"Stroma-induced phenotypic plasticity offers phenotype-specific targeting to improve melanoma treatment"

Kotryna Seip[a], Kjetil Jørgensen[a]*, Marco Vincent Haselager[a]*, Marco Albrecht[b], Mads Haugland Haugen[a], Eivind Valen Egeland[a], Philippe Lucarelli[b], Olav Engebraaten[a,c,d], Thomas Sauter[b], Gunhild Mari Mælandsmo[a,e] and Lina Prasmickaite[a]

* The authors contributed equally.

[a] Department of Tumor Biology, Oslo University Hospital, The Norwegian Radium Hospital, Oslo, Norway;

[b] Life Sciences Research Unit, University of Luxembourg, Luxembourg;

[c] Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway;

[d] Department of Oncology, Oslo University Hospital, Oslo, Norway;

[e] Department of Medical Biology, Faculty of Health Sciences, UiT/The Arctic University of Norway, Tromsø, Norway.

Corresponding author:

Dr. Lina Prasmickaite

Department of Tumor Biology, Institute for Cancer Research

Oslo University Hospital, The Norwegian Radium Hospital

Montebello 0310, Oslo

Norway

e-mail: linap@rr-research.no

Tel.: +47-22781868

Fax.:+47-22781795

Abstract

Cancer cells' phenotypic plasticity, promoted by stromal cells, contributes to intra-tumoral heterogeneity and affects response to therapy. We have disclosed an association between fibroblast-stimulated phenotype switching and resistance to the clinically used BRAF inhibitor (BRAFi) vemurafenib in malignant melanoma, revealing a challenge in targeting the fibroblast-induced phenotype. Here we compared molecular features and drug sensitivity in melanoma cells grown as co-cultures with fibroblasts *versus* mono-cultures. In the presence of fibroblasts, melanoma cells switched to the dedifferentiated, mesenchymal-like, inflammatory phenotype that showed reduced sensitivity to the most of 275 tested cancer drugs. Fibroblasts, however, sensitized melanoma cells to PI3K inhibitors (PI3Ki) and particularly the inhibitor of GSK3, AR-A014418 (GSK3i), that showed superior efficacy in co-cultures. The proteome changes induced by the BRAFi+GSK3i combination mimicked changes induced by BRAFi in mono-cultures, and GSK3i in co-cultures. This suggests that the single drug drives the response to the combination treatment, depending on fibroblast presence or absence, consequently, phenotype. We propose that the BRAFi and GSK3i (or PI3Ki) combination exemplifies phenotype-specific combinatorial treatment that should be beneficial in phenotypically heterogeneous tumors rich in stromal interactions.

Keywords: Phenotype switching, melanoma, cancer-associated fibroblasts, drug resistance, tumor-stroma interactions.

Abbreviations: CAF, Cancer associated fibroblasts; CDSS, Cancer drug sensitivity screening; EMT, Epithelial-to-mesenchymal transition; FACS, Fluorescence-activated cell sorting; GFP, Green fluorescent protein; GSK3, Glycogen synthase kinase 3; LUC, Luciferase; MFI, Mean fluorescence intensity; MITF, Microphthalmia-associated transcription factor; RPPA, Reverse-phase protein array; SWI, Simple western immunoassay.

1. Introduction

Solid tumors are heterogeneous and comprise, besides malignant cells, various stromal cells like cancer-associated fibroblasts (CAFs) and immune cells. Stromal cells can provide soluble factors or matrix components that increase cancer cell survival and growth [1]. They can also promote phenotypic plasticity in cancer cells and help to acquire a more aggressive phenotype [2]. In carcinomas, phenotype switching is known as epithelial-to-mesenchymal transition (EMT) characterized by loss of epithelial features and gain of mesenchymal characteristics [3]. Melanoma undergoes similar phenotype switching when the melanocytic lineage program is suppressed and exchanged with mesenchymal programs [4]. The drivers of phenotype switching are not known, but inflammation or CAFs are very likely candidates [5, 6]. The mesenchymal-like phenotype in melanoma is often described as $MITF^{low}/AXL^{high}$ to indicate down-regulation of microphthalmia-associated transcription factor (MITF) - the master regulator of the melanocytic lineage, and up-regulation of AXL receptor-tyrosine kinase - a marker of mesenchymal cells [5, 7]. In addition, a number of other signaling pathways/transcription factors have been linked to this phenotype, among them NF-kB [8], c-Jun [9], AP-1 [10], or suppressed Wnt/β-catenin signaling [11].

The mesenchymal phenotype/EMT is traditionally linked to enhanced cellular motility and invasion [3], although recently its role in therapy resistance has become apparent [4, 12, 13]. The microenvironment conditions that promote the mesenchymal phenotype and contribute to phenotypic heterogeneity can lead to heterogeneity in drug sensitivity and thereby facilitate resistance. This might require phenotype-directed therapies, i.e. a combination of drugs that act on cancer cells of distinct phenotypes.

Malignant melanoma is one of the most metastasizing and drug resistant cancer types, resulting in high mortality in this patient group. Modern therapeutic options, like immunotherapy and targeted therapy with BRAF inhibitors (BRAFi), significantly improved clinical management of this cancer [14]. About 50% of melanomas harbor a mutation in BRAF and are therefore eligible for treatment with BRAFi, although, despite strong initial responses, most patients develop resistance [15]. Innate resistance to BRAFi has also been observed particularly in $MITF^{low}/AXL^{high}$ tumors [7, 8, 16], and the role of CAFs has been reported [17, 18]. We have shown in a previous study that the presence of CAFs fosters melanoma cells with reduced sensitivity to BRAFi, and this effect was associated with the phenotypic plasticity [19]. In the

current study, we compared molecular features and drug sensitivity in melanoma cells with and without CAFs, with the aim to characterize the CAF-induced phenotype and identify potential nodes for targeting.

2. Materials and Methods

2.1. Cell lines and drugs

Malignant melanoma cell lines, HM8 (derived from brain metastases) and Melmet 5 (derived from lymph node metastases), were established from melanoma patients at Oslo University Hospital, The Norwegian Radium hospital (Oslo, Norway), as described previously [20] (approved by the Norwegian Research Ethics Committee 2011/2183, S-01252, 2.2007.997). The melanoma cells were stably labeled with green fluorescent protein (GFP) - luciferase (LUC) construct, described previously [21] and kindly provided by Dr. Glenn Merlino (NIH, MD). Human lung fibroblasts WI-38 were obtained from ATCC (Rockville, MD). Melanoma cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) and 2 mM L-Alanyl L-Glutamine (all from Sigma-Aldrich, St. Louis, MO). WI-38 fibroblasts were cultured in EMEM medium (ATCC, Manassas, VA), supplemented with 10% FCS. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and were routinely tested for mycoplasma and cell ID.

The BRAF inhibitor vemurafenib, PI3K inhibitor BKM120 and GSK3 inhibitor AR-A014418 were from Selleck Chemicals (Houston, TX). All drugs were dissolved in DMSO.

2.2. Co-cultures

Co-cultures were prepared by seeding out GFP-LUC-labeled melanoma cells together with non-labeled WI-38 fibroblasts in the supplemented RPMI medium at a ratio of 1:4 (1:2.5 for cancer drug sensitivity screening) and total cell density (number/well) of: 3.5 x 10^3 (384-wells), 8 x 10^3 (96-wells), 10 x 10^4 (24-wells) or 75 x 10^4 (T25 flask). The same cell density was used in the mono-cultures. The co-cultures were pre-incubated for one-two days before treatment for 24-72 hrs, depending on the application (specified in the figure text).

2.3. Tumor cell-specific bioluminescence assay for measuring melanoma cell viability

Melanoma cell viability was determined by measuring bioluminescence generated by the tumor cell-specific label, luciferase, as described previously [22] and validated in our cell cultures [19]. The cell cultures were prepared in white 96-well plates (Costar, Corning, NY) one day before drugs were added. After 72 hrs, D-luciferin (Biosynth AG, Staad, Switzerland) was added to a

final concentration of 0.1 mg/mL, and 10 min later bioluminescence was measured by a plate reader Victor^2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA). The viability of treated melanoma cells was determined by normalizing the bioluminescence values in the treated samples to the values in the respective (mono-cultures or co-cultures) non-treated controls (set to 100%).

2.4. Cancer drug sensitivity screening

The cancer drug sensitivity screening (CDSS) was performed on the high throughput Chemical Biology screening platform at the Biotechnology Center, University of Oslo (Norway), by using Selleck Chemicals Cambridge cancer compound library (384 compounds) provided by the Center. HM8 mono-cultures and co-cultures were prepared in white tissue coated 384-well plates (Greiner Bio-One, Frickenhausen, Germany) one day before drugs were added. The drugs (0.4 μM BRAFi together with 5 μM of each compound, or DMSO for BRAFi alone; the non-treated controls received only DMSO) were added by an automated liquid handling system (Hamilton Bonaduz AG, Bonaduz, Switzerland). After 72 hrs, cancer cell viability was measured by adding D-luciferin and reading bioluminescence on an EnVision Multilabel reader (Perkin Elmer). The relative cell viability in the treated samples was calculated as above. Compounds that in both cultures enhanced the bioluminescence signal (e.g. stimulated LUC or cell proliferation) or were too toxic (i.e. reduced cell viability in both culture conditions to levels below 10 %) were excluded from further analysis.

2.5. Intracellular flow cytometry

For analysis of single cells, flow cytometry was used. All collected cells were fixed for 15 min in 1.6% paraformaldehyde (PFA) at room temperature (RT) and permeabilized with 100% ice-cold methanol. Four samples (representing non-treated and differently treated samples) were barcoded with different concentrations of Pacific Orange or Pacific Blue dye (both ThermoFisher Scientific, Waltham, MA), as described previously [19] and illustrated in Supplementary Figure S1. Following incubation at RT for 30 min and a washing step, the four barcoded samples were combined into one tube and stained simultaneously with different antibodies for 30 min at RT: anti-pS6-Alexa Fluor 647 (#4851) 1:200 and anti-p-c-Jun (#3270) 1:200 (both from Cell Signaling Technology, Danvers, MA); anti-Ki67-PE (#556027) 1:6 and anti-fibronectin-Alexa Fluor 647 (#563098) 1:40 (both from BD Pharmingen, San Jose, CA); anti-Melan-A (M7196) 1:66 (DakoCytomation, Glostrup, Denmark). The stained samples were analyzed on a LSR II

flow cytometer controlled by BD FACS Diva™ software (BD Bioscience, San Jose, CA). The data was analyzed by using Flow Jo software (FlowJo, Ashland, OR). The melanoma cells were distinguished from the fibroblasts based on the GFP signal, and only the GFP^+ cancer cells were analyzed for the levels of proteins of interest, as illustrated in Supplementary Figure S1.

2.6. Reverse phase protein array and Simple western immunoassay

For protein analysis in cell lysates, the melanoma cells from the co-cultures were separated from the fibroblasts by fluorescence-activated cell sorting (FACS) collecting GFP^+ melanoma cells. The stringent gating on GFP (illustrated previously [19]) assured the purity of the melanoma cell fraction. The mono-cultured melanoma cells were handled identically i.e. also run though the FACS machine.

The sorted cells were lysed in lysis buffer (1% Triton X-100, 50mM HEPES, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na_3VO_4, 10% glycerol; supplemented with protease- and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany), followed by ultrasonication.

The cell lysates were analyzed by reverse-phase protein array (RPPA) at MD Anderson Cancer Center RPPA core facility (Houston, TX). In brief, serial dilutions of the lysates were arrayed on nitrocellulose-coated slides. A primary antibody (specified at the core facility‘s home page; available upon request) was added to probe each slide, followed by a biotin-conjugated secondary antibody. A DAB colorimetric reaction was used to detect the signal. After scanning the slides, spot intensities were determined by analysis with the MicroVigene software (VigeneTech, Carliste, MA). Each dilution series was fitted with a logistic model (“Supercurve Fitting”) to obtain a dilution curve in log 2 scale. The data in log2 scale were transformed to linear and median-centered values. We clustered the data with the public available R-package Clustermap (Lingjærde OC and Steen CB) in R (version 3.2.2) using R-Studio (Version 1.1.447). Rows and columns were subjected to hierarchical clustering with Euclidean distances. We used Partitioning Algorithm based on Recursive Thresholding (PART) [23] to estimate the number of clusters, but also manually identified specific clusters to find biologically relevant groups. Cluster significance was determined with Fisher's exact test.

Simple western immunoassay (SWI) was performed on a PeggySue™ instrument (ProteinSimple, San Jose, CA). The protein lysates (1 μg/μL) were separated by using a 12-230 kDa size separation master kit according to the manufacturer’s protocol. All settings were kept

on default, except primary antibody incubation time, which was set to 60 min. The Compass software (ProteinSimple, version 2.7.1) was used to program the instrument and analyze the results. The following antibodies from Cell Signaling Technology were used: anti-PDGFRβ (#3169) 1:50, anti-AXL (#8661) 1:50, anti-p-c-Jun (#3270) 1:50, anti-pSTAT3 (#9145) 1:50, anti-MITF (#12590) 1:50, and anti-β-catenin (#19807) 1:50. Anti-β-actin (A5316) 1:100 was from Sigma-Aldrich.

2.7. TCF/LEF-luciferase reporter assay

To measure Wnt/β-catenin signaling activation, we used a TCF/LEF responsive luciferase reporter plasmid pGL4.49 [luc2P/TCF-LEF RE/Hygro], (Promega, Madison, WI), which was transfected into non-labeled HM8 melanoma cells by the help of X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland). After 24 hrs, the cells were treated with the GSK3 inhibitor AR-A014418 for 24 hrs, and the collected samples were analyzed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA), following the manufacturer's protocol. Luminescence was measured by using a TD−20/20 Luminometer (Turner Designs Inc, San Jose, CA, USA).

2.8. Statistical analysis

Two-tailed Student's unpaired t-test was used in all statistical analysis except the RPPA-data analysis, which is specified below. Differences were considered significant if p-values were below 0.05.

The detection of significantly changed protein levels, identified by RPPA, between the two cell culture conditions was based on two-way analysis of variance (ANOVA). The "cell line" (HM8 and Melmet 5) is independent variable one, and the "cell culture condition" (+/- BRAFi and +/- CAFs) is independent variable two. The "RPPA measurements" represent the dependent variable. Only the impact of the variable "cell culture condition" on the "RPPA measurements" was assessed using the *anova()* function of the stats package, available for the programming language R [24]. The indicated fold change (FC) is the average of the cell line specific fold changes. The false discovery rate was derived from p-values with the R package q-value [25].

3. Results

3.1. The presence of CAFs enriches for BRAFi-insensitive melanoma cell subpopulations

We demonstrated previously that the presence of CAFs affects melanoma cell response to BRAFi [19]. Using HM8 and Melmet 5 melanoma cell lines grown as mono-cultures or co-

cultures with CAFs, we validated an impaired response to BRAFi in the presence of CAFs (Fig.1 A). To assess whether the elevated resistance was due to CAF-dependent insensitivity to BRAFi or activation of additional resistance pathways, we compared protein signatures in BRAFi treated melanoma cells from mono-cultures and co-cultures. The GFP-tagged melanoma cells were isolated by FACS and analyzed by RPPA to detect the levels of approximately 300 cancer relevant proteins. Using the significance level $p < 0.05$ and $q < 0.05$, we identified 43 proteins modulated by BRAFi in the mono-cultures *versus* 12 proteins in the co-cultures in both HM8 and Melmet 5 (Supplementary Table S1). Reduced levels of proliferation/protein translation/cell cycling-related proteins, such as phosphorylated RPS6 and RB1, DUSP4, CCNB1 and CDK1, are known indicators of good response to BRAFi [16, 26] and were observed in the mono-cultures, but to a much lower degree or not at all in the co-cultures (Fig. 1B and Supplementary Table S1). To note, we did not detect BRAFi-induced reduction of pERK, a common marker of BRAFi effect, since this protein was sensitive to FACS so that the reduction could not be captured in FACS-processed samples (data not shown). No activated additional resistance pathways were identified in the treated co-cultures. This suggests that the impaired response to BRAFi in the co-cultures is due to general insensitivity of melanoma cells to this drug in the presence of CAFs.

To assess how individual cells respond to BRAFi, we used intracellular flow cytometry and measured the levels of proliferation-linked markers in single melanoma cells. We scored Ki67, a clinical marker of proliferation, and phosphorylated ribosomal protein S6 (RPS6, further referred to as pS6), a regulator of protein translation that is known to be suppressed in BRAFi sensitive (but not resistant) cells [27], as also observed in our models (Fig. 1B). Based on pS6/Ki67 levels, we defined four cellular states (Q1-Q4), where the proliferative $pS6^{high}/Ki67^{high}$ (Q1) and the quiescent $pS6^{low}/Ki67^{low}$ (Q3) states are of particular interest (Fig. 1C). The control cells were primarily in a proliferative state (Q1) in both mono-cultures and co-cultures (Fig. 1C). After treatment with BRAFi, the vast majority of the mono-cultured cells accumulated in the quiescent state (Q3). In the treated co-culture, however, a notable fraction of cancer cells remained in the proliferative state (Q1), indicating the BRAFi-insensitive melanoma cell subpopulation (Fig. 1C).

3.2. Molecular characteristics of the CAF-induced phenotype

Hierarchical clustering analysis of the RPPA data revealed that the melanoma cells clustered according to their growth conditions (+/- CAFs) and not the treatment (+/- BRAFi) (Fig. 2A). The dominant effect of the difference between the co-cultured and the mono-cultured melanoma cells is attributable to 52 proteins affected by CAFs with the significance level $p < 0.05$ and $q < 0.05$ (Supplementary Table S2). Among the top up-regulated proteins were several markers of EMT (PDGFR, FN1, SERPINE1, AXL, MYH9), proteins involved in the JNK/AP-1/STAT3 network (p-c-JUN, FOSL1, pSTAT3), as well as a gap-junction component GJA1 and an apoptosis inhibitor MCL1 (Fig. 2B and Supplementary Table S2). The down-regulated proteins included regulators of melanocytic lineage (TYRO3), PI3K signaling (GAB2) and metabolism (FASN, ACACA). We validated the up-regulation of mesenchymal markers (PDGFR, AXL, FN1) and the down-regulation of factors associated with melanocytic differentiation (MITF, Melan-A and β-catenin) (Supplementary Fig. S2). In addition, we validated the up-regulation of p-c-Jun and p-STAT3, proteins known to be linked to an inflammatory state (Supplementary Fig. S2). "Inflammatory" features were assessed further by measuring the expression of pro-inflammatory cytokines. As shown in Figure 2C, the co-cultured melanoma cells expressed elevated levels of all measured cytokines: IL6, IL8, IL10, CCL2, CCL5 and CXCL1. Altogether, this indicates that melanoma cells in the presence of CAFs undergo an EMT-like switch to a dedifferentiated, mesenchymal-like, inflammatory phenotype that shows poor response to BRAFi.

3.3. Cancer drug sensitivity screening in co-cultures *versus* mono-cultures

To identify compounds that enhance treatment response in the presence of CAFs, we employed a Selleck Chemicals Cambridge cancer compound library and performed cancer drug sensitivity screening (CDSS), comparing HM8 cell viability in co-cultures *versus* mono-cultures. The cell cultures were treated with each compound in the presence of BRAFi, and the effect was determined by measuring tumor cell-specific bioluminescence. In this experimental setup, BRAFi alone reduced melanoma cell viability to 52±4% in the mono-cultures *versus* 64±7% in the co-cultures. We excluded 109 of 384 compounds from further analysis because they either enhanced the bioluminescence or were too toxic in both culture conditions (see Materials and Methods 2.4). The efficacy of the remaining 275 compounds is depicted in Figure 3A. The compounds were ranked based on their efficacy in the mono-cultures (from the lowest to the highest), shown in black, and the efficacy of the corresponding treatments in the co-cultures is

indicated in blue. The majority of the treatments showed lower effect in the co-cultures compared to the mono-cultures. Only 36 compounds were more effective in the co-cultures. These compounds were ranked based on the effect difference in the co-cultures *versus* the mono-cultures (Fig. 3B). AR-A014418, an inhibitor of Glycogen Synthase Kinase-3 (GSK3), showed the biggest difference in efficacy, inducing a superior effect in the co-cultures. Eleven of 36 compounds are inhibitors of PI3K/mTOR signaling (Fig. 3B). Ten other inhibitors of the PI3K/mTOR pathway, including the clinically relevant BKM120 (known as buparlisib), induced an effect in the co-cultures that approached the effect seen in the BRAFi sensitive mono-cultures. Only two PI3K/mTOR inhibitors from the library did not show any "therapeutic benefit" in the co-cultures. Based on the CDSS data, we chose the PI3K inhibitor BKM120 (further referred to as PI3Ki) as a representative of a drug class of PI3K inhibitors, and our "best hit", the GSK3 inhibitor AR-A014418 (further referred to as GSK3i), for further investigation.

3.4 Effects of PI3K inhibitor BKM120 in co-cultures *versus* mono-cultures

The effect of the PI3Ki+BRAFi treatment *versus* BRAFi alone was tested by assessing: i) cancer cell viability, ii) a fraction of cells positive for cleaved PARP ($cPARP^{+}$) as a measure of apoptosis, and iii) a fraction of cells in a proliferative/quiescent state as defined by pS6/Ki67 levels.

In the HM8 co-cultures, the PI3Ki+BRAFi combination reduced melanoma cell viability more than BRAFi alone (Fig. 4A), which was confirmed also in Melmet 5 models (Supplementary Fig. S3). In concordance, the fraction of apoptotic $cPARP^{+}$ cells increased from 5% to 20% when PI3Ki was combined with BRAFi (Fig. 4B). The PI3Ki+BRAFi combination eliminated the proliferative $pS6^{high}/Ki67^{high}$ melanoma cell fraction (Q1) and enriched the quiescent $pS6^{low}/Ki67^{low}$ cell fraction (Q3) (Fig. 4C). In the mono-cultures, the PI3Ki+BRAFi treatment induced a similar increase in the $cPARP^{+}$ cell fraction as in the co-cultures (Fig. 4B), but there was no clear "benefit" of adding PI3Ki according to the cell viability assays (Fig. 4A and Supplementary Fig. S3) or cellular state analyses (Fig. 4 C). The response to PI3Ki alone was also stronger in the co-cultures than the mono-cultures (Fig. 5 and Supplementary Fig. S3). To note, the effect of PI3Ki on fibroblasts was very low (~ 20% based on the cell viability assays, data not shown). This indicates that the presence of CAFs sensitizes melanoma cells to PI3K-targeted treatment.

3.5. Effects of GSK3 inhibitor AR-A014418 in co-cultures *versus* mono-cultures

GSK3 is a "multitasking" kinase downstream from PI3K/AKT. Among the best described signaling pathways involving GSK3 is Wnt/β-catenin, which was shown to be stimulated by GSK3 inhibition [28, 29]. By using a Wnt/β-catenin reporter, TCF/LEF –luciferase, we confirmed that the GSK3 inhibitor AR-A014418 activates luciferase, indicating stimulation of Wnt/β-catenin signaling (Supplementary Fig. S4).

In the HM8 co-cultures, the GSK3i+BRAFi combination reduced cell viability more than BRAFi alone, and resulted in a much stronger effect than in the mono-cultures (Fig. 6A), in agreement with the CDSS data. A similar response was observed in the Melmet 5 models (Supplementary Fig. S5). In line with this, the GSK3i+BRAFi combination elevated a fraction of apoptotic $cPARP^{+}$ cells more in the co-cultures than the mono-cultures (Fig. 6B). Furthermore, the GSK3i+BRAFi treatment reduced the proliferative $pS6^{high}/Ki67^{high}$ cell fraction (Q1) in the co-cultures, but not in the mono-cultures (Fig.6C). In the mono-cultures, the GSK3i+BRAFi combination enriched the $pS6^{high}/Ki67^{low}$ cell fraction (Q2), which became dominant (Fig. 6C). This was unexpected since BRAFi alone promoted the quiescent $pS6^{low}/Ki67^{low}$ cellular state (Q3), as shown in Figure 1C. To investigate this further, we measured pS6 levels over the course of the treatment. We observed a suppression of pS6 in both mono-cultures and co-cultures after 24hrs treatment (Supplementary Fig. S6). However, after 48hrs treatment with the BRAFi+GSK3i combination, the level of pS6 was restored in the mono-cultures, but not in the co-cultures, further strengthening the notion about CAF-dependent differences in response.

When we measured the effect of GSK3i alone, we also detected a better response in the co-cultures than the mono-cultures (Fig. 7 and Supplementary Fig. S5). Thus, GSK3i reduced HM8 cell viability down to 10% in the co-cultures, but only to 55% in the mono-cultures (Fig. 7A); the fraction of apoptotic $cPARP^{+}$ cells increased to 46% and 22%, respectively (Fig. 7B). The proliferative $pS6^{high}/Ki67^{high}$ cell fraction (Q1) was lower in the GSK3i treated co-cultures than the mono-cultures (Fig. 7C). To note, the effect of GSK3i on fibroblasts was very low (below 10%, data not shown). Taken all together, this indicates a differential response to treatment containing GSK3i in the presence and absence of CAFs, and suggests that CAFs strongly sensitize melanoma cells to GSK3i.

To compare the effects of GSK3i, BRAFi and their combination at a proteome level, HM8 melanoma cells were isolated by FACS, and analyzed by RPPA. The protein levels in the treated cells were normalized to the levels in the respective non-treated controls to identify changes

induced by each treatment. The list of proteins modulated by BRAFi in this dataset (not shown), was comparable to the lists from the previous dataset presented in Supplementary Table S1, indicating the consistency of the treatment effect. The normalized data were subjected to the hierarchical clustering analysis as shown in Figure 8. Thus, in the co-cultures, the GSK3i+BRAFi sample clustered together with the GSK3i sample, indicating similar proteome changes induced by these treatments. In contrast, in the mono-cultures, the GSK3i+BRAFi sample clustered together with the BRAFi sample, indicating their similarity. Thus, the response to the combination treatment is driven by the response to the single drug, and it depends on the interactions with CAFs, consequently, phenotype, whether GSK3i or BRAFi is decisive.

4. Discussion

Intra-tumoral heterogeneity (genetic and/or phenotypic) creates substantial obstacles to the clinical management of cancer patients. It has been demonstrated that cancer cells of distinct phenotypes respond to drugs differently, which can influence the overall efficacy of the treatment. In the current study, we investigated the impact of CAFs on melanoma cells phenotypic plasticity and drug sensitivity. We demonstrated that the presence of CAF induced phenotype switching that was associated with resistance to various anti-cancer drugs, including the clinically used BRAF inhibitor vemurafenib. This defines the CAF-induced phenotype as a challenge for treatment, and signifies the need for phenotype-tailored therapies. Better characterization of the CAF-induced phenotype is a prerequisite for design of such therapies. Using the co-culture models, we demonstrated that melanoma cells reprogram their proteome in the presence of CAFs. Among the changes was a reduction in the levels of MITF/β-catenin and elevation in the levels of AXL/PDGFR. This observation is in line with Tirosh et al. [5], who showed that CAF-rich tumors preferentially display the $MITF^{low}/AXL^{high}$ gene signature, reflecting their dedifferentiated, mesenchymal-like state. Moreover, we observed elevated levels of phosphorylated c-Jun and STAT3, suggesting potentiated JNK/STAT3 signaling. Both JNK and STAT3 are known promoters of inflammatory responses [30, 31], and their up-regulation might indicate activated inflammatory programs in the CAF-induced phenotype. Interestingly, Riesenberg et al. [9] reported on a central role of c-Jun in promoting the $MITF^{low}$ state and simultaneously elevating production of inflammatory cytokines. In line with this, we observed up-regulation of numerous cytokines (IL6, IL8, CCL2, CCL5 and CXCL1) in co-cultured melanoma cells. Collectively, this data supports the notion that in the presence of CAFs,

melanoma cells switch to the dedifferentiated, mesenchymal-like phenotypic state with strengthened inflammatory traits.

The association between the $MITF^{low}/AXL^{high}$ phenotype and resistance to the MAPK pathway inhibitors is relatively well documented now [5, 7] and is in line with our observation in the co-culture models. The components of inflammatory signaling, STAT3 and c-Jun, were also linked to the resistance, though as a part of the adaptive response to the treatment [32, 33]. Our data on elevated p-c-Jun/pSTAT3 in non-treated co-cultures suggests that stimulated JNK/STAT3 might be a characteristic of an intrinsically resistant phenotype, as also proposed by Ramsdale et al. [34]. One possibility of how inflammation-related factors can contribute to resistance is generation of an inflammatory microenvironment that can engage macrophages. It has been shown that the cooperation between melanoma, fibroblasts and macrophages, as well as the macrophage-provided cytokine TNFα, can facilitate resistance to the MAPK pathway inhibitors [35, 36]. Given that the CAF-induced phenotype gets benefits from inflammatory microenvironment, targeting its inflammatory traits might be an attractive therapeutic option. The EMT - inflammation alliance is a field of increasing interest, and it has been proposed that anti-inflammatory therapeutic strategies might be particularly effective in EMT-like tumors [37, 38]. The significance of inflammatory traits for the CAF-induced phenotype is thus an interesting topic for future research.

The cancer drug sensitivity screening, testing several hundred compounds of which most are "targeted drugs", revealed a lower effect in the co-cultures, indicating that the CAF-induced phenotype is generally more refractory to treatment. However, the presence of CAFs sensitized melanoma cells to PI3K/AKT/mTOR inhibitors, which showed better efficacy in the co-cultures. The emergence of the PI3K-linked signaling as a potential target in the CAF-induced phenotype is in agreement with our previous results, where we showed that LY294002 (an early generation inhibitor of PI3K) and everolimus (a clinically used inhibitor of mTOR) improved the anti-cancer effect in the co-cultures [19]. A similar improvement was also observed with the clinically relevant PI3K inhibitor BKM120 investigated in the current study. Altogether, this strengthens our conclusion that the CAF-induced phenotype is addicted to PI3K/AKT/mTOR rather than MAPK signaling and therefore shows enhanced response to inhibitors of the former. In concordance, other studies also suggested that resistant cancer cells with an EMT signature might be targeted with PI3K/AKT inhibitors [39].

Our “best hit”, the inhibitor AR-A014418, targets GSK3β that also belongs to the PI3K/AKT signaling network. It has been reported that GSK3β can regulate melanoma growth and invasion, which could be prevented by GSK3 inhibitors like AR-A014418 [29, 40], and that GSK3 inhibition could reverse the “EMT-like” state [41]. The superior efficacy of GSK3i in the co-cultures could be due to a GSK3 role in Wnt/β-catenin signaling. Inhibition of GSK3 leads to activation of canonical Wnt/β-catenin [29], and the latter is a recognized driver of the $MITF^{high}$ phenotype [11, 42]. In contrast, suppression of Wnt/β-catenin is a characteristic of the mesenchymal-like, the $MITF^{low}$ phenotype [11, 42]. Thus, GSK3i-induced activation of Wnt/β-catenin might work against the mesenchymal-phenotype that is enriched in the co-cultures. Another possibility is that GSK3i abrogates the melanoma cell interactions with fibroblasts, as shown previously [41], which could eliminate the supportive influence of CAFs. Regardless of the exact mechanism, the GSK3i data can be looked upon as a “proof-of-principle” of identification of drugs effective in the the highly resistant CAF-induced phenotypes. The BRAFi and GSK3i combination might represent an example of a phenotype-specific combinatorial treatment that acts on distinct (CAF-independent and CAF-dependent) phenotypes. Traditionally, combinatorial treatments seek to achieve drug synergy or additivity, though an early rationale for combination therapy was based on the hypothesis that cancer cells being resistant to one drug might be killed by a second [43]. The comparison of the proteome changes induced by the GSK3i+BRAFi combination in the two cultures conditions, nicely demonstrates that the single drug drives the effect, and the CAFs presence or absence determines which drug is decisive. The *in vivo* experiments comparing the effect of combination versus single treatment in e.g. early-stage lung metastases with extensive melanoma-fibroblast contacts, would be an important validation of our *in vitro* observations. Using such a model and assessing pS6/Ki67 levels (as a measure of treatment efficacy) in tissue sections by immunostaining, we can identify which melanoma cells (adjacent or distant from the stroma) respond to the treatment, as shown previously [19].

In conclusion, we demonstrated that the presence of CAFs induced a dedifferentiated mesenchymal-like melanoma phenotype with up-regulated inflammatory features and enhanced resistance to BRAFi and multiple other drugs. The inhibitors of PI3K-linked signaling, and particularly GSK3, showed better efficacy in the presence of CAFs and might represent drugs that target the CAF-induced phenotype. The up-regulated inflammatory traits observed in the

presence of CAFs, might be another potential target. By combining phenotype-specific drugs, an improved net therapeutic effect could be achieved in phenotypically heterogeneous tumors rich in stromal interactions.

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Conflict of Interest

The authors state no conflicts of interest.

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Figure legends

Figure 1: BRAFi effect on the viability, proteome and proliferative state of melanoma cells with/without CAFs. HM8 and Melmet 5 cells were grown as mono-cultures and co-cultures with WI-38 fibroblasts and treated with BRAFi of different concentrations (A) or 1 μM (B, C); controls received DMSO. **(A)** Relative viability of melanoma cells treated for 72 hrs; average ±

SEM (n > 3); *, p < 0.05 by unpaired t-test. **(B)** The RPPA-analysis identified proteins modulated by BRAFi (24 hrs treatment) with high significance level (p-value < 0.01 and q-value < 0.01 by two-way ANOVA) in both HM8 and Melmet 5 cells. The proteins were ranked based on the fold-change (FC); blue and red indicate down-regulated and up-regulated proteins, respectively. (**C**) Flow cytometric analysis of a proliferative state in individual melanoma cells treated with BRAFi for 48hrs. Representative contour plots (numbers indicate cell percentages) show HM8 cell subpopulations defined by pS6/Ki67 levels. The four quadrants (Q1-Q4) reflect four different cellular states with respect to the pS6/Ki67 levels. The bar-graphs indicate cell fraction in each state, average ± SEM (n=6 for HM8 and n=4 for Melmet 5); *, p< 0.05 by unpaired t-test.

Figure 2. Molecular changes induced by CAFs in melanoma cells. Melmet 5 and HM8 cells were grown as mono-cultures or co-cultures with WI-38 fibroblasts for 72 hrs. The melanoma cells were separated by FACS, and their proteome was analyzed by RPPA (A, B); cytokine expression was analyzed by real-time PCR (C). **(A)** A heat-map of the RPPA data from three independent experiments with hierarchical clustering of the conditions. Rows indicate a specific protein; columns indicate different samples. Proteins with high and low levels are indicated in red and blue, respectively. **(B)** A list of proteins modulated in the melanoma cells from the co-cultures *versus* the mono-cultures with a high significance level (p-value < 0.01 and q-value < 0.01) in both cell lines. The proteins were ranked based on the fold-change (FC); red and blue indicate up-regulated and down-regulated proteins, respectively. **(C)** Relative expression of cytokine genes in the melanoma cells from the co-cultures *versus* the mono-cultures (set to 1); average ± SEM for HM8 (n=3) or ± St.Dev. for Melmet 5 (n=2).

Figure 3: Cancer drug sensitivity screening in melanoma cells with/without CAFs. HM8 melanoma cells were grown as mono-cultures or co-cultures and treated with 0.4 μM BRAFi combined with 5 μM of different drugs from the Selleck Chemicals Cambridge compound library. After 72 hrs, melanoma cell viability was detected by measuring tumor cell-specific bioluminescence and shown as % relative to the respective non-treated controls (set to 100). **(A)** The compounds (275) were ranked based on their efficacy (from the lowest to the highest) in the mono-cultures along the X-axis and displayed as black circles; the efficacy of the corresponding treatment in the co-cultures is indicated as blue circles. The effect of the GSK3i AR-A014418 and the PI3Ki BKM120 in the co-cultures is indicated in red. **(B)** A list of compounds (and their

molecular targets) that reduce melanoma cell viability more in the co-cultures than the mono-cultures; the compounds were ranked based on the difference in efficacy (Δ).

Figure 4: Effect of PI3Ki+BRAFi combination *versus* BRAFi alone in melanoma cells with/without CAFs. HM8 mono-cultures and co-cultures were treated with 0.5 μM BRAFi (A) or 1 μM BRAFi (B, C) alone or in combination with 0.6 μM PI3Ki. **(A)** After 72 hrs, melanoma cell viability was determined by measuring tumor cell-specific bioluminescence. Data indicate average ± SEM (n=4). *, $p<0.05$ by unpaired t-test. (**B**) Flow cytometry-based analysis of apoptotic $cPARP^+$ cells after 48 hrs treatment. Representative histograms show cPARP levels (MFI) and indicate the percentage of $cPARP^+$ cells in the presented experiment. (**C**) Flow cytometric analysis of a proliferative state in individual melanoma cells treated for 48 hrs. Representative contour plots show cell subpopulations (numbers indicate percentages in the shown experiment) defined by pS6/Ki67 levels. The four quadrants (Q1-Q4) reflect four different cellular states with respect to the pS6/Ki67 levels as illustrated in Fig.1B. The bar-graphs show the fraction of cells in each state; average ± SEM (n=3); *, $p<0.05$ by unpaired t-test.

Figure 5: Effect of PI3Ki in melanoma cells with/without CAFs. HM8 mono-cultures or co-cultures were treated with 0.6 μM PI3Ki. (**A**) Melanoma cell viability was determined after 72 hrs treatment by measuring tumor cell-specific bioluminescence. Data indicate average ± SEM (n=4); *, $p<0.05$ by unpaired t-test. (**B**) Flow cytometric analysis of apoptotic $cPARP^+$ cells after 48 hrs treatment. Representative histograms show cPARP levels (MFI) and indicate the percentage of $cPARP^+$ cells in the presented experiment. (**C**) Flow cytometric analysis of a proliferative state in individual melanoma cells treated for 48 hrs. Representative contour plots show cell subpopulations (numbers indicate percentages) defined by pS6/Ki67 levels. The four quadrants (Q1-Q4) reflect four different cellular states with respect to the pS6/Ki67 levels as illustrated in Fig.1B. The bar-graphs show the fraction of cells in each state; average ± SEM (n=3); *, $p<0.05$ by unpaired t-test.

Figure 6: Effect of GSK3i+BRAFi combination *versus* BRAFi alone in melanoma cells with/without CAFs. HM8 mono-cultures or co-cultures were treated with 1 μM BRAFi alone or in combination with 4 μM (A) or 5 μM (B, C) GSK3i. **(A)** Melanoma cell viability was determined after 72 hrs treatment by measuring tumor cell-specific bioluminescence. Data

indicate average ± SEM (n=4); *, $p<0.05$ by unpaired t-test. **(B)** Flow cytometric analysis of apoptotic $cPARP^+$ cells after 48 hrs treatment. Histograms show cPARP levels (MFI) and indicate the percentage of $cPARP^+$ cells in the presented experiment. **(C)** Flow cytometric analysis of a proliferative state in individual melanoma cells treated for 48 hrs. Representative contour plots show cell subpopulations (numbers indicate percentages) defined by pS6/Ki67 levels. The four quadrants (Q1-Q4) reflect four different cellular states with respect to the pS6/Ki67 levels as illustrated in Fig.1B. The bar-graphs show the fraction of cells in each state; average ± SEM (n=3); *, $p<0.05$ by unpaired t-test.

Figure 7: Effect of GSK3i in melanoma cells with/without CAFs. HM8 mono-cultures or co-cultures were treated with 4 μM (A) or 5 μM (B, C) GSK3i. **(A)** Melanoma cell viability was determined after 72 hrs treatment by measuring tumor cell-specific bioluminescence. Data indicate average ± SEM (n=4); *, $p<0.05$ by unpaired t-test. **(B)** Flow cytometric analysis of apoptotic $cPARP^+$ cells after 48 hrs treatment. Histograms show cPARP levels (MFI) and indicate the percentage of $cPARP^+$ cells in the presented experiment. **(C)** Flow cytometric analysis of a proliferative state in individual melanoma cells treated for 48 hrs. Representative contour plots show cell subpopulations (numbers indicate percentages) defined by pS6/Ki67 levels. The four quadrants (Q1-Q4) reflect four different cellular states with respect to the pS6/Ki67 levels as illustrated in Fig.1B. The bar-graphs show the fraction of cells in each state; average ± SEM (n=3); *, $p<0.05$ by unpaired t-test.

Figure 8. Clustering of proteome changes induced by different treatments in the presence and absence of CAFs. HM8 melanoma cells were grown as mono-cultures or co-cultures for 48 hrs before treatment with 1 μM BRAFi, 5 μM GSK3i or their combination for 24 hrs; controls were exposed to DMSO. The melanoma cells were isolated by FACS, and their proteome was analyzed by RPPA. The protein levels in the treated samples were normalized to the levels in the controls. The normalized data were subjected to hierarchical clustering and presented as a heat-map, where rows indicate a specific protein and columns indicate different samples. Proteins with high and low levels are indicated in red and blue, respectively.

Supplementary Figure S1: A strategy for flow cytometric analysis. Four samples (control, treated with drug #1, drug #2, and their combination (Combo)) from mono-cultures and co-cultures were barcoded with increasing concentrations of a dye like pacific orange. The four

barcoded samples were mixed together and stained with antibodies of interest. Based on GFP signal, GFP-positive melanoma cells are distinguished from GFP-negative fibroblasts (illustrated in **A**). Based on pacific orange signal, the four differently-treated samples are distinguished, so that the level of the proteins in each of them can be analyzed. **A1** and **A2** illustrate the levels of pS6-Alexa Fluor 647 vs. Ki67-PE (shown as contour plots (**A1**) and cPARP-PE (shown as histograms) (**A2**) in the "Combo" sample. The other samples were analyzed identically.

Supplementary Figure S2: The levels of selected proteins in mono-cultured and co-cultured melanoma cells as detected by SWI and intracellular flow cytometry. HM8 and Melmet 5 melanoma cells were grown as mono-cultures or co-cultures for 72 hrs. (**A**) The melanoma cells were isolated by FACS and analyzed for the indicated proteins by Simple Western immunoassay. Representative electropherograms and quantifications of relative levels (RL) of the proteins are shown. The Y-axis in the electropherograms indicates chemiluminescence signal intensity and reflects the amount of the protein while the X-axis shows the protein size (kDa). The bars present protein level in co-cultures compared to mono-cultures (set to 1). The protein levels were quantified by integration of the peak area and normalization to the loading control β-actin. (**B**) The melanoma cells were stained for the indicated proteins and analyzed by flow cytometry. Histogram overlays indicate the protein levels in co-cultured and mono-cultured melanoma cells.

Supplementary Figure S3: Effect of treatment containing PI3Ki compared to BRAFi in Melmet 5 melanoma cells in mono-cultures and co-cultures. Melmet 5 cells were grown as mono-cultures or co-cultures and treated with 0.5 μM BRAFi, 0.6 μM PI3Ki or their combination for 72 hrs. Melanoma cell viability was determined by measuring tumor cell-specific bioluminescence; average ± SEM (n=3). *, $p<0.05$ by unpaired t-test.

Supplementary Figure S4: GSK3i stimulates Wnt/β-catenin signaling in HM8 melanoma cells. Non-labeled HM8 cells were grown as mono-cultures and transfected with a Wnt-reporter, a plasmid carrying TCF/LEF-luciferase. The cells were treated with 5 μM GSK3i for 24 hrs before luciferase activity was assessed by measuring luminescence, shown on the Y-axis as relative luminescence units (RLU). The non-transfected cells treated with GSK3i, and transfected but non-treated cells, served as controls.

Supplementary Figure S5: Effect of treatment containing GSKi compared to BRAFi in Melmet 5 melanoma cells in mono-cultures and co-cultures. Melmet 5 melanoma cells were grown as mono-cultures or co-cultures and treated with 1 μM BRAFi, 15 μM GSK3i or their combination for 72 hrs. Melanoma cell viability was determined by measuring tumor cell-specific bioluminescence; average ± SEM (n=3). *, $p<0.05$ by unpaired t-test.

Supplementary Figure S6: The influence of GSK3i on pS6 levels in BRAFi-teated mono-cultured and co-cultured melanoma cells. HM8 mono-cultures and co-cultures were treated with 1 μM BRAFi alone or in combination with 5 μM GSK3i (controls were not treated). After 24 hrs- and 48 hrs- treatment, the cells were stained for pS6 and analyzed by flow cytometry. The levels of pS6 (MFI) in controls, BRAFi-treated and GSK3i+BRAFi-treated melanoma cells are presented as histogram overlays.