SIRT1/PGC1α dependent increase in oxidative phosphorylation supports chemotherapy resistance of colon cancer

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Many metastasized colorectal tumors are intrinsically resistant to chemotherapy, while others acquire drug resistance during treatment. With the current therapeutic modalities disease progression is virtually inevitable. The design of therapeutic strategies that target drug resistance needs to be based on a thorough understanding of its mechanistic principles. In the present study we used transcriptomics data of chemotherapy-exposed and chemo-naive liver metastases to identify a novel pathway of drug resistance, involving an increased dependency on mitochondrial biogenesis and oxidative phosphorylation. We have subsequently used colonosphere cultures to demonstrate a causal relationship between chemotherapy treatment and altered energy metabolism and have identified SIRT1 and PGC1α as key factors in the process. Suppression of these factors increases the efficacy of chemotherapy in vitro and in mice bearing colonosphere-initiated human tumor xenografts. We propose that therapeutic targeting of oxidative energy metabolism should be explored as a strategy to prevent acquired drug resistance.
Abstract

Purpose: Chemotherapy treatment of metastatic colon cancer ultimately fails due to development of drug resistance. Identification of chemotherapy-induced changes in tumor biology may provide insight into drug resistance mechanisms.

Experimental design: We studied gene expression differences between groups of liver metastases that were exposed to pre-operative chemotherapy or not. Multiple patient-derived colonosphere cultures were used to assess how chemotherapy alters energy metabolism by measuring mitochondrial biomass, oxygen consumption and lactate production. Genetically manipulated colonosphere-initiated tumors were used to assess how altered energy metabolism affects chemotherapy efficacy.

Results: Gene ontology and pathway enrichment analysis revealed significant upregulation of genes involved in oxidative phosphorylation (OXPHOS) and mitochondrial biogenesis in metastases that were exposed to chemotherapy. This suggested chemotherapy induces a shift in tumor metabolism from glycolysis towards OXPHOS. Indeed, chemo-treatment of patient-derived colonosphere cultures resulted in an increase of mitochondrial biomass, increased expression of respiratory chain enzymes and higher rates of oxygen consumption. This was mediated by the histone deacetylase sirtuin-1 (SIRT1) and its substrate, the transcriptional co-activator PGC1α. Knockdown of SIRT1 or PGC1α prevented chemotherapy-induced OXPHOS and significantly sensitized patient-derived colonospheres, as well as tumor xenografts to chemotherapy.

Conclusion: Chemotherapy of colorectal tumors induces a SIRT1/PGC1α-dependent increase in OXPHOS that promotes tumor survival during treatment.
phenomenon is also observed in chemotherapy-exposed resected liver metastases, strongly suggesting that chemotherapy induces long-lasting changes in tumor metabolism that potentially interfere with drug efficacy. In conclusion, we propose a novel mechanism of chemotherapy resistance that may be clinically relevant and therapeutically exploitable.
Introduction

Colon cancer is one of the most common and deadliest types of cancer. Mortality is mainly the consequence of metastatic growth in secondary organs like liver and lungs. Chemotherapeutic drugs that have proven to be effective in the treatment of metastatic colon cancer are 5-fluorouracil (5-FU), oxaliplatin and irinotecan which together prolong median survival from ~6 months to ~2 years (1). However, most tumors are either intrinsically resistant to these drugs or acquire resistance during treatment. Consequently, disease progression following systemic therapy occurs in the vast majority of cases (1).

In order to rationally develop therapeutic approaches that can overcome drug resistance, it is essential to understand the underlying mechanisms. ‘Omics’ technologies are nowadays increasingly used to determine the (epi-)genetic underpinnings of drug resistance (2, 3). In colon cancer this has resulted in the identification of gene signatures that can identify intrinsically drug-resistant tumor subtypes (4-7). In the current study we use such technologies to study how drug resistance develops in colon cancer patients that have been treated with chemotherapy.

Normal, healthy cells mainly use OXPHOS for energy production. Cancer cells, however, face an impressive metabolic challenge due to rapid cell growth and frequent divisions, which forces them to adjust their energy metabolism in order to meet these demands (8). In addition to the (in-)activation of specific oncogenes and/or tumor suppressor genes, the bioenergetic status of tumor cells is influenced by micro-environmental factors such as availability of oxygen (hypoxia) and nutrients (9-11). These factors also induce changes in the metabolic state of the cells, allowing them to
survive the harsh tumor environment. All this results in a predominant shift in metabolism of tumor cells from OXPHOS towards aerobic glycolysis (Warburg effect) (12). Although in various tumors glycolysis prevails as the main, and in some cases even exclusive source of ATP, it is important to note that intense glycolysis is not mandatory for all tumor types and some cancers do rely on OXPHOS for ATP production (13, 14). Tumor metabolism has received increased attention over the last decade, mainly in relation to proliferation and specific metabolic alterations. Only recently the metabolic state has been implicated in tumor drug resistance (15, 16). Targeting tumor metabolism is now actively being studied as an alternative approach to overcome this problem. Drugs that limit the uptake of nutrients or interfere with their use in anabolic pathways have shown efficacy as resistance-modulating agents in several pre-clinical models (17-20). However, the link between tumor metabolism and drug resistance is highly complex, it depends on multiple parameters such as oxygen and nutrient availability or the specific drugs that are being used, and the underlying mechanisms still remain to be elucidated (15). In the present study we used transcriptomics data of liver metastases from chemotherapy-treated and chemo-naive colon cancer patients to identify a novel pathway of drug resistance, involving a change in energy metabolism. We show that upon chemotherapy, cancer cells shift their metabolism from glycolysis towards OXPHOS. This process is regulated via SIRT1-PGC1α signaling pathway and, as a consequence, increases the resistance of cells to chemotherapy.
Materials and Methods

Cell culture

Human colorectal tumor specimens were obtained from patients undergoing colon or liver resection for primary or metastatic adenocarcinoma respectively, in accordance with the local ethical committee on human experimentation (protocol #09-145). Informed consent was obtained from all patients. Human colonosphere cultures were isolated and propagated as described (21).

Flow cytometry

300nM Mitotracker Green (Invitrogen) was added to the cultures for 30’. Cells were resuspended in PBS containing 0.5µg/ml PI to exclude dead cells and analyzed with FACSCalibur (BD). Experiments were performed in triplicates, and the results are depicted as an average of at least three independent experiments. Data represented as mean ± SD.

Antibodies and reagents

WB: anti-SIRT1 (Millipore), anti-cleaved caspase-3 and anti-cleaved PARP (Cell Signaling), MitoProfile Total OXPHOS Rodent WB antibody cocktail (Abcam), anti-β-actin (Novus Biologicals). Immunohistochemistry: COXIV (Abcam). SIRT1 inhibitors: nicotinamide (Sigma), EX527 (Sigma), Tenovin-6 (Cayman Chemical).
Lentiviral transduction

shSIRT1 plasmids were obtained from Sigma [TRCN0000018979] and [TRCN0000018983]. shSCR plasmid (SHC002) was used as a control. shPGC1α construct was a kind gift from Prof. P. Puigserver from the Dana-Farber Cancer Institute. Lentiviral particles were generated by transfecting 293T cells with pLKO and packaging vectors using Fugine (Roche). Transduced cells were selected with puromycin.

Rescue experiment

CRC29 cells were transfected with expression vectors encoding shSIRT1 (TRCN0000018983) and FLAG-SIRT1 either alone or combined using standard manufacturer Lipofectamine3000 (LifeTechnologies) protocol. 48h following transfection, cells were treated with chemotherapy for 24h and mitochondrial content was analysed using Mitotracker as described previously.

Immunohistochemistry

COXIV staining was performed on formalin-fixed paraffin embedded sections from colorectal liver metastasis. Samples were deparaffinized and rehydrated. Citrate buffer (pH6.0) was used for antigen retrieval. Primary antibody: anti-COXIV (Abcam).

Quantitative Real Time Polymerase Chain Reaction

Total RNA from colonosphere cultures or tissue samples was isolated using manufacturer's protocol (RNeasy Mini Kit, Qiagen). cDNA was synthesized using iScriptcDNA Synthesis Kit (Bio-Rad Laboratories). The amplification was performed in
an iCycler thermocycler (Bio-Rad Laboratories) using iQ SYBR Green Supermix (Bio-Rad Laboratories). mRNA expression levels were quantified using iCycler software (Bio-Rad Laboratories) and were normalized to RPL13a. The primers used for RT-qPCR are listed in Supplementary table S1. All samples were analyzed in triplicates.

**Animal experiments**

Colonospheres were dissociated into single cell suspension with Accumax and 5x10⁴ cells expressing shSCR or shSIRT1 were mixed with Matrigel (BD) at 1:1 ratio and injected subcutaneously into the flanks of CBy.Cg-Foxn1nu/J mice. Tumor growth was measured weekly and tumor volumes were calculated \( V = axb^2 \times 0.5263 \), ‘a’ being the maximal width and ‘b’ maximal orthogonal width. Six weeks after the injection we started the chemo-treatment. Chemotherapy was administered weekly intraperitoneally, oxaliplatin (10mg/kg) and 5-FU (100mg/kg). Control mice received PBS only. When tumors reached a volume of 1500mm³ mice were sacrificed. For tumor analysis outliers (±>3 times SD from the mean) were excluded. For analysis of necrosis, the slides were digitized and analyzed via Aperio Imagescope (Leica Biosystems). The surface of necrotic areas (excluding the necrotic centre) were measured and calculated as a percentage of the total area (excluding the necrotic centre). All experiments were performed in accordance with University of Utrecht institutional animal welfare guidelines.
Cell death analysis

Colonospheres were plated in 96-well plate and either treated or not with oxaliplatin (10μg/ml) plus 5FU (10μg/ml) for 48h. Prior to the measurement, total cell population was labelled with DRAQ5™ (Abcam) and live cells were labelled with Calcein Green AM (LifeTechnologies). Fluorescence was measured using the Arrayscan (ThermoScientific). The percentage of dead cells was calculated by normalizing the levels of intensity to and expressed as a relative percentage of the plate-averaged vehicle treated control. Experiments were performed in triplicates, and the results display the average of at least three independent experiments. Data represented as mean ± SD.

Bioinformatics Analyses

We used our previously generated dataset containing gene expression profiles of 119 liver metastases (22) deposited at Array Access under accession number: E-TABM-1112. The dataset was uploaded into the R2 microarray analysis and visualization platform and analyzed. Differential gene expression between tumor groups was performed with the ‘Find differential expression between groups’ option (single gene ANOVA p<0.05), and selecting each available clinical variable separately. To find differentially represented pathways in chemotherapy-treated versus non-treated tumors the resulting gene list was analyzed for enrichment of Gene Ontology terms in chemotherapy-treated versus naïve tumor subgroups with the Gene Set Analysis option. In addition, differentially represented pathways were analyzed with the ‘KEGG pathway finder’ (Minimal t test p<0.0001).
**OCR and ECAR measurement**

The Seahorse XF96 Extracellular Flux Analyzer (Seahorse Biosciences) was used to obtain real-time measurements of OCR and ECAR in cells. Colonospheres treated with or without chemotherapy were dissociated into single cells using Accumax. Cells were reconstituted in culture medium and seeded in 96-well Seahorse culture plates at a density of 40,000 cells/well. For analysis of ECAR, cells were reconstituted in Seahorse medium. Cells were allowed to settle for 1h prior to measurements. OCR and ECAR were analyzed using 2’ mix followed by 3’ measurement cycle. Oligomycin (Sigma) was injected at a final concentration of 2μM, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma) at 3μM, antimycin A (Sigma) at 5μM and rotenone (Sigma) at 2μM. Basal OCR is the mean OCR from 0’, 5’, 10’, 15’ and maximal OCR is the mean OCR from 37’, 42’, 47’. For ECAR measurements 2-deoxyglucose (Sigma) was injected at a final concentration of 100mM. OCR and ECAR were normalized to protein or DNA content determined by BCA assay or Cyquant assay (Invitrogen). Data are presented as mean ± SEM.

**Statistics**

All values are presented as mean ± SEM or SD. The Student t-test (unpaired, 2-tailed) or one-way analysis of variance (ANOVA) was performed to analyze if differences between the groups are statistically significant. Differences with a $P$ value of less than 0.05 were considered statistically significant.
Results

Chemotherapy of colorectal liver metastases induces lasting changes in gene expression

The majority of patients with metastatic colorectal cancer receive chemotherapy, either alone or in combination with surgery. To identify mechanisms that could underlie chemotherapy resistance we used gene expression profiles of 119 liver metastases of which 64 had received neo-adjuvant chemotherapy (i.e. prior to resection) (22). Of all clinical variables tested, neo-adjuvant chemotherapy was by far most strongly associated with changes in gene expression (Supplementary table S2). Variables that were not correlated with significant changes in gene expression included various clinical scoring systems, tumor recurrence, tumor size and location, CEA levels, extent of resection and disease-free and overall survival (Supplementary table S2). We identified 481 genes whose expression was significantly (p<0.05) down-regulated and 613 genes whose expression was significantly up-regulated in chemotherapy-exposed tumors (Fig. 1A, Supplementary table S3). Interestingly, since the mean interval time between the last cycle of chemotherapy and resection of the metastases was 17 weeks (5-35), the observed differences in gene expression are persistent and long-lasting.
Genes regulating mitochondrial biogenesis and OXPHOS are upregulated in chemotherapy-exposed liver metastases

To identify the biological pathways that are represented by the differentially expressed genes between chemotherapy-exposed and chemotherapy-naive tumors, we performed gene ontology analysis. This showed that the ‘mitochondrial respiration chain’ (GO0005746) was most significantly different between the two tumor groups (p=2.2e-5). In addition, the ‘KEGG pathway finder’ identified OXPHOS as the most significantly up-regulated pathway in chemotherapy-treated tumors (p=4.1e-4; Supplementary table S4). Similar results were obtained with Ingenuity Pathway Analysis (Supplementary table S4). The 14 differentially expressed OXPHOS pathway genes were all up-regulated in chemo-treated tumors. The electron transport chain consists of 5 distinct enzyme complexes. Chemo-treated metastases expressed high levels of multiple components of complexes I, III, IV and V (Supplementary table S5 and Fig. 1B). In addition, we found that mitochondrial biogenesis genes, in particular mitochondrial ribosomal proteins, like Mrpl11, Mrps16 and Mrps12, were also expressed to significantly higher levels in chemotherapy-treated tumors when compared to non-treated tumors (Supplementary table S5 and Fig. 1B).

Next, we sought to validate the above results by performing immunohistochemistry analysis of the expression of COXIV, a component of mitochondrial complex IV and a frequently used marker for mitochondrial content, in pathological tissue sections. For this, we used an independent series of colorectal liver metastases that had either received neo-adjuvant chemotherapy (n=8) or not (n=8). Indeed, we identified a markedly higher intensity of COXIV staining in sections from liver
metastases that were treated with neo-adjuvant chemotherapy as compared to chemo-naive tumors (Fig. 1C and Supplementary Fig. S1). Double-blind scoring of the stained sections confirmed that COXIV staining was significantly higher in treated compared to non-treated metastases (Fig. 1D). These findings establish that electron transport chain pathway is significantly upregulated in chemotherapy-treated liver metastases from two independent patient cohorts both at the mRNA and protein level.

Chemotherapy treatment induces oxidative energy metabolism in patient-derived colonospheres

Upregulation of mitochondrial biogenesis and electron transport chain pathways suggested that chemotherapy may alter energy metabolism in colorectal tumors by shifting cellular metabolism towards mitochondrial OXPHOS. To test this experimentally, we made use of a panel of colonosphere cultures established from primary colorectal tumors (CRC09, CRC16 and CRC29) and liver metastases (L145 and L167) (21). First, we assessed how exposure of colonospheres to oxaliplatin and 5FU, the first line cytotoxic treatment for metastatic colorectal cancer, alters mitochondrial mass in these cells. Control and drug-exposed colonospheres were stained with the fluorescent Mitotracker probe and mitochondrial content was determined by FACS analysis. Chemotherapy significantly increased mitochondrial mass in 5 distinct colonosphere cultures, irrespective of whether they were derived from primary tumors (n=3) or liver metastases (n=2) (Fig. 2A). Furthermore, similar to the upregulation of COXIV staining in chemotherapy treated tumors in vivo, protein levels of complex IV as well as complex I, II and III were strongly up-regulated following
chemotherapy exposure (Fig. 2B), demonstrating that the colonosphere cultures reflect the changes observed in vivo.

Chemotherapy-induced increase in mitochondrial content and expression of OXPHOS enzymes suggested that chemotherapy-exposed tumor cells may shift from a glycolytic to a more oxidative energy metabolism. To test this directly, we determined oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) of colonosphere cells by Seahorse analysis. Both basal and maximum OCRs were significantly increased in chemotherapy-treated colonospheres in three independent cultures (Fig. 2C). In parallel, ECAR was significantly reduced. 2-deoxyglucose injection normalized the ECAR levels of both chemo-treated and non-treated cells to baseline levels. Both maximal and basal ECAR where reduced, demonstrating that the overall glycolytic capacity of the cells was reduced (Fig. 2C). Combined, lowered ECAR and enhanced OCR show that chemotherapy-treated colonospheres rely more on mitochondrial OXPHOS than non-treated ones. To further explore the effect of chemotherapy on glycolysis we analyzed the expression of glycolytic enzymes, respiratory chain components, and SIRT1 following chemotherapy exposure of two independent colonosphere lines (L145; CRC09) by RT-qPCR analysis. We found that chemotherapy reduced the expression of the glycolytic enzymes PKM2, PFKP and hexokinase 2, while the respiratory chain components NDUFS3 and ATP5G1 as well as SIRT1 showed increased expression (Supplementary Fig. S2). It should be noted however, that a correlation between mRNA and protein levels is not always found (23, 24). ATP synthesis rates through glycolysis and OXPHOS respectively were calculated (Supplementary table S6; 25, 26). Glycolysis was the predominant source of ATP
production in two independent non-treated colonosphere lines (L145, CRC09). Assessment of the generality of this phenomenon would require analysis of glycolysis- and OXPHOS-dependent ATP production rates in a large series of colonosphere lines. However, while chemotherapy treatment reduced glycolytic ATP synthesis, it increased ATP synthesis through OXPHOS (Supplementary table S6).

**Chemotherapy induces SIRT1 to promote oxidative energy metabolism**

To identify potential regulators of the observed chemotherapy-induced metabolic changes, we re-analyzed the gene list that was upregulated in chemotherapy-treated tumors and searched for factors that could regulate mitochondrial biogenesis and/or OXPHOS. SIRT1 encodes sirtuin-1, a NAD+-dependent histone deacetylase which is activated in response to DNA damage and plays a key role in mitochondrial biogenesis (27, 28). SIRT1 was also in the top 5% of most significantly up-regulated genes following chemotherapy (Supplementary table S3; Fig. 3A). For these reasons we hypothesized that SIRT1 could play a causative role in altering cellular energy metabolism following chemotherapy. None of the other Sirtuins (SIRT2-7) were expressed to higher levels in chemotherapy-treated tumors. Treatment of colonosphere cultures with oxaliplatin and 5FU also greatly enhanced SIRT1 protein levels, thus demonstrating a causal relationship between chemotherapy and increased SIRT1 expression (Fig. 3B). The SIRT3 enzyme regulates mitochondrial respiration by directly de-acetylating respiratory enzymes and could thereby control changes in energy homeostasis following chemotherapy exposure. However, neither SIRT3 mRNA nor protein levels were changed in tumors or colonospheres exposed to chemotherapy (Fig. 3C, D).
Next we analyzed whether inhibition of SIRT1 would interfere with the increase in mitochondrial mass triggered by chemotherapy. To this end we used the sirtuin inhibitors nicotinamide (NAM), EX-527 and Tenovin-6 (TV-6). All three compounds impaired the chemotherapy induced enhancement of mitochondrial mass (Fig. 3C). Importantly, NAM also completely prevented the chemotherapy-induced increase in basal and maximum OCR (Fig. 3D and supplementary Figure S3).

Next, we analyzed whether suppression of SIRT1 expression by RNA interference would have an impact on the chemotherapy-induced increase in mitochondrial mass. We suppressed SIRT1 in CRC29 and L145 colonospheres by expressing two independent short-hairpin RNAs (shRNAs) and used scrambled (SCR) hairpins as controls (Fig. 4A). Consistent with the SIRT1 inhibitor data, we found that SIRT1 knockdown (KD) prevented the chemotherapy-induced elevation in mitochondrial mass (Fig. 4A). Furthermore, expression of complex I-IV was upregulated by drug treatment, but was not induced in SIRT1 KD cells (Fig. 4B). Because NAM suppressed the increase in OCR upon drug-treatment, we performed similar Seahorse experiments in cells with KD of SIRT1. Eliminating SIRT1 expression did not impact OCR in non-treated cells, whereas in chemotherapy treated cells OCR was greatly reduced (Fig. 4C), again demonstrating that SIRT1 is required for the induction of mitochondrial OXPHOS by chemotherapy. To control for non-specific off-target RNAi effects we performed a rescue experiment using the SIRT1-targeting shRNA#2 (which targets a non-coding region in the SIRT1 mRNA) and an expression vector encoding FLAG-tagged SIRT1 which is not targeted by shRNA#2. 48h after transfection colonosphere cultures were treated with oxaliplatin (10 μg/ml) and 5FU (10μg/ml) for 24 hours and
mitochondrial content was analysed. Consistent with the previous results, SIRT1 KD blocked the chemotherapy-induced increase in mitochondrial biomass. Co-expression of FLAG-SIRT1 completely restored the chemotherapy-induced increase in mitochondrial biomass, demonstrating that the effect of the KD vector was indeed due to SIRT1 suppression (Fig. 4D).

SIRT1 controls mitochondrial biogenesis by deacetylating and activating PGC1α, which is a master regulator of mitochondrial function (29). PGC1α has been shown to be essential for directing the transcriptional program for enhancing mitochondrial biogenesis and function (30). To test whether mitochondrial biogenesis in chemotherapy-treated colonospheres is indeed activated via the SIRT1/PGC1α-axis we expressed shRNAs directed against PGC1α in CRC29 colonospheres, which resulted in efficient suppression of PGC1α protein expression (Fig. 5A). Again, chemotherapy caused an increase in mitochondrial mass and stimulated expression of OXPHOS enzymes in control cells, but not in colonospheres in which PGC1α was suppressed (Fig. 5B,C). Together, these findings demonstrate that SIRT1 and PGC1α are necessary for triggering mitochondrial biogenesis in response to chemotherapy in patient-derived colonosphere cultures.

**SIRT1 and PGC1α protect colon cancer cells against chemotherapy**

In addition to mitochondrial biogenesis, SIRT1 is involved in multiple DNA repair processes and both SIRT1 and PGC1α play a role in the defense against reactive oxygen species (ROS) generated in high amounts by chemotherapy (31). Furthermore,
chemotherapy-induced DNA damage greatly increases the ATP demand (32). Therefore we studied whether suppression of SIRT1 or PGC1α would affect the sensitivity of cancer cells to chemotherapy. Strikingly, already after 16 hours of chemotherapy treatment of PGC1α KD cells, cleaved PARP and cleaved caspase-3 were detected, whereas in control cells only after 48 hours low levels of cleaved products became apparent (Fig. 6A). Also in SIRT1 KD cells, drug treatment resulted in markedly increased levels of both cleaved PARP and cleaved caspase-3 as compared to treated control cells (Fig. 6A). Increased levels of cleaved PARP and cleaved caspase-3 indicate that SIRT1/PGC1α KD cells might commit to a cell death program faster than control cells. Therefore, we analyzed chemotherapy-induced cell death using Arrayscan analysis. Exposure of control cells to chemotherapy for 48 hours resulted in ~35% cell death. Strikingly, in both PGC1α and SIRT1 KD cells, the percentage of cell death was approximately two-fold higher (Fig. 6B), demonstrating that PGC1α and SIRT1 make the colonospheres more resistant to chemotherapy.

Finally, to evaluate the impact of SIRT1 KD on tumor growth and chemosensitivity in vivo, control and SIRT1 KD colonospheres were subcutaneously injected in nude mice. Mice were treated with oxaliplatin in combination with 5FU 6 weeks after injection of cells. As shown in Fig. 6C, SIRT1 KD tumors had a marked regression in response to oxaliplatin/5FU treatment. By contrast, chemotherapy did not induce regression of control tumors. Overall effect of chemotherapy at the end of the experiment was significantly more pronounced in SIRT1 KD compared to control tumors (Fig. 6D). Western blot analysis showed that SIRT1 KD was maintained in the tumors throughout the experiment (Fig. 6C). Histochemical analysis of tumor tissue sections
further showed that chemotherapy induced significantly more tumor necrosis in SIRT1 KD tumors when compared to control tumors (Fig. 6D). Collectively, these results demonstrate that chemotherapy induces a shift in tumor energy metabolism from glycolysis to OXPHOS via SIRT1/PGC1α and that this protects tumor cells from cytotoxic damage.

Discussion

Preventing disease progression by overcoming drug resistance is a major goal in medical oncology. In the present study we have identified OXPHOS and the SIRT1/PGC1α pathway as an important contributor to therapy resistance and a potential therapeutic target.

Cells may rapidly adapt their metabolic pathways in response to changes in the microenvironment, including the availability of growth factors, nutrients and oxygen (33). Our data indicate that genotoxic stress also alters energy metabolism. We show that in response to chemotherapy colonospheres engage a SIRT1/PGC1α-dependent (partial) reversion of the Warburg effect, shifting glycolysis to OXPHOS, and that this supports survival after treatment. This is in line with previous reports correlating increased mitochondrial oxidative energy metabolism to chemoresistance of tumor cells. Metabolic characterization of glioma cells revealed an enhanced oxidative metabolism in drug-resistant cells when compared to drug-sensitive cells (34). Importantly, we show that tumors of chemotherapy-treated cancer patients show lasting gene expression changes that reflect activation of the OXPHOS program. This strongly suggests that
chemotherapy-induced gene expression alterations could potentially underlie tumor resistance in a long run, making our data clinically relevant.

Why would cancer cells shift their metabolism in favor of OXPHOS? Under normal conditions the amount of ATP produced through aerobic glycolysis is sufficient to support tumor cell growth and basal DNA repair activity. However, cellular ATP demand is greatly increased following chemotherapy as many enzymes involved in DNA repair, drug efflux and drug detoxification require ATP (35, 36). As OXPHOS is the most efficient way to generate ATP, it is not surprising that cancer cells would increase this pathway at times of high ATP demand. The role of SIRT1 in cancer is still controversial as evidence for both tumor-promoting and -suppressing activities have been found and the impact of SIRT1 on cell viability seem to be highly context-dependent (37). In the present report we show that all tumor-derived colonosphere cultures and xenografts in which SIRT1 (or PGC1α) was suppressed, were sensitized to drug treatment. This is in line with previously published data showing that SIRT1 KD sensitized Saos2 osteosarcoma cells to doxorubicin although this study did not identify increased OXPHOS as the critical downstream SIRT1 effector pathway (38). Likewise, high expression of PGC1α in melanoma cells causes an increase in oxidative energy metabolism, increased expression of ROS-detoxifying enzymes and resistance to ROS-inducing drugs (39). Upon PGC1α inhibition, such OXPHOS-dependent melanoma cells reverted to a glycolytic energy metabolism (40).

Mechanistically, chemotherapy-induced DNA damage results in increased expression of SIRT1, possibly via the multifunctional DNA repair protein and transcriptional co-activator APE1 (41). SIRT1-mediated de-acetylation reactions
consume and require NAD+ as a substrate (42). NAD+ levels rise when cells experience an energy deficit, for instance during fasting or exercise, and presumably also after chemotherapy-induced DNA damage (43). Indeed, SIRT1 is recruited to sites of DNA damage and participates in several DNA repair processes (44, 27). In addition, SIRT1 de-acetylates PGC1α, leading to its activation as a transcriptional co-activator (45). PGC1α acts in concert with several transcription factors to stimulate the expression of genes involved in mitochondrial biogenesis and respiration, resulting in increased OXPHOS (46, 47).

Taken together our study provides insight into how colorectal tumors shift their energy metabolism when challenged with chemotherapy. We demonstrate that chemotherapy induces OXPHOS in colon cancer cells via the SIRT1/PGC1α axis to help them survive treatment. Further investigations aimed at targeting this program in combination with chemotherapy deserve further attention and may ultimately increase response rates in the treatment of colon cancer.
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Figure legends

Figure 1. Chemotherapy of colon cancer liver metastases is associated with increased expression of genes involved in mitochondrial biogenesis and OXPHOS. A, Overview of the study design. B, Analysis of the interaction between genes that were significantly upregulated in chemotherapy-treated tumors by using the Search Tool for the Retrieval of Interacting Genes/Proteins. The clusters reflecting OXPHOS and mitochondrial biogenesis are shown. C, Cytochrome c oxidase (COXIV) immunohistochemistry on paraffin-embedded tissue sections of liver metastasis either treated or not with chemotherapy. D, Quantification of the COXIV staining intensity (n=8 per group). Significance was determined with the Student’s t-test. ****p<0.0001.

Figure 2. Chemotherapy increases OXPHOS in colonosphere cultures. A, Colonosphere cultures derived from five distinct tumors were exposed to chemotherapy (oxaliplatin 10μg/ml plus 5FU 10μg/ml) for 72h. Mitotracker Green was used to determine chemotherapy-induced changes in mitochondrial content. FACS dot plots show an increase in mitotracker signal in colonospheres upon chemotherapy. Quantification of the FACS data is shown on the right. B, Colonosphere cultures were treated as in A for 48h. WB demonstrates an increase in OXPHOS complexes after chemotherapy. C, Colonosphere cultures were treated as in A for 48h. The basal and maximal OCRs and basal ECARs were measured on the Seahorse Bioanalyzer. Significance was determined with the Student’s t-test. * p<0.05; **p<0.01; ***p<0.001.
Figure 3. SIRT1 inhibition prevents chemotherapy-induced OXPHOS. A, The gene expression profiles of 119 liver metastases (22) were used to analyse SIRT1 and SIRT3 expression in liver metastases that had been exposed to chemotherapy (n=64) or not (n=55). The p-value was determined by ANOVA. B, Colonosphere cultures were treated with oxaliplatin (10μg/ml) plus 5FU (10μg/ml) for 24h. WB shows an increase in SIRT1 protein levels upon the treatment. SIRT3 protein levels did not alter upon chemotherapy. C, Colonosphere cultures were treated as in B for 48h, with or without SIRT1 inhibitors nicotinamide (NAM; 30mM), EX527 (30μM) or TV6 (10μM). Mitochondrial content was assessed as in Figure 2A. D, CRC29 were treated with chemotherapy and NAM as in B for 48h. The basal and maximal OCRs are from the same experiment as depicted in Fig 2C. The basal and maximal OCRs were determined on the Seahorse Bioanalyzer. Significance was determined with the Student’s t-test. * p<0.05; **p<0.01.

Figure 4. SIRT1 KD prevents chemotherapy-induced OXPHOS. A, Colonosphere cultures were transduced with lentiviral vectors expressing two independent shRNAs targeting SIRT1 or a control vector expressing a scrambled shRNA (SCR). SIRT1 protein levels were determined by WB. SIRT1 KD and SCR were treated with oxaliplatin (10μg/ml) plus 5FU (10μg/ml) for 48h and mitochondrial content was analysed as in Figure 2A. B, SIRT1 KD and SCR were treated as in A. Expression levels of OXPHOS complexes were measured by WB. C, SIRT1 KD and SCR were treated with chemotherapy as in A. The basal OCRs were measured on the Seahorse Bioanalyzer. L145-scr shows an increased basal OCR when compared to parental cells (Fig 2C),
possibly/most likely due to the transduction and/or selection process. D, Colonospheres were transfected with expression vectors encoding shSIRT1 and FLAG-SIRT1 either alone or in combination as indicated. 48h after transfection cultures were treated with oxaliplatin (10μg/ml) plus 5FU (10μg/ml) for 24h and mitochondrial content was analysed as in Figure 2A. Significance was determined with the Student’s t-test. *p<0.05; **p<0.01.

**Figure 5.** PGC1α KD prevents chemotherapy-induced OXPHOS. A, CRC29 were transduced with lentiviral vector expressing shPGC1α or shSCR. PGC1α mRNA levels were determined by qRT-PCR. B, PGC1α KD and SCR were treated with oxaliplatin (10μg/ml) plus 5FU (10μg/ml) for 48h and mitochondrial content was analysed as in Figure 2A. C, PGC1α KD and SCR were treated as in B. Protein levels of OXPHOS enzymes were measured by WB. Significance was determined with the Student’s t-test. *p<0.05, ***p<0.001.

**Figure 6.** SIRT1 and PGC1α protect colon cancer cells against chemotherapy. A, SIRT1 KD, PGC1α KD and SCR were treated with chemotherapy for 16h and 48h. Cleaved PARP and cleaved caspase-3 levels were determined by WB. Arrowheads indicate the cleaved forms of caspase-3. B, SIRT1 KD, PGC1α KD and SCR were treated with chemotherapy for 48h and percentage of dead cells was measured by cytotoxicity assay, as described in Materials and Methods. C, SIRT1 KD and SCR were injected subcutaneously into nude mice. From 6 weeks on, mice were treated with oxaliplatin plus 5FU or PBS as described in Materials and Methods. The experiment
was terminated when the tumors reached 1500 mm$^3$ volume. The tumor growth curves are plotted as mean ± SEM (n = 9 per group). Arrows indicate start of treatment. SIRT1 protein levels in SIRT1 KD and SCR xenograft tumors were determined by WB. D, Absolute change in tumor volume from start treatment (week 6) until stop treatment (week 8). Quantification of the necrotic areas in tumor tissue sections from SIRT1 KD and SCR tumors as described in Material and Methods. Significance was determined with the Student’s t-test. **p<0.01; ***p<0.001.
gene expression profiles
119 liver metastases
correlation clinical variables with
differential gene expression
genes correlated with neo-adjuvant
chemotherapy (613 UP, 481 DOWN)
gene ontology
oxidative phosphorylation
mitochondrial biogenesis

chemo-naive
neo-adjuvant chemotherapy

mean intensity score COXIV

0
1
2
3
4
5

* * * *

NO       YES

n=8 n=8

mean intensity score COXIV

0
1
2
3
4
5

****

n=8 n=8

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

A. 

B. 

C. 

D. 

- **Mitotracker high cells (%)**
  - C NAM C EX527 TV6
  - CRC29 CRC09

- **OCR (pmol/min/10^6 cells)**
  - basal max
  - control NAM

- SIRT1 SIRT3
  - CRC29 L145
  - actin
  - - + - + OX/5FU

- -0.6 -0.4 -0.2 0 0.2 0.4 0.6
- chemotherapy

- -0.4 -0.2 0 0.2 0.4 0.6
- chemotherapy

- p=5.1e-6
- p=0.13

- * ns
- * ns
- ns
- ns

- p=5.1e-6
- p=0.13
Figure 5

A. Relative PGC1α expression (%)

- SCR
- PGC1α KD

B. Mitotracker high cells (%)

- control
- OX/5FU

C. Western blot analysis

- SCR
- PGC1α KD

OX/5FU

actin
**Figure 6**

A. Immunoblot analysis showing cleaved PARP and cleaved caspase-3 levels in different conditions. Actin is used as a loading control.

B. Graph showing the percentage of dead cells across different conditions.

C. Graphs illustrating the change in tumor volume (mm³) over time (weeks) for different conditions.

D. Graphs showing the change in tumor volume (week 6-8) and tumor necrosis (%) for different conditions.
SIRT1/PGC1α dependent increase in oxidative phosphorylation supports chemotherapy resistance of colon cancer

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