was performed using one dose for all screened drugs, and if this dose was too high or too low, we might have missed a potential hit. One should also have in mind that many targeted drugs are not specific and induce off-target effects. Their specificity should be validated by measuring the levels of target proteins. In our studies, the level of pS6, a target of mTOR, was measured by WB and flow cytometry to evaluate specificity and activity of an mTOR inhibitor (**papers II**). We also used Wnt/β-catenin reporter construct, TCF-LEF responsive luciferase, to validate that a GSK3 inhibitor stimulates Wnt/β-catenin signaling (**paper III**). In brief, inhibition of GSK leads to nuclear translocation of β-catenin, which then binds to TCF-LEF transcription factors, leading to enhanced activity of luciferase.

4.2.2. Studies on cell signaling by measuring phosphoproteins

Phosphoproteins work as signaling molecules, transferring messages from one protein to another. Studies on phosphoproteins are challenging since phosphate groups can easily be "removed" because of various factors, such as phosphatases, proteases, time delays, differences in temperature, etc [232]. In our work, caution was taken to preserve the phosphate groups. For flow cytometry and IF analysis (**papers II** and **III**), samples were fixated in paraformaldehyde/formalin immediately after harvesting to halt kinase and phosphatase activity [233]. For WB/SWI/RPPA, cell lysates were prepared in lysis buffer supplemented with compounds preserving phosphate groups (**papers II** and **III**). Multiple freezing and thawing were avoided. Preparation of tumor cell lysates from co-cultures requires tumor cell separation from stromal cells by FACS. Being aware of the fact that phosphorylation might be affected by this "harsh" procedure, mono-cultured cells were also "sorted" through the FACS machine. Eventually, we compared the levels of (phospho)proteins in sorted and non-sorted cells from the mono-cultures to be sure that these levels were not affected by the FACS procedure (**papers II** and **III**). One of the proteins where phosphorylation was affected during FACS, was ERK. For this reason, pERK activity in this study was evaluated by other means, i.e. flow cytometry and IF.

4.2.3. Multi-parameter intracellular flow cytometry

Multi-parameter flow cytometry allows measurement of several proteins of interest at once in each cell. We primarily used this technique to discriminate GFP^+ tumor cells from GFP^- stromal cells and simultaneously measure the levels of 2-3 phosphoproteins in the tumor fraction. Importantly, we employed a barcoding strategy, where the samples to be compared were given a certain "barcode" before they were mixed and stained "as one" with specific antibodies. In this way, we avoided tube – tube staining variations, and even small differences in protein levels observed between the barcoded samples could be considered "real".

Another important advantage of flow cytometry is its ability to discriminate cell subpopulations and thereby explore the diversity of the cellular system. Conventional analysis

of pooled cells, e.g. by WB/SWI, measures average levels of proteins; cell subpopulations with exceptional levels of the protein will not be captured. Flow cytometry can discriminate cell fractions with defined characteristics. For example, in our case, we identified cell subpopulations with different levels of pS6, and showed that they differ with respect to cell cycle (**paper II**). However, some limitations apply also to this method. Due to spectral overlap between different fluorochromes that are conjugated to antibodies, a limited number of different proteins can be measured at once (we analyzed maximum 5). In addition, challenging fluorescence compensation has to be performed in order to avoid spectral overlap between fluorochromes to avoid false positive signals. The above mentioned limitations are avoided in the new generation of cytometry, mass cytometry, which is based on mass-labeled, instead of fluorescently-labeled, antibodies [234]. This technique allows measuring of up to 35 surface- and functional-proteins in one cell and will be implemented in our system for future studies.

4.2.4. Immunofluorescence (IF)

Another antibody-based method implemented in this thesis (**papers I-III**) was IF. This technique enables visualization of intracellular (e.g. cytoplasm *versus* nucleus) or spatial (e.g. tumor cell localization with respect to stromal cells) localization of proteins. For *in vitro* studies, we have developed asymmetric co-cultures, where tumor cell regions with or without fibroblast as "neighbors" are generated in the same dish. IF analysis of such cultures allowed us to reveal the differences in protein levels depending on tumor cell localization **(papers II** and **III**).

5. RESULTS AND DISCUSSION

The knowledge about TME in cancer progression and therapy is relatively new, but it is an increasingly recognized topic in the field of cancer research. We hypothesize that the ability of malignant melanoma to establish metastases and resist therapies might be associated with its intrinsic capability to exploit the microenvironment. Thus, the crosstalk between melanoma cells and microenvironmental factors has been the main underlying topic throughout all three papers presented in this thesis. The thesis highlights the role of tumor – stroma interactions in shaping the melanoma phenotype towards a more aggressive state, with enhanced pro-metastatic and drug-resistant characteristics. Specifically, we revealed soluble factor-mediated communication between tumor cells and inflammatory myeloid cells, which promoted melanoma growth and invasion (**paper I**). We also disclosed cell – cell contact-mediated crosstalk between melanoma and fibroblasts that resulted in melanoma phenotype switching and, consequently, resistance to BRAF inhibition (**papers II** and **III**). Several ways to target such stroma-protected melanoma cells were evaluated (**papers II** and **III**). In summary, this thesis presents original data on how cancer cells exploit cellular and soluble factors of the microenvironment to potentiate melanoma aggressiveness, and suggests targetable nodes for phenotype-directed therapies.

5.1. Stroma-regulated phenotype switching – a reason for resistance and a target for therapy

In **paper I**, melanoma cells' response to the pro-inflammatory factor S100A4 and their further interactions with inflammatory stromal cells were investigated. We revealed that S100A4-stimulated melanoma cells secrete a plethora of cytokines that recruit and educate myeloid cells, such as monocytes/TAMs. The educated myeloid cells act back on the tumor cells and potentiate their aggressive properties. Recently, Riesenberg et al. [235] demonstrated that pro-inflammatory factors, such as TNF, stimulate melanoma cells to switch their phenotype to the de-differentiated $MITF^{low}$ state, known as the invasive phenotype. This phenotypic state was

associated with an elevated production of cytokines and enhanced recruitment of myeloid cells. Such interconnectivity between an inflammatory milieu and phenotype switching made the authors suggest that myeloid cell-directed therapies might work against the invasive phenotype [235]. How S100A4 influences the phenotypic state in melanoma was not investigated in **paper I**. However, one of our later studies revealed an induction of the $MITF^{low}$ invasive phenotype in S100A4 stimulated melanomas [236]. Thus, the S100A4 potentiated recruitment of pro-tumorigenic myeloid cells might be associated with S100A4-induced phenotypic switching in melanoma. In this respect, our study supports the notion that the invasive phenotype is more pro-inflammatory and more prone to engage monocytes/TAMs, which might be its Achilles' heel from a therapeutic perspective (discussed later in chapter 5.1.2).

In **papers II** and **III,** we focused on stroma-dependent melanoma resistance to BRAF inhibition and how it could be overcome. We observed that melanoma cells in co-cultures with fibroblasts undergo a phenotypic switch to the invasive state, characterized by reduced levels of MITF and elevated levels of mesenchymal markers. We suggest that such a phenotype transition is one of the main mechanisms for how stromal cells protect adjacent melanoma cells from BRAF inhibitors. Consequently, we propose that by targeting the invasive phenotype cells, we could potentiate the efficacy of melanoma therapy in phenotypically heterogeneous tumors. Several recent review papers discussed phenotypic plasticity as a resistance mechanism and therapeutic target in melanoma [237, 238]. It has also been reported that epithelial cancers undergoing EMT show lower susceptibility to different treatments [239]. Collectively, this indicates that the invasive phenotype plays an important role in tumor resistance and signifies the need to understand biological mechanisms supporting this role, particularly in the context of stroma. This knowledge should help to tailor phenotype-directed therapies. Thus, the important questions are as follows: can we disclose intrinsic features or extrinsic interactions that are vital for the invasive phenotype, and can they be further exploited as therapeutic targets? In the following sections, several options will be presented, where both phenotype-specific intrinsic and extrinsic properties will be discussed from a therapeutic perspective.

5.1.1. Intrinsic properties: signaling pathways

Tumor cell functions are usually governed through signaling pathways, and phenotype transition seems to not be an exception. As we discussed in **paper II,** when cancer cells undergo phenotypic switch, they rewire their signaling networks. Such reprogramming might explain why targeted drugs aimed at a specific signaling pathway (e.g. BRAF inhibitors) work against one phenotype, but not the other. The fact that the $MITF^{low}$ invasive phenotype shows poor response to MAPK pathway inhibitors has also been reported by others [207, 208]. Those studies, though, did not address the significance of stroma for this phenomenon. However, a very recent study by Tirosh et al. [240], performed on melanoma patient material, revealed that fibroblast-rich tumors preferentially display the $MITF^{low}$ mesenchymal-like phenotype. This observation is in line with our results, suggesting that the presence of fibroblasts shape melanoma cell phenotype and can thereby modulate their sensitivity to targeted therapy.

Based on the molecular studies in **paper II** and CDSS in **paper III**, we anticipate that the CAF-interacting melanoma, i.e. the invasive phenotype, might utilize PI3K/mTOR signaling and therefore be resistant to BRAF inhibition. This notion was supported by functional studies, where improved therapeutic response in BRAF inhibitor-treated co-cultures was observed after co-treatment with PI3K or mTOR inhibitors. In contrast, inhibitors of other targets in the MAPK pathway did not improve the effect in the co-cultures, arguing against MAPK-reactivation as a resistance mechanism in the presence of CAFs (**paper II**). This data led us to propose that the invasive phenotype of melanoma might prefer the PI3K/mTOR pathway instead of MAPK. Rewiring of signaling towards PI3K was also reported for epithelial cancers undergoing EMT [241], and this effect was linked to alternate RTKs. We also identified up-regulation of several RTKs in the co-cultured melanoma cells. Two of them, PDGFR and AXL, which are known to potentiate PI3K signaling [242, 243], were explored as therapeutic targets in **paper III**. However, neither sunitinib, an inhibitor of multiple RTKs, nor BGB324, an AXL inhibitor, improved the anti-cancer effect in BRAF inhibitor-treated co-cultures. Thus, targeting more common signaling nodes down-stream from RTKs (like PI3K/mTOR) instead of specific RTKs might be a more efficient approach.

In **paper III**, we employed CDSS, aiming to identify compounds that induce anti-cancer effects more efficiency in co-cultures than mono-cultures. The most efficient compound was AR-A014418, an inhibitor of GSK3β. This suggested that GSK3β activity might be vital for co-cultured melanoma cells, i.e. the invasive phenotype. One of the best-described roles of active GSK3β is to suppress Wnt/β-catenin signaling. Inhibition of GSK3β "rescue" β-catenin from degradation, leading to potentiation of Wnt/β-catenin signaling [244]. Thus, our data suggest that co-cultured melanoma might prefer inactive Wnt/β-catenin, while its activation via GSK3 inhibition might be detrimental for such cells. This is in line with what is known about the role of Wnt in regulating phenotypic switch. Canonical Wnt/β-catenin signaling is a recognized driver of the $MITF^{high}$ differentiated phenotype. In contrast, suppression of such signaling is a characteristic of the $MITF^{low}$ invasive phenotype [159, 245]. Although targeting the invasive phenotype through modulation of Wnt/β-catenin might seem as an attractive approach, it also raises important concerns. Since this pathway plays an opposite role in the distinct phenotypes, its modulation might induce opposite therapeutic outcomes depending on the phenotype. Indeed, in **paper III** we observed that the GSK3β inhibitor impaired the effect of the BRAF inhibitor in mono-cultured melanoma cells, i.e. the differentiated phenotype. This might be problematic in a clinical setting if phenotypically heterogeneous tumors were treated with a combination of BRAF and GSK3 inhibitors.

In summary, we identified that suppression of PI3K, GSK3β or mTOR pathways enhances the anti-cancer effect in CAF-interacting melanoma, which shows resistance to MAPK inhibitors. We propose that the PI3K-GSK-mTOR signaling network might be preferred by cells of the invasive phenotype. Further clarification of signaling preferences of each phenotype, and disclosure of critical nodes, should help to tailor phenotype-specific targeted therapies.

5.1.2. Extrinsic properties: engagement of CAFs and immune cells

The TME harbors numerous cellular and soluble/ECM factors that are able to trigger phenotypic switch. This raises the question of whether such factors could be targeted in order to eliminate the invasive phenotype. In **paper II**, we demonstrated the importance of

fibroblasts in promoting melanoma resistance, which was also observed by Hirata et al. [113], and made us inquire whether we can target CAFs to overcome this resistance? This requires identification of specific, targetable features in CAFs. Generally, CAFs are too similar to normal fibroblasts, and, therefore, targeting them is challenging. One factor, though, fibroblast activation protein (FAP) α, has predominantly been identified in CAFs [246]. However, clinical trials targeting FAPα gave disappointing results [246]. The current thesis has not investigated CAFs characteristics, mainly due to difficulties in isolating a pure (not contaminated with melanoma cells) fraction of CAFs. However, this issue was addressed by Hirata et al., who identified up-regulation of ECM remodeling factors in CAFs from treated co-cultures [113]. The authors highlighted alterations within ECM, specifically FN, as an essential event in CAF-mediated resistance to BRAF inhibitors. The modulated ECM affected signaling in melanoma. In addition, Fedorenko et al. has observed that melanoma resistance is associated with ECM/FN-affected RTK/PI3K/AKT signaling [204]. Altogether, this signifies the role of CAFs/CAF-produced ECM in modulating melanoma signaling, and consequently, response to targeted therapy. The mentioned studies, though, have not analyzed whether or how modulated ECM/FN affected the melanoma phenotype. However, it has been reported by others that exposure to exogenous FN stimulates EMT responses and motility in epithelial cancer cells [55, 247]. It should be noted, that we also observed that CAFs deposit a dense network of FN in co-cultures, which surrounds the adjacent melanoma cells (Figure 10). Such an ECM architecture could significantly influence signaling, and consequently, phenotype and function of the embedded melanoma cells. This issue, though, has not been investigated in detail in this thesis. However, we did identify ILK signaling among the most affected pathways in co-cultured melanoma cells (**paper II**). Since FN is a ligand for integrins, it is likely that the observed potentiation of the ILK pathway is due to influence from the FN network. Due to FN's role and abundance in TME at the tumor periphery, it has been considered a target for tumor therapy. For example, coupling therapeutic agents to anti-FN antibodies has been tested for selective delivery to TME [248].

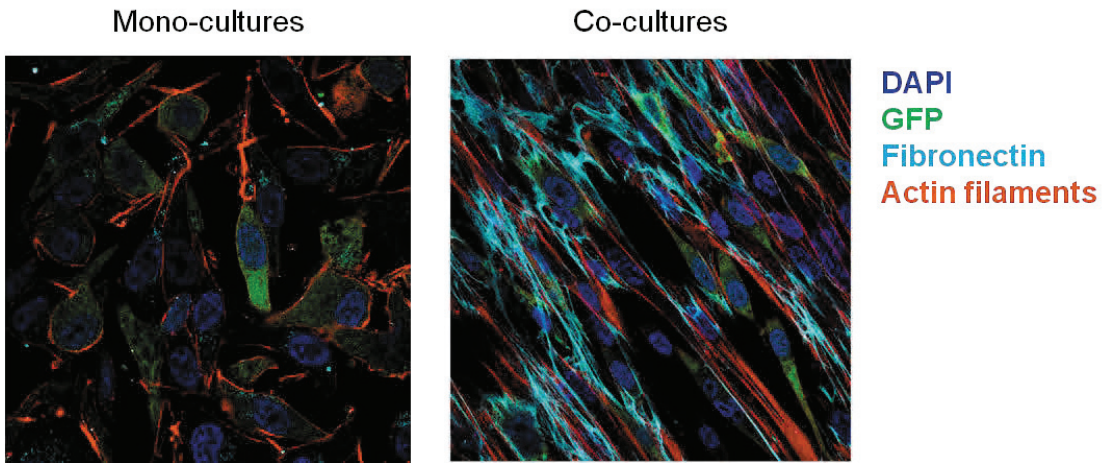

Figure 10. Melanoma cells get embedded in a FN network in co-cultures. GFP^+ melanoma cells and GFP^- fibroblasts were co-cultured for 72 hrs before IF co-staining for FN, GFP, actin filaments and nuclei (DAPI). A dense network of FN with trapped green melanoma cells is seen in co-cultures, but not in mono-cultures, where no FN mesh could be seen. IF was performed by Marco Haselager.

Altered engagement of immune cells might represent another extrinsic property of the invasive phenotype that could be explored from a therapeutic perspective. As discussed above, our data (**paper I** and [236]) and the study by Riesenberg et al. [235] suggest that the invasive phenotype is more prone to recruit, educate and benefit from inflammatory myeloid cells, such as TAMs. Furthermore, we observed stimulation of inflammatory signaling pathways, STAT3 and JNK, in co-cultured melanoma cells (**paper III**). This observation further supports the notion that fibroblast-induced transition to the invasive phenotype might foster an inflammatory milieu. Similarly, Su et al. [249] also reported on the strong bidirectional crosstalk between breast cancer cells undergoing EMT and TAMs. Collectively, this suggests that by interrupting tumor – TAM communication, we could target the invasive phenotype.

A number of strategies have been suggested to target TAMs or their recruitment. These include inhibition of colony stimulating factor 1 and its receptor, or neutralization of CCL2 (reviewed in [250]). In addition, reprogramming of M2 TAMs towards the anti-tumorigenic M1 state has been proposed. For example, it was shown that inhibition of PI3Kγ stimulates the M1 TAM phenotype and potentiates tumor regression [83].

Besides an influence on innate immunity cells, the invasive phenotype appears to affect the functions of adaptive immunity, i.e. T-cells. The association between mesenchymal transition/MITF^{low} state and impaired T-cell functions/immune suppression has been observed in both melanoma and epithelial cancers [240, 251]. This topic, however, has not been investigated in our experimental systems, as they do not allow recapitulation of T-cell responses. However, some of our results might be interesting to discuss from an immune interactions/therapy perspective. First of all, melanoma cells in different phenotypic states might have different abilities to express and present melanocytic antigens, which might influence how they are recognized by the immune system. It has been reported that the inflammation-induced de-differentiated state down-regulates melanocytic antigens and abrogates immune recognition by T cells [164]. Based on transcriptome analysis, we identified antigen-presentation as the most affected pathway in the co-cultured melanoma cells. We found up-regulation of multiple members of major histocompatibility complex class I and II, which would favor antigen presentation. This observation, however, has not been further explored, but is an interesting topic for future research. Secondly, it has been reported that the tumor-intrinsic signaling pathways, such as PI3K/AKT/mTOR and Wnt/β-catenin, can regulate immune evasion [252-254]. Having in mind that we found these networks to be associated with the invasive phenotype, we can speculate that they could contribute to phenotype-associated evasion from the immune system. Thirdly, both CAFs and TAMs that accompany the invasive phenotype also produce immune suppressive factors, such as PD-L1 [186, 188]. For example, Tirosh et al. [240] has reported that CAFs, which favored the MITF^{low} invasive phenotype, simultaneously regulated T-cell exhaustion. Furthermore, it has been reported that induction of EMT leads to up-regulation of PD-L1 [255]. Taken together, this suggests that it might be beneficial to target the invasive phenotype via immune therapy with checkpoint inhibitors, which remains to be explored.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Communication between tumor cells and factors of the microenvironment has recently gained increasing attention. This could eventually help designing improved strategies for metastasis treatment. To achieve this, an increased understanding of how the crosstalk between a tumor and its microenvironment at distant sites is executed and how it changes properties of both counterparts is required. The present thesis addresses these issues using cellular systems of different complexity *in vitro* and *in vivo*. We demonstrate how melanoma cells can exploit soluble and cellular factors of the microenvironment to potentiate features that melanomas are notorious for, i.e. metastatic propensity and drug resistance.

We showed that under the influence of stroma, melanoma cells undergo phenotype switching and acquire different characteristics, which are absent or less pronounced without stromal support. Such stromal influence leads to phenotypic heterogeneity in the tumor, where both stroma-dependent and independent melanoma cells co-exist and need to be targeted (Figure 11). Importantly, we demonstrate that the phenotypic heterogeneity is associated with diversity in response to therapy that is currently in use. Specifically, we showed that the stroma-dependent phenotype lose sensitivity to BRAF inhibition, which signifies the need to tailor phenotype-specific targeted therapies. Several targetable nodes in the stroma-induced phenotype were disclosed through our studies (e.g. PI3K, mTOR or GSK3β). In future research, it would be advantageous to perform system-level analysis of our "-omics" and drug-screening data. The aim would be to model phenotype-specific signaling networks and their perturbations upon treatment. This would help to predict targets that were not disclosed by manual approaches. Eventually, a validation of all identified candidate targets in more complex *in vivo* metastasis models and clinical cohorts should be done.

An association between a phenotypic state and immune interactions is another highly interesting topic for future research. This would help to clarify whether stroma-dependent tumor cells are eligible for immune therapy. Our data suggests that the stroma-dependent and stroma-independent phenotype prefer different signaling pathways. On the other hand, the literature reports that tumor-intrinsic signaling pathways can regulate immune responses [235,

249]. Taken together, this motivates studies on phenotype-dependent immune interactions and how they are modulated by relevant targeted drugs. This is especially important in melanoma, where immune therapy shows great promise.

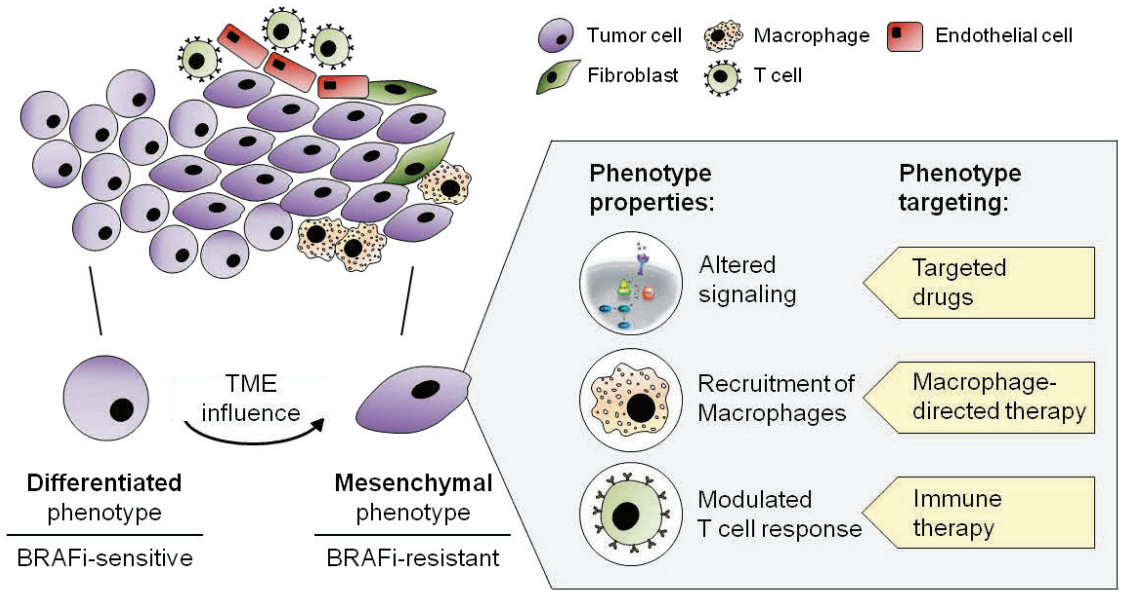

Figure 11. Illustrative summary of the main observations and therapeutic options discussed in the thesis. The figure is inspired by ©Science Shaped.

Finally, the data presented in this thesis support the concept of a relationship between inflammation and metastasis. We show how the metastasis-promoting factor S100A4 induces an inflammatory milieu involving myeloid cells, which are exploited by metastatic cells to potentiate their aggressive properties. The involvement of inflammation/TAMs raises new possibilities for therapy, which remains to be explored. However, increased knowledge on phenotype-specific influences on TAMs and vice versa in different contexts or treatments are required to pinpoint targetable nodes.

In conclusion, through our studies we revealed diversity and plasticity in the interplay between tumor and stroma, forming a well-functioning ecosystem that assures growth and survival of the malignant cells. The complexity and phenotypic plasticity within such a cancer ecosystem creates both challenges and new opportunities for anti-metastatic therapy.

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