Metabolic reprogramming supports the invasive phenotype in malignant melanoma

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Abstract

Invasiveness is a hallmark of aggressive cancer like malignant melanoma, and factors involved in acquisition or maintenance of an invasive phenotype are attractive targets for therapy. We investigated melanoma phenotype modulation induced by the metastasis-promoting microenvironmental protein S100A4, focusing on the relationship between enhanced cellular motility, dedifferentiation and metabolic changes. In poorly motile, well-differentiated Melmet 5 cells, S100A4 stimulated migration, invasion and simultaneously down-regulated differentiation genes and modulated expression of metabolism genes. Metabolic studies confirmed suppressed mitochondrial respiration and activated glycolytic flux in the S100A4 stimulated cells, indicating a metabolic switch towards aerobic glycolysis, known as the Warburg effect. Reversal of the glycolytic switch by dichloracetate induced apoptosis and reduced cell growth, particularly in the S100A4 stimulated cells. This implies that cells with stimulated invasiveness get survival benefit from the glycolytic switch and therefore, become more vulnerable to glycolysis inhibition. In conclusion, our data indicates that transition to the invasive phenotype in melanoma involves dedifferentiation and metabolic reprogramming from mitochondrial oxidation to glycolysis, which facilitates survival of the invasive cancer cells. Therapeutic strategies targeting the metabolic reprogramming may therefore be effective against invasive phenotype.

Keywords: Melanoma, Phenotype switch, Warburg effect, S100A4, Metabolic reprogramming.

Abbreviations: DCA, dichloracetate; LDH, lactate dehydrogenase; MITF, Microphthalmia-associated transcription factor; NMR, nuclear magnetic resonance; PGC1-α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha. PPP, pentose phosphate pathway; TCA, tricarboxylic acid.

1. Introduction

Metastasis relies on cancer cells with phenotypic plasticity to enable accomplishment of all steps in the metastatic cascade. An important example of such plasticity is the transition to an invasive (mesenchymal) phenotype, which not only facilitates dissemination from a primary tumor and extravasation at distant sites [1], but also can promote therapy resistance [2]. Invasiveness is consequently considered as a hallmark of aggressive, metastatic cancer cells, and factors involved in acquisition or maintenance of the invasive phenotype might be highly relevant targets in anti-metastasis therapy.

Malignant melanoma is one of the most aggressive forms of human cancer, and melanoma cells exhibit considerable phenotypic plasticity [3]. Comparison of melanoma cell lines with high *versus* low invasive capacity revealed that the melanocyte differentiation genes controlled by the master regulator of the lineages, Microphthalmia-associated transcription factor (MITF), were among the signature genes clearly distinguishing the two phenotypes. While highly expressed in poorly invasive cells, low expression in the counterpart indicated that invasive cells are less differentiated [4, 5]. Further signifying the importance of the dedifferentiation state for melanoma aggressiveness, is the observation that less differentiated melanoma cells show higher resistance to therapy [6]. Although an association between aggressiveness and invasive dedifferentiated phenotype has been acknowledged, the mechanisms involved in acquisition of such phenotype are not fully clarified. It has been reported previously that melanoma cells can switch between proliferative/differentiated and invasive/dedifferentiated phenotypes during metastasis progression [7, 8]. Further, it was proposed that the tumor microenvironment plays a significant role in regulation of the phenotype switch [7], due to a multitude of factors present in

the microenvironment that may influence the malignant phenotype [9]. Among such factors are members of the S100-protein family that affect many cellular processes including cell migration and invasion [10]. A number of these proteins, such as S100A4 (also known as metastasin (Mts1) or fibroblast-specific protein 1 (FSP1)) are associated with metastasis and poor prognosis in several cancer types, including melanoma [11]. Both cancer cells and stromal cells express and secrete S100A4, actualizing the protein as an important factor in the tumor microenvironment [12-16]. Extracellular S100A4 has been shown to induce motility in several cancer types [17, 18]. The protein is also known as a regulator of epithelial-mesenchymal transition (EMT), being particularly enriched in mesenchymal, stem cell-like subpopulations of carcinoma [2, 19].

Metabolic plasticity has emerged as an important feature to aid cancer cells during tumor progression. Cancer cells can utilize glycolysis rather than oxidative phosphorylation even in the presence of oxygen (known as the Warburg effect) [20, 21]. Aerobic glycolysis assures not only supply of energy and building blocks for fast proliferating cells, but can also be beneficial for invasive, metastatic cells [22]. However, the relationship between metabolism and metastasis is poorly understood, and e.g. mitochondrial oxidation has been linked with both pro-metastatic [23] and anti-metastatic [22, 24] effects. Interestingly, it has been recently shown that the main regulator of melanocyte differentiation, MITF controls an important regulator of mitochondrial metabolism, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) [25, 26]. These reports suggest interconnectivity between differentiation and metabolic pathways, and further propose that development of the invasive dedifferentiated phenotype could involve metabolic reprogramming.

In the present study, we investigated phenotype modulation induced by the pro-metastatic protein S100A4 in malignant melanoma *in vitro,* focusing on the relationship between enhanced cellular motility, differentiation status and metabolic alterations. We revealed that upon stimulation with S100A4, melanoma cells acquire the invasive dedifferentiated phenotype and simultaneously switch their metabolism to glycolysis. This data supports the concept of glycolytic flux as a metastasis-associated mechanism, and proposes metabolic inhibitors as a promising approach against metastatic cells.

2. Materials and methods

2.1. Cell lines

The malignant melanoma cell lines Melmet 1 and Melmet 5 were established at the Norwegian Radium Hospital (Norway) as described previously [27]. The cells were cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 10 % fetal calf serum (FCS) (PAA, Austria) and 2 mM GlutaMAX (Gibco, UK). All cells cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and were routinely tested for mycoplasma contamination and cell ID.

2.2. Compounds

Human recombinant S100A4 was produced as described previously [18], and routinely used at 2 μg/ml to stimulate (typically for 48 hrs) the melanoma cells growing in RPMI with 5 % FCS. Dichloracetate (DCA) was purchased from Sigma-Aldrich (St.Louis, MO) and dissolved in PBS to a 2M stock solution.

2.3. Migration and invasion assays

Cell migration and invasion was measured in a 24-well trans-well plate (triplicate wells) with 8 μm pore size filter inserts (Costar, Cambridge, MA). For the invasion assay, the inserts were

coated with 50 μg Matrigel (BD Biosciences, Bedford, MA). Fifty thousand [^{3}H] thymidine (PerkinElmer, Waltham, MA)-labeled melanoma cells were seeded out in the upper compartment, and medium with/without S100A4 was applied in the lower chamber. After incubation for 48 hrs, the cells from the upper and the lower side of the filter were harvested separately and analyzed in a liquid scintillation analyzer (Packard Instrument Company, Chicago, IL) to measure [^{3}H] counts. The migration/invasion was evaluated by quantification of the [^{3}H] counts from the lower side compared to the total [^{3}H] counts from both sides of the inserts. Additionally, the migration was scored by the wound healing assay as described in the Supplementary Methods.

For separation of the migrated and non-migrated cell fractions, the migrated cells from under the filter and the non-migrated cells remaining above the filter were harvested as illustrated in Fig. 2D, and transferred to separate tubes with TRI Reagent® (Invitrogen, Carlsbad, CA) for isolation of RNA as described below.

2.4. Cell proliferation and survival

To follow cell proliferation over time, 4000 melanoma cells were seeded out in 96-well plates (Falcon Corning, Durham, NC) and incubated with/without S100A4, tracking the cell growth by IncuCyte™ live cell imaging system (IncuCyte, Essens Bioscience, U.K.). Phase contrast pictures were collected every 2-3 hrs, and the percentage of cell confluence was calculated as a measure of cell proliferation. To evaluate the effect of DCA, the cells were pre-incubated with/without S100A4 before DCA was added for additional 2 day-treatment. The cell growth was followed by IncuCyte. Finally, cell survival was measured by the CellTiter® 96 AQ_{ueous} Non-radioactive Cell Proliferation Assay (Promega, Madison, WI), measuring absorbance at 490 nm using a Victor plate reader (Wallac Oy, Turku, Finland).

2.5. Detection of apoptosis by Annexin V

For detection of apoptosis, 70000 cells were seeded out in a 12-well plate, pre-incubated with/without S100A4 for 48 hrs before DCA was added for additional 1 day-treatment. After harvesting, the cells were re-suspended in 100 µl staining buffer with 5 µl Annexin V FITC (BD Pharmingen, San Jose, CA) and stained at room temperature for 15 min followed by a quick staining with 1 µg/ml propidium iodide (PI). The samples were analyzed on LSRII flow cytometer (BD Bioscience), and the data were analyzed using FlowJo software (FlowJo, Ashlan, OR).

2.6. Measurements of lactate, glucose, LDH enzymatic activity and ATP

The level of lactate and glucose in culture medium was measured by the nuclear magnetic resonance (NMR) analysis as described below, or by blood gas analyzer GEM Premier 4000 (Instrumentation Laboratory, Bedford, MA). Additionally, the lactate concentration was measured by Lactate Colorimetric Assay (Biovision, Milpitas, CA) following the manufacturer instructions. The enzymatic activity of intracellular lactate dehydrogenase (LDH) was measured in 96-well plates by using the Pierce LDH Cytotoxicity Assay Kit (Thermoscientific, Rockford, IL) following the manufacturer instructions. For ATP, the cells were cultured in 96-well white plates (Corning, NY), and the ATP level was detected by CellTiter-Glo® Luminescent Cell Viability-assay (Promega, Madison, WI) measuring ATP-dependent bioluminescence on a Victor plate reader.

2.7. Gene expression analysis

Melmet 1 and Melmet 5 cells were cultured with/without S100A4 for 48hrs, and total RNA was isolated using TRI Reagent®. For global gene expression analysis, Illumina microarray hybridization and data analysis was performed as previously described [15]. Briefly, total RNA

(750 ng) were used for labeling and hybridization according to manufacturers protocol (Illumina Inc, San Diego, CA) using Illumina Human HT-12v4 Expression BeadChip. Preprocessed data was imported into J-Express v2012 (www.molmine.com) to identify differently expressed genes by Significance of Microarray (SAM) analysis. Genes were considered significant if fold change FC ≥ 1.5 and false discovery rate FDR ≤ 5%. The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE65897 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65897).

The expression of selected genes was examined by qPCR. One ug RNA was reverse transcribed using qScript cDNA Syntesis Kit (Quanta Biosciences, Gaithersburg, MD) and qPCR reactions were performed on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All qPCR reactions were run in duplicates in 25 µl volume containing 25 µg cDNA, 200 nM FAM-labeled probe, 300 nM of each primer and 1x Perfecta qPCR Supermix (Quanta BioSciences). All primers were designed using the probe finder software from Roche Applied Science available online at the Universal Probe Library Assay Design Center. The probes were from the Universal Probe Library collection (Roche Applied Science, Germany). The primer sequences and probe numbers are listed in the Supplementary Table S1. Relative gene expression was calculated by the ΔΔCt method.

2.8. Immunoflourescence

Melanoma cells were grown on glass cover-slips with/without S100A4 for 48 hrs, fixed in 4% paraformaldehyde for 15 min on ice before staining over night with mouse anti-MITF (Thermo Scientific, Waltham, MA) diluted in PBS/0.05% saponin 1:100. After staining for 60 min with donkey anti-mouse 488 (Jackson ImmunoResearch, West Grove, PA) diluted 1:500, the cover-slips were mounted in Prolong Gold mounting medium containing DAPI (Life Technologies,

Carlsbad, CA). Fluorescent images were obtained using Zeiss LSM710 confocal microscope equipped with Plan-Apochromat X 63/1.4 Oil DICII objective and analyzed using the ZEN 2011 software and Adobe Photoshop CS5.

2.9. Immunoblotting

Protein lysates were prepared in lysis buffer (50mM Tris-HCl, 150 mM NaCl and 0.1% NP-40, pH 7.5) supplemented with the protease- and phosphatase-inhibitor cocktails, CompleteMini and PhosSTOP (Roche, Manmheim, Germanys). Total cellular proteins (20 μg) were separated by SDS-PAGE, in a NuPAGE® Novex Bis-Tris Gel, 4-12% (Invitrogen) and subsequently electro-transferred to a 0.45 μm PVDF membrane (Merck Millipore, Darmstadt, Germany). The membrane was incubated overnight at 4 °C with the primary antibodies: PGC1-α (#4259, Cell Signaling Technology, Danvers, MA) diluted 1:1000 and α-tubulin (#CP06, Calbiochem, Merck Millipore) diluted 1:5000. Appropriate horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) were used for visualization in a G:BOX instrument (Syngene, Cambridge, UK) using the GeneSnap software. Relative PGC1-α level normalized to α-tubulin was quantified using the Gene Tools densitometry software (Syngene, Cambridge, UK).

2.10. Oxygen consumption and extracellular acidification rate

The oxygen consumption rate (OCR, pmol/min) and extracellular acidification rate (ECAR, mpH/min) were measured using XF^{e}96 or XF^{e}24 Extracellular Flux Analyzers from Seahorse Bioscience (North Billerica, MA) following the manufacturer's protocol. Briefly, the melanoma cells were seeded out at a density 5000-7000 cells/96-well (minimum 10 parallels for each treatment) or 15000 cells/24-well (minimum 5 parallels for each treatment) and incubated with/without S100A4 for 48 hrs. Before the analysis, the cells were re-suspended in the Seahorse

assay medium, and the drugs from the XF Cell Mito Stress test kit (Seahorse Bioscience) were added: oligomycin (1μM), FCCP (1 μM and 0.5 μM for Melmet 1 and Melmet 5, respectively) and rotenone/antimycin A mix (1 μM each). To evaluate the effect of DCA, the cells were pre-incubated with/without S100A4 for 48 hrs before DCA was injected through an injection port, giving a final concentration of 10mM. The data were analyzed by XF^{e} Wafe software (Seahorse Bioscience).

2.11. NMR analysis of cell culture medium

Melmet 1 and Melmet 5 cells were incubated with/without S100A4 for 48 hrs, and 500 μl aliquots of culture medium were mixed with 100 μl of D_2O. The MR spectroscopy was performed at using a Bruker Avance III Ultrashielded Plus 600 MHz spectrometer (Bruker Biospin GmbH, Germany), equipped with a 5 mm QCI Cryoprobe with integrated, cooled preamplifiers for 1H, 2H and 13C. Proton spectra were acquired using 1D NOESY (Bruker: noesygppr1d) with presaturation and spoiler gradients as described previously [28]. The spectra were collected with 32 scans and 4 dummy scans. The acquisition time was 2.65 sec, measuring the FID via collection of 64 K complex data points. Sweep width was 20.5682 ppm. Spectra were Fourier transformed to 128 K after 0.3 Hz exponential line broadening. The glucose signal at 5.24 ppm and the lactate signal at 1.33 ppm were identified. After baseline correction, the amount of glucose and lactate in the culture medium was assessed by integration of the signals.

2.12. NMR analysis of cell extracts

Melmet 5 cells were pre-incubated with or without S100A4 for 48 hrs (estimated to reach a cell number of ~50 millions) and subsequently incubated for 6 hrs in RPMI medium, where glucose was substituted with 2 g/L [1,2 -13C] glucose (Sigma). The cells were harvested in ice-cold 80% methanol, and spun at 18 000 rpm for 15 min at 4 °C. The cell extracts were vacuum dried and

stored at -80 °C until NMR analysis. The extracts were reconstituted in 680 µl PBS/D_2O buffer solution containing 1 mM trimethyl-silyl propionic acid (TSP) as a chemical shift reference. Proton spectra were acquired using 1D NOESY (Bruker: noesygppr1d) sequence as described above. Proton decoupled 13C spectra were acquired using a power gated coupling sequence with a 30º pulse angle (Bruker: zgpg30). The spectra were collected with 16 K scans and 16 dummy scans. The acquisition time was 1.65 sec, measuring the FID via collection of 96 K complex data points over a sweep width of 197.175 ppm. All the experiments were carried out at a constant temperature of 300 K. Spectral assignments (Supplementary Table S2) were done on the basis of 1D: NOESY, 2D: HSQC (Supplementary Fig. S1) and COSY (Supplementary Fig. S2) spectra, human metabolome database (HMDB; www.hmdb.ca) and previously published data [29]. The 13C spectra were Fourier transformed with a 3.0 Hz exponential line broadening and the chemical shift was calibrated to the TSP peak (δ0 ppm). After baseline correction, the amounts of [4-13C] glutamate, [3-13C] glutamate, [2,3-13C] lactate, [3-13C] lactate, [3-13C] serine, [2-13C] glycine, [2,3-13C] alanine and [2-13C] α-glucose in the extracts were assessed by integration of the signals and normalized to the amount of protein.

2.13. Statistical analyses

Statistical analysis were performed using two-tailed Student`s t-test (specified in the figure legend when paired analysis was used). The threshold for statistical significance was defined as $p \leq 0.05$.

3. Results

3.1. S100A4 stimulates motility in poorly migratory melanoma cells

Given the importance of invasiveness in the metastatic process, we investigated whether melanoma cells gain invasive properties under the influence of S100A4. Two melanoma cell

lines, Melmet 1 and Melmet 5, representing the highly invasive and poorly invasive phenotype, respectively [30], were utilized. By use of a trans-well chamber assay, we showed that both migration and invasion were stimulated by S100A4 in the poorly motile Melmet 5 cells, with a fold increase of 1.8 and 2.4, respectively (Fig. 1A) (from 14% to 24% migration, and from 0.5% to 1.3% invasion, Supplementary Fig. S3A). No changes were seen in the highly motile Melmet 1 cells (~ 60 % migration and invasion regardless S100A4). The S100A4 mediated increase of migration in Melmet 5, and no effect in Melmet 1 was confirmed by the wound healing assay (Supplementary Fig. S3B). S100A4 did not change the proliferation rate in any of the cell lines, indicating no effect on cell growth (Fig. 1B). Taken together, these results indicate that extracellular S100A4 stimulates invasiveness in poorly motile melanoma cells.

3.2. S100A4-induced cell migration correlates with dedifferentiation

To further elucidate how extracellular S100A4 can contribute to the acquisition of the invasive phenotype, we performed a global gene-expression analysis of S100A4 stimulated *versus* non-stimulated Melmet 1 and Melmet 5 cells. We focused on the 105 genes defined by Hoek *et al.* [5] as the non-invasive/invasive signature genes. The most apparent S100A4 induced alterations were observed in Melmet 5, where we found down-regulation of melanocyte differentiation genes controlled by the master regulator of the lineage, MITF (Fig. 2A). The Melmet 1 cells, which generally express low levels of the differentiation genes, showed no clear changes in response to S100A4. Analysis by qPCR confirmed the down-regulation of MITF and its target genes, MLANA and TYR in Melmet 5 (Fig. 2B), but not in Melmet 1 (Supplementary Fig. S4A). Furthermore, the down-regulation of MITF protein in Melmet 5 was validated by immunofluorescence, where the number of highly positive MITF nuclei was reduced in S100A4 stimulated cells (Fig. 2C). No S100A4 dependent changes in the MITF protein level (which was

very low) were seen in Melmet 1 (Supplementary Fig. S4B). S100A4-mediated down-regulation of MITF was also seen in several other melanoma cell lines (Supplementary Fig. S4C), but this was not further pursued in the study.

To further investigate the molecular characteristics of the migrated *versus* the non-migrated Melmet 5 cells, we separated these cell fractions from trans-well inserts as illustrated in Fig. 2D. We found significantly reduced expression of MLANA and TYR in the migrated cells (Fig. 2E), validating the notion that motile cells are less differentiated. The lowest level of the differentiation genes was observed in the migrated cells stimulated with S100A4 (Fig. 2E). Taken together, these observations suggest that extracellular S100A4 promotes a dedifferentiated state associated with increased cell motility.

3.3. S100A4 modulates the expression of genes associated with metabolism

In addition to the differentiation genes, our global gene expression analysis revealed S100A4 influence on metabolism genes in Melmet 5 cells (Table 1). Some of these genes, including NNMT, KYNU and AKR1C1/2 have been linked to the invasive transcriptional signature identified by Hoek *et al.* [5] and Jeffs *et al.* [4]. Interestingly, NNMT, which appears to regulate cell migration [31], was one of the top genes that correlated with S100A4 in breast cancer patients when analyzed by bc-GenExMiner (data not shown). The S100A4-mediated regulation of genes involved in different metabolic activities was validated by qPCR, revealing much higher changes in Melmet 5 cells compared to Melmet 1 (Fig. 3A). The reactive oxygen species (ROS) detoxification genes (SOD-2 and metallothioneins), the critical glycolysis-associated gene (LDHA), and the genes regulating NAD^+/NADH levels (NNMT, NAMPT and KYNU), were significantly up-regulated in Melmet 5 cells (Fig. 3A). On the contrary, the gene involved in mitochondrial biogenesis/activity, PGC1-α was down-regulated in Melmet 5, which was further

verified by Western blotting (Fig. 3B). Furthermore, the lower expression of PGC1-α was identified in the migrated *versus* the non-migrated Melmet 5 cells, and the lowest PGC1-α expression was observed in the migrated cells stimulated with S100A4 (Fig. 3C). Based on these results, we hypothesized that extracellular S100A4 adjusts cellular metabolism to support the invasive phenotype.

3.4. S100A4 suppresses mitochondrial activity and potentiates glycolysis

The observed differences in metabolism genes motivated further studies on relevant metabolic parameters in S100A4 stimulated *versus* non-stimulated cells. Oxygen consumption rate (OCR), reflecting mitochondrial respiration, was evaluated by an Extracellular Flux Analyzer. OCR was measured under basal conditions and after sequential addition of compounds targeting mitochondrial oxidation: oligomycin (inhibits ATP synthase), FCCP (uncouples ATP synthesis from the electron transport chain) and an antimycin A/rotenone mix (inhibits complex III and I in the respiration chain). In Melmet 5, but not Melmet 1 cells both basal respiration and maximal respiration were significantly lower in the S100A4 stimulated cells compared to the non-stimulated controls (Fig. 4A and B). The same differences were observed when the OCR data were normalized to DNA content in each well (data not shown). The observation that S100A4 reduces OCR correlates nicely with the S100A4 mediated down-regulation of PGC1-α (Fig. 3), together suggesting a suppressive influence of the protein on mitochondrial activity in Melmet 5 cells.

Extracellular acidification rate (ECAR), which reflects glycolysis-associated proton release, was enhanced in S100A4 stimulated Melmet 5 cells, but not Melmet 1 (Fig. 4C). Furthermore, the ratio between ECAR and OCR was enhanced in Melmet 5 upon stimulation with S100A4

(Fig. 4 D), indicating a metabolic shift towards more glycolytic phenotype (Supplementary Fig. S5).

To validate S100A4 effect on glycolysis, we measured the level of remaining glucose and released lactate in the cell culture medium from S100A4 stimulated *versus* non-stimulated cells by proton NMR spectroscopy. As visualized in the example spectrum in Figure 5A, Melmet 5 cells, but not Melmet 1 consumed more glucose (less glucose left in the medium) (Fig. 5B) and produced more lactate (Fig. 5C) upon stimulation with S100A4. Furthermore, the ratio between lactate and glucose levels was increased in S100A4 stimulated Melmet 5 cells (Fig. 5D), indicating potentiated conversion of glucose to lactate. Similar changes in lactate and glucose levels were observed when analyzing the culture medium using a blood gas analyzer or a Lactate Colorimetric Assay Kit (Supplementary Fig. S6). The latter also revealed that S100A4 dependent elevation in lactate secretion in Melmet 5 was detectable as late as day 2 after the protein addition (Supplementary Fig. S6C). This suggests that S100A4 stimulated potentiation of glycolysis is a relatively slow/late event.

We exclude the possibility that the observed effects on OCR, ECAR, glucose and lactate levels could be due to a higher number of cells in the S100A4-samples. First, S100A4 does not influence cell proliferation, as shown in Fig. 1B. Secondly, we counted the cells (Supplementary Fig. S7), evaluated total DNA content by staining with PicoGreen (data not shown) and measured total protein amount (absorbance at 280 nm) by a NanoDrop Instrument (data not shown) in S100A4 stimulated *versus* non-stimulated samples. No significant differences in any of these parameters were observed, confirming that S100A4 does not increase cell number/proliferation.

To note, we did not detect any differences in the levels of ROS (detected by the ROS sensitive probe DCFH-DA), NAD^+/NADH (detected by Abcam NAD^+/NADH assay kit) or ATP (detected by CellTilter-Glo assay) in cells stimulated with S100A4 *versus* non-stimulated controls (data not shown).

3.5. Exploring glucose metabolic flux by 13C NMR

Since analysis of the culture medium indicated increased glucose consumption upon S100A4 stimulation, the metabolic fate of glucose was further examined using 13C NMR. Tracing downstream metabolites of [1,2-13C] glucose in cell extracts from S100A4 stimulated *versus* non-stimulated Melmet 5 cells allowed assessment of glycolytic flux as well as the utilization of glucose in different biochemical pathways as illustrated in Figure 6. Although, we saw a slight increase in the level of intracellular lactate, the difference was not statistically significant (Fig. 7). This might be due to rapid secretion of the produced lactate out from the cells, and the significantly elevated level of lactate in the culture medium (Fig. 5) supports this possibility. Importantly, we found that the levels of [4,5-13C] glutamate, a product of the tricarboxylic acid (TCA) cycle, were significantly reduced ($p=0.038$) in the S100A4 stimulated cells (Fig. 7). This indicates that S100A4 suppresses the activity of the TCA cycle, in accordance with the reduced oxygen consumption observed in Figure 4. TCA cycle turnover rate was assessed by comparing the relative enrichment of 13C in the 4-C and 3-C (not formed until glutamate has completed at least one full turn in the TCA cycle) positions of glutamate. No changes in their relative levels were observed, indicating that S100A4 did not have any effect on the turnover rate.

Glucose has several alternative metabolic fates, and we therefore explored the flux of glucose into secondary pathways. As illustrated in Figure 6, lactate is not only formed through glycolysis, but also via the pentose phosphate pathway (PPP). Glycolysis yields [2,3-13C]

lactate, whereas PPP yields [3-13C] lactate. The ratio between these isotopomers was not significantly altered by S100A4, indicating that the protein does not alter the fraction of glucose entering PPP. In addition, we measured the levels of [3-13C] serine and [2-13C] glycine. These amino acids are formed when glucose exits glycolysis at the level of 3-P-glycerate through the action of phosphoglycerate dehydrogenase (PHGDH). Even though we saw reduced expression of PHGDH in response to S100A4 (Table 1), no significant change in the levels of [3-13C] serine and [2-13C] glycine in cell extracts was observed (Fig. 7B). Finally, we found that the levels of [2,3-13C] alanine, which is formed from pyruvate, were unchanged by S100A4. This further supports the notion that the primary metabolic effect of S100A4 stimulation is increased production of lactate, likely through up-regulation of LDH, which generates lactate from pyruvate. To validate the involvement of LDH, we measured its enzymatic activity in S100A4 stimulated cells *versus* non-stimulated controls. We observed a small, but statistically significant increase of 7 ± 1.8 % ($p=0.01$ by paired t-test, $n=4$) (data not shown).

In summary, the NMR analysis suggests that S100A4 selectively regulates the metabolic fate of glucose at the level of pyruvate, favoring aerobic lactate formation and suppressing the entry into the TCA cycle.

3.6. S100A4 stimulated cells are more vulnerable to glycolysis inhibition

To further explore the significance of the potentiated glycolysis for the S100A4 stimulated cells, we treated the cells with DCA aiming to reverse the Warburg effect. DCA inhibits pyruvate dehydrogenase kinase, thereby shifting pyruvate metabolism from lactate (glycolysis) into the TCA cycle [32]. To validate the DCA effect on metabolism, we followed changes in ECAR and OCR in Melmet 5 cells. We observed DCA-induced reduction in ECAR and increase in OCR, which was accompanied by elevation in the ATP level (Fig. 8A-C). Collectively, this validates

that DCA shifts the metabolism from glycolysis to mitochondrial oxidation, i.e. the opposite direction than seen with S100A4.

To investigate whether the reversal of the glycolytic switch by DCA prevents S100A4 effects on cell motility and dedifferentiation, we treated Melmet 5 cells with DCA followed by S100A4 and scored cellular migration and expression of the differentiation genes. The DCA treated cells demonstrated doubling in migration and down-regulation of the differentiation genes in response to S100A4 (data not shown), similar to the cells without DCA (shown in Fig. 1A and 2B). This indicates that DCA-reduced glycolysis and potentiated mitochondrial oxidation does not prevent the transition to the invasive phenotype.

Next, we asked how the reversal of the glycolytic switch by DCA affects survival and growth of S100A4 stimulated cells compared to non-stimulated controls. We observed that S100A4 pre-stimulation potentiated the DCA effect on apoptosis and growth inhibition in Melmet 5 cells (Fig. 9), but not in Melmet 1 cells (Supplementary Fig. S8). Thus, we found that S100A4 stimulated Melmet 5 cells showed slower growth (Fig. 9A), reduced cell survival (Fig. 9B) and contained a substantially higher fraction of apoptotic cells (Fig. 9C and D) compared to the non-stimulated controls after DCA treatment. This indicates that S100A4 stimulated cells that undergo phenotype transition, gain survival benefit from the glycolytic switch and, consequently, become more vulnerable to glycolysis inhibition.

Collectively, our results indicate that S100A4 promotes the invasive dedifferentiated phenotype and also alters cellular metabolism by attenuating mitochondrial activity and potentiating the glycolytic pathway. Pharmacological interference with such metabolic reprogramming forces the S100A4 stimulated cells into apoptosis/growth arrest, signifying further studies on metabolic inhibitors as a promising approach against metastatic cells.

4. Discussion

In the present study, we explored how melanoma cells may switch from a non-invasive state to an invasive phenotype, and revealed cell dedifferentiation and metabolic reprogramming as important features related to this transition. In well-differentiated, poorly motile melanoma cells, we observed enhanced migration/invasion, dedifferentiation and a metabolic switch to glycolysis upon stimulation with the metastasis-promoting protein S100A4. None of these properties were induced by S100A4 in highly invasive melanoma cells. On this basis, we hypothesized that enhanced cellular motility and alterations in differentiation and metabolism may be interconnected.

Our conclusion that S100A4 stimulated cells increase glycolytic flux and reduce mitochondrial oxidation was supported at both, a metabolic and a transcriptional level. The increase in glucose uptake, secretion of lactate, ECAR and LDHA gene expression indicates stimulated glycolysis. The decrease in OCR, entry of glucose into the TCA cycle and PGC1-α gene expression indicates reduced mitochondrial oxidation. Consistent with our findings, several studies have linked attenuation of mitochondrial metabolism/down-regulated PGC1-α to tumor promotion and metastasis [24, 33]. In contrast, LeBleu *et al.* have shown that PGC1-α/oxidative phosphorylation stimulate cell migration and metastasis in breast cancer [23]. The contribution of mitochondrial oxidation could be context-dependent, and therefore different in breast cancer and melanoma. A such difference has been reported previously; it was shown that Wnt5a (a known driver of the invasive phenotype in melanoma) activates glycolysis in melanoma in contrast to oxidative phosphorylation in breast cancer [34].

In line with our observations that pro-metastatic factor S100A4 activates glycolysis, Liu *et al.* reported that an anti-metastasis factor KISS1 activates mitochondrial oxidation [24]. The reverse

metabolic switch induced by the metastasis suppressor KISS1, and the metastasis promoter S100A4, strongly proposes an association between regulation of metastasis and metabolism. Both studies support the concept of glycolysis as a metastasis-associated mechanism. This is in agreement with the fact that high serum levels of LDH (a critical enzyme of lactate production in the final stage of glycolysis) is a biomarker for poor prognosis in different cancers, including melanoma, where LDH is also used as a part of staging for metastatic disease [35, 36]. Switching from oxidative phosphorylation to glycolysis may be beneficial for metastatic cells due to several reasons. First, a common explanation why cancer cells exploit aerobic glycolysis is the supplement of "building blocks" needed for proliferation. However, S100A4 neither increased cell proliferation nor enhanced shunting of glucose through the PPP and the glycine-serine pathways that provide nucleotides and amino acids. This proposes that S100A4 stimulated cancer cells adjust their metabolism to support invasiveness, not proliferation. Second, the reliance on glycolysis may provide survival benefits in episodes of restricted oxygen supply faced by metastatic cells during dissemination and organ colonization. Third, matrix-detached disseminating cells are particularly sensitive to ROS, a consequence of oxidative phosphorylation [37]. By switching to glycolysis and thereby reducing accumulation of damaging ROS, the invading cells could achieve survival benefit. In line with this, we observed that S100A4 stimulated cells up-regulate antioxidants like SOD-2, indicating a combined attempt to reduce oxidative stress. Fourth, extracellular acidification/lactate release can modulate the tumor microenvironment to promote metastasis e.g. activate proteases that facilitate invasion [38], or "educate" pro-tumorigenic stromal cells [39]. Such "education" takes place upon stimulation with S100A4, as we have shown previously [15]. Finally, glycolysis might support a dedifferentiation state, and less differentiated melanoma cells are more invasive as shown in this

study, and demonstrated by others [7, 8]. It is generally accepted that glycolysis supports stemness, while oxidative phosphorylation potentiates lineage differentiation [40]. On the other hand, recent studies disclosed that the differentiation factor MITF positively regulates PGC1-α and, consequently, mitochondrial oxidation [25, 26]. In line with this, we observe that S100A4-induced down-regulation of MITF is accompanied by down-regulation of PGC1-α and reduced mitochondrial oxidation. Such complex interplay between metabolism and differentiation may be important for the maintenance of the invasive phenotype.

In the present study, we used the metastasis associated protein S100A4 to stimulate invasiveness. The pro-metastatic functions of S100A4 are not fully clarified, although S100A4-mediated activation of proteolytic enzymes, like matrix metalloproteinases, has often been pointed out [17, 18]. Our presented results allow speculations that S100A4 might act as a broader regulator, which modulates essential programs of differentiation and metabolism. To explain the association between S100A4-induced invasive phenotype and activated glycolysis, at least two scenarios can be foreseen. Our initial assumption was that S100A4 activates glycolysis and thereby drives/initiates invasiveness. However, this possibility is unlikely, because: a) increase in the glycolysis product lactate was a relatively late event; b) suppression of a glycolytic switch by DCA did not suppress the S100A4 stimulated invasiveness or dedifferentiation. An alternative scenario, where activated glycolysis can be seen as a form of metabolic adaptation that supports the invasive phenotype, is more probable. Switching to glycolysis might be essential for energy production and, thus, survival in invasive cells with reduced mitochondrial oxidation. In line with this scenario, we observed that reversal of the glycolytic switch by DCA, sensitizes the S100A4 stimulated cells to undergo apoptosis and reduce cell growth. The fact that metastatic, less-differentiated cells are more vulnerable to DCA than well-differentiated cancer cells, has

also been observed by others [41]. Altogether, this strengthens the notion that cancer cells with enhanced invasiveness benefit from a glycolytic switch i.e. become dependent on the metabolic reprogramming. This suggests that such cells could be targeted by metabolic inhibitors. Drugs targeting lactate production/release are under rapid development [42], and attempts to combine them with other anti-melanoma agents, like BRAF inhibitors or pro-oxidants are emerging [43, 44]. Increased knowledge of metabolic alterations associated with the invasive phenotype may point to novel drug combinations to target metastatic cells.

In conclusion, we have shown that transition to the invasive phenotype in melanoma is accompanied by dedifferentiation and metabolic reprogramming. The metabolic switch from mitochondrial oxidation to glycolysis is beneficial for survival/growth of cancer cells that undergo such phenotype transition, which makes these cells particularly vulnerable to metabolic inhibitors. We propose that drugs targeting cellular metabolism, particularly glycolysis might be a promising approach against metastatic cells.

Acknowledgements

The project was supported by the Research Council of Norway, the Norwegian Cancer Society, the K.G.Jebsen Foundation, Jeanett and Søren Bothners Legacy, the South East Regional Health Authority and the Liaison Committee between the Central Norway Regional Health Authority (RHA) and the Norwegian University of Science and Technology (NTNU). We thank Linda Langdahl for measurements by blood gas analyzer and Prof. Peter Hersey (Royal North Shore Hospital, University of Sydney, Australia) for providing Patient-3-pre melanoma cell line. The MR spectroscopy was performed at the MR Core Facility, Norwegian University of Science and Technology (NTNU).

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Figure captions

Figure 1. S100A4 stimulates melanoma cell motility without affecting proliferation. (A) Effect of S100A4 on migration and invasion in Melmet 1 and Melmet 5 cells was evaluated in a trans-well chamber by stimulating the cells for 48 hrs, and calculating fold change in the fraction of migrated cells compared to the non-stimulated controls (where migration was set to 1). Error bars indicate SD (Melmet 1, n=1, 3 parallel wells) and SEM (Melmet 5, n=3); * $p<0.05$. (B) Proliferation of melanoma cells with/without S100A4 was tracked by IncuCyte and presented as fold increase in cell confluence as a function of time. Representative graphs from at least three independent experiments are shown; error bars indicate SD (from at least 5 parallel wells) in the presented experiment.

Figure 2. S100A4 induces dedifferentiation in the melanoma cells. (A) Microarray based heat-map of hierarchical clustering of 102 genes associated with invasive/proliferative phenotype (based on the gene list from Hoek *et al.* [5]) in Melmet 1 and Melmet 5 cells stimulated for 48 hrs with S100A4 compared to non-stimulated controls (n=3). The red and green colors represent expression levels ranging from high (red) to low (green) on a log2-transformed scale. An area with primarily the most significant affected genes is shown enlarged with annotations, revealing an over-representation of melanocyte differentiation genes. (B) Relative expression of melanocyte differentiation genes, MITF, MLANA and TYR in S100A4 stimulated Melmet 5 cells compared to non-stimulated controls (where the expression was set to 1), as detected by qPCR. (C) Immunofluorescence staining of MITF (green, right panels) and nucleus stained with DAPI (blue on the phase contrast (PH) background, left panels) in Melmet 5 cells with/without S100A4 stimulation for 48hrs; scale bar, 50 μm. (D) Illustration how the separation of the non-migrated and migrated cell fractions from a trans-well chamber was performed. (E) Relative

expression of the MITF, MLANA and TYR genes in migrated and non-migrated Melmet 5 cell fractions (separated as shown in Fig. 2D) with/without S100A4 stimulation. Error bars indicate SEM (n=3), * $p<0.05$ (paired t-test in Fig. 2E).

Figure 3. S100A4 modulates expression of metabolism-associated genes. (A) Relative expression of gene associated with various metabolic processes (specified under the X-axis) in S100A4 stimulated cells compared to non-stimulated controls (where the expression was set to 1). Error bars indicate SEM ($n \geq 3$). $p<0.05$ for all genes unless denoted NS, non-significant. (B) Western immunoblot analysis of PGC1-α (α-tubulin as a loading control) in Melmet 1 and Melmet 5 cells with/without S100A4 stimulation. Relative PGC1-α level (normalized to α-tubulin) was quantified by densitometric analysis (error bars indicate SD, n=2). (C) Relative expression of the PGC1-α gene in migrated and non-migrated Melmet 5 cell fractions (separated as shown in Fig. 2D) with/without S100A4 stimulation. Error bars indicate SEM (n=3), * $p<0.05$.

Figure 4. S100A4 reduces oxygen consumption and elevates extracellular acidification. (A) OCR in control and S100A4 stimulated (for 48 hrs) Melmet 1 and Melmet 5 cells under basal conditions and after sequential addition of oligomycin (O), FCCP and antimycinA/rotenone (A+R). Representative graphs from three independent experiments are shown for Melmet 5 (a single experiment using 11 parallel wells was performed on Melmet 1); error bars indicate SD in the presented experiments. OCR (B), ECAR (C) and ECAR/OCR ratio (D) under basal conditions in control and S100A4 stimulated cells. Error bars indicate SD (Melmet 1, n=1, 11 parallel wells) and SEM (Melmet 5, n=5, paired t-test). * $p<0.05$.

Figure 5. S100A4 activates glucose consumption and lactate secretion. Melanoma cells were cultured with/without S100A4 for 48 hrs, and the culture medium was analyzed by proton NMR.

(A) Illustrative proton NMR spectra of S100A4 stimulated (blue) and non-stimulated control (grey) Melmet 1 samples, and S100A4 stimulated (red) and non-stimulated control (black) Melmet 5 samples. The enlarged spectral regions show S100A4-induced differences in glucose and lactate levels in Melmet 5, but not in Melmet 1 cells. Relative glucose (B) and lactate (C) levels calculated by integrating the glucose (5.24 ppm) and lactate (1.33 ppm) signals in the NMR spectra and setting the value in the control samples to 1. Error bars indicate SEM (n=3),* $p<0.05$. (D) Ratio between the levels of lactate and glucose. Data from three independent experiments are shown.

Figure 6. Utilization of [1,2-13C] glucose in different metabolic pathways. Metabolic flow chart of 13C-labelled metabolite patterns representing glycolysis, the pentose phosphate pathway (PPP), glycolytic metabolism-linked biosynthesis of amino acids, and tricarboxylic acid (TCA) cycle. The circles symbolize the carbon backbone of the molecules. Dark red circles mark the position of the label. Blue circles mark the position of the label after conversion in the PPP and re-entry to glycolysis. Light red circles indicate that 50% or less of the molecules formed are labeled in that position. The pathway-associated enzymes, whose expression was found to be modulated by S100A4 (Table 1), are indicated in grey boxes.

Figure 7. S100A4 induced changes in the metabolic fate of [1,2-13C] glucose. Melmet 5 cells with/without S100A4 pre-stimulation were labeled with [1,2-13C] glucose for 6 hrs, and cell extracts were analyzed by 13C NMR. (A) Illustrative 13C NMR spectra, where the red and the black lines indicate S100A4 stimulated and non-stimulated samples, respectively. The enlarged spectral region show reduced amount of glutamate in the stimulated cells. (B) Signal intensity arbitrary units (a.u.) of the selected metabolites (indicated on the X axis) in the 13C NMR

spectra from three independent experiments; horizontal lines indicate average, and error bars indicate SEM (n=3), * $p<0.05$.

Figure 8. DCA suppresses glycolysis and potentiates mitochondrial oxidation. (A) Changes in ECAR and OCR in Melmet 5 cells after injection of 10 mM DCA (indicated by the arrow). A representative graph from three independent experiments is shown, where error bars indicate SD of 5 parallel wells in the presented experiment. (B, C) DCA-induced change in basal ECAR and OCR (B) and the ATP level (C) in Melmet 5 cells treated for 2 hrs. Error bars indicate SEM (n=3), * $p<0.05$.

Figure 9. S100A4 potentiates the DCA effect on apoptosis and growth inhibition in Melmet 5 cells. (A) Growth of Melmet 5 cells with/without S100A4 pre-stimulation and with/without treatment with 20 mM DCA was tracked by IncuCyte, and shown as changes in cell confluence as a function of time. The DCA addition moment is indicated by the arrow. A representative graph from three independent experiments; data indicates average from 3 parallel wells, where 3 regions per well were tracked; *, $p<0.05$ at the end point. (B) DCA effect on survival of Melmet 5 wells with/without S100A4 pre-stimulation. Cell viability was scored by the MTS assay, and relative survival was calculated setting the values in the respective “No DCA” cells to 100%. Error bars indicate SEM (n=4); * $p<0.05$. (C and D) DCA-induced apoptosis as detected by Annexin V-FITC staining. Cells with/without S100A4 pre-stimulation were treated with 30mM DCA for 24 hrs before the flow cytometric analysis for apoptosis. The percentages of apoptotic (Annexin-positive) cells under each condition are shown in (C), where error bars indicate SEM (n=4); * $p<0.05$. The representative dot-plots are shown in (D), where the numbers in the right-quadrants indicate % of early-apoptotic (lower quadrant) and late-apoptotic (higher quadrant) cells in the presented experiment.

A**dditional files.**

Supplementary Figure S1. 2D Heteronuclear (13C-1H) single-quantum correlation (HSQC) spectrum of cell extracts from Melmet 5 cells incubated with [1,2-13C] glucose for 6 hrs. On the top the proton spectrum is shown and on the left hand side the 13C spectrum is shown. Dotted red lines show the cross peak assignments between proton and 13C spectra.

Supplementary Figure S2. 2D Homonuclear (1H-1H) Shift Correlation (COSY) spectrum of cell extract from Melmet 5 cells incubated with [1,2-13C] glucose for 6 hrs. The proton spectra are shown both on top and on left hand side. The correlated cross peaks in the COSY spectra are shown with dotted red lines.

Supplementary Figure S3. Effect of S10A4 on melanoma cell motility. (A) Chemo-tactic migration and invasion of Melmet 1 and Melmet 5 cells in a trans-well chamber incubated with/without S100A4 for 48 hrs. Bars indicate percentage of migrated cells; error bars indicate SD (Melmet 1, n=1, 3 parallel wells) and SEM (Melmet 5, n=3). (B) Relative wound density in percent (Y axis) as a function of time (X axis) for the Melmet 1 and Melmet 5 cells with/without S100A4 stimulation in a wound healing assay. Representative graphs from at least three independent experiments are shown; error bars indicate SD from 10 parallel wells.

Supplementary Figure S4. Effect of S100A4 on expression of the melanocyte differentiation genes in Melmet 1 and several other melanoma cell lines. (A) Relative expression of MITF, MLANA and TYR in Melmet 1 cells stimulated with S100A4 compared to non-stimulated controls (where the expression was set to 1), as detected by qPCR. Error bars indicate SD (n=2); *n.d.,* non-detectable. (B) Immunofluorescence staining of MITF (green, right panels) and nucleus (blue, left panels) in Melmet 1 cells with/without S100A4 stimulation; scale bar, 50 μm. (C) Relative expression of MITF in a panel of melanoma cell lines (specified in the X axis) with/without S100A4 stimulation.

Supplementary Figure S5. S100A4-induced metabolic shift in Melmet 5. OCR versus ECAR in the control and the S100A4 stimulated cells. ECAR and OCR values from five independent experiments are plotted, where each experiment is coded by a symbol+color; filled symbols represent control samples without S100A4, while unfiled symbols of the same shape/color represent the S100A4 stimulated samples.

Supplementary Figure S6. Effect of S100A4 on glucose and lactate levels in the culture medium from cells with/without S100A4 as detected by blood gas analyzer or Lactate Colorimetric Assay. Relative levels of glucose (A) and lactate (B) measured 2 days after addition of S100A4 (controls - no S100A4) as measured by a blood gas analyzer. Error bars indicate SEM (n=3), * $p<0.05$. (C) Relative levels of lactate detected in Melmet 5 cell culture medium at different time points. Aliquots were collected at 6, 24 hrs (denoted as < 24hrs) and 48, 72 hrs (denoted as > 48) after the addition of S100A4 and analyzed by the Lactate Colorimetric Assay. Error bars indicate SEM (n=5), *$p<0.05$.

Supplementary Figure S7. S100A4 does not influence cell proliferation as measured by counting Melmet 1 and Melmet 5 cells with/without S100A44 stimulation. The results from three independent experiments are shown, where symbols indicate average, and error bars indicate SD of 6 parallel wells in each experiment.

Supplementary Figure S8. S100A4 does not influence the DCA effect on apoptosis and growth inhibition in Melmet 1 cells. (A) Growth of Melmet 1 cells with/without S100A4 pre-stimulation and with/without 10 mM DCA treatment was tracked by IncuCyte, and shown as changes in cell confluence as a function of time. A representative graph from two independent experiments; data indicates average from 5 parallel wells, where 3 regions per well were tracked; (B) DCA effect on survival of Melmet 1 cells with/without S100A4 pre-stimulation. Cell survival was scored by

the MTS assay, and relative survival was calculated setting the values in the respective "No DCA" cells to 100%. Error bars indicate SD (n=2); (C and D) DCA-induced apoptosis as detected by Annexin V-FITC staining. Cells with/without S100A4 pre-stimulation were treated with 30mM DCA for 24 hrs before the flow cytometric analysis for apoptosis. The percentages of apoptotic (Annexin-positive) cells under each condition are shown in (C), where error bars indicate SD (n=2). The representative dot-plots are shown in (D), where the numbers in the right-quadrants indicate % of early-apoptotic (lower quadrant) and late-apoptotic (higher quadrant) cells in the presented experiment.

Supplementary Table S1. Primer sequence and probes used for qPCR.

Supplementary Table S2. Metabolite assignments in 1H and 13C NMR spectrum

Supplementary Methods.