Combination Treatments to Overcome Fibroblast-Associated Resistance to BRAF Inhibitors in Malignant Melanoma

Use of the PeggySue Technology to Explore Drug Responses at a Protein Level

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Abstract

Malignant melanomas are one of the most devastating forms of cancers with high mortality rate. The melanoma cells readily spread to distant organs, where the cancer cells interact with stromal cells. Such interaction can induce protection against treatment practiced in the clinics. This project has verified a protection against the BRAF inhibitor (BRAFi) Vemurafenib when metastatic melanoma cells were grown as co-cultures together with the stroma cells, fibroblasts.

Previously, it has been proposed by our group that the BRAFi resistance in the co-cultures can be associated with the PI3K-pathway. By combining the BRAFi with the PI3K inhibitor buparlisib, an enhanced anti-cancer effect in the co-cultures was observed. To investigate molecular responses to buparlisib in our in vitro-systems, we chose to utilize a previously unused method called PeggySue Charge, and attempted to detect AKT levels as an indicator of PI3K-pathway activity. However, further optimization is needed to get reliable results on AKT, or other phospho-proteins, by this method. Thus, verification of PI3K-pathway involvement in stroma-dependent BRAFi resistance at the molecular level is still lacking.

Phenotypic-switching is a common characteristic of metastatic melanomas, and the mesenchymal phenotype has been associated with treatment-resistance. With a new method called PeggySue Size, we have managed to detect increased levels of mesenchymal phenotype-related proteins in melanoma cells cultured with fibroblasts. Inhibition of a WNT-pathway’s negative regulator GSK-3β, by the drug AR-A014418, induced a strong anti-cancer effect in fibroblast associated melanoma cells. However, the molecular mechanism behind this effect has not been disclosed.

In conclusion, this work addressed both, a biological question on stroma-mediated resistance to BRAFi, and a technical question, how to analyze proteins with a new technology PeggySue. When optimized, the latter can contribute as a convenient tool to explore mechanisms involved in stroma-mediated resistance.
Acknowledgements

This work was performed in the time period August 2015 to December 2016 at the Norwegian Radiumhospital, Institute of Cancer Research, in the Department of Tumor Biology lead by Gunhild Mari Mælandsmo.

Firstly, I’d like to give my biggest gratitude to my supervisor Lina Prasmickaite. Lina has been the best supervisor I could have hoped for. She has guided my project with an exceptional eye to detail, and her enthusiasm towards research has been highly contagious. She has encouraged greatness, yet showed patience.

Secondly, the majority of the practical skill-sets I’ve mastered in this project emerge from Kotryna Seip’s excellent supervision. Her guidance in the lab, and her eye for perfection, has been detrimental for my project. She has been available every time I was in need of support or guidance.

Furthermore, I owe my thanks to prof. Gunhild Mari Mælandsmo, and the rest of the people at the Department of Tumor Biology, for making the work enjoyable in a social, as well as a professional, context. I would especially like to thank Mads Haugen and Siri Tveito, for their support in developing the PeggySue results.

Finally, I’d like to thank my family and friends; you have been patient and supportive.

Kjetil Jørgensen

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List of abbreviations

Ab(s) – antibody (ies)
AKT – protein kinase B
AR – AR-A014418
ATP – adenosine-tri-phosphate
AUC – area under the curve
AXL – tyrosine-protein kinase receptor UFO
BRAF – rapidly accelerated fibrosarcoma protein kinase b
BRAFi – BRAF inhibitor
BMDC – bone marrow-derived cells
CAF(s) – cancer associated fibroblast(s)
Charge – PeggySue Charge
DMSO – dimethyl sulfoxide
ECM – extracellular matrix
EDTA – ethylenediaminetetraacetic acid
EMEM – eagle’s minimal essential medium
EMT – epithelial-mesenchymal transition
ERK – extracellular signal regulated kinase
EV(s) – extracellular vesicle(s)
FACS – fluorescence activated cell sorting
FBS – fetal bovine serum
GFP-luc – green fluorescent protein-luciferase
GSK-3β – glycogen synthase kinase 3 beta
H3 – histone 3
HRP – horseradish peroxidase
MAPK – mitogen-activated protein kinases
MEK – mitogen-activated protein kinase kinase
MET – mesenchymal-epithelial transition
MES buffer – 2-(n-morpholino)ethanesulfonic acid buffer
MITF – microphthalmia-associated transcription factor
mTORC1 – mammalian (mechanistic) target of rapamycin complex 1
mTORC2 – mammalian (mechanistic) target of rapamycin complex 2
MTS – tetrazolium dye
PBS – phosphate-buffered saline
PI3K – phosphoinositide3-kinase
pS6 – phospho S6
pAKT – phospho AKT
pGSK – phospho GSK
pERK – phospho ERK
PDGFR – platelet-derived growth factor receptors
pI – isoelectric point
PTM – post-translational modification
PVDF – polyvinylidene fluoride
RPMI – Roswell Park Memorial Institute
RTK – receptor tyrosine kinase
SDS – sodium dodecyl sulfate
Size – PeggySue Size
SEM – standard error of the mean
St.dev – standard deviation
TBST – tris-buffered saline and tween
TDSF – tumor-derived secreted factors
TME – Tumor micro-environment
VEGF(R) – Vascular endothelial growth factor (receptor)
WB – western immunoblot
WNT – wingless-related integration site
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1 Introduction

1.1 Cancer

Cancer is a common term of several hundred diseases involving rapid and uncontrolled cell division with the potential to metastasize to distant organs. The lethality of every cancer diagnosis varies amongst individual cases and the origin of the cancer. Traditionally, cancer has been understood as a disease of the genome, where alteration to highly regulated machinery controlling cell division/growth, is the main driver to irregular cell behavior leading to the disease. The final products of coding genes, proteins, are necessary to ensure cell division and cell survival. The critical genes involved in cancer progression can be classified in two brackets, the oncogenes and the tumor-suppressor genes [1]. The oncogenes and their products, oncoproteins, drive the proliferation and cell survival. In general, amplification of oncoproteins signaling or loss of negative regulation, is cancer causing. On the contrary to oncoproteins, the proteins deriving from tumor-suppressor genes inhibit proliferation and cell survival. Loss of tumor-suppressor protein-function is another leading step to cancer. The list of proteins classified as either onco- or tumor-suppressor proteins continue to grow [2]. Furthermore, various other conditions must be met to lead up to a cancerous disease [3]. For example, lately interactions with tumor micro-environment have emerged as an important step contributing to disease progression.

Traditionally, cancer treatment includes surgery, radiation therapy and a broad spectrum of cytotoxic drugs, which target all rapidly dividing cells. In addition to a low success rate of chemotherapy in many of the cancer types, it also inflicts damage to healthy tissue. This demonstrates the importance to search for alternative treatment methods, which targets the exact mechanism(s) through which the cancer operate, meanwhile preserving healthy tissue. Inhibition of known oncogenic signaling by the help of targeted drugs may eventually make it possible for a more personalized medicine, instead of the “One size fits all” paradigm currently practiced in the clinics.
1.2 Metastasis and pre-metastatic niche

Metastasis

Metastasis is a term given to tumor cells which have managed to travel from tissue of origin (a primary tumor), to a distant site and manifested itself as a secondary tumor. In general, 5-year survival for cancer patients is dependent on metastases location. Some locations have worse survival outcome than others, and e.g. metastasis to the brain has one of the worst odds of survival [4, 5]. The metastatic cascade involves three crucial steps (reviewed in [6]); intravasation from a primary tumor to circulation, extravasation from circulation to distant site and finally settlement and colonization of distant organs. Intravasation is the result of changes in cell phenotype, when the cell goes from an epithelial to a mesenchymal state known as epithelial-mesenchymal transition (EMT). EMT makes the cell more motile, invasive, and less proliferative (reviewed in [7]). Extravasation occurs when the circulating tumor cells leave the circulation and invade a preferable tissue. The final step for a successful metastasis is settlement/colonization which leads to tumor growth (micro/macro metastasis) at a secondary site. This involves a reversal of EMT, classified as a mesenchymal-to-epithelial transition (MET). Formation of micro- and eventually macro-metastases is the most inefficient step in the metastatic cascade [8, 9].

Pre-metastatic niche

How cancer cells choose specific sites for metastatic growth remains uncertain. In some instances it might be due to blood circulation characteristics, whereas others propose the existence of a pre-metastatic niche (reviewed in [10]). Pre-metastatic niche means that distant organs are primed to accept and nurture cancer cells as they arrive to these organs as illustrated in figure 1.1. A pre-metastatic niche is believed to be formed in distant organs due to signals from the primary tumor. Such signals include tumor-derived secreted factors (TDSFs) and extracellular vesicles (EVs), which can recruit bone marrow derived cells (BMDC) and blood vessels making the site hospitable for cancer cells [11]. TDSFs and EVs (such as exosomes) remodel the organ, which produces mediators such as cytokines, chemokines and extra-cellular remodeling enzymes that makes the conditions favorable for the incoming tumor cells [12, 13].
Figure 1.1: Formation of a pre-metastatic niche. TDSFs and EVs recruit BMDC and formation of blood vessel, which makes it hospitable for cancer cell colonization. Various stromal cells are involved in this remodeling such as immune cells and fibroblasts. Modified figure from Lui, Yang. et. al. [10].

1.3 Tumor micro-environment (TME)

The tumor micro environment

The tumor micro-environment (TME) comprises three major constituents; extracellular matrix (ECM), various soluble factors and vast amount of stromal cells (reviewed in [3, 6, 14]). A general overview of the TME is illustrated in figure 1.2.
ECM generally consists of a fibrous network such as collagen and fibronectin that provide structural support, as well as proteins bonded to polysaccharide groups called proteoglycans. Furthermore, ECM helps retain soluble factors involved in proliferation and survival within the TME [15]. Soluble factors (such as growth factors, cytokines, chemokines) produced by cancer or stromal cells can mediate cell-cell communication and stimulate growth [16]. Vasculature (blood vessel) is critical for tumor growth by supplying nutrients and eventually oxygen in late stage tumor progression. Generally speaking, stromal cells are a classification given to all non-cancerous cell populations. This includes a spectrum of cells derived from the immune system, as well as non-immune cells such as fibroblasts and endothelial cells. Interactions between the cancer cells and TME are believed to be supportive for tumor proliferation and/or survival in various ways (reviewed in[14]). Exactly how the TME constituents influence each other and how they affect cancer cells remains elusive.

**Cancer associated fibroblasts**

Fibroblasts are one of the most dominant stromal cell types in TME. Fibroblasts produce ECM and growth factors and normally functions in wound healing. They usually alter between a quiescent and activated secretory phenotype through external signal such as stress, growth factors and chemokines. However, when fibroblasts becomes associated with cancer (cancer associated fibroblasts, CAFs) they irreversible change to a secretory phenotype supporting itself and surrounding tissue with cell to cell contact and growth factors involved
in proliferation and migration [17]. In addition, the fibroblasts show extraordinary ability to support and protect cancer cells from treatment [18-21]. The role of fibroblasts in regulating drug responses was in focus in this project.

1.4 Melanoma

Clinical facts

Malignant melanomas is a skin cancer type which is considered as one of the most devastating forms of cancers with high mortality and a low six months survival rate when detected in a late stage [22, 23]. Malignant melanoma is an easily metastasizing cancer type. If the primary tumors are discovered too late, the cancer has often metastasized to various locations, such as lungs, brain and skin. Prevalence seems to be on a rise, and an increased number of people die from the disease every year. Evidence points towards a somatic disease acquired from environmental exposure such as excessive UV-radiation. Melanoma has a higher prevalence on Caucasians compared to non-white, emphasizing the necessity of sun protection [23, 24]. In the year 2015, 2001 individuals got diagnosed with malignant melanoma in Norway [25]. Although primary tumors are easy to remove through surgery, metastases are most often inoperable and resistant to treatment [26].

Melanoma progression

The initial steps in melanoma progression are shown in figure 1.3. The transformation starts in melanocytes, a melanin-forming cell. Formation of a benign nevus in the basement membrane is characterized by increased proliferation with symmetrical appearance and uniform color of the skin. As the disease progresses to a dysplastic nevus, the symmetry is lost and skin coloring appears uneven. In the radial-growth phase the melanocytes gain the ability to mobilize in the dermis, and this is often associated with losing the asymmetry observed in the previous stage. The vertical-growth phase is the final step before melanoma initiates metastasis. In this stage the lesion can proliferate inside dermis and fat tissues, and furthermore initiate conditions needed for intravasation.
Figure 1.3: Malignant melanoma progression. The main stages involved in metastatic development are showed. Modified figure from Miller, A et. al [23].

Phenotype switching in melanoma

It has been reported that melanoma cells can undergo phenotype switching, a phenomenon resembling EMT [27-29]. Phenotype switching seems to be involved in metastasis, as well as drug resistance (reviewed in [30]). In melanomas high expression of the microphthalmia-associated transcription factor (MITF), and low expression of the receptor tyrosine kinase AXL, is associated with the differentiated phenotype. In contrast, low expression of MITF and high expression of AXL is associated with the mesenchymal phenotype [29]. Recently, Tirosh et.al reported that melanoma cells with MITF low/AXL high mesenchymal phenotype are more difficult to treat [31]. It has been shown that different phenotype cells are driven by different signaling pathways. For example, it has been reported that active WNT signaling drives the differentiated phenotype, while the mesenchymal phenotype shows suppressed signaling in this pathway (reviewed in [32]), resulting in decreased levels β-catenin [28].

Main driver mutation and treatment of melanoma

One of the most common genetic mutations and a driver of malignant melanomas occur in 40-60% of the cases on the BRAF protein acting in the MAPK pathway [33]. The BRAF V600
mutation affects the crucial regulatory seat of the BRAF protein, making it active at all times. While MAPK pathway is active, the cell experience increased protein synthesis contributing to proliferation and cell survival. The ATP-competitive inhibitor Vemurafenib (BRAFi) targets mutated BRAF and shows great promise by blocking the sustained signaling from BRAF downstream the MAPK pathway. However, the good initial response is met by rapid resistance after a few months [34]. To overcome the resistance, new clinical approaches are needed. These include combination treatment with targeted agents such as BRAF and MEK inhibitors, and immunotherapy. All though the new generation of treatment in the clinics has increased the overall six months survival rate, patients still experience resistance, and furthermore toxicity towards immunotherapy treatment (reviewed in [35, 36]).

### 1.5 Drug resistance

Drug resistance is not yet fully understood. However, some general resistance mechanisms have been identified, which have been classified into two main brackets of resistance; tumor cell-intrinsic resistance and TME-mediated resistance (reviewed in [37]). Subcategories of tumor cell-intrinsic resistance includes 4 main mechanisms; i) mutations or amplification of drug target, ii) alterations upstream or downstream in the targeted pathway, iii) activation of a compensatory pathway (e.g PI3K in BRAF inhibitor treated melanomas) and finally iv) a phenotypic state [38, 39]. TME-mediated resistance can occur through remodeling of vasculature by soluble factors such as VEGF, which increases interstitial fluid pressure of tumors [40] and thereby reduces drug delivery. Furthermore, cells of the TME can secrete various soluble factors, like hepatocyte growth factor, which can induce resistance to various targeted drugs [41], and also BRAF inhibitors [42]. The relevance of cell-cell adhesion/contact between melanoma and fibroblast have also been linked to BRAF inhibitor resistance [18]. The fibrous network in ECM has been shown to provide a BRAF inhibitor tolerant environment through integrin signaling [19].

### 1.6 MAPK and PI3K pathway in melanomas

Normally both PI3K- and MAPK-pathways gets activated by ligand binding to receptor tyrosine kinases (RTKs). RTK starts a signal transduction by phosphorylation of down-stream elements (hence “kinase”) which leads to proliferation and cell survival (Figure 1.4).
MAPK-pathway role in proliferation and cell survival have been known for decades, and mutations on BRAF leading to uncontrolled signaling occurs in many different cancers, especially melanomas [43, 44]. Phosphorylation on MEK and ERK proteins are indicators of MAPK-pathway activity.

PI3K-pathway is another commonly activated signaling-pathway in melanomas [45]. PI3K-pathway primarily signals through phosphorylation of AKT-protein [46], and AKT can phosphorylate GSK-3β [47] and mTORC1 [48]. The latter can continue signaling through phosphorylation of S6 [49]. AKT protein is a complex protein with three main isoforms [50], however, measuring the phosphorylation levels of AKT at sites Ser473 and Thr308 are a common way of determining PI3K-pathway activity [51, 52]. Phosphorylation at Thr308 site is a common indicator of PI3K-protein mediated activation [53]. Normally, active mTORC1 is an inhibitor of mTORC2. However, if mTORC1 is suppressed it releases its inhibition on mTORC2, and mTORC2 can phosphorylate AKT on Ser473 site which reactivates the pathway [54, 55].

The signal transduction and the proteins involved in both PI3K and MAPK-pathway are more complex than illustrated in figure 1.4. However, it shows a general consensus on how these two pathways can cross-talk. Active PI3K-pathway is the main regulator mTORC1 and S6, leading to the phosphorylation. However, active MAPK-pathway has also been reported to be able to phosphorylate mTORC1 and S6 [18, 56]. High levels of phosphorylated S6 upon treatment have been suggested as an indication of BRAFi resistance [56]. PI3K-pathway activation has been observed in BRAFi treated melanomas which has metastasized to the brain [5]. Our group has suggested that PI3K/mTOR-pathway could be involved in stroma-supported phenotype which is resistant to BRAF inhibitors [18]. The latter made us interested in inhibitors against RTKs and PI3K-pathway, as an approach to target stroma induced resistance to BRAFi.
Figure 1.4: Simplified schematic drawing of PI3K- and MAPK- pathway. The figure shows the intracellular region of a cell (light blue). The arrows indicate the direction of signal transduction. A “Stop” sign indicates inhibitory effect.

1.7 Immunoassays for detection of (phospho) proteins

The proteome from cell lysates can be studied in immunoassays by the use of antibodies. Usually the proteins in the cell lysates are separated prior to detection. The proteins can be separated based on various qualities, most prominently by their size or charge. Traditionally, size separation includes some sort of separation matrix which influences the speed larger proteins travel through the matrix compared to smaller proteins. An example of this method is the western immunoblotting (WB). After separation by WB, the entire proteome is primed with an antibody towards a specific protein or a protein with a specific modification such as phosphorylation.

When proteins are separated based on their charge, we exploit the acidic or basic properties of the amino acid side-chains. Seven of the naturally occurring twenty amino acids have ionizing properties on their side chain. This means that they can either loose or gain a hydrogen atom at a specific pH value. At specific pH-values these side chains are neutrally charged. This is
called the isoelectric-point (pI). A very long peptide (protein) has a pI-value which is unique and based on the amino acid composition. Furthermore, a post-translational modification (PTM) such as a phosphorylation on a protein will introduce a change in the pI-value. An old method which utilizes this separation method is the 2D gel electrophoresis, however this is time consuming. A new method has been developed by the ProteinSimple™ Company, called PeggySue charge, which combines pI-separation of proteins with immuno-probing. This makes it possible to detect phospho-proteins with the use of pan antibodies, instead of phospho-specific antibodies.
1.8 Aims of the study

Previously, our group has observed resistance towards BRAFi when metastatic malignant melanoma cells were grown in proximity to stromal cells, specifically fibroblasts. The group has also identified the PI3K/AKT/mTOR-pathway as a possible resistance mechanism. This MSc thesis was a continuation of the work done by the group and had a dual aim:

1) Investigate functional and molecular responses to targeted drugs in melanoma cells with/without presence of fibroblasts. Aim 1) had the following sub-goals:

   i) Investigate the activity of different inhibitors of PI3K-pathway and RTKs on melanoma cells grown as mono-cultures or co-cultures with fibroblasts in vitro.

   ii) Investigate molecular responses at a protein level to drug(s) (selected in i)), alone or in combination with BRAFi, in melanoma cells.

2) Optimize the PeggySue technology (Charge and Size) for detection of (phospho) proteins in melanoma samples from Aim 1).
2 Materials and Methods

2.1 Cells and cell handling

2.1.1 Cell lines

In this project two metastatic melanoma cell-lines and one stroma cell-line were used in the *in vitro* studies. The cell-lines are presented in Table 2.1.

Table 2.1: Cell-lines used in this project. Name of cell-lines, tissue of origin and location of origin for cell lines used in this project.

<table>
<thead>
<tr>
<th>Name</th>
<th>Tissue of origin</th>
<th>Geological Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melmet5</td>
<td>Melanoma</td>
<td>Norway, Radiumhospitalet</td>
</tr>
<tr>
<td></td>
<td>lymph-node</td>
<td></td>
</tr>
<tr>
<td></td>
<td>metastasis</td>
<td></td>
</tr>
<tr>
<td>HM8</td>
<td>Melanoma</td>
<td>Norway, Radiumhospitalet</td>
</tr>
<tr>
<td></td>
<td>brain metastasis</td>
<td></td>
</tr>
<tr>
<td>WI38</td>
<td>Lung fibroblasts</td>
<td>ATCC. Product number CCL-75,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lot number:58483158</td>
</tr>
</tbody>
</table>

2.1.2 Cell culturing

Melmet5 or HM8 melanoma cells and fibroblasts were grown as a cell monolayer in a tissue-culture flask and placed in incubators holding 37°C and a constant CO2 level of 5%. The melanoma cells were grown in RPMI 1640 medium (Sigma® Life Sciences, Cat# R0883) supplemented with 2mM glutamax (Sigma® Life Sciences, Cat# G8541) and 10% FBS (Sigma® Life Sciences, Cat# F7524), further refer as RPMI++. WI38 fibroblasts were cultured as cell monolayer in EMEM medium (ATCC, Cat# 30-2003) supplemented with 10% FBS. Cell stocks were stored in 50% medium, 40% serum and 10% dimethyl sulfoxide (DMSO) (Sigma® Life Sciences, Cat# D2650) in liquid nitrogen. When needed, the cells
were quickly thawed then transferred to preheated 37°C RPMI 1640 medium, spun to remove DMSO and then cultured in a flask until 80-90% confluence.

2.1.3 Cell subculturing

Once cell confluence reached approximately 90%, cells were detached from the culture flask using ethylenediaminetetraacetic acid (EDTA) 0.02% (Sigma® Life Sciences, Cat# E8008) (for HM8 and Melmet5) or trypsin and EDTA-solution (Sigma® Life Sciences, Cat# T3924) (0.5g/L and 0.2g/L, respectively) (for WI38 fibroblasts and co-cultures). The cells were then resuspended in fresh RPMI++ medium, and transferred to a new flask. If needed, cell lines were seeded out in medium containing 1% penicillin and 1% streptavidin (Sigma® Life Sciences, Cat# P4458).

2.1.4 Cell counting

An aliquot of 10 µL of cell suspension was mixed with 10 µL trypan blue (Gibco life Technologies, Cat# 15250-061), and the live cells were counted in countess™ to determine cell concentration in the cell suspension. Trypan blue penetrates the pores in the membrane of dead/dying cells, and thereby allow discriminating the dead cells that appear dark-blue and are excluded from the counting.

2.1.5 Cell culturing with drug treatment

For mono-cultures treatment with a drug in T25 flasks (Nunc, Cat# 157400), cells were seeded at a density 8*10^5 cells/flask in 2.5 mL medium. Drugs were added to the cells 48 hours after seeding, in various concentrations to a total volume of 5 ml per flask. For treatment in 96-well-plates (Falcon, Cat# 353072) cells were seeded out in a density of 7000 cells/well for HM8, 5000 cells/well for Melmet5 in 100 µL medium. Drugs were then added 24 hours after seeding, to a total volume of 200 µL per well. In co-cultures, cells were seeded in a ratio of 1:5 (HM8/Melmet5:fibroblasts) to a density not higher than 10^4 Cells/well in 96-well-plates and 8*10^5 for T25 flasks. If needed in co-cultures, due to fibroblast cell death from drug treatment, extra fibroblasts were seeded out compensating for cell death.

2.1.6 Drugs
BRAFV600E inhibitor vemurafenib (Selleckchem, Cat# S1267) (Stock concentration: 20mM in DMSO) was used in a final concentration of 0.1-5 µM.

PI3K class 1 inhibitor buparlisib (Selleckchem, Cat# S2247) (Stock concentration: 10 mM in DMSO) was used in a final concentration of 0.5-2 µM.

Receptor tyrosine kinase inhibitor Sunitinib (Selleckchem, Cat# 1611-25) (Stock concentration: 750 µM in DMSO) was used in a final concentration of 0.1-10 µM.

AKT inhibitor Afuresertib (Selleckchem, Cat# S7521) (Stock concentration: 50mM in DMSO) was used in a final concentration of 0.001-30 µM.

GSK3β inhibitor AR-A014418 (Selleckchem, Cat# S7435) (Stock conc.: 50000 µM in DMSO) was used in a final concentration of 3-100 µM.

AXL inhibitor BGB324 (BerGenBio, Norway) (Stock conc.: 50000 µM in DMSO) was used in a final concentration of 0.1-10 µM.

All Control (non-treated) samples for the corresponding drugs above were exposed to the medium containing DMSO (since all drugs were dissolved in DMSO) corresponding to the DMSO concentration as in the highest drug concentration samples.

### 2.2 Cell viability/proliferation assays

#### 2.2.1 MTS Cell proliferation assay

Tetrazolium dye (MTS) cell proliferation assay measures the reduction of MTS by viable cells to a color compound soluble in cell medium. MTS was added to the cell in a ratio of 40 µL MTS/200 µL medium per 96-well, incubated for 30-60 minutes at 37°C. Absorbance (at 490nm) was measured in the Victor™ instrument (Wallac).

#### 2.2.2 Bioluminescence assay

Bioluminescence assay measures changes in bioluminescence levels generated by cells expressing luciferase following addition of the luciferase substrate, D-luciferin. For this, HM8 and Melmet5 cells were previously labeled with a gene expressing luciferase. Medium was
removed from the cells in light-isolated 96-well-plates (Corning Costar, Cat#3610) and new medium consisting of 199 µL RPMI and 1 µL Luciferin (Stock: 20mg/mL) were added. After 10 minutes incubation at room temperature, bioluminescence was measured in the Victor™ instrument.

### 2.2.3 CellTiter-Glo luminescent cell viability assay

CellTiter-Glo™ measures changes in ATP levels when luciferase and luciferin are added to viable cells producing ATP. Light-isolated 96-well plates were emptied of medium. Equal volume of CellTiter-Glo Reagent (Promega, Cat# G7570) and medium was added to the 96-well plates, to the final volume of 40 µL. Then the contents were mixed for 2 minutes. The plate was incubated at room temperature in the dark for 10 minutes and measured for luminescence in the Victor™ instrument.

### 2.2.4 Incucyte Live Cell Analysis

Incucyte is an automatic live imaging system measuring cell-confluence in a mono-layer of cells. Cells were seeded in 96-wells plates and placed in Incucyte. The Incucyte measures cell density every 3 hours. In the lag period of measurement, plates were taken out for the addition of drugs.

### 2.3 Proteomic analysis

#### 2.3.1 Preparation of Cell lysates and protein concentration measurement

Cells used to make protein lysates were grown in T25 flasks. Growth medium was removed, and the cells were washed once with either EDTA or trypsin with EDTA-solution accordingly to cell type (see chapter 2.1.3). The cells were detached from the flask with EDTA (for monocultured HM8 and Melmet5). Cells were centrifuged and supernatant was removed. Cell pellets were washed with 2ml PBS (Sigma Life Sciences, Cat#D8537) and centrifuged, and then supernatant was removed. This step was repeated 2 times and where aim was to remove all the remaining proteins from the culture medium. The cell pellet was lysed in a lysis buffer (which was dependent on method) containing protease (Roche, Cat# 04693159001) inhibitors.
and with or without phosphatase (Roche, Cat# 4906845001) inhibitors. The samples were incubated on ice for 10 minutes before vortexing the samples. The same procedure was repeated two times. Preparation of fine homogenate was made using ultra-sound at +4°C. The cell lysates were centrifuged for 15 minutes at 13000 rpm at +4°C to remove cell debris. The supernatant was collected and the protein concentration was measured according to protocol in Pierce™ BCA protein assay kit (Thermo Scientific, Cat#23227). This kit measures the reduction of Cu²⁺ to Cu⁺⁺ by protein (peptide-bonds) in an alkaline medium (the biuret reaction). The cell lysates were stored at -80°C before use.

2.3.2 Dephosphorylation of protein lysates by FastAP

FastAP contains a thermo-sensitive Alkaline Phosphatase (1 U/µL) (Fermentas Life sciences, Cat# EF0654) which catalyzes the release of phosphate groups from DNA, RNA, nucleotides and proteins. Dephosphorylation of protein lysate was done according to protocol for FastAP™ Thermosensitive Alkaline Phosphatase. Samples were incubated with (dephosphorylated) or without (non-dephosphorylated) FastAP enzyme for 1 hour in a water bath at +37°C. The reaction was stopped by addition of a final concentration of 10 mM sodium orthovandate (Na₃VO₄) in the samples.

2.3.3 Western immunoblot

Western immunoblotting(WB) lysate buffer contained: 1% Triton X-100, 0,05M HEPES(pH 7,4, 1M), 0,15M NaCl, 0,0015M MgCl₂, 0,001M EGTA, 0,1M NaF, 0,01M NaPyrophosphate, 0,001M Na₃VO₄ and 10% Glycerol.

20µg of protein in WB-lysate was mixed with a loading buffer, reducing agent and water to a total volume of 20µL, and then heated at +75°C for 5 minutes.

- The samples were run in a Nu-PAGE 4-12% Bis-Tris 1.0mm x 12 well gel (Novex for life sciences, Invitrogen, Cat# NP0322 BOX). When applied to a voltage of 150V, and under the influence of reducing agent SDS, the proteins will travel slowly to the positively charged pole, and become separated based on the size (kDa). Larger proteins will move slower through the pores of the gel. Gels were run for 1-2 hours in MES solution (Invitrogen, Cat# NP0002-02). To track the size of the proteins a See Blue Plus 2 prestained standard was applied in all runs (Invitrogen, Cat# LC5925).
- The proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane in a semi-dry transfer in a transfer buffer (3g Tris, 14,4G Glycin, 200 mL MeOH, 800 mL H₂O). For this the PVDF membrane was activated with MeOH. A “sandwich” was made with layers of sponges and whatman papers with the gel and membrane in between. Electric current of 400mA was applied to the sample to force the proteins from the gel to the PVDF membrane.

- Blocking of unspecific antibody-binding to PVDF membrane was done with 10% dry milk in TBST buffer (20 mL 1M Tris-HCL, 30mL 5M NaCl, 5 mL Tween-20 and 945 mL H₂O) for 60 minutes. TBST containing Tween maintains a stable pH at 7.6 and helps on non-specific antibody binding.

- The membrane was incubated with various primary antibodies in 5% dry milk TBST buffer overnight at +4°C (see table 2.2).

- Washing step with TBST varied between 3 x 10 minutes and 3 x 7 minutes.

- Secondary antibody (see table 2.2) was applied at room temperature for 1-2,5 hours.

- For detection, the membrane was placed into a Syngene™ instrument and Super Signal West Dura kit™ (Thermo Scientific, Cat# A 34076F) was applied to the bands of interest. The machine then measured emitted light in the GeneSnap program.
2.3.4 Peggy Sue (phospho) protein separation and detection

PeggySue Size separation (Denatured Proteins)

PeggySue size assay is a semi-quantitative measurement method for detecting protein in protein lysates by separating the proteins based on their size (kDa), and then immunoprobe against your protein of interest. Every step in the PeggySue Size assay protocol, serve the same purpose as the steps in the WB protocol. The main difference between this method and WB is that PeggySue Size is semi-automatic and done by a machine. Furthermore, every step in the protocol from beginning to end is located inside one capillary, which can room a small amount of volume. The PeggySue machine always maneuver 12 capillaries (1 ladder and 11 samples) per one set of detection through coordinates which represent wells in a 384-microplate. When the capillaries are located in the right location, the machine sucks up the contents in the well, which are pipetted into the 384-microplate prior to starting the run. Guidance of these capillaries, and various technical parameters, are predefined by the user in a program called Compass prior to the run. The main steps from protein samples to detection in PeggySue Size assay are shown in figure 2.1. In general PeggySue Size is considered to have 6 major steps to detect proteins levels which are described below.

1. The first step the machine does is to suck up a separation matrix and a stacking matrix. Separation matrix works as a physical barrier, separating proteins based on size. Large proteins travel slower through the separation matrix than small proteins.

2. The machine loads denatured protein samples into the capillaries, into a region where the proteins accumulate prior to separation, called the stacking matrix.

3. After protein loading the machine introduce electric voltage through the capillaries which forces the denatured proteins to travel towards the positive end pole of the capillaries, meanwhile traveling different distances in a time period based on their size.

4. To make sure that the proteins do not migrate further, the proteins are immobilized by covalently binding to capillary walls through UV-fixation technology. Fixation is followed by a blocking step to hinder unspecific binding of antibodies.

5. After step 4, capillaries have fixated proteins attached throughout the column, which are then immunoprobed against your protein of interest with a primary antibody of your
choosing. This is followed by an incubation period. Typically, a secondary HRP-labeled secondary antibody (mostly anti-mouse/rabbit) against your primary antibody is then sucked into the capillaries, which forms a detection module for your protein of interest.

6. For detection a Luminol-peroxide mix is sucked into the capillaries which react with HRP-label on the secondary antibody. The reaction produces a bioluminescence signal which is detectable by photosensitive elements in the machine. This signal is proportional to protein levels, and the amount of protein can be quantitatively calculated by area under the curve (AUC). Normally, AUC is calculated automatically by the Compass program. The Compass program plots the bioluminescence signal against the position in the capillary. The position of the bioluminescence signals in the 11 sample capillaries are compared to loading control with known protein size, through overlapping the standards mixed in sample preparation. The position in the sample capillaries is then translated into protein size.
Sample preparation prior to a PeggySue Size Run

Prior to every PeggySue size run, reagents and samples are prepared. Protein samples were prepared by mixing 1 part fluorescent master mix and 4 parts sample buffer (1X) with protein lysate (conc. 0.2-1 μg/μL protein) to a final volume of 5 μL (Standard Pack 1, 12-230 kDa, Cat# PSST01-8). Samples and biotinylated ladder were vortexed and denatured at +95°C for 5 min, then spun and stored on ice. Primary antibodies (See table 2.2) were diluted 1:50-1:200 in antibody diluent 2. All reagents except primary-antibodies were provided by the ProteinSimple™ company. HRP-labeled Secondary antibody (mouse, Cat# 042-205 or Rabbit, Cat# 042-206) was readily provided by the ProteinSimple™ company. All Samples, primary and secondary antibody, antibody diluent 2(ProteinSimple, Cat# 042-203), biotinylated ladder, streptavidin-HRP(Cat# 042-414), Luminol-s (Cat# 043-311)- Peroxide (Cat#043-379), separation matrix 2(Cat# 041-247), stacking matrix 2(Cat# 041-248) and
water were pipetted in volumes according to schematics provided by ProteinSimple™ Company into a 384-well microplate (Cat# 040-663) shown in figure 2.2. Prior to the run, the prepared 385-microplate was centrifuged at 1k G at room temperature to remove bubbles in the wells. The remaining bubbles were removed with a needle.

Figure 2.2: PeggySue 384-microplate layout. Pipetting scheme prior to PeggySue Size run on a 384-microplate. (Figure is from ProteinSimple™ company)

**Technical parameters for PeggySue Size assay used in this thesis**

All the settings required to run the assay are predefined by the user in the Compass program prior to the run. Proteins were loaded in a containment region of the capillaries occupied with stacking matrix 2. All samples were separated in separation matrix 2 which separated proteins in the size range of 12-230 kDa. The separation time was set to 40 minutes, with voltage kept at 250 V. Blocking by antibody diluent 2 varied from 20-40 minutes and was followed by a washing step 2 times. Primary antibody incubation time was 120 minutes, and was followed by 2 washing steps. Secondary antibody incubation was 60 minutes, and was followed by 2 washing steps. Signal detection time varied from 5-480 seconds.

**PeggySue Charge separation (Naïve proteins)**

Similar to PeggySue Size assay, PeggySue Charge assay (Charge) is fully automated, separation occurs within capillaries and technical parameters are programmed into the machine prior to the run. However, separation of proteins through Charge method is fundamentally different to Size. Charge assay separate naïve proteins based on the state in which the protein has a net zero charge, otherwise known as the isoelectric point (pI). For most proteins, the pI-value is influenced by post-translational modifications, and a shift in the
pI-value of modified versus non-modified protein can be enough to separate the proteins. The pI-shift differences can be especially noticeable when proteins undergo phosphorylation or dephosphorylation. The applications for this method are broad. In this thesis, PeggySue Charge method was used to observe the relative levels of phosphorylated versus non-phosphorylated protein state across treatment regimes or growth conditions. The 5 major steps involved in Charge assay are shown in figure 2.3 and explained below.

1. Prior to loading the protein lysates into the capillaries, the protein lysates are mixed with a solution of carrier ampholytes and a pI standard ladder. The ampholytes consists of a mix of zwitterions, each with a very narrow pH range. There are various choices of ampholyte mixes available, which form a separation gradient in the pH range of your choosing. The pI standard ladder contains zwitterions with known pI-values, and reports position in the capillary to translate the position into a pI-value.

2. When an electrical current is applied to the capillaries, the ampholytes moves towards either the anode (+) or the cathode (-) end of the capillaries until the zwitterions becomes neutrally charged. The orientation of zwitterions forms a pH gradient in the capillaries. Similarly, the proteins in the sample will orient themselves in the pH gradient until they are at zero charge, thereby separating the proteins in the capillaries based on pI.

3. Similar to Size method, proteins are immobilized by covalently binding to capillary walls through UV-light technology.

4. For immunoprobing in PeggySue Charge method I refer to PeggySue Size method. The only noticeable difference in Charge compared to Size method, is that Charge can detect phosphorylated-protein state by the use of non-phospho antibodies, simply due to shift in pI-location. Albeit, a pan antibody does not give information regarding site specific phosphorylation on a particular protein.

5. Signal detection in PeggySue Charge is similar to PeggySue Size and I refer to previous Size separation method.
Prior to a PeggySue Charge Run

Protein sample lysates were diluted to 4X final protein concentration with Bicine/CHAPS (ProteinSimple, Cat# 040-764) containing 4X DMSO inhibitor (ProteinSimple, Cat# 040-510) (Stock: 50X) to a final volume no less than 6 µL. A stock of final separation gradient was made out of 176 µL Premix G2 (ProteinSimple, Cat# 040-973) (pH 5-8 separation gradient) and 4 µL of pI standard ladder 1 (ProteinSimple, Cat# 040-644) (Stock: 60X). 4 µL of protein lysate samples were mixed with 12 µL final separation gradient. The final protein concentration in the samples was in the range of 0.05-1 µg/µL depending on protein of interest. Mixed samples with final separation gradient were vortexed for ~30 seconds. All samples and reagents were kept on ice throughout preparation. Primary antibody (see table 2.2) was diluted in a range of 1:25-100 in Antibody Diluent (ProteinSimple, Cat# 040-309), and mixed by vortexing. Secondary antibody (anti-mouse/rabbit, Cat# 040-655/040-656) was
diluted to 1:100 with Antibody Diluent, and then mixed by vortexing. Samples, primary and secondary antibody and luminal/peroxide was pipetted according to Compass coordinates into a 384-microplate, programmed prior to the run. The 384-microplate was centrifuged for 5 minutes at 1k G at room temp to remove bubbles in the chambers. The remaining bubbles were removed with a needle.

**Technical parameters for PeggySue Charge assay used in this thesis**

Separation profile was typically 40 minutes under 21000 microwatts. Immobilization time was 100 seconds. Primary antibody incubation time varied from 1-4 hours depending on protein of interest. Secondary antibody incubation time was typically 60 minutes. Samples were washed 2 times in all washing steps. Detection of signal was through 7 exposures in a range of 15-960 seconds depending on protein of interest.
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2.4 Data and Statistical analysis

Statistical analyses and normalization were performed on all cell-culturing experiments which measured proliferation/cell-survival.

To normalize the results, raw data from treated samples were divided by raw data from non-treated samples. This gave the relative effect of treated samples compared to non-treatment samples.

Due to time/cost restraints some experiments do not have $\geq 3$ biological replicates, and the conclusions based on these results should be cautioned. In these experiments error-bars represent standard deviation (st.dev). This method measured the variation in sample preparation in $\geq 3$ technical parallels.

In experiments reproduced more/equal to 3 times, error-bars represent standard error of the mean (SEM). This method estimates how far the sample is likely to be from the population mean. Significance of the findings was analyzed by Student’s t-test for unpaired samples. A T-test determines if two sets of data are significantly different from each other and not only due to chance. Unpaired samples means that the samples were independently prepared, otherwise identical.
3 Results

3.1 Sensitivity of melanoma cells grown as monocultures to BRAF inhibitor

The BRAF inhibitor Vemurafenib, hereby referred to as BRAFi, has been extensively used in the research group the last 3 years. At the start of this project a new batch of BRAFi was made by one of the group members. To verify potency of the new drug batch compared to the old drug batch, a cell survival assay was performed on a common melanoma cell-line, the HM8 cell-line. The cell survival was scored by use of the MTS assay which measure metabolic activity of cells. From figure 3.1 the new and the old batch of BRAFi showed similar restrain on relative cell survival, indicating that both batches were equally potent. These data indicate that cell survival is reduced by ~50% at a dose of around 0,5 µM of BRAFi.

![Figure 3.1: BRAFi effect on HM8.](image)

To verify potency of BRAFi in another commonly used melanoma cell line, Melmet5 we measured Melmet5 cell survival after BRAFi treatment by the MTS method. Figure 3.2 show that Melmet5 responds to BRAFi similar to HM8 cells, where 0,5 µM BRAFi reduces cell survival by ~50%.
Figure 3.2: BRAFi effect on Melmet5. Cell survival was assayed by the MTS method. Melmet5 cells were treated with BRAFi at various concentrations for 3 days. Treated samples were normalized to non-treated (0 µM BRAFi) controls. Error-bars indicate +/- standard-error measurement (SEM) from 3 different experiments (n=3). Statistically significant at 0.5 and 1 µM, p-value < 0.05 by unpaired t-test.

In upcoming analysis of co-culture, consisting of tumor and stroma cells, we are only interested in measuring drug effect on cancer cells, not stromal cells. One way to discriminate the response in cancer cells from stromal cells in co-cultures is by the bioluminescence assay. In this assay HM8 and Melmet5 cells were previously labeled with luciferase which produces bioluminescence upon addition of luciferin. By measuring the intensity of bioluminescence, it is possible to evaluate the treatment effect on tumor cells only. We wanted to validate that the cell response to BRAFi as detected by the bioluminescence method, is comparable to what is measured by the conventional MTS method. Figure 3.3 shows that relative survival of HM8 cells, grown at different densities and assayed by bioluminescence method, was similar to what was observed using the MTS method (figure 3.1).

Figure 3.3: Detection of cell survival by the bioluminescence method. HM8 cells were treated with BRAFi at various concentrations for 3 days. High density indicates 7500 cells/well, and Low density indicates 6500 cells/well. Error bars indicate st.dev from 3 technical parallels.
3.2 Validation of fibroblast-mediated protection from BRAFi

The presence of fibroblast can significantly reduce the melanoma cell response to BRAFi, as previously reported by the group [18]. To verify this observation, HM8 and Melmet5 cells were seeded with or without fibroblasts (as co-culture or mono-cultures, respectively), and treated with BRAFi. As can be seen in figure 3.4A, HM8 cells in co-cultures show almost no response to BRAFi in a dose, which in mono-cultures kills 50% of the cells. For MM5, treatment in the presence of fibroblasts resulted in approximately 60% survival compared to 30% survival seen in the absence of fibroblasts (figure 3.4B). Fibroblast-dependent decrease in BRAFi effect, underlines the importance of finding another drug that targets the fibroblast-protected melanoma cells.

Figure 3.4: Fibroblast-mediated BRAFi protection. Melanoma cell-response to BRAFi in the presence (co-cultures) or absence (mono-cultures) of fibroblasts. After 3 days-treatment, melanoma cell-survival was measured by the bioluminescence method. (A) HM8 cells were treated with 0.5 µM BRAFi. (B) Melmet5 cells were treated with 1 µM BRAFi. Treated samples were normalized to non-treated (Ctr). Error bars indicate +/- SEM, n = 3. * indicated significance, p-value <0.05, by unpaired t-test.
3.3 Sensitivity of melanoma cells grown as mono-cultures to various targeted drugs

The fibroblast induced protection observed in the previous chapter can have various explanations. One possible explanation is that the melanoma cells in the presence of fibroblasts, switch to an alternative signal pathway, e.g. PI3K, which reduces their sensitivity to MAPK suppression by BRAFi. Therefore, our aim was to explore the efficiency of drugs targeting the PI3K/AKT pathway or upstream RTKs. Several clinically-relevant drugs were tested. Figure 3.5 illustrates a simplified view of the PI3K/AKT and MAPK pathways, and indicates the targets of the chosen drugs. Sunitinib, a pan RTK inhibitor acts on PDGFR/VEGFR/C-kit [57]. BGB324 blocks the AXL receptor. Buparlisib is a drug targeting PI3K [58]. Finally, Afuresertib targets AKT [59]. Before combining these drugs together with BRAFi in co-cultures, we needed to find suitable drug concentrations for Melmet5 and HM8 cell lines. The suitable drug concentration was defined as a dose which reduces cell survivability/proliferation by 10-50% in mono-cultures.

Figure 3.5: The used drugs and their targets. Simplified schematic drawing of MAPK and PI3K signaling pathways with indication where the used drug acts. Arrows points the direction of signal cascade. A “stop” sign means inhibitory effect.
We used the MTS assay for assessing cell survival following treatment with the different drugs at different doses. In addition we employed cell proliferation assay on Incucyte to track cell responses over time. Based on the MTS data (figure 3.6, 3.7) a suitable range of doses for each drug in both cell lines were identified and presented in table 3.1. Proliferation assay by the Incucyte generally reflected responses observed by the MTS method (figure 3.6 and figure 3.7).

Table 3.1: Suitable drug concentrations for PI3K- and RTK- inhibitors in HM8 and Melmet5 cells as detected by the MTS method (see also figure 3.6 and 3.7).

<table>
<thead>
<tr>
<th>Drug</th>
<th>HM8</th>
<th>Melmet5</th>
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<tr>
<td>Buparlisib</td>
<td>0.5-1 µM</td>
<td>0.3-0.6 µM</td>
</tr>
<tr>
<td>BGB324</td>
<td>1-3 µM</td>
<td>3 µM</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>4-8 µM</td>
<td>2-5 µM</td>
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<td>Afuresertib</td>
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Figure 3.6: Efficacy of PI3K-pathway inhibitors in HM8 cells. HM8 cell survival and proliferation were evaluated by MTS and Incucyte, respectively. Buparlisib (A, B), BGB324 (C, D), sunitinib (E, F) and afuresertib (G, H). Error-bars indicate st.dev from 3 technical parallels.
Figure 3.7: Efficacy of PI3K-pathway inhibitors in Melmet5 cells. Melmet5 cell survival and proliferation were evaluated by MTS and Incucyte, respectively. Buparlisib (A, B), BGB324 (C, D), sunitinib (E, F) and afuresertib (G, H). Error-bars indicate st.dev from 3 technical parallels.
3.4 Treatment effect in co-cultures versus mono-cultures

To investigate if the PI3K/RTK-inhibitor could abolish BRAFi resistance observed in the presence of fibroblasts, we treated the co-cultures or mono-cultures with BRAFi and the drug of interest. We chose the drug-doses from Table 3.1. The drugs selected for further analysis were buparlisib, BGB324 and sunitinib. Afuresertib was excluded from further analysis, since a WB showed up-regulation of phospho-AKT, and AKT was supposed to be suppressed by this drug (Supplementary Figure S1). Relative cell survival of mono- and co-cultured HM8 cells treated with two different concentrations of Buparlisib alone or in combination with BRAFi is shown in figure 3.8. The results indicate that fibroblast-associated BRAFi resistance was reduced in the combination treatment. Thus, when almost no response to BRAFi alone was seen in the co-cultures, addition of buparlisib reduced cell survival by 40-60% depending on the buparlisib concentration.

Figure 3.8: Effect of Buparlisib in combination with BRAFi. HM8 cell response to buparlisib alone, 1 µM BRAFi or combination of both drugs in co-cultures and mono-cultures. (A) Buparlisib concentration was 0.6 µM. (B) Buparlisib concentration was 1 µM. After 3 days-treatment, HM8 cell-survival was measured by the bioluminescence method. Treated samples were normalized to non-treated controls. Error-bars indicate st.dev from 3 technical parallels.

Relative cell survival of HM8 cells in mono- and co-cultures, treated with BGB324 alone or in combination with BRAFi is shown in figure 3.9. In co-cultures, combination lowered HM8 cell survival when compared to BRAFi alone. However, results from other group members repeated multiple times, did not validate this observation. Then it has been observed that the cell survival in combo-treated versus BRAFi-treated co-cultures was quite similar (Seip et al.
manuscript in preparation). Taken all this together we concluded that BGB324 does not overcome the fibroblast-mediated protection from BRAFi.

![Figure 3.9: Effect of BGB324 in combination with BRAFi. HM8 cell response to 2.5 μM BGB324 alone, 0.5 μM BRAFi or combination of both drugs in co-cultures and mono-cultures. After 3 days-treatment, HM8 cell survival was measured by the bioluminescence method. Treated cells were normalized to non-treated cells. Error-bars indicate st.dev from 3 technical parallels.

In figure 3.10 we can see the relative cell survival of HM8 treated with Sunitinib alone or in combination with BRAFi. In co-cultures, Sunitinib and BRAFi combination did not reduce HM8 cell survival compared to BRAFi alone. However, it should be noticed that Sunitinib alone induced a very low effect on mono-cultured cell survival at the used dose in this particular experiment.

![Figure 3.10: Effect of Sunitinib in combination with BRAFi. HM8 cell response to 5 μM Sunitinib alone, 0.5 μM BRAFi or combination of both drugs in co-cultures or mono-cultures. After 3 days-treatment, HM8 cell-survival was measured by the bioluminescence method. Treated cells were normalized to non-treated cells. Error-bars indicate st.dev from 3 parallels.]
3.5 Molecular characterization of buparlisib treated melanoma cells

A decision was made to proceed with Buparlisib treated cell analysis, because Buparlisib reduce the protection towards BRAFi in the co-cultures. To clarify the Buparlisib effect on HM8 cells at the protein level, the levels of phospho-proteins involved in the PI3K/AKT and MAPK pathways were analyzed. All chosen proteins are kinases, thus modify other proteins by adding phosphate groups. Phosphorylated protein state is indicated with the prefix “p” in front of the protein name, and sites of phosphorylation are indicated in the suffix. Usually, phosphorylation means activation of the protein, with the exception of pGSK(Ser9) whose activity gets inhibited by phosphorylation. Figure 3.11A shows western blot results of protein lysates from HM8 mono-cultures treated with BRAFi, Buparlisib and their combination. The HM8 cells treated with BRAFi shows a reduction in phosphorylation of the downstream element in the MAPK signaling pathway, pERK(Thr202/Tyr205), as well as the mTORC1 activity marker, pS6(Ser234/236). This verifies that BRAFi blocks the MAPK signal cascade and induces a good response under these treatment conditions. Buparlisib showed an effect on pERK and pS6, albeit lower than what was observed in BRAFi treatment. Furthermore, Buparlisib in solo and combination treatment, showed an up-regulation of pAKT(Thr308), which was unexpected. Buparlisib inhibits PI3K pathways, which is known to phosphorylate AKT on the Thr308 site. To evaluate the unexpected effect of Buparlisib on pAKT further, we chose to analyze Buparlisib treated HM8 cells over various time points from treatment initiation (figure 3.11B). This result indicates that Buparlisib treated HM8 cells consistently maintain a higher pAKT(Thr308) status compared to non-treated samples. This was particularly pronounced in the 4h-sample, suggesting a strong initial response which decreases over time. Our original intention was to perform a similar western blot analysis on the co-cultured melanoma cells exposed to the same treatments as above. However, the preparation of samples from the co-cultures is challenging, since melanoma cells have to be separated from fibroblasts by fluorescence activated cell sorting (FACS) before the tumor cell-lysates can be made. FACS is a time-consuming procedure that requires special qualifications; it does not allow to collect high amount of tumor cells required for western blotting. Unfortunately, due to time- and resource-limits, we were unable to prepare the protein lysates from the Buparlisib-treated co-cultures within the frame of this MSc thesis.
Figure 3.11: (Phospho)protein levels in mono-cultures HM8 cells exposed to different treatments. (A) HM8 cells were treated with 1 µM BRAFi, 2µM Buparlisib or their combination for 24 hours, and cell protein lysates were analyzed by Western Blotting (B) HM8 cells were treated for different time points with 2µM Buparlisib (all time points except 48h-sample, where 0,75 µM Buparlisib was used), and cell protein lysates were analyzed by Western Blotting.

3.6 Melanoma cell response to AR-A014418 in mono-cultures versus co-cultures

In parallel to this MSc thesis, other members of the group performed a drug-screening, where melanoma cell responses to 384 drugs in co-cultures versus mono-cultures were compared. The results pointed at the GSK inhibitor AR-A014418 (AR) as a candidate for further analysis, since AR was particularly effective in the co-cultured melanoma cells (Seip et al., manuscript in preparation). Therefore, we included AR and performed similar studies as on the other drugs above. First we screened for a suitable dose-range. Relative cell-survival of mono-cultured HM8 and Melmet5 cells treated with AR is shown in figure 3.12. Based on these results we decided that suitable range of drug concentration for AR was 4-6 µM for HM8 and 12-18 µM for Melmet5.
Figure 3.12: Effect of AR in mono-cultured HM8 and Melmet5 cells. Cell-survival was evaluated by MTS for HM8 (A) and CellTiter-Glo for Melmet5 (B). Both cell-lines were treated with the indicated concentrations of AR for 3 days. Error-bars indicate st.dev from 3 technical parallels.

Figure 3.13 shows relative cell survival of HM8 and Melmet5 cells in mono-cultures and co-cultures treated with AR alone or in combination with BRAFi (HM8 results were generated in co-operation with Kotryna Seip and Marco Haselager). In HM8 co-cultures, the combination treatment induced a significantly stronger anti-cancer effect compared to BRAFi alone (Figure 3.13A). Furthermore, the combination treatment was more potent in the co-cultures than the mono-cultures (Figure 3.13A). However, it should be mentioned that in co-cultures, AR alone reduced cell survival to less than 10% compared to non-treated cells. Similar effect was observed in the Melmet5 cell-line (figure 3.13B). This indicates that AR is particularly efficient in the co-cultured melanoma cells, and is an interesting drug to proceed with molecular analysis.

Figure 3.13 Effect of AR in HM8 (A) and Melmet5 (B) cells treated with AR, 1 µM BRAFi or the combination of both drugs in the co-cultures and the mono-cultures. AR concentration was 4 µM (A) and 15 µM (B). After 3 days-treatment, cell-survival was measured by the bioluminescence method. Treated samples were normalized to non-treated controls. Error bars indicate SEM. N = 3. * significant, p-value < 0.05, by unpaired t-test.
3.7 Optimization of the PeggySue Charge (Charge) method for phospho-protein detection

Drug treated mono-cultures might show a different PI3K-pathway response compared to drug treated co-cultures. Due to above-mentioned problems related to sample preparation from the co-cultures, we chose to utilize a new method called PeggySue Charge (further called Charge). This method allows to significantly reduce the amount of protein lysate needed for the analysis. Therefore it would be an ideal tool for analysis of the tumor cells from the co-cultures. Charge had not been used at the institute before, and one of the aims of this thesis was to test various conditions for best possible detection of phospho-proteins by this method. We chose to analyze AKT for optimization due to our interest in the PI3K pathway. The initial parameter we needed to determine was optimal protein concentration. Figure 3.14 shows an AKT peak-profile when different amount of protein lysates was applied to the Charge method. The distinguishable peaks could be observed in samples of 0,8 and 0,4 µg/µL, and a lower concentration gave no clearly distinguishable peaks. The 0,4 µg/µL sample gave peaks matching the pI-values observed by others [51, 52, 60]. Based on these results, we need at least ~0,4 µg/µL protein to detect phospho-AKT. A protein concentration lower than 0,4 µg/µL was insufficient for detecting AKT peak(s) by this method.

Figure 3.14: AKT detection by PeggySue Charge. An AKT peak-profile from isoelectric point separation by Charge. Different concentrations of protein lysate were analyzed on pH 5-8 ampholyte mix. Primary antibody was against AKT1/2/3, * indicate pI shift possibly due to high salt concentration. Y-axis shows chemiluminescence signal, X-axis shows isoelectric point (pI).
To measure pAKT levels in melanoma cells with versus without fibroblasts, we analyzed FACS-sorted (Sorting was performed by Core facility at the Radium Hospital) melanoma cells from co-cultures and mono-cultures by Charge. Figure 3.15 shows AKT peak-profile of BRAFi treated mono-cultures and co-cultures, which, as we saw previously (figure 3.4), demonstrate different sensitivity to the drug. Both samples showed a very similar peak-profile, with small differences in peak heights. Peak height differences were particularly noticeable in the region 5.3-5.6 pI. However, we were uncertain which AKT isoforms/phospho-states these peaks represent.

![Figure 3.15: AKT peak-profile in mono-cultures vs. co-cultures as detected by Charge. Protein concentration in samples was 0.8 µg/µL. Top graph shows BRAFi treated mono-cultures; bottom graph shows BRAFi treated co-cultures. Y-axis shows chemiluminescence signal, X-axis shows pI.](image)

To identify which peaks represent phosphorylated forms of AKT, we dephosphorylated the protein lysate samples with FastAP enzyme. Dephosphorylating the lysate would ideally lead to three remaining peaks, one for each non-phosphorylated isoform of AKT. For FastAP treated lysates, all peaks were abolished at pI values <5.4 pI (Figure 3.16). Based on that we concluded that peaks <5.4 pI represented phosphorylation peaks. However, in FastAP treated samples, we observe 5-6 overlapping peaks instead of expected 3 peaks that should represent 3 AKT isoforms. Based on these data we concluded that AKT is a problematic protein to be analyzed for method optimization.
Figure 3.16: AKT peak-profile in FastAP treated and non-treated protein lysates. HM8 cell-lysates were treated with (top) or without (bottom) FastAP dephosphorylation enzyme and analyzed by Charge. Primary antibody was against AKT1/2/3. Protein concentration in samples was 0.8 µg/µL. Y-axis shows chemiluminescence signal, X-axis shows pI.

To proceed with further optimization of the method, we switched to samples provided by the manufacturer as positive controls for ERK. The aim was to test whether we can get nice peak separation in these samples. The positive controls were lysates of HeLa cells with high and low phosphorylation status, and the results for ERK are shown in figure 3.17. The pI location for ERK-peaks with or without phosphorylation has been identified in these samples, and pI-location is indicated with vertical lines in figure 3.17. The results show a clear difference in phosphorylated ERK1/2 in High vs. Low phospho-HeLa cell lysates. In Low, only 2 peaks could be observed, corresponding to non-phospho ERK. Thus, we concluded that by using the positive control samples we are able to detect nicely separated total- and phospho-ERK peaks.
Next, we analyzed our protein lysates with respect to ERK. We utilized two previously made samples – non-treated and BRAFi treated HM8 – that based on WB results (figure 3.18 insert) showed a clear difference in phospho-ERK levels. As can be seen in figure 3.18, Charge analysis showed that BRAFi treated samples had slightly larger non-phosphorylated ERK-peaks, compared to non-treated samples. However, these differences in phospho-ERK peaks were minor compared to what was observed in WB. In addition, the peak-profile was quite different from the profile observed in the positive control samples presented in Figure 3.17. Thus, when we compare our samples to HeLa positive controls samples, we concluded that there must be parameters in our lysates that account for the differences observed.
Figure 3.18: ERK peak-profile in previously made protein lysate samples. HM8 cells were non-treated (top) or BRAFi treated (bottom), and the cell lysates were analyzed by Charge with a Pan-ERK primary antibody. Y-axis shows chemiluminescence, and X-axis show pI. Vertical lines indicate ERK1/2 peaks; p means single phosphorylation, pp means double phosphorylation. Insert shows the WB results from same lysates.

One possibility could have been sample degradation. Freshly made HM8 cell lysates with or without BRAFi treatment, was run on Charge (figure 3.19). The new lysates gave a slightly better peak-profile compared to the previously-made lysates shown in figure 3.18. Furthermore, the differences in phosphorylation levels between non-treated and BRAFi treated samples were more apparent. However, we were still not satisfied with the peak-profile. In addition, the BRAFi effect on ERK phosphorylation-status as detected by Charge was less obvious compared to the Western Blotting data.
Figure 3.19: ERK peak-profile in freshly made protein lysate samples. HM8 cells were non-treated (top) or BRAFi treated (bottom), and the cell lysates were analyzed by Charge with a Pan-ERK primary antibody. Y-axis shows chemiluminescence, and X-axis pI. Vertical lines indicate ERK1/2 peaks; p means single phosphorylation, pp means double phosphorylation. Insert shows the WB results from same lysates.

Another parameter that distinguishes our lysates from the lysates of positive controls was that the later were made in a specialized lysis buffer (Bicine/CHAPS) recommended by the manufacturer. We speculated that our lysis buffer, originally optimized for WB, might not be optimal for Charge. Therefore, we made new samples, where HM8 cells with or without BRAFi treatment, were lysed in the recommended lysis buffer for Charge. As seen in Figure 3.20, the peak-profile for ERK resembled the peak-profile obtained with positive control HeLa lysates (figure 3.17). In addition, we observed a clear effect of BRAFi on ERK phosphorylation-status. The peaks representing the phospho-ERK were absent in the BRAFi-treated samples (figure 3.20). Based on this data, we concluded that Charge results are highly dependent on the lysis buffer used for making protein lysates, and that our lysis buffer, optimized for western blotting is not optimal for Charge.
Figure 3.20: ERK peak-profile in protein lysates made in recommended Bicine/CHAPS buffer. HM8 cells were non-treated (top) or BRAFi treated (bottom); the protein lysates were made Bicine/CHAPS and analyzed by Charge with a Pan-ERK primary antibody. Y-axis shows chemiluminescence, and X-axis shows pI. Vertical lines indicate ERK1/2 peaks; p means single phosphorylation, pp means double phosphorylation.

3.8 PeggySue Size for (phospho)protein detection

Having experienced problems with Charge, we switched to PeggySue Size (further called Size) hoping that the lysis buffer would have less impact on the results. Similar to Charge, Size requires less amount of sample compared to Western Blotting, but uses protein size (kDa) instead of pI as a separation method. We used samples prepared from mono-cultured and co-cultured HM8 cells treated with BRAFi, AR or the drug combinations. Since AR targets GSK-3β, which regulates β-catenin degradation, we decided to measure the levels of pGSK-3β(Ser9) and β-catenin. The results are shown in figure 3.21. For pGSK(Ser9) nice peaks were detected at the right size (Figure 3.21A). However, measuring area under the curve (AUC), we could see no striking differences in pGSK(Ser9) levels for either of the conditions (Table 3.3). β-catenin was hardly detected as a peak, making it difficult to quantify AUC (Figure 3.21B). This indicates that more protein should be loaded for detection of β-
catenin. GAPDH was used as a loading control (figure 3.21C). For mono-cultures, GAPDH levels were similar in all treatments, albeit with a slight up-regulation in BRAFi treated samples (Table 3.3). Furthermore, in the co-cultures, GAPDH levels were reduced ≥2-fold in BRAFi- and combination-treated samples (Table 3.3). However, a western blotting performed on the same samples could not confirm the variations in protein concentrations (Supplementary figure S2). Taken all together, this made us doubt whether GAPDH is a suitable loading control for Size.

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<th>Co-cultures</th>
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<td><strong>A)</strong></td>
<td></td>
<td>pGSK3-β(Ser9)</td>
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<td></td>
<td>pGSK3-β(Ser9)</td>
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<tr>
<td>BRAFi -&gt;</td>
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<th><strong>B)</strong></th>
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<td></td>
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<tr>
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Figure 3.21: Analysis of pGSK(Ser9), β-catenin and GAPDH by PeggySue Size method. HM8 melanoma cells were FACS-isolated from mono-cultures or co-cultures treated with 1 µM BRAFi, 5 µM AR or their combination for 24 hours. Y-axis shows chemiluminescence, and X-axis shows protein size (kDa).
Table 3.2: The levels of pGSK(Ser9) and GAPDH as measured by AUC from Figure 3.21.

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<tr>
<td></td>
<td>pGSK(Ser9)</td>
<td>pGSK(Ser9)</td>
<td>GAPDH</td>
<td>GAPDH</td>
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<td>(Mono-Cultures)</td>
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<tr>
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<td>98360</td>
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<td>Combination</td>
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<td>148152</td>
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To address the question of a loading control, we analyzed non-treated and BRAFi treated samples for 4 different proteins commonly used as loading controls. Figure 3.22 shows HM8 cells with or without BRAFi treatment, analyzed for β-actin, GAPDH, α-tubulin and H3. The nicest peaks were identified for β-actin and GAPDH (figure 3.22A and B, respectively). However, the GAPDH signal in BRAFi treated sample was so strong that the peak surpassed the top detection limit of the system. Reaching this top detection limit of the system is observable when a peak top drops, which is referred to as a “burn-out”. Taken all together, we decided to use β-actin as a loading control for our further studies.
3.9 Detection of phenotype-related proteins in co-cultured and mono-cultured melanoma cells by Size method

Finally, we used the non-treated co-cultured and mono-cultured melanoma cells to detect a number of proteins – markers of the mesenchymal and differentiated phenotype – by Size. Based on the previous results obtained by the group [18] we expected to see up-regulation of mesenchymal-phenotype associated proteins and down-regulation of the melanocytic proteins in the co-cultured cells. Figure 3.23 present the results for PDGFR and AXL, reported previously as mesenchymal-associated proteins. We observed slightly higher peaks for both of these proteins in the co-cultured cells compared to the mono-cultured. In contrast, the melanocytic markers MITF and β-catenin were reduced in the co-cultured cells compared to the mono-cultures. Thus, we were able to detect phenotype-associated proteins by using PeggySue Size and could validate a switch towards a mesenchymal state in melanoma by the presence of fibroblasts.
Figure 3.23: Size-based analysis of phenotype-related proteins (specified in the figure). Protein lysates were prepared from HM8 and Melmet5 cells grown as mono-cultures and isolated by FACS. X-axis indicates size (kDa). Arrows indicate peaks representing the protein of interest.
4 Discussion

4.1 Stroma (fibroblast) induced treatment protection

Traditionally, cancer resistance towards various treatment regimes has been viewed as an intrinsic quality of the cancer cells. This means that as the disease progresses or under pressure of the treatment, cancer cells undergo genetic or phenotypic alterations, leading to a new population of cells which are more resistant towards treatment. However, lately there has been a bigger focus on the role of tumor micro environment (TME) in mediating treatment resistance, as reviewed by Joyce [6]. The results in this thesis have verified a protection against BRAF inhibitor when normally responsive malignant melanomas are grown as co-cultures in proximity to the TME component, fibroblasts [18, 19]. As reviewed by Kalluri et.al, the function of fibroblast in various settings varies [17]. The exact mechanism by which fibroblasts protect our malignant melanoma cells from the drug is unknown. Yet, we propose that utilization of PI3K-pathway by fibroblast-protected melanoma could be a major contributor to resistance from BRAFi[18]. This was a motivating factor to explore different inhibitors of PI3K or PI3K-activating RTKs aiming to enhance an anti-cancer effect in melanoma-fibroblast co-cultures.

In concordance with other studies on the PI3K inhibitor Buparlisib efficacy on BRAF mutant melanoma tumors [61], we did see an inhibitory effect on cell survival/proliferation in our melanoma models in vitro. It should be noted that we could not validate that Buparlisib suppresses PI3K signaling, when we analyzed pAKT levels as an indicator of the PI3K pathway activity. However, this analysis has been performed only on the mono-cultured melanoma cells, where we could not see reduction in pAKT levels. In fact, we observed enhanced levels of pAKT at sites Thr308 and Ser473 after Buparlisib treatment, which might be due to release of feedback inhibition [62]. In 2005, Sarbassov et. al. showed that by using shRNA against Raptor 1/2 that suppresses mTOR signaling, pAKT(Thr308) is up-regulated [54]. It should be mentioned that other groups also reported that pAKT levels may remain unchanged after the treatment with Buparlisib [63]. Thus, another marker reflecting PI3K pathway status might be necessary to accurately measure the activity of this signaling pathway. For example, by measuring phosphorylation status of the PI3K signaling lipid product, phosphoinositide [64]. How the co-cultured cells respond to Buparlisib with respect
to pAKT has not been investigated in this thesis. It might be that the co-cultured cells have different PI3K signaling regulation mechanisms than the mono-cultured cells, which is an interesting topic for further research.

The observed changes in phenotype-associated proteins in co-cultured versus mono-cultured melanoma cells, indicate a switch towards a mesenchymal state when our cancer cells were grown in the presence of fibroblasts. This cellular dynamic leading to EMT and MET during cancer progression has been known for years, and has been usually linked to cancer cell motility/invasion [65]. Interestingly, our results indicate that the sole presence of fibroblasts seems to be sufficient to introduce a switch from a melanocytic to a mesenchymal-like phenotype, and that this switch is associated with resistance to BRAFi. The existence of cancer cells in different phenotypic states signifies the need for phenotype-directed drugs, which target either mesenchymal or melanocytic state cells [66]. While melanocytic phenotype can be efficiently targeted by BRAFi as seen by our group [18] as well as others [66], to target a mesenchymal phenotype is a challenge [38]. Our results indicate that the GSK3-β inhibitor AR-A014418 (AR), which had such a big effect on co-cultured melanomas, could be a drug against the mesenchymal phenotype. AR is supposed to increase WNT signaling by inhibiting GSK. WNT-signaling as well as its substrate, β-catenin, has been reported to be low in the mesenchymal phenotype [67]. In our co-cultures we observed reduction of β-catenin, which might indicate reduction in WNT signaling, although the latter has not been validated in this thesis. It might be that a strong AR-mediated anti-cancer effect in the co-cultures might be associated with AR-induced WNT activation, which is detrimental for the mesenchymal state.

### 4.2 PeggySue Charge-based studies on phospho-protein levels

In this MSc thesis it has been shown that we can detect changes in levels of cancer-related signaling proteins, such as phospho-ERK by the isoelectric point separation in small sample volume by the Charge method. This indicates that Charge can be a valuable method to determine phosphorylation-status on various proteins in smaller sample volumes than needed for other conventional methods for protein analyses. However, there are major challenges ahead. Our results indicate that the separation of different phospho-protein forms is highly dependent on the buffer the sample is prepared in, as well as sample handling. Protein lysates
made in the buffer optimized for other proteomic analysis methods (like western blotting), do not give satisfactory results on Charge. This means that previously made cell lysates are not suitable for optimal analysis by this method. Charge method requires dedicated samples, at least for optimal detection of some proteins.

The peak-profile and the peak-separation are highly dependent on the degree of complexity of individual proteins. For example, as seen from our results, AKT detection is challenging due to existence of several AKT isoforms and different phosphorylation sites, which was also observed by others [51, 52, 60]. Depending on which protein is investigated, future work will require more optimization of the method. Eventually, there will be a need to identify all peaks detected by the Charge methods. We tried to discriminate phospho- from non-phospho- AKT by dephosphorylation of samples, but we did not observe expected number of peaks (i.e. 3) representing each isoform of AKT. However, there are numerous methods other than dephosphorylation, for peak identification. For example, we can use cell-lines known to express only specific isoforms of a protein [60], or knock-down specific isoforms in our own cell-lines. With editing tools, such as CRISPR/CAS9, we can edit phosphorylation sites, albeit this might influence pI-value of the protein. We can stimulate the pathway, consequently enhance phosphorylation levels by adding e.g. growth factors [68].

There are various technical possibilities to optimize the analysis, which due to cost/time were not addressed in this thesis. The protein lysates were run on an ampholyte mix which contains zwitterions that separate proteins in a gradient from 5-8 pH. For better separation of peaks, zwitterions of various pI can be mixed [60]. Furthermore, pI-ladders can accurately pin-point the exact location of peaks with specific pI. While we used Pan-antibodies to detect all phosphorylation sites of our protein, some groups have also had success with phosphorylation-site-specific antibodies [51, 52, 60]. As a final remark, there are various tools available to calculate theoretical pI-values of any given protein in their denatured state with or without phosphorylation. From own experiences, these theoretical values rarely match observed values. This could be because appearances of peaks are influenced by salt concentration in our lysates, and/or unknown PTMs on our proteins of interest. Furthermore, all pI separations in this thesis were done on proteins in Naïve form. It would be quite interesting to investigate if protein detection improves by denaturing proteins prior to the pI separation.
5 Future perspectives

So far, we focused primarily on analysis of molecular responses in melanoma cells from mono-cultures. It will be highly interesting to analyze melanoma cells from the co-cultures with respect to PI3K-associated proteins. The aim will be to verify the significance of PI3K pathway for stroma-supported melanoma cells and thereby to increase understanding of the molecular mechanism of stromal protection from BRAFi.

A GSK inhibitor AR-A014418 showed enhanced efficacy in the co-cultures i.e. mesenchymal phenotype making it highly interesting to analyze the molecular mechanism behind. One possibility would be to explore the role of WNT-pathway by analyzing WNT-related proteins such as AXIN by PeggySue Size technology.

This thesis was a first attempt to utilize PeggySue technology, primarily PeggySue Charge, for analysis of proteome of melanoma cells from \textit{in vitro} models. Although the Charge method could detect phospho-proteins by using pan- antibodies, the detection was not optimal. It will be very interesting to make dedicated samples and further optimize the Charge method as discussed in the Discussion part.
6 Conclusions

In this thesis we have shown that the presence of fibroblast influences phenotypic state and drug-responses of melanoma cells. This highlights the need for combination treatments that target stroma-dependent and independent cancer cell subpopulations within a tumor. Advanced methods like PeggySue Charge/Size can help in search of targetable nodes for better treatment. Specifically, we have shown:

- Combining BRAFi with PI3K inhibitor Buparlisib enhances an anti-cancer effect in melanoma-fibroblast co-cultures, which show resistance to BRAFi. This indicates that Buparlisib reduces fibroblasts-associated protection from BRAFi. However, verification of PI3K-pathway involvement in BRAFi protection at the molecular level is lacking.

- Fibroblasts induce an up-regulation of mesenchymal phenotype-related proteins in the melanoma cells, indicating a phenotype switch in the co-culture conditions. This effect can be captured by studying phenotype-specific protein levels by the PeggySue Size method.

- Inhibition of GSK-3β by AR-A014418 induces a stronger anti-cancer effect in melanoma-fibroblast co-cultures than mono-cultures. This indicates that AR-A014418 is more potent against melanoma cells in the mesenchymal phenotype. The molecular mechanism behind this effect has not been disclosed.

- PeggySue Charge method can detect phospho-proteins in cell lysates made for western blotting, however the detection is not optimal and ideally requires dedicated samples and further optimization.
References


Supplementary figures

Supplementary figure S1: The level of pAKT in HM8 cells treated with Afuresertib.

Supplementary figure S2: AR-treated Mono- and Co-cultured HM8 cells. Mono- and co-cultured were treated with 1 μM BRAFi (Vemurafenib), 5μM AR-A014418 or combination for 24 hours.