MSc in Cancer, Stem cells and Developmental Biology

Major Research Project

"Multiple metabolic hit therapy against cancer"

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LAYMAN SUMMARY

Cancer cells are highly proliferative cells that display distinct metabolic features compared to healthy cells. These metabolic alterations are necessary for cancer cells to secure all the metabolic intermediates necessary to maintain high proliferation rates. One of the most profound changes is the one correlated to glucose metabolism. As opposed to normal cells, cancer cells are characterized by increased uptake of glucose. Cancer cells do not use glucose efficiently for energy production, however, they convert it into lactate, despite the adequate presence of oxygen.

In order to use glucose efficiently for energy production, glucose has to enter the mitochondria in the form of pyruvate. However, in order for pyruvate to enter the mitochondrial membrane a specific transporter is necessary. The Mitochondrial Pyruvate Carrier (MPC) plays the role of the transporter, which consists of two subunits, the MPC1 and MPC2. The expression of the MPC1 gene is downregulated in many cancer types and correlated with poor survival rates in patients. As such, increasing the expression of MPC1 in cancer cells can exhibit anti-cancer properties. To increase the expression levels of MPC1 we transfected the cells with modRNA, which efficiently delivers genetic material inside the cells and avoids any permanent changes in the genomic DNA.

Another difference observed in cancer cells is the elevated synthesis of fatty acids. Thus, we hypothesized that by inhibiting the pathway that leads to the *de novo* synthesis of fatty acids, cancer cells would struggle to survive. One of the enzymes that play a critical role in fatty acid (FA) synthesis is the SCD1 enzyme, which adds a double bond in the saturated form of FA, giving rise to the unsaturated form. Only the unsaturated FA can be used by the cells to satisfy their need for lipids. Inhibiting the SCD1 enzyme causes the accumulation of saturated FA inside the cells, leading to cell death. To further stimulate the effects of the SCD1 inhibition cells were cultured additionally in high concentrations of saturated FA.

The increased glucose uptake in cancer cells is interconnected with high levels of lactate produced, which is known to coordinate important functions for cancer cell progression and survival. Furthermore, lactate works as an energy source. In order for lactate to fuel the energetic demands of cancer cells it has to be converted to pyruvate, a function catalyzed by the LDHB enzyme. Inhibition of LDHB, prevents the usage of lactate for the generation of energy, leading to unfavorable results for cancer cells that depend on this pathway.

Exploiting the differential intrinsic metabolism of cancer cells could result in possible targeted metabolic anti-cancer therapies. Therefore, the study aims to incorporate all the above metabolic hits into one single approach and to test it on highly proliferative cell lines and organoids.

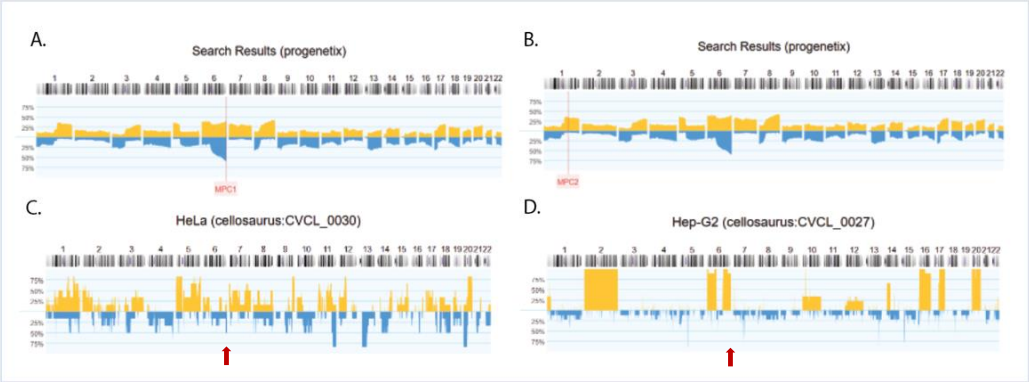
INTRODUCTION

Cancer leads to approximately 11 million deaths each year globally, standing as the second most prevalent factor leading to death among adults and children (Siegel *et* al., 2017). The modern lifestyle in combination with the increased lifespan will lead to approximately 27.5 million new cases of cancer every year until the year 2040, according to the World Health Organization's International Agency for Research on Cancer Global Cancer Observatory (GLOBOCAN). Despite ongoing worldwide efforts to decrease the prevalence of cancer, such as population screens, the development of tools that allow early diagnosis and preventive measures, cancer has remained a fundamental health problem worldwide (Hulvat *et* al., 2020). Several treatment options for cancer have been proven effective, such as chemotherapy, radiotherapy, surgery, and hormonal therapies. However, none of these therapies work for all cancer types. To achieve an optimal effect, a combination of treatments is usually preferred (Wang *et* al., 2018). One of the most common types of cancer treatment is chemotherapy using cytostatic drugs. However, various cancer types are unaffected by existing treatment methods (Morgan *et* al., 2004). Moreover, chemotherapy is highly toxic for the human body, causing severe short-term and long-term side effects (Schirrmacher, 2019).

Cancer cells display distinct biological features compared to healthy cells. Advancements in the understanding of cancer biology have enabled various innovative targeted treatments, such as nanobodies and small molecule inhibitors, which can exploit these differences (Liu *et* al., 2023; Verhaar *et* al., 2021). One of the most distinctive characteristic of cancer cells is their adaptation to high biosynthetic and metabolic needs that can be exploited for the development of targeted anti-cancer therapies.

First of all, according to the nutrient availability of the cells' microenvironment and the energetic and anabolic demands, cells can exploit different glucose catabolic pathways. In the presence of oxygen, cells derive energy from aerobic oxidation using pyruvate, while under hypoxic conditions cells shift their metabolism towards glycolysis and the phosphate pentose pathway (Zhang *et* al, 2023). However, cancer cells prefer glycolysis over oxidative phosphorylation (OXPHOS), even when adequate amount of oxygen is present, a phenomenon first described by Otto Warburg *et* al., in 1927. According to this 'Warburg effect,' increased glucose consumption leads to increased levels of pyruvate, which gets reduced to lactate by lactate dehydrogenase A (LDHA). Accumulating evidence suggests that glycolysis is required to satisfy the high anabolic demands of cancer cells and can provide cancer cells with the necessary building blocks required for rapid growth (Margetis, 2023). The glycolysis-dependent metabolic program, in which the acquisition of building blocks takes precedence over energy production, seems to apply to most of the cancer types described as well as to some tumor-initiating cells (Boroughs & Deberardinis, 2015; Sancho *et* al., 2016).

Pyruvate enters mitochondria through the mitochondrial pyruvate carrier (MPC) complex, consisting of two subunits, the MPC1 and MPC2. Both proteins are required for MPC activity, because the loss of one of the proteins causes destabilization and loss of the other protein (Bricker DK *et* al., 2012, Herzig *et* al., 2012). As mentioned, cancer cells exhibit lower pyruvate oxidation rates as opposed to normal cells, which is thought to be the result of downregulation of MPC1/2. Accordingly, the genomic region 6q27 surrounding the *MPC1* is one of the most commonly deleted loci in cancer, more frequently compared to the region of MPC2 (Cai *et* al., 2014). Additionally, the downregulation or absence of MPC1 is correlated with poor survival rates in lung, colon and kidney clear cell carcinoma (Aguirre-Gamboa *et* al., 2013; Schell *et* al., 2014). Low *MPC* expression correlates with enhanced survival and proliferation in cancer cells (Schell *et* al., 2014) and over-expression of *MPC1* showed antitumor effects by inhibiting cell growth, cell stemness and metastasis in gastric cancer cells (Zhou *et* al., 2017). Together, this indicates that *MPC1* regulation is significantly correlated to cancer cell proliferation.



1.Histoplot generated from Progenetix database displaying the frequencies of regional gains and losses. A. CNV of MPC1 gene across 1000 cancer samples. B. CNV of MPC2 gene across 1000 cancer samples. C. CNV across all chromosomes of HeLa cells. D. CNV across all chromosomes of HepG2 cells. The red arrows indicate the region of MPC1.

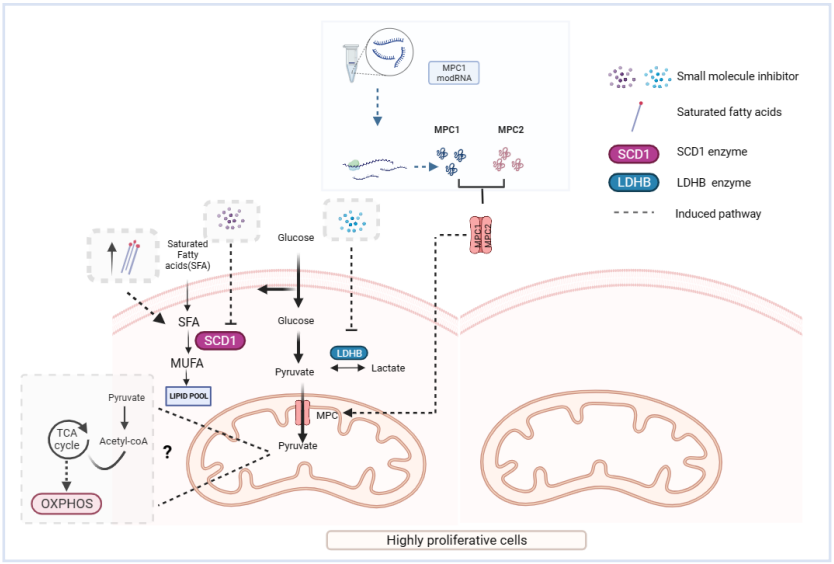
Another important factor in glycolysis is lactate production. LDHA, responsible for conversion of pyruvate into lactate, has been identified as an important enzyme to allow tumor growth. Potent inhibitors have been developed for LDHA to treat cancer, while the role of lactate dehydrogenase B (LDHB) in cancer has not been studied (Sharma *et* al, 2022). LDHB expression is increased in several cancer types ?leading to more aggressive behavior of cancer cells (Satoh *et* al., 2017). A selective LDHB inhibitor (AXKO-0046) has been identified recently by Shibata *et* al, 2021. AXKO-0046 binds to an allosteric binding site of LDHB, which is not present in LDHA, and can be used to target cancer metabolism.

Elevated de novo production of fatty acids (FA) is another cancer-associated metabolic reprogramming mechanism associated with poor prognosis (Brusselmans & Swinnen, 2009). Cancer cells have increased demands for lipids due to their high proliferation rates (DeBerardinis *et* al., 2008). Additionally, de novo lipogenesis can also protect the membranes of cancer cells against oxidative stress–induced cell death by decreasing the percentage of monounsaturated lipids which are more susceptible to death induced by lipid peroxidation. Another advantage that elevated de novo productions of FAs confers is the change in the biophysical properties of the membranes which influences the response of cancer cells to chemotherapeutic agents (Rysman *et* al., 2010). Increased FA synthesis is mediated by the hyperactivation of lipogenic enzymes. Among the lipogenic enzymes that are highly activated in cancer is the stearoyl-CoA desaturase enzyme 1 (SCD1), which is responsible for converting the dietary or endogenous saturated FA and their Δ9-monounsaturated counterparts into their monounsaturated form so that they can be further utilized by the cell (Miyazaki & Ntambi, 2003). SCD1 enzyme is upregulated in cancer cells, promoting the aggressive phenotype (Liu *et* al., 2018).

Inhibition of the lipogenic enzymes slows down the proliferation and results in cancer cell death (Pizer *et* al., 1996). The SCD1 enzyme has been considered an attractive target, which is why several pharmaceutical companies have shown interest in developing potent SCD1 inhibitors. Chemical inhibitors for SCD1 that have been tested have shown promising results when it comes to suppressing the growth of lung cancer, hepatocellular carcinoma, colorectal cancer, and renal carcinoma (Lai *et* al., 2017; Chen *et* al., 2016; Noto *et* al., 2013; von Roemeling *et* al., 2013). SCD1 inhibitors, such as A939572 and T-3764518 have been used in colorectal cancer (CRC) mouse models and showed promising results regarding tumor proliferation (Nishizawa *et* al., 2017). Treating cancer cells with the SCD1 inhibitor simultaneously with other targets

can maximize the effectiveness of the SCD1 inhibition. One example is the combinational administration of SCD1 inhibitors with Saturated FAs. The inhibition of SCD1 reduces the de novo biogenesis of unsaturated FAs causing the accumulation of cytotoxic Saturated FAs (Beloribi-Djefaflia *et* al., 2016).

In this work, we aimed to exploit the cancer-specific cell metabolism to inhibit proliferation and/or induce cell death in cancer cells. We targeted different metabolic pathways, including glucose, lactate and fatty acid metabolism. We explored the effectiveness of both single- and multiple so-called 'metabolic hits' as potential anti-cancer therapy. First, we tested the overexpression of MPC1 in three different highly proliferative cell lines (human hepatoma HepG2 cells, human embryonic kidney HEK293T, human cervical HeLa cells) with different characteristics regarding metabolism and copy number variation of the *MPC1* (Figure 1). We established MPC1 overexpression using modified RNA, which allows the transient and potent overexpression of MPC1. We show that the MPC1 overexpression can either suppress cell growth or cause cell death, depending on the cell line. Additionally, we inhibited the SCD1 enzyme using a small molecule inhibitor and showed that it has a slightly stronger effect on the cells in combination with the MPC1 overexpression. Lastly, we combined LDHB inhibition and MPC1 overexpression, which resulted in increased cells death. Taken together, we establish a framework for the exploitation of metabolic weaknesses specific to cancer cells.



2. A metabolic approach to cancer therapy: schematic representation of the changes induced in cell metabolism after the metabolic therapy.

MATERIALS & METHODS

1. Primers
I. qrt-PCR (MPC1)
MPC1_FW1 GGACTATGTCCGAAGCAAGG MPC1_RV1 AAATGTCATCCGCCCACTGA
MPC1_FW2 GTCCGAAGCAAGGATTTCCG MPC1_RV2 CAAATGTCATCCGCCCACTG
MPC1_FW3 GGACTATGTCCGAAGCAAGG MPC1_RV3 AAATGTCATCCGCCCACTGA
II. IVT PCR
XU-F1-pcDNA4 CTACCGGTCGTACAGAAGCTAATACG XU-T120 TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTCCTACTCAGGCTTTATTCAAAGA CCA

2. Chemicals/Antibodies
I. MPC1 primary antibodies
Western Blot: MPC1 (D2L9I) Rabbit mAb (Cell Signaling Technology)
Immunofluorescence (IF): MPC1 (D2L9I) Rabbit mAb (Cell Signaling Technology)
II. Fatty acids
Oleic acid (diluted in DMSO, MCE MedChemExpress)
Stearic acid (diluted in DMSO, MCE MedChemExpress)
Palmitic acid (diluted in DMSO, MCE MedChemExpress)
Palmitoleic acid (diluted in DMSO, MCE MedChemExpress)
III. Inhibitors
SCD1 inhibitor: A939572 (Sigma-Aldrich)
LDHB inhibitor: AXKO-0046 (MCE MedChemExpress)

Cell culture

HepG2, HEK293T and HeLa cell lines were cultured in DMEM + GlutaMax (1x, Gibco) supplemented with 10% FBS (Gibco) and 1% Penicillin/Streptomycin. For maintenance, the cells were cultured in T75 flasks at 37ºC and 5% CO_2 and splitted twice per week. To grow the cells in different concentrations of glucose, DMEM (1x, Gibco) medium was used supplemented with 10% FBS, 1% GlutaMax (100x, Gibco) and 1% Penicillin/Streptomycin with the desired amount of glucose.

modRNA production

For the production of modRNA, the plasmids that contain the gene of interest need to get linearized. For the linearization of the plasmids, SpeI (NEB) was used as the restriction enzyme for 1h at 37ºC followed by a 5min. inactivation step at 80ºC. The product was loaded on an agarose gel together with the undigested plasmid to check if the plasmid was successfully linearized. After purification of the digested plasmid with the NucleoSpin Gel and PCR Clean-up kit (Macherei-Nagel), a PCR reaction was performed for the addition of a polyA tail in a final volume of 200ul. The Q5 hot start high fidelity polymerase was used

together with the XU-F1-pcDNA4 and XU-T120 primers. The PCR product was purified using the RNeasy Mini kit (QIAGEN) and loaded on agarose gel. Only the product with the polyA tail attached was isolated from the gel and purified with the NucleoSpin Gel and PCR Clean-up kit (Macherei-Nagel), to be used for the in vitro transcription reaction. For the in vitro transcription the T7 MegaScript kit (ThermoFischer) was used. To prepare the reaction the purified product was mixed with a 3'-O-Me-m7G cap analog and a mix of all the NTPs. The reaction was incubated for 3h in a Thermocycler. Next, 4ul of turbo DNAse (ThermoFischer) were added for 40ul of reaction and left to incubate for 15min. at 37ºC. After purifying the reaction using the RNeasy Mini kit (QIAGEN), the produced mRNA was incubated for 30min at 37ºC together with 11ul of 10x antarctic phosphatase buffer and 2ul of antarctic phosphatase (NEB). The reaction was purified once again and the concentration of the modRNA was measured with Qubit. The modRNA was immediately stored at -80 ºC.

Cell Transfections

Cells were plated to be at 70% confluency the day of the transfection. For imaging and functional assays cells were plated at 20.000 cells/well in a 96-well plate and the day after were transfected with either 120 ng/well or 240 ng/well of modRNA. For immunofluorescence imaging the transfection occurred the same day as the plating and the amount of modRNA used for transfection was either 60ngr/well or 120ngr/well respectively. For the exCELLigence experiment 5.000 cells/well in 96-well plates were plated and transfected 24h after with either 60ngr/well or 120ngr/well. For qPCR and Western blot 16 times more cells were plated in a 6-well plate and transfected with 16 times more modRNA, compared to the amount used in the 96-well plate. HepG2 and HeLa cells were transfected with modRNA using Lipofectamine3000 (Invitrogen), while HEK293T cells were transfected using Lipofectamine2000 (Invitrogen), following the manufacturer's instructions. The transfection mixes were prepared on ice inside the RNAse-free hood and left to incubate for 15-20 min at room temperature (RT). 10ul/well of the transfection mix were then added in a 96-well plate. In the 6-well plate 250ul were added per well for HEK293T and 400ul for HepG2 and HeLa cells.

SCD1 inhibitor and Fatty acids treatment

Cells plated on a 96-well plate were treated with the SCD1 inhibitor with or without 50uM fatty acids. The SCD1 inhibitor was dissolved in dimethylsulfoxide (DMSO), heated at 37 ºC and added to an ultrasound sonicator bath for 5 min. Subsequently, both the inhibitor and the fatty acids were diluted in prewarmed DMEM medium separately before adding them to the wells at a final concentration of 200 nM and 50uM respectively.

LDH inhibitor treatment

Cells plated on a 96-well plate were treated with 10uM, 1uM or 0,1uM LDHB inhibitor. To prepare the different concentrations the inhibitor was first dissolved in dimethylsulfoxide (DMSO) and then diluted in DMEM medium. The cells were first transfected with modRNA, then treated with the SCD1 inhibitor and at last the LDH inhibitor was added. The cells were left to incubate for 48h before imaging.

Organoid culture

Human liver organoids (EMC18 line) were plated in 6-well plate with GF$^-$ (Advanced DMEM/F-12, 1x, Gibco) and BME and cultured in 2ml of human liver expansion media supplemented by forskolin (2000x), A83 (2000x) and primocin (1000x). Organoids formed in BME were chemically disrupted by TryplE and passaged from 1 to 6 wells once a week.

Electroporation

To investigate the function of MPC1 overexpression in organoids we electroporated them using modRNA. 2 wells of organoids from a 6 well-plate were used per condition. The organoids were normally splited and resuspended in OptiMEM (1x, Gibco). The cells were then mixed with the modRNA and transferred in the electroporation cuvettes. The electroporated cells were transferred into 1ml of fresh GF^- and left to rest for 20 min. After that the cells were spun down and resuspended in GF^- and BME, before plating them in a 96-well plate (1x10ul drop/well).

Fluorescence Microscopy

The assessment of the proliferation and viability of the cells was performed by live-dead staining using Hoechst (Invitrogen, 1/1000) and PI (Invitrogen, 1/20). A mix of the two stains was prepared and added to the wells on top of the medium. The cells were incubated at 37ºC for 15 min. in the dark and imaged immediately after using the Thunder fluorescent microscope. Pictures were taken from the whole well and then were merged into a single one using the Leica software. To analyze the images obtained the blue (Hoechst) and red (PI) channels were merged using the Fiji software and positive cell detection was performed on Qupath, to detect the total number of cells (Hoechst+) in the well and the number of dead (PI+) cells out of them.

Immunofluorescence staining

Cells transfected with modRNA were grown on 96-clear bottom well plates coated with 50ul Poly-D-Lysine (Thermo Fischer). After 1 hour of incubation time at room temperature the Poly-D-Lysine was aspirated and the wells were washed 3x with 100ul PBS0, before plating. The transfection occurred on the same day. 24h or 48h prost transfection the cells were first stained with the mitochondrial stain MitoTracker® Deep Red (Cell Signaling Technology), according to the manufacturer's instructions. After that the cells were chemically fixed with 4% formaldehyde for 20 min at RT. Before adding the antibodies the cells were permeabilized with Triton-x-100 0,1% (Sigma-Aldrich) diluted in PBS0 for 15 min. For the antibody staining, 4% BSA (Sigma-Aldrich) blocking buffer diluted in PBST (PBS+ 0.1% Tween-20, Sigma-Aldrich) was added to the wells. After 45 min. at RT the blocking buffer was removed the cells were incubated overnight with the MPC1 antibody at 4ºC. After removing the MPC1 primary antibody, an anti-rabbit-FITC antibody (1/100) was used as the secondary antibody together with a Phallodin-TRITC antibody (1/100) to stain the cytoskeleton. A mix of the two antibodies was prepared using 1% BSA. For nuclear staining Hoechst (1/1000) was used. After 15 min. the cells were washed 3 times for 5 min. in the dark with PBS0. Before imaging, 150ul PBS0 were added in the wells. A confocal microscope was used for imaging.

xCELLigence

To measure cell proliferation cells were plated in E-plate 96 PET plates, compatible with the xCELLigence the real-time cell analyzer and transfected with modRNA. The plate was left in the xCELLigence machine for either 5 or 7 days and after that the data were extracted and analyzed.

Western Blot

Cells were plated in 6-well plates so that on the day of transfection they would be at 2/3 of confluency. 24h or 48h post transfection cells were harvested for protein extraction. To harvest the cells Pierce™ Ripa Buffer (Thermo Scientific) was used as a lysis buffer supplemented with Halt™ Protease inhibitor (100x, Thermo Scientific). After centrifugation, the supernatant was collected and the protein concentration was determined using the Biochioninic Acid assay) (BCA assay) (ThermoFisher).After preparing the samples

to a final volume of 20ul using Laemli buffer and 1ul of SDS sample buffer were run on a Mini-PROTEIN TGX Stain-Free Gel (4-20% gradient, BIO-RAD) at first for 10 min. at 120V and afterward for 45 min. at 160V, with TGS(Tris/ Glycine/ SDS) buffer as running buffer. To transfer the proteins to the membrane (immobilin-FL) wet transferring was performed. Before transferring the membrane was activated for 2 min. in ethanol. After constructing the cassette and filling the blot box with Tris/Glycine, 10% methanol) blot buffer it run for 45 min. at 90V at 4ºC. After transferring, the membrane was incubated for 1h with 5ml 5% ELK blocking buffer diluted in TBS-T (TBS buffer + 0.1% Tween-20) at RT. After blocking, the membrane was incubated with the MPC1 primary antibody (1:1000, Cell Signalling) at 4°C overnight. The membrane was washed three times for 5 min. each with TBST and incubated further with HRP conjugated goat antirabbit secondary antibody (1:2000, ThermoFisher) for 1 hour at RT. Both the primary and secondary antibody were diluted in 5% ELK blocking buffer. After washing the membrane three times for 5 min. each with TBST, 500ul of the SuperSignal West Pico substrate (Thermo Fischer Scientific) were spread on the membrane at the height the MPC1 protein is expected (12kDa). One minute after, the immunoblot was visualized by chemiluminescence using the ChemiDoc Imaging system (BioRad). If the signal was not strong enough the time of exposure was increased or more sensitive substrates such as the SuperSignal West Dura Extended Duration substrate (Thermo Fischer Scientific) or the SuperSignal West Femto Maximal Sensitivity substrate (ThermoFischer Scientific) were used. β-actin (manufacturer), was used as a housekeeping gene.

RNA extraction and qRT-PCR

For the purpose of qPCR, 320.000 HEK293T cells/well were plated in a 6-well plate, transfected after 24h with 120ng modRNA and left to incubate for 24h or 48h. Total RNA was extracted from the cells using the PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. The extracted RNA was used as a template for cDNA synthesis using the reverse transcription reagents (iSCRIPT Cdna Synthesis kit, BIO-RAD). A -RT reaction was also prepared using only RNA sample and water. The resulting cDNA was subjected to qRT-PCR with SYBR green. The primers used are listed in Table 2.1. The relative mRNA expression of MPC1 was determined based on the cycle threshold (Ct), using the $2^{-\Delta\Delta Ct}$ formula and normalized against two housekeeping genes (β-actin, GADPH). The samples were run in triplicate.

Functional assay: LDH activity assay

To measure the amount of intracellular LDH enzyme the CyQUANT™ LDH Cytotoxicity Assay-Fluorescence (Invitrogen) was performed. First, the medium was aspirated to prevent the measurement of the extracellular LDH. Fresh medium was added to the wells and the cells were lysed with Lysis Buffer (10x). After a 45 min. incubation time at 37ºC, 50ul of the lysate were transferred to a new plate and on top of that 50ul Reagent Mix were added. After a 10 min. incubation time at RT, 50ul Stop Solution were added and the fluorescence was measured by a microplate reader (Clariostar, Excitation:560nm/Emission:590nm). All the reagents used were provided by the manufacturer.

Functional assay: ATP production assay

To measure the amount of ATP produced by the cells the ATPlite™ assay was performed, following the manufacturer's instructions. A white plate was used to transfer the cell lysate to. The microplate reader (Clariostar) was used to measure the luminescence.

Functional assay: Lactate measurement

To measure the lactate excreted by the cells, the medium was collected after 24h or 48h incubation time with the modRNA. The samples were then sent to be biochemically analyzed and determine the levels of lactate produced.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad software, mention version, La Jolla, CA, USA). Data were analyzed by Student's t test and differences were considered significant at $p<0.005$.

RESULTS

The downregulation of *MPC1* in cancer cells has been shown to result in enhanced malignancy and lower survival rates in patients (Li *et* al., 2017). Therefore, we hypothesized that the overexpression of *MPC1* could partially reverse the malignant phenotype. To evaluate the anti-cancer potential of *MPC1*, several studies tried to overexpress it by establishing stable lines and observed repressed cell growth (Schell *et* al., 2014). As described, modRNA holds several advantages, as it enables large-scale production and effective transfection. Therefore, we produced MPC1 modRNA and assessed the effects of *MPC1* overexpression in HepG2, HEK293T and HeLa cells. We included a non-functional modRNA and lipofectamine as controls. Western blot and qRT-PCR results confirmed the overexpression of MPC1 induced by the modRNA (Figure 1A; Figure 1B and C).

Intriguingly, western blot from HEK cells showed an increase in the levels of MPC1 24 hours after transfection with MPC1 modRNA, but not after 48 hours, despite the presence ofMPC1 RNA, which was confirmed by qRT-PCR. This suggests the presence of some kind of mechanism specifically targeting the MPC1 protein. Moreover, we tested several *in* vitro transcription kits and found that the highest levels of MPC1 were detected in the samples that were transfected with MPC1 modRNA produced by the VENI kit. From the western blot performed from protein samples gathered from HeLa cells, there is still overexpression 48h post transfection (Figure 1B and C).

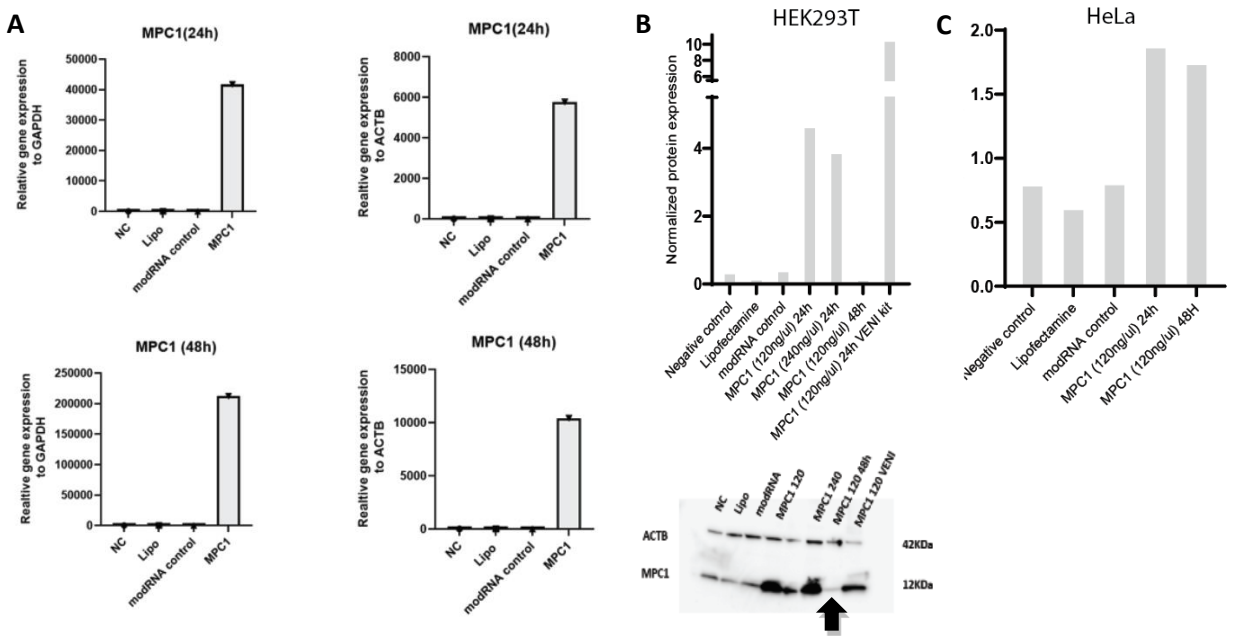


Figure 1: MPC1modified messenger RNA induces overexpression of MPC1.

A.qRT-PCR analysis of HEK293T cells with MPC1 overexpression 24h and 48h post transfection with MPC1 modRNA B. Western blot of MPC1 in HEK293T cells. With the black arrow is indicated the absence of MPC1 in the 48h condition C. Western blot of MPC1 in HeLa cells after overexpression.

As a first step, we measured the effect of *MPC1* overexpression on HepG2 and HEK293T cell proliferation. For this experiment, two concentrations of modRNA were used, one of 60ng/ul and one of 120ng/ul. We observed a drop in the proliferation of the MPC1-transfected cells in both concentrations compared to the modRNA control. In HepG2 cells, the higher concentration of 120ng/ul of MPC1 modRNA exhibited a

stronger effect on proliferation as opposed to the 60ng/ul. However, the cell index of the MPC1-transfected cells was similar to the one of the negative control (Figure 2A). HEK293T cells exhibited lower proliferation rates in the MPC1 condition compared to both the modRNA control and the negative control, however, this time we did not observe lower cell index when the concentration of MPC1 modRNA increased (Figure 2B).

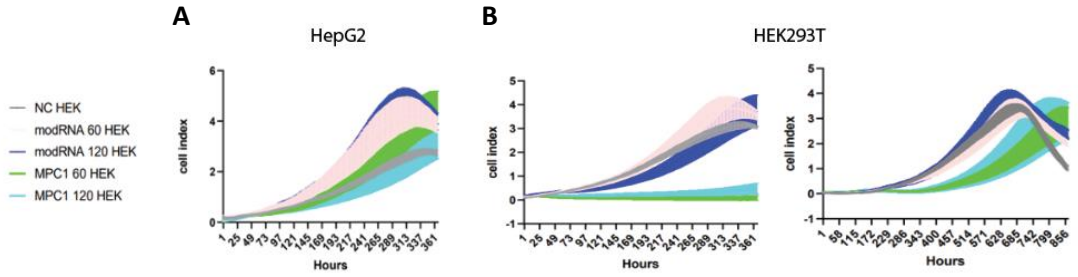


Figure 2: Cell proliferation curve for HepG2 B. Cell proliferation curve for HEK293T. Overexpression of MPC1 slowed down cell proliferation.

After confirming that the produced modRNA effectively overexpressed MPC1 slowing down proliferation rates, we further evaluated the effects of MPC1 overexpression on cell viability. We set up a cell-count experiment by staining all the transfected cells with Hoechst, and dead cells with PI. Imaging the total number of cells (Hoechst+) and the number of dead cells (PI+) revealed that overexpression of MPC1 inhibited cell proliferation in both HepG2 and HEK293T cells without affecting cell viability. Specifically, the fold change of the PI+ cells over the Hoechst+ cells in the MPC1 condition was twice as high compared to the control due to the lower number of cells observed in the MPC1 wells (Figure 3A and B).

While in HepG2 and HEK293T cells the differences in the number of dead cells between MPC1 and control were small, in HeLa cells we observed an increased number of dead cells after MPC1 overexpression ($p<0.05$). In HeLa cells, 120ng/ul of MPC1 modRNA did not seem to affect growth rate but induced high levels of cell death. Similarly, the fold change of PI+ over Hoechst+ cells in the MPC1 condition was four times higher compared to control, but this time due to the higher number of PI+ cells (Figure 3C). All these data were retrieved 24h post transfection and the modRNA used was produced using the MEGAscript kit.

When HEK293T cells were transfected with modRNA produced by the VENI kit, the cellular response mimicked the response in HeLa cells, showing a slight drop in cell growth and a high number of dead cells (Figure 3D). Taking into consideration the Western blot results, the increased lethality caused by the VENI MPC1 might correlate with the overproduction of MPC1 we observed, causing cell stress.

Next, we investigated the effects of MPC1 overexpression on primary human liver organoids in expansion medium. Liver organoids electroporated with 10ugr MPC1 modRNA showed decreased proliferation compared to control. Cell numbers were measured in three different timepoints (24h, 48h, 72h). Although there was a small increase in the number of Hoechst+ cells overtime, the overall number of cells for each timepoint remained lower in the MPC1 condition compared to the control. The MPC1 overexpression did not influence the viability of the organoids, since 24h after the incubation with MPC1 the number of PI+ cells were lower than the control. The PI+ cells of the MPC1 condition slightly increased overtime, as expected (Figure 3E).

Collectively, all the above provide a strong indication that MPC1 overexpression effectively reduces cell proliferation and cell viability in HepG2, HEK293T, human liver organoids and HeLa cells respectively (Figure 3).

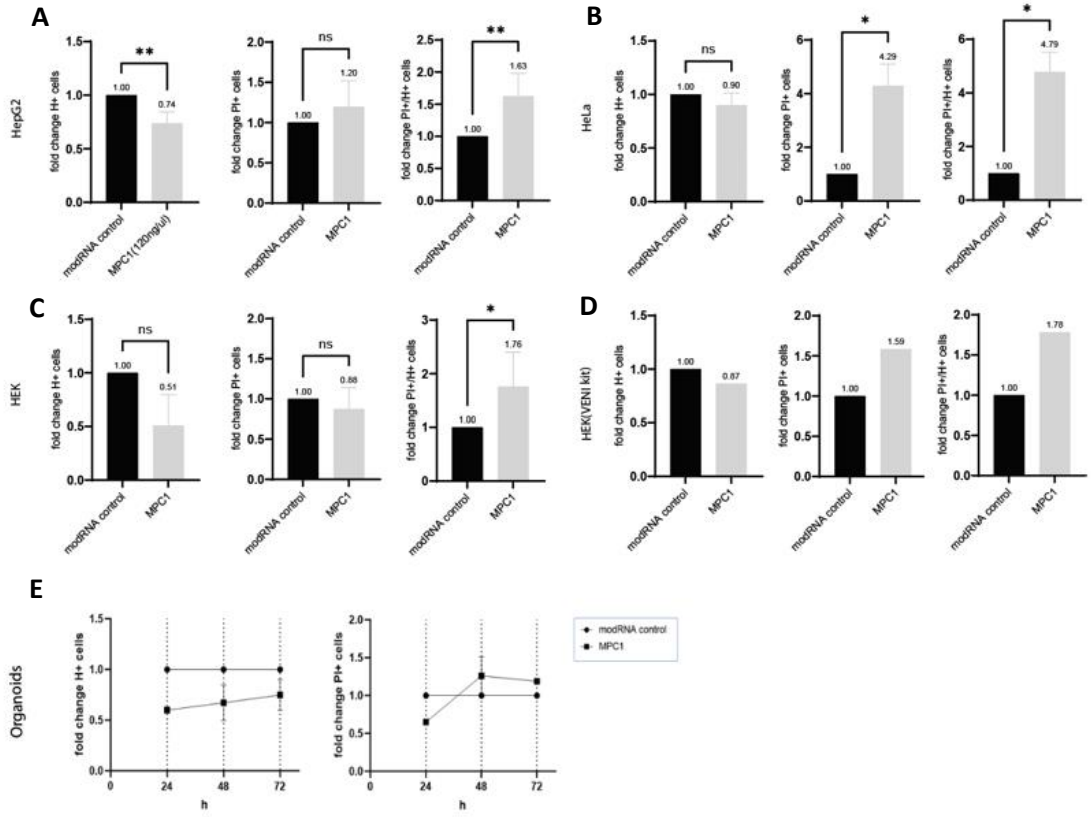


Figure 3: The effect of MPC1 overexpression is cell-type specific.

Cell number and cell viability determined by Hoechst/PI in A. HepG2 B. HeLa C. HEK293T D. HEK293T cells transfected by MPC1 modRNA produced by the VENI kit E. human liver organoids.

To confirm proper localization of MPC1 in the cells, we stained mitochondria and MPC1 in HeLa and HEK293T cells. The images confirmed the co-localization of the MPC1 with the mitochondria (Figure 4A) and the overexpression of MPC1. The mitochondrial pattern in HEK cells remained the same 24h and 48h post transfection with MPC1 modRNA (Figure 4B). The green signal of MPC1 was higher upon the overexpression, and it was still detected after 48h post transfection on both HeLa and HEK cells (Figure 4C and D). This was surprising, since we did not observe MPC1 expression with western blot 48hours post transfection in HEK cells.

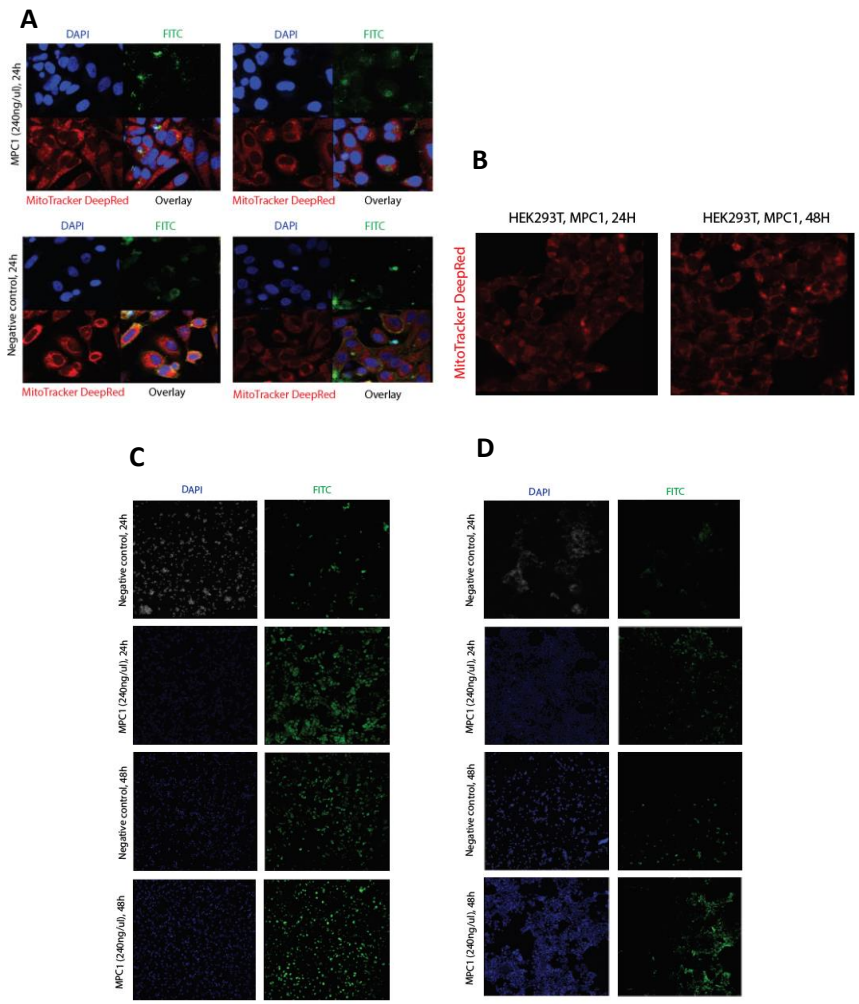


Figure 4: Immunofluorescence microscopy of negative control and MPC1-treated cells.

A. Co-localization of MPC1 with the mitochondria in MPC1-treated HeLa cells (63x) B. Same mitochondrial pattern in HEK293T cells transfected with MPC1 modRNA 24h and 48h post transfection. C. Increased expression of MPC1 (green) in MPC1-treated HeLa cells D. Increased expression of MPC1(green) in MPC1-treated HEK293T cells

To further elucidate the effects of MPC1 overexpression we performed a series of functional assays. First, we measured the lactate excreted in the medium. In the medium that was collected 24h after transfection with MPC1 modRNA, the levels of lactate were not significantly lower compared to the modRNA control, for both HepG2 and HEK cells. However, 48h post transfection the levels of lactate showed a higher drop, mostly for HepG2 cells (Figure 5A). The ATP assay revealed less ATP present 24h post transfection in HEK cells, but no significant differences in the levels of ATP in HepG2 (Figure 5C). Next, we measured the levels of active LDH inside the cells. The data showed no statistically significant decrease 24h and 48h post transfection for both HepG2 and HEK cell lines, however there appeared to be less LDH production over time (Figure 5B).

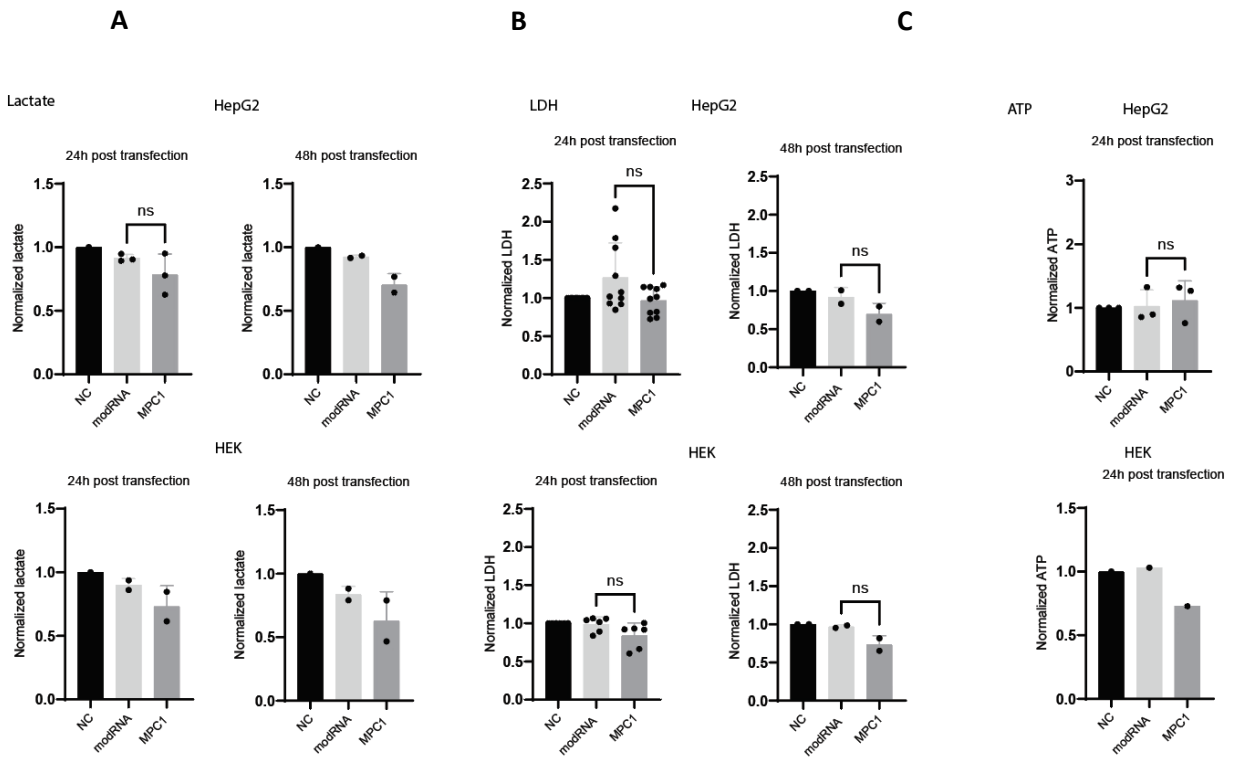


Figure 5: MPC1 overexpression effect on cell metabolism.

Effects of MPC1 overexpression on A. Lactate production B. LDH activity C. ATP production in the indicated cell lines.

To further investigate metabolic targeting of cancer cells, we treated highly proliferative cells with an SCD1 inhibitor, A939572. However, addition of the SCD1 inhibitor to the medium caused highly variable results. Upon addition of the SCD1 inhibitor, cell proliferation was slightly suppressed, and cell death increased. However, the differences between the modRNA and MPC1 combined with A939572 were mostly subtle. Overall, the combination of MPC1 overexpression and SCD1 inhibition caused a slightly stronger effect on both HEK and HeLa cells in terms of cell growth and cell viability, as shown from the %PI+/H+ graphs (Figure 6).

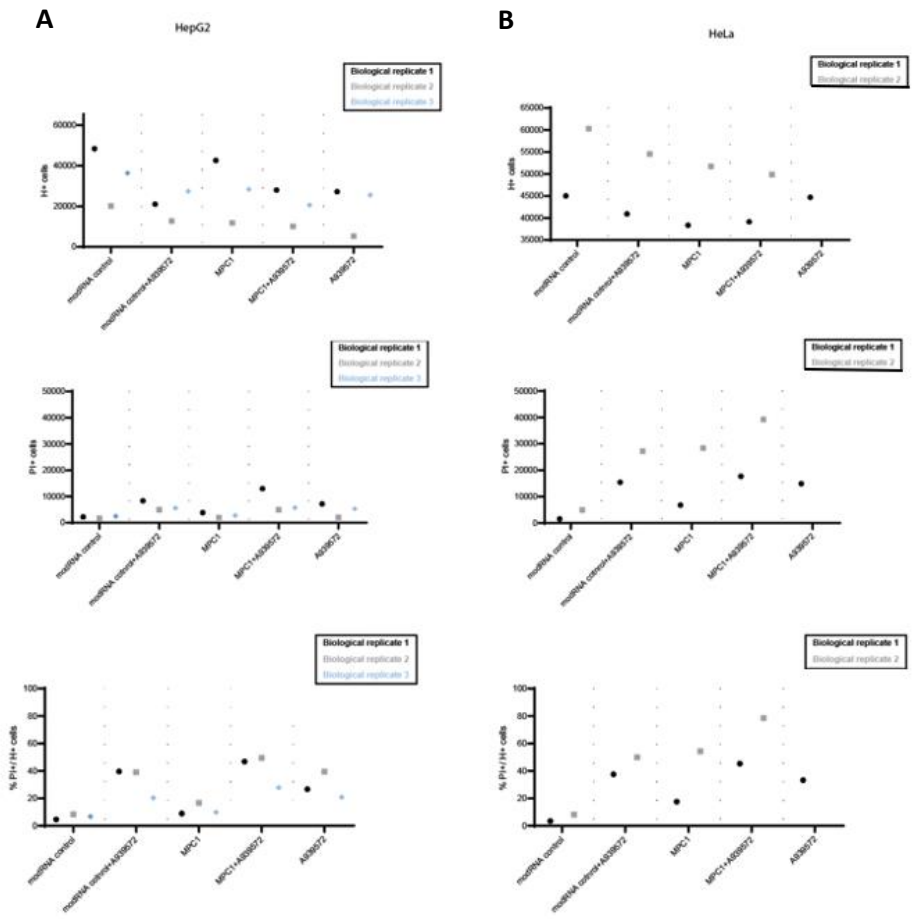


Figure 6: Cell number and cell viability determined by Hoechst/PI after inhibition of the SCD1 enzyme in A. HepG2 B. HeLa.

Previously, other studies observed that the addition of fatty acids could enhance the role of the inhibitor (Potze *et* al., 2016). More specifically, culturing the cells in SFAs, such as stearic or palmitic acid, could expose the cells to higher stress levels, because of the SCD1 inhibition, while the addition of unsaturated FAs, such as oleic or palmitoleic acid, is expected to ameliorate this phenotype. We only observed an increase in the number of dead cells when FAs were added to HEK293T cells, independent of saturated or unsaturated FA addition. From the experiment performed on HepG2, oleic acid together with the SCD1 inhibitor was the one to perform better in terms of cell death. Lastly, the stearic acid together with the SCD1 inhibitor showed more promising results on HeLa cells. No rescue effect was observed from any of the FAs examined and for none of the cell lines (Figure 7).

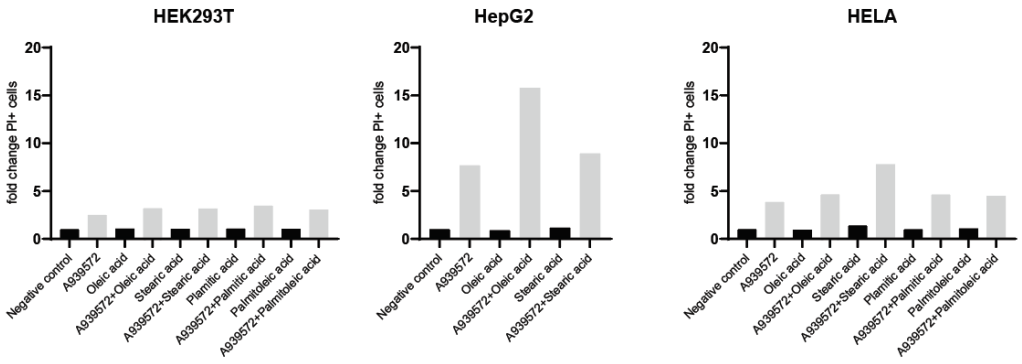


Figure 7: Cell number and cell viability determined by Hoechst/PI after inhibition of the SCD1 enzyme in cells cultured in high concentrations of fatty acids

Next, to evaluate the effects of LDHB inhibition we tested different concentrations of the LDHB inhibitor, AXKO-0046. When using the highest concentration (10uM), almost all the cells died, irrespective of the condition examined. As the concentration of the LDHB inhibitor dropped, the lethality dropped as well, with the biggest differences in the lowest LDHB concentration (0.1uM). Interestingly, the lethality did not drop for the MPC1 condition, with or without the SCD1 inhibitor. However, the addition of the SCD1 inhibitor did not show any additional effect on the cells (Figure 8).

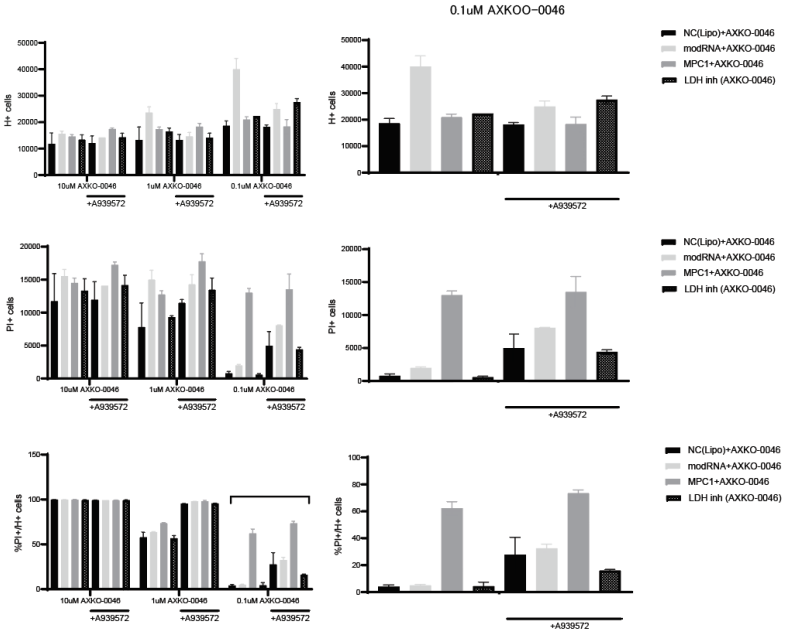


Figure 8: MPC1 overexpression and LDHB inhibition have a modest synergistic effect

Cell number and cell viability determined by Hoechst/PI in MPC1-treated HeLa cells, after inhibition of the LDHB and SCD1 enzyme. Different concentrations of 10uM, 1uM and 0.1uM of the LDHB inhibitor (AXCO-0046) have been tested.

Finally, we investigated the effect of glucose concentrations on MPC1 overexpression. To test this, we cultured the cells in different concentrations of glucose, ranging from 0mM to 50mM. We observed no increase in proliferation with higher glucose concentration in the MPC1-overexpressed cells as opposed to the control. Intriguingly, lower glucose caused more cell death in the control cells, however in the MPC1-overexpressed cells the level of cell death in high glucose concentration remained almost as high as when no glucose was added in the medium. This indicates that the effect of MPC1 on cell proliferation and cell viability seems to be independent of the amount of glucose and therefore the effect that we observe 48h post transfection is indeed due to the MPC1 overexpression and not due to the lower glucose concentration.

Furthermore, we also tested the effect of different glucose concentrations on cells treated with both MPC1 modRNA and the SCD1 inhibitor. Similarly, higher levels of glucose promoted cell growth in the control+SCD1 inhibitor condition but not in the MPC1+SCD1 inhibitor. The combination of MPC1+SCD1 inhibition led to a surprisingly higher number of dead cells in comparison to control+SCD1 inhibition, which decreased only slightly over exposure of the cells to more glucose. This suggests that in the presence of glucose the effect of MPC1+SCD1 inhibitor becomes more prominent (Figure 9).

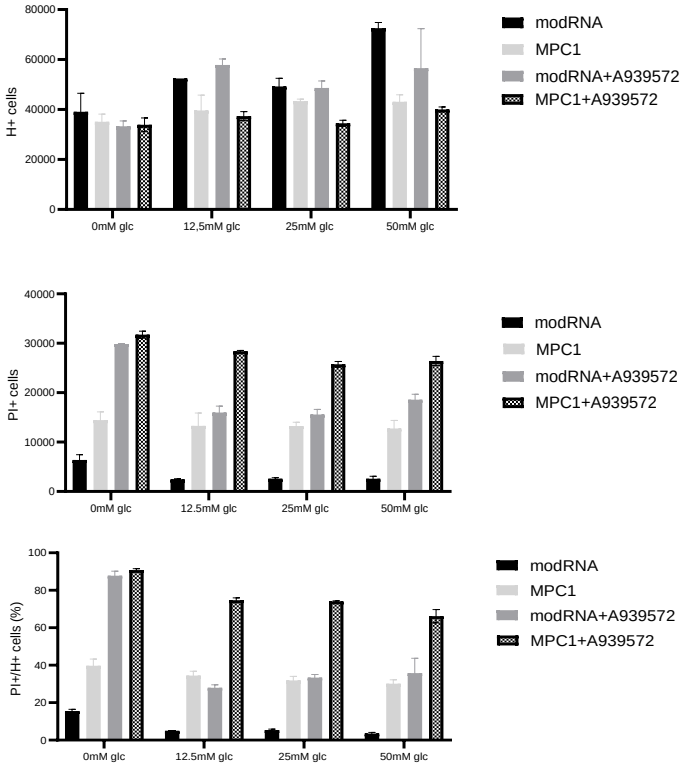


Figure 9: The effect of MPC1 overexpression in different glucose concentrations

Cell number and cell viability determined by Hoechst/PI in MPC1-treated HeLa cells with or without the SCD1 inhibitor in different concentrations of 0Mm, 12,5mM, 25Mm (DMEM) and 50mM glucose.

DISCUSSION

Cancer is a disease influenced by numerous different pathways and cellular mechanisms, which makes the development of a universal treatment difficult. Identifying a set of potential therapeutic targets that are crucial in many cancer types would be highly beneficial in terms of cancer treatment. In this study we identified MPC1 as a possible target. The MPC1 modRNA effectively overexpressed MPC1 in cells. The increased expression of MPC1 led to suppressed cell growth or reduced viability, depending on the cell-model used. Additionally, the combination of MPC1 overexpression with the inhibition of the SCD1 enzyme enhanced only slightly the effect of the MPC1 overexpression. A subtle synergistic effect was exhibited when MPC1 overexpression was combined with the inhibition of the LDHB enzyme.

To begin with, MPC1 is downregulated or depleted from the genome of various cancer types and is a key molecule of mitochondrial metabolism. Using modRNA to successfully increase the expression of MPC1 in highly proliferative cells strongly affects proliferation. The MPC1 overexpression inhibited cell growth of HepG2 and HEK cells as well as of human liver organoids, without impairing viability. Putatively, this is due to decreased glycolysis and consequential reduced formation of building blocks for rapid proliferation. However, when HeLa cells were subjected to MPC1 overexpression it resulted in increased cell death and minimal impairment of cell growth. If we hypothesize that the overexpression of MPC1 leads to more pyruvate inside the mitochondria and increases OXPHOS, then cell death could be attributed to the increased levels of ROS produced by OXPHOS, driving cells to apoptosis (Tai *et* al., 2018). To measure the ROS in the presence of MPC1 either MitoSox red or Reactive Oxygen species assay kits could be used.

Overexpression of MPC1 thus diverged between different cell types. These cell-type specific responses can be asserted to the differences in the intrinsic metabolism of each cell line. According to the Human Protein Atlas the expression levels of MPC1 vary between the different cancer cell lines. HepG2 cells have a relatively high expression compared to HEK293T and HeLa, with the HeLa cells having a very low expression of 37.9 Ntpm. This difference could partially explain the diverse effect of MPC1 overexpression on HeLa cells and highlights the necessity of carefully choosing the cell line to target. Cancer cell lines with very low MPC1 activity that could be promising targets are those derived from prostate cancer, bladder cancer, rhabdoid, testis cancer, adrenocortical cancer, kidney cancer and gallbladder cancer. In normal mammalian tissues the expression of MPC1 seems to be high in the heart, muscle, liver, and kidney. Interestingly, the expression of MPC1 is high in kidney but low in kidney cancer cell lines, which suggests that there is a mechanism activated that tightly downregulates MPC1 expression. Therefore, by testing cell lines with differential expression of the MPC1 protein we confirm that indeed the intrinsic levels of MPC1 can affect the reaction of the cells to the overexpression and that when it comes to metabolic therapies it is crucial to fully investigate the metabolism of the model you use. However, that becomes more challenging with tumors because of their intrinsic heterogeneity**.** Namely, the MPC1 re-expression on adherent colon cancer cells did not impose any negative effect on their growth, while that was not the case for adherent gastric cells (Schell *et* al., 2014).

Another difference between the cell lines was observed after performing Western Blot on protein samples derived from cells treated with MPC1 modRNA for 48h. For HEK cells we observed the absence of MPC1 protein 48h after the transfection with the MPC1 modRNA, while for HeLa cells the MPC1 protein remained detectable. It has been previously described that either MPC1 or MPC2 protein, when expressed alone, fail to accumulate despite the high levels of mRNA present (Schell *et* al., 2014). However, in our case accumulation of the protein occurred 24h post transfection but was lost 48h later. Therefore, another possibility could be that the mitochondria in HEK293T cells are degraded after 48h, explaining the absence of the MPC1 protein. However, the images obtained from IF showed that the mitochondria remained intact. Therefore, if we repeat the western blot and observe the same pattern, we could hypothesize that a tightly

regulated MPC1-specific protein degradation mechanism is responsible for the observed effects. This hypothesis could be supported by the findings that continued passage of HCT15 cells that overexpress MPC1 and MPC2 lose the MPC1 protein, which proves the existence of a mechanism that selectively acts against the expression of the MPC1 protein (Schell *et* al, 2014).

To better understand the complex response of highly proliferative cells after overexpression of MPC1, the levels of innate metabolic products were measured. The levels of lactate and LDH enzyme seemed to be reduced overtime, albeit not significantly, both in HepG2 and HEK293T cells. The ATP produced after the MPC1 overexpression in HepG2 cells was similar to the control, while in HEK cells, it was lower. The data obtained from the functional assays do not allow us to draw any conclusions and further investigation of the metabolic alterations caused by the MPC1 overexpression is necessary. One more direct way to gain insight in cell metabolism is analyzing OXPHOS and glycolysis by measuring the levels of oxygen consumed by the cells and the rate of extracellular acidification, respectively. This can be done using a Seahorse assay. A pyruvate colorimetric assay kit could also be performed to localize pyruvate in the cell and thereby determine the activity of the MPC transporter. This was done in MPC1-/- cells (Xaoli *et* al., 2017), where they found that pyruvate accumulated in the cytoplasm and not in the mitochondria. These approaches would help to clarify whether more pyruvate enters the mitochondria upon the overexpression of MPC1 and if the pyruvate is used for additional OXPHOS or is just trapped inside the mitochondria. An isotope labeled flux analyses could also be performed to look at how much of glucose derivatives are used as a source for the citric acid cycle.

The instability of the synthetic modified RNA molecules highlights the necessity of combining the MPC1 overexpression with other types of treatment to prolong the anti-tumor effects. The pharmacologically induced reduction of the activity of the SCD1 enzyme could be one, as it disrupts cancer cells lipid metabolism. Treating the cells with the SCD1 inhibitor impaired both survival and growth of HepG2, HEK and HeLa cells. However, the effects of SCD1 inhibition were only subtle after combining it with MPC1 overexpression. Subsequently, the addition of unsaturated FA did not rescue the phenotype of SCD1 inhibition, as expected. So far, we have only tested the SCD1 inhibitor on highly proliferative cell lines. However, it would be interesting to try the inhibition on organoids. Skrypek *et* al., 2021 have proven the degeneration of tumor organoids after inhibition of the SCD1 enzyme and the phenotype was restored by unsaturated FA.

Treating cells with MPC1 and the inhibitor of LDHB simultaneously had a synergistic effect with regards to cell viability, as opposed to combining MPC1 and SCD1. MPC1 overexpression with the inhibition of the LDHB enzyme could have a similar effect on the cells by depleting them from pyruvate. Higher doses of the LDH inhibitor caused increased lethality, however in the lowest dose used in our studies (0.1uM), the LDH inhibitor alone did not lead to cell death. Only when combined with MPC1, cell death increased. This synergistic effect between LDH inhibition and MPC1 overexpression could be of great importance for cancer therapies. Since the LDH enzyme is present in high concentrations in cancer cells, high doses of the inhibitor would be required for effective inhibition of the enzyme, which limits use of an LDH-based anti-cancer therapy. By combining the LDH inhibitor with the MPC1 overexpression, we could use lower doses of the inhibitor, and make the treatment more cancer-cell specific. When the MPC1-treated cells were also treated with the LDHB inhibitor and the SCD1 inhibitor no stronger effect was observed. A reason for that could be the drop in the Ph caused by the accumulation of lactate because of the LDHB inhibition, which can possibly affect the efficiency of the SCD1 inhibitor. Another approach would be to use inhibitors, such as galloflavin, which target both LDHB and LDHA to see how cancer cells would react after depriving them from lactate (Manerba *et* al., 2012).

To make sure that the effects we see 48h post MPC1 transfection are due to the overexpression of MPC1 and not because of the decreasing availability of glucose with time, we cultured the cells in a gradient of glucose concentrations. The results indicated that supplementation of the media with glucose did not mediate the alterations caused by the MPC1 overexpression. When cells were cultured in lower glucose concentrations, less pyruvate and other metabolites were available driving cells to death. As the concentration of glucose increases the intracellular sources of pyruvate and of the metabolites that the cells need for proliferation increase as well, contributing to the overall survival and higher growth rates of the cells. However, when MPC1 is overexpressed, the proliferation remains low and cell death remained high despite the high concentrations of glucose. This observation could be explained by the fact that when MPC1 is present the pyruvate gets inserted into the mitochondria and is not available to participate in other metabolic processes.

As a final remark I would like to discuss the complexity that characterizes cells metabolism. Upon inhibition of one pathway, there are anaplerotic mechanisms that can compensate for the loss of it, allowing the cells to survive. Because of all these interconnected mechanisms it becomes vital to recognize and target costly reversible or irreversible pathways that cancer cells have developed during the process of oncogenesis. These adaptations make cancer cells less flexible, allowing us to selectively kill them. The process of developing a successful metabolic therapy becomes even more complicated when the heterogeneity of cancer cells is added. The type of cancer as well as the position of cancer cells inside a tumor are only some of the parameters that can influence their either glycolytic or oxidative metabolism. Similarly, the type of cell-model used to test these metabolic therapies will determine the outcomes. For the *MPC1* gene specifically, since it is genetically epistatic to the *APC* gene, overexpressing it in APC knock out colon cancer cell lines or organoids could be a promising strategy to sensitize these cells.

Overall, the multiple metabolic hit approach that we propose presents an opportunity for a therapeutic intervention, based on the alterations observed in cancer cell metabolism. Further investigation is needed to elucidate the efficacy and the clinical potential of the approach as well as to determine the specificity of it by testing it on both malignant and benign cells. A targeted metabolic therapy against cancer cells that spares normal cells could be a strong weapon in the fight against cancer.

SUPPLEMENTARY DATA

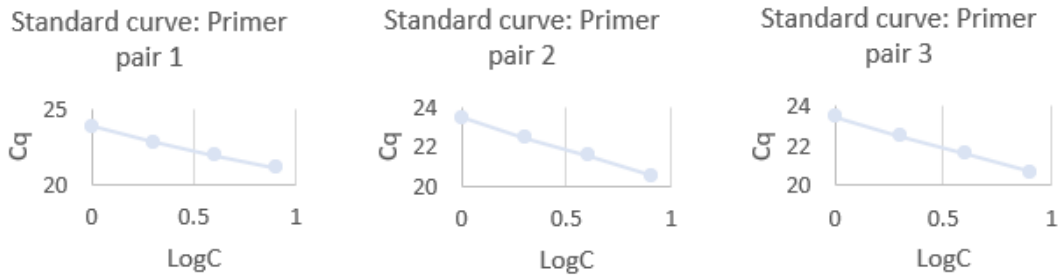
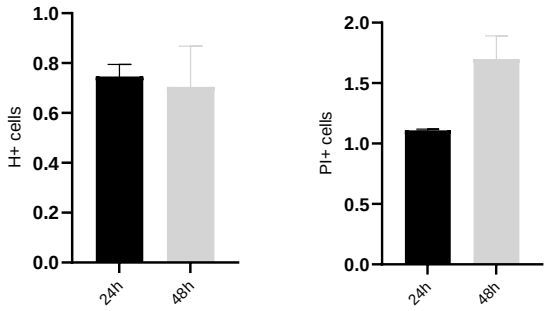
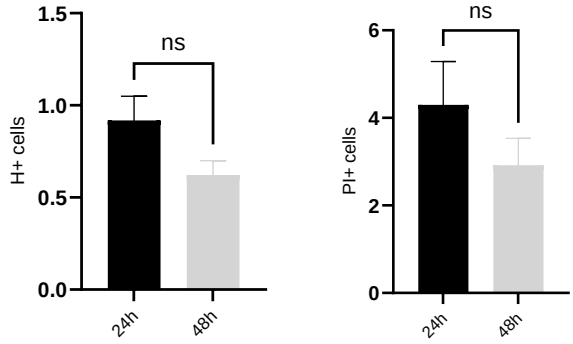


Figure 1: Standard curves of primers used for the amplification of MPC1.

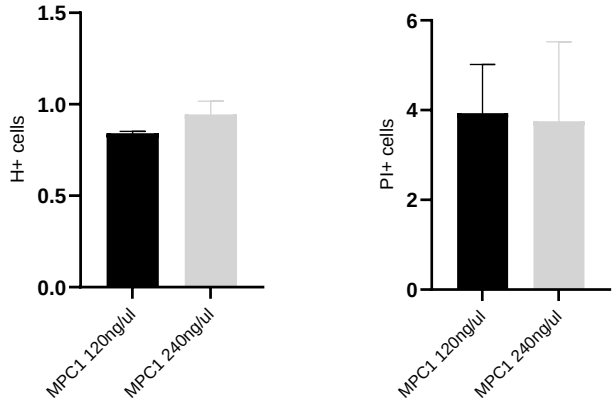
I.HepG2: Cell number and cell viability determined by Hoechst/PI 24h and 48h post transfection.



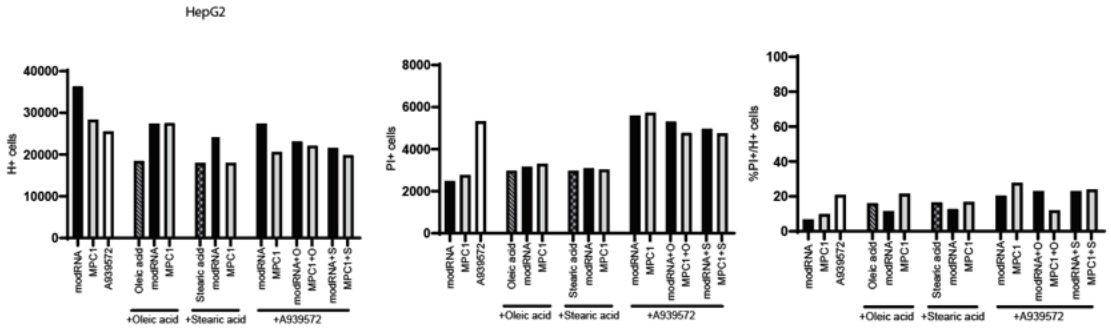
I.HeLa: Cell number and cell viability determined by Hoechst/PI 24h and 48h post transfection.



I.HeLa: Cell number and cell viability determined by Hoechst/PI after transfection with 120ngr/well and 240ngr/well



Cell number and cell viability determined by Hoechst/PI after inhibition of the SCD1 enzyme in MPC1-treated cells cultured in high concentrations of fatty acids.



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