ÖNSÖZ

The project was designed to evaluate the differences in response to metformin treatment in paired colorectal cancer cell lines SW620 and SW480 that are derived from the same patient but from the primary tumor (SW480) and from a metastatic lymph node (SW620). These cells show a greater reliance on mitochondrial respiration rather than aerobic glycolysis. Inhibition of complex 1 of the electron transport chain with metformin and removal of anaplerosis into the Tricarboxylic acid (TCA) cycle with L-glutamine withdrawal resulted in extensive death. However, the surviving cells were found to be more stem cell like showing more extensive nuclear localization of β-catenin, higher expression of the Wnt target c-Myc and a lobular appearance of stem cell enriched spheroids, especially in SW620 cells. Our data suggest that extensive inhibition of mitochondrial bioenergetics may lead to an enrichment of stem cell population in surviving cells.

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Güncel çalışmalar kanser hücrelerinde değişen metabolizmanın kanser hücrelerinin hayatta kalmasına katkıda bulunabileceğini ileri sürmektedir. Kanser hücrelerinin Warburg etkisinde oksijen varlığında bile glikozu tercihen glikoliz yoluyla tercihen glikoz metabolize ederler. Glikolizin tercih edilmesi, biyokütleyi artırabilen metabolik ara ürünler sağlamanın yanı sıra hücrelere hızlı enerji sağlar. Kanser hücrelerinin, hızla büyümek ve çoğalmak için anabolizma ve katabolizmayı aynı anda kullandığı bilinmektedir. Bu nedenle kanser hücrelerinin metabolik zayıflıklarını anlamak, terapötik faydalar sağlayabilir.

Metformin, tip 2 diyabet (T2DM) için birincil tedavi olarak kullanılan klasik bir biguanid ilacıdır. Metformin, mitokondriyal elektron taşıma zincirindeki Kompleks I proteinlerinin inhibisyonu yoluyla kalori kısıtlamasını taklit eder, böylece ATP sentezini azaltır ve AMP Kinaz'ı (AMPK) aktive eder. L-glutamin, anabolik yollara hem karbon hem de nitrojen sağlayabilen esansiyel olmayan bir yağ asididir. L-glutamin, Glutaminaz 1 (GLS1) ile glutamata dönüştürülür ve sonrasında α-ketoglutarat'a dönüştürülerekKreb döngüsüne girebilir.

SW480 ve SW620 hücreleri, sırası ile aynı hastadan primer kolorektal tümör ve lenf nodu metastazından izole edilen iki hücre hattıdır. Aynı hastadan izole edilmiş olmalarına rağmen, enerji kullanımında ve hareket etme ve metastaz yapma yeteneğinde birbirlerinden farklı özellikler gösterirler. Bu hücre hatları galaktoz içinde büyütüldükleri için mitokondriyal enerji üretimine bağımlıdır. Bu hücre hatlarının metformin muamelesi ve L-glutamin yoksunluğuna farklı şekilde tepki vereceğini ve Wnt yolağının aktivasyonu ile kök hücre karakterinin farklılık göstereceği hipotezinde bulunmuştuk.

Verilerimiz tek başına metformin muamelesinin veya tek başına L-glutamin yoksunluğunun hücrelerin proliferasyonunu ve hareketliliğini azalttığını göstermektedir. Bununla birlikte, ikisinin kombinasyonu hayatta kalan SW620 hücrelerinde WNT yolağı sinyalinde ve kök hücre karakterlerinde artışa sebep olmuştur ve bu artış SW480 hücrelerine kıyasla daha büyük ölçüde

olmuştur. CB839 (metformin gibi oral olarak alınan biyoyararlı bir GLS1 inhibitörü) metformin kombinasyonu dikkatli kullanılmalıdır.

Anahtar kelimeler: metabolizma, metformin, glutamat, Wnt yolağı, kök hücre karakteri

ABSTRACT

Growing evidence suggests that altered metabolism in cancer cells can contribute towards survival of cancer cells. Cancer cells are known to undergo Warburg effect, whereby cells preferentially use glucose via glycolysis even in the presence of oxygen. Such a mechanism provides rapid energy to cells, along with metabolic intermediates that can enhance biomass. Cancer cells are known to utilize anabolism and catabolism simultaneously to grow and proliferate rapidly. Understanding metabolic vulnerabilities of cancer cells can therefore enhance therapeutic benefits.

Metformin is a classic biguanide drug that is used as a first line treatment for type 2 diabetes (T2DM). Metformin mimics calorie restriction via inhibition of Complex I proteins in the mitochondrial electron transport chain, thereby reducing ATP synthesis and activating AMP Kinase (AMPK). L-glutamine is a non-essential fatty acid that can provide both carbon and nitrogen to anabolic pathways. It is converted to glutamate with Glutaminase 1 (GLS1); glutamate can be converted to α-ketoglutarate and enter into the Kreb's (Tricarboxylic acid, TCA) cycle.

SW480 and SW620 cells were isolated from the primary colorectal tumor and its lymph node metastasis from the same patient. Nonetheless, these cell lines show variation in energy utilization and ability to move and metastasize. These cells are grown in galactose and therefore are reliant on mitochondrial energy generation. We hypothesized that these cell lines will respond differentially to metformin and L-glutamine withdrawal and focused on differences in stemness and activation of the Wnt pathway.

Our data suggest that metformin or L-glutamine withdrawal alone reduced the proliferation and motility of cells. The combination of the two, however, enhanced Wnt signaling and stemness behavior of the surviving SW620 cells and to a lesser extent in the surviving SW480 cells. A combination of orally bioavailable GLS1 inhibitors such as CB839 and metformin should be used with caution.

Keywords: Metabolism, metformin, glutamine, stemness, Wnt pathway

1. INTRODUCTION

Quiescent cells are more catabolic and utilize the tricarboxylic acid (TCA) cycle to break down fuels such as glucose, glutamine, and fatty acids for complete oxidation to carbon dioxide (Boroughs ve DeBerardinis, 2015). Reduced electron carriers such as NADH and $FADH_2$ carry the chemical energy of these fuels and use them to generate ATP in the mitochondria in order to sustain basic cellular processes. Cancer cells are highly proliferating cells that need to rewire their metabolism to activate anabolic pathways and generate macromolecules such as proteins, lipids, and nucleic acids necessary for cell proliferation (Lunt ve Vander Heiden, 2011). Consequently, cells take up large amounts of nutrients such as glucose and glutamine that are used to support cell growth. Metabolic intermediates generated during glycolysis and the TCA cycle supply the building blocks for the synthesis of nucleic acids, amino acids, and fatty acids. Glutamine catabolism maintains a steady supply of nutrients into the TCA cycle (anaplerosis), thus preserving the TCA cycle integrity despite continual efflux of metabolites to support growth (Heiden vd., 2009).

Epithelial to mesenchymal transition (EMT) is a series of genetic, epigenetic and metabolic changes that cells undergo in order to convert from a less motile more proliferating epithelial phenotype to a highly motile less proliferating mesenchymal phenotype that can metastasize to distant organs and also develop chemoresistance (Lamouille vd., 2014). Thus, the metabolic activity of highly proliferative cells in the primary tumor (epithelial type) versus the circulating tumor cell (CTC) (mesenchymal type) that are generally less proliferative, are expected to be distinct. Non-transformed epithelial cells, upon detachment from the extracellular matrix, lose their ability to take up glucose. This results in a decrease in ATP concentration and increase in ROS production, both of which lead to anoikis (Schafer vd., 2009). Epithelial cells in the primary tumor show increased glycolytic flux and lactic acid release, reduced OXPHOS, and increased activation of the pentose phosphate pathway (PPP); the latter two events can also reduce ROS production and thereby increase cell survival. The choice between glycolysis or TCA cycle followed by oxidative phosphorylation (OXPHOS) in primary versus metastatic tumors, however, is not simple. Thus, increased oxidative metabolism has also been reported to regulate metastasis (Danhier vd., 2017; Payen vd., 2016).

A balance between ATP production and consumption is very critical to sustain motility and invasive capacity since both EMT and metastasis cascade are highly energy-consuming processes. Therefore, an increase in the AMP:ATP ratio in the cell and the subsequent activation of AMPK can regulate the EMT and metastasis cascades. AMPK inhibits anabolic pathways and stimulate catabolic pathways to maintain energy homeostasis under ATP depletion (Hardie, 2015). Although it is no doubt a critical regulator, the role played by AMPK in the EMT process remains controversial. It was reported that the anticancer activity of metformin conferred via AMPK activation resulted in a metabolic crisis, along with the repression of proliferation, migratory capacity and EMT (Banerjee et al., 2016). Depletion of glutamine or inhibition of the enzyme glutaminase 1 (GLS1) that converts glutamine to glutamate was shown to reduce the stem cell population in hepatocellular, lung and pancreatic cancer, along with an inhibition of signaling via β-catenin and enhanced oxidative stress (Li vd., 2019; Liao vd., 2017).

SW480 and SW620 CRC cells as models for primary and disseminated tumors:

SW480 and SW620 colorectal cancer cell lines were derived respectively from primary and lymph node metastasis of CRC from the same patient. SW480 cells exhibit an epithelial morphology, while SW620 cells exhibit a fibroblast-like morphology *in vitro.* This unique feature, therefore, allows scientists to examine changes that appear late in colon cancer progression such as EMT, stemness and metastatic behavior (Thuringer vd., 2015). A comparison of cancer stem cells (CSC) derived from metformin treated SW620 and HT29 cells indicated that SW620 CSCs were resistant to the OXPHOS inhibitory capacity of metformin through compensatory use of glutamine as precursor for metabolism (Kim vd., 2018). In this study, however, the authors did not compare the metabolic profile or sensitivity of CSCs derived from SW480 and SW620.

1.1 Aims of the research

SW620 cells are of a relatively stronger mesenchymal phenotype and were shown to rely primarily on mitochondrial ATP generation when compared to SW480 cells, which are more epithelial and rely on glycolysis (Lin vd., 2018). Both cell lines are grown in galactose containing Leibovitz L-15 medium, which relies more on the mitochondria for its metabolism (Robinson vd., 1992). Metformin inhibits mitochondrial ATP synthesis (Rena vd., 2017) and therefore both cell lines; and

SW620 to a greater extent may be susceptible to metformin, particularly in the absence of glutamine (Kim vd., 2018). In this project, we aimed to discover whether the differential responses of SW480 and SW620 cells to metformin can alter their ability to undergo epithelial to mesenchymal transition and acquire stemness properties.

To address this hypothesis, we have identified two major aims:

1. To determine changes in cellular migration/motility, stemness behavior and spheroid forming ability and changes in EMT related genes in SW480 and SW620 cells treated with metformin in the presence or absence of L-glutamine.
2. To discover the signaling pathways that are associated with the differential responses of SW480 and SW620 cells to metformin.

2. SUMMARY OF THE LITERATURE

2.1 Metabolism and cancer

Quiescent cells are more catabolic and utilize the tricarboxylic acid (TCA) cycle to break down fuels such as glucose, glutamine, and fatty acids for complete oxidation to carbon dioxide (Boroughs ve DeBerardinis, 2015). Reduced electron carriers such as NADH and $FADH_2$ carry the chemical energy of these fuels and use them to generate ATP in the mitochondria in order to sustain basic cellular processes. Cancer cells are highly proliferating cells that need to rewire their metabolism to activate anabolic pathways and generate macromolecules such as proteins, lipids, and nucleic acids necessary for cell proliferation (Lunt ve Vander Heiden, 2011). Consequently, cells take up large amounts of nutrients such as glucose and glutamine that are used to support cell growth. Metabolic intermediates generated during glycolysis and the TCA cycle supply the building blocks for the synthesis of nucleic acids, amino acids, and fatty acids. Glutamine catabolism maintains a steady supply of nutrients into the TCA cycle (anaplerosis), thus preserving the TCA cycle integrity despite continual efflux of metabolites to support growth (Heiden vd., 2009).

2.2 Metformin and cancer

Metformin is a classic biguanide drug that is used as a first line treatment for type 2 diabetes (T2DM). Metformin, which mimics calorie restriction, is known to regulate cellular metabolism at multiple levels (Pierotti vd., 2013). The primary mechanism of action of metformin is the inhibition of Complex I proteins in the mitochondrial respiratory chain (Rena vd., 2017). This reduces ATP synthesis, enhances the ADP:ATP and AMP:ATP ratio and leading to the activation of AMP-activated protein kinase (AMPK) that is an important regulator of cellular energy homeostasis (Pierotti vd., 2013). Phosphorylated AMPK can cause downstream activation of tumor suppressors such as TSC1/2 and p53, which in turn promote pathways such as apoptosis and autophagy. The inhibition of mTOR upon AMPK activation can also inhibit proliferation by perturbing synthesis of mitogenic Wnt pathway target proteins such as MYC (Dowling vd., 2007;

Shen vd., 2018). In general, activation of AMPK leads to the inhibition of anabolic pathways and activation of catabolic pathways, which, in turn can produce ATP to restore homeostasis.

Use of metformin by Type-2 diabetes patients was shown to reduce their risk for CRC (Chang vd., 2018). Subsequently, a number of epidemiological and basic research studies have shown that metformin has anti-cancer properties with the inhibition of several hallmarks of cancer, justifying the re-purposing of metformin as a cancer drug (Higurashi ve Nakajima, 2018).

Oral metformin was specifically shown to accumulate in the intestines, liver, kidney and bladder. The levels of metformin in jejunal samples were reported to be 30-300 times higher than the plasma levels. Therefore, the GI tract is likely to be an important target for metformin (Rena vd., 2017). Metformin was also reported to increase anaerobic glucose metabolism in enterocytes, resulting in reduction in net glucose uptake and increased lactate delivery to the liver (Bailey vd., 1994). Additionally, metformin can act on systemic levels of insulin and glucose thereby affecting cancer cell growth indirectly (Pierotti vd., 2013).

2.3 L-Glutamine

Immortalized cancer cells grown in culture have long been known to require the non essential amino acid L-glutamine for sustained survival (Earle vd., 1956). PIK3CA encodes the catalytic subunit of phosphatidylinositol 3-kinase α (PI3Kα) and is mutated in a wide variety of human cancers including ~30% of CRCs (Samuels vd., 2004). Colorectal tumors with PIK3CA mutations were found to be addicted to L-glutamine; the amino acid was primarily utilized via its conversion to glutamate (with GLS1) and then to α-ketogluarate (via glutamate pyruvate transaminase 2), which can then enter the TCA cycle and provide energy (Y. Zhao vd., 2019).

2.4 Epithelial to mesenchymal transition

Activation of invasion and metastasis is one of the hallmarks of cancer (Hanahan ve Weinberg, 2011). Cancer cells undergo epithelial-to-mesenchymal transition (EMT) during invasion and

metastasis. Epithelial cells can transform to a mesenchymal phenotype through a very dynamic process. It is, however, implicated in tumor growth and metastatic spread, as well as the generation of tumor cells with stem cell like features, which play a key role in cancer therapy resistance. In cancer cells, EMT can be partial, and tumor cells can be seen in multiple transitional states, expressing a mix of epithelial and mesenchymal genes. Disruption of cell–cell adhesion and cellular polarity, cytoskeleton remodeling, and changes in cell–matrix adhesion are all part of the EMT process (Roche, 2018).

The expression of the transmembrane protein E-Cadherin is directly linked to the regulation of EMT. E-cadherin is required for cell-to-cell contact stabilization, which allows the cell to maintain its epithelial state and polarity. When the levels of E-Cadherin are reduced, the non-epithelial adhesion molecule N-Cadherin is usually upregulated. N-Cadherin increases ß-catenin activity and FGFR signaling, which promotes tumor growth (Van Roy, 2014). Vimentin is a type III intermediate filament that is involved in cell migration, motility, and adhesion as well as maintaining cell integrity. Vimentin promotes EMT and, eventually, metastasis in solid tumors when it is overexpressed (Wu vd., 2018).

The metabolic state of cells in the primary tumor, circulation after metastasis and at the pre-metastatic niche are all known to be different suggesting very high metabolic plasticity (Dorsch vd., 2021). SW480 and SW620 cells are also known to have distinct metabolic profiles with SW480 more reliant on glycolysis than SW620 cells (Lin vd., 2018). It is also known that detachment of cells from the matrix reduces ATP levels in cells, causing oxidative stress and anoikis (Dorsch vd., 2021). Cells therefore need to modify their metabolism to OXPHOS and high cellular generation of NADPH (to mitigate oxidative stress) during EMT. Therefore, the inhibition of OXPHOS with metformin should reduce EMT characteristics in cancer cells. Supporting this, recent studies from the literature claim that metformin has the capacity to inhibit EMT in several malignancies (Lei vd., 2019; Qu vd., 2014). Metformin inhibits the HIF-1/PKM2 signaling pathway, which prevents hypoxia-induced EMT in keloid fibroblasts (Lei vd., 2019). It is also reported that metformin has the ability to re-sensitize 5-FU-induced multidrug-resistant breast cancer cells and reverse their EMT phenotype. This impact could be related to the AMPK signaling pathway being activated (Qu vd., 2014).

2.5 Wnt pathway and stemness

One of the key regulators of stemness in CRC is the Wnt pathway (Ben-Ze'ev vd., 2016). GSK3 (found as two isoforms α and β) is a key regulatory protein which stands at the crossroad of many essential pathways including gluconeogenesis and Wnt signaling. GSK-3β is known to be basally active and unlike other kinases; phosphorylation at Ser-9 or Ser-21 via extracellular stimulation inhibits its kinase activity (Lee ve Kim, 2007). Phosphorylation of S-9 in GSK3β causes the N-terminal tail of GSK-3β to act as a pre-phosphorylated substrate, or pseudosubstrate (Frame vd., 2001). This phosphorylated S-9 tail self-associates in the primed-substrate binding pocket, hindering the binding of actual primed substrates, and thus diminishing primed-substrate phosphorylation by GSK-3β. In its active form, GSK-3β can phosphorylate β-catenin after a priming phosphorylation by casein kinase 1; this event targets β-catenin to the proteasomal degradation machinery. Wnt ligands induce disruption of the complex, which prevents GSK3 from phosphorylating β-catenin, allowing the accumulation and nuclear import of β-catenin (Frame vd., 2001).

Metformin was shown to reduce stem cell characteristics of spheroids generated from HCT116 cells in a dose dependent manner (C. Zhang ve Wang, 2019). On the other hand, depletion of glutamine or inhibition of the enzyme glutaminase 1 (GLS1) was shown to reduce the stem cell population in hepatocellular, lung and pancreatic cancer, along with an inhibition of signaling via β-catenin and enhanced oxidative stress (Li vd., 2019; Liao vd., 2017).

Based on the information available in the literature, we hypothesized that a combination of L-glutamine withdrawal (or use of a glutaminase inhibitor) and metformin may further enhance the vulnerability of these cells. Moreover, based on previous studies suggesting that SW480 cells are more glycolytic than SW620 cells (Lin vd., 2018) we expected to observe differential outcomes with both L-glutamine withdrawal and metformin treatment in these cell lines.

3. MATERIALS AND METHODS

3.1 Cell culture and treatments

The colorectal cancer (CRC) cell lines SW480 and SW620 were purchased from American Type Culture Collection (ATCC) (Middlesex, UK). SW480 and SW620 cells were grown in Leibovitz L-15 medium (Biological Industries, Israel) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin in a humidified incubator with 100% air at 37ºC. In experiments where L-glutamine withdrawal was carried out, certified L-glutamine free Leibovitz L-15 medium was used. Plastic sterile consumables used in cell culture were purchased from Sarstedt (Germany). The cell lines were routinely tested for mycoplasma using a PCR method (Young vd., 2010). A maintenance dose of plasmocin was used in the culture medium. The passaging and storage of cells were carried out using well- established standard techniques. Metformin and CB839 were dissolved in PCR grade sterile water and used at a concentration of 1, 5 or 10mM for metformin and a standard dose of 1μM for CB839.

3.2 Proliferation with an MTT assay

To determine the viability of SW480 and SW620 cells with various doses of metformin in the presence or absence of L-glutamine, the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was conducted according to the manufacturer's instructions (Sigma Aldrich, USA). 7,500 cells were seeded in each well of three separate 96 well plates to determine the proliferation at three different time points (24-48-72 hours). At the end of each time point, 10% MTT (dissolved in PBS) containing 100 μl medium was applied directly into the cells to enable the formation of formazan crystals in the metabolically active (and thereby proliferating cells). After 4 hours of incubation, 1% SDS-0.01M HCl was introduced to each well to solubilize the formazan crystals. Each plate was then incubated for 16 hours at 37°C in a humidified incubator. The absorbance values at 570 nm from each well were determined using a Multiskan-GO microplate reader (Thermo Fisher Scientific, USA).

3.3 Colony Formation Assay

To determine the effects of metformin and CB-839 on colony formation capacity and long term proliferative of SW480 and SW620 cells, a colony formation assay was carried out according to previously published protocols (Feoktistova et al., 2016). SW480 and SW620 cells were seeded to 6 well plates at a density of 1,000 cells/well. The cells were cultured with treatments (1 or 5 mM metformin, 1 μM CB839 or L-glutamine withdrawal, as indicated in the relevant results) for 10 - 14 days in a humidified incubator with 100% air at 37ºC until the colonies (in the control wells) became visible to the naked eye. The medium of the cells was changed every 3 days. At the end of the experiment, the colonies were washed with PBS once and fixed with 4% Paraformaldehyde (PFA) solution (Sigma Aldrich, USA) for 15 minutes at room temperature. Following the fixation process, the PFA solution was removed from the wells, and cells were rewashed with PBS once. After this step, 1 ml of 0.5% crystal violet dissolved in methanol (Sigma Aldrich, USA) was applied directly to the wells and incubated for 20 minutes at room temperature on a bench-type rocker. Next, the plates were carefully washed with a gentle stream of tap water 4 times and left to air-dry overnight. The next day, stained colonies were imaged by using Chemi-Doc MP (BioRad, USA). The obtained images were analyzed with ImageJ (NIH, USA) software Threshold/Subtract method.

3.4. RNA isolation

Total RNA isolation was conducted by using the NucleoSpin RNA Extraction Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions on scraped cells. Approximately 500,000 cells were collected for the total RNA isolation procedure. NanoDrop (Biochrom, UK) was used for RNA quantification. A260/280 ratio close to 2.0 and A260/230 ratio between 2.0 and 2.2 were accepted as acceptable RNA samples. All samples were stored at -80ºC after isolation and quantification.

3.5 cDNA Synthesis

Total RNA samples were treated with RNase-free DNase I (Thermo Fisher Scientific, USA). 1 µg of RNA sample was used for each cDNA synthesis reaction with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Random hexamers were used, and all reactions were conducted according to the manufacturer's instructions. Synthesized cDNA samples were stored at -20ºC.

3.6 Real time Polymerase Chain Reaction

To evaluate the expression of genes relevant to EMT, Wnt pathway and stemness, quantitative real-time PCR (qRT-PCR) assay was used. Primers used in this study are shown in Table 1 while the NCBI reference numbers for the primer pairs indicating the transcript that the primer pair was designed to amplify is shown in Table 2. The final volume of the PCR reaction was set to 10 µl and contained 1 µl of cDNA (1:10 diluted) and 9 µl reaction mix [0.5-1 µM of Forward and Reverse primers, 5 µl GoTaq qPCR Master Mix (Promega, USA) and dH2O to complete the volume]. Standard curves were generated (with 1:10, 1:50, 1:100, 1:250, 1:500, 1:1000 and 1:10000 dilutions of 500 ng cDNA) for each primer pair for quantification. β-actin or GAPDH were used as internal controls. The reactions were carried out in Rotor-Gene Q (Qiagen, Netherlands) qRT-PCR equipment. Expression values of individual genes were determined using the Pfaffl method, which implies relative quantification based on target genes and reference genes representing physiological differences in the expression level difference (Pfaffl, 2001).

Table 1. Primer sequences used in the current study

Gene Name	**Forward (5'-3')**	**Reverse (5'-3')**	**T_m (ºC)**
EPCAM	GTCATTTGCTCAAAGCTGGC	GCTCTCATCGCAGTCAGGAT	53
CDH1	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC	59
VIM	CCAGCCGGAGCTACGTGACTA	GTGCGGGTGTTCTTGAACTCG	59

MYC	CTTCTCTGAAAGGCTCTCCTTG	GTCGAGGTCATAGTTCTTGTTG	56
LGR5	CAACCTCAGCGTCTTCACCT	CATCCAGACGCAGGGATTGA	56
ACTB	CAGCCATGTACGTTGCTATCCAGG	AGGTCCAGACGCAGGATGGCATG	60

Table 2. NCBI reference numbers for each primer pair used in the current study.

Gene Name	**NCBI Reference Sequence**
EPCAM	NM_002354.3
CDH1	NM_001317185.2, NM_001317186.2, NM_001317184.2, NM_004360.5
VIM	NM_003380.5
MYC	NM_002467.6, NM_001354870.1
LGR5	NM_001277226.2, NM_003667.4, NM_001277227.2
ACTB	NM_001101.5, NM_001199954.3, NM_001614.5, NM_001613.4, NM_001141945.2, NM_001320855.1, NM_001083538.3, NM_001371926.1, NM_001277083.2, NM_001277406.2, NM_001099771.2, NM_005159.5, NM_001100.4, NM_001145442.1, NM_001017992.4

3.7 Protein isolation

Total protein isolation was carried out using Mammalian Protein Extraction Reagent M-PER (Thermo Fisher Scientific, USA) containing PhosSTOP Phosphatase Inhibitor and c0mplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Germany) according to the manufacturer's instructions.

Measurement of protein amount was carried out with the Bradford assay using Coomassie Protein Assay Reagent (Thermo Fisher Scientific, USA) and a standard curve generated with bovine serum albumin (BSA). 5 µl of total protein was added to 1.5 ml of Coomassie Protein Assay Reagent in a plastic cuvette and absorbance value was obtained by using Multiskan-GO microplate reader (Thermo Fisher Scientific, USA) at 595 nm. Quantified amount was later determined by applying the reading to a standard curve.

3.8 Western blot

Total proteins were separated using reducing SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) at 95 V by loading 30 µg protein per well in 10% Acrylamide/Bis-acrylamide gels. As a marker for the 10-250 kDa range, PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, USA) was used. Before separation, a wet-transfer of proteins from the gel to a Polyvinylidene Fluoride (PVDF) membrane was carried out at 115 V and 4ºC for 75 minutes. The membrane was blocked with TBS-T containing 5% skimmed milk. Incubation with primary antibodies was carried out overnight at 4ºC with shaking. The membrane was extensively washed with TBS-T, followed by incubation with the appropriate secondary antibody at room temperature for 1 hour and then washed again with TBS-T. The membranes were next incubated with Clarity ECL Substrate (BioRad, USA) for 1 min for visualization and imaged in a Chemi-Doc MP (BioRad, USA). When necessary, the membranes were stripped by incubating in a mild stripping buffer (100 mM β-mercaptoetanol, 2% SDS, 62.5 mM Tris-HCL, pH 6.8) for 10 minutes at 60ºC and washed extensively with TBS-T before reprobing with a different antibody. For a list of antibodies used in the current study, please see Table 1. Alpha Tubulin or GAPDH antibody was used as a loading control to ensure equal protein loading.

Table 3. Antibodies used for western blot in the current study

Description	Size (kDa)	Catalog Number	Origin	Brand	Dilution
p-S6	32	2211S	Rabbit	Cell Signaling	1:1000
p-AMPK	62	2535S	Rabbit	Cell Signaling	1:3000
Total AMPK	62	sc-74461	Mouse	Santa Cruz	1:1000
p-GSK3β	46	9315S	Rabbit	Cell Signaling	1:2000
Beclin-1	60	sc-48341	Mouse	Santa Cruz	1:1000
E-Cadherin	135	sc-8426	Mouse	Santa Cruz	1:1000
β-Catenin	92	sc-133240	Mouse	Santa Cruz	1:1000
Vimentin	57	sc-7558	Goat	Santa Cruz	1:1000
α-Tubulin	52	HRP-66031	Mouse	Proteintech	1:5000
GAPDH	37	sc-47724	Mouse	Santa Cruz	1:4000

3.9 Wound Healing Assay

To assess the effect of metformin or CB839 on the motility of SW480 and SW620 a wound healing assay was carried out. The cells were seeded at the density of 1,000,000 cells/well on 6 well plates and cultured in a humidified incubator with 100% air at 37ºC to confluency. Next, cells were washed with PBS once, and 1 ml of fresh PBS was added to each well. Wounds were created in each well by using a 100 µl pipette tip. After this step, PBS was aspirated, the cells were cultured in complete medium containing 1 mM metformin or 1µM CB839 or a combination of both along with 0.5 µM Mitomycin C (Serva Biochemicals, Germany). Mitomycin C was used to inhibit proliferation. The cells were imaged every 24 hours for a total time of 96 hours and the obtained images were analyzed by the edge-detection/thresholding method on ImageJ software.

3.10 Spheroid Formation Assay

To determine the capacity of SW480 and SW620 cells to form spheroids upon treatment with metformin (2mM), CB-839 (1μM) or a combination of both, the cells were cultured in culture medium containing these drugs in Ultra-Low Attachment (ULA) plates (Corning, USA). To enrich for stem cells, a stem cell enrichment medium (SSM) containing serum free DMEM/F-12(HAM)1:1 medium with 2% serum free supplement B27 (Thermo Fisher, Hempstead, UK), 20 ng/ml epidermal growth factor (EGF), 0.4% bovine serum albumin and 4 μg/ml insulin was used. SW480 and SW620 cells were seeded in 50,000 cells/well density to 96 well ULA plates (Corning, USA) and cultured in a humidified incubator with 5% CO_2 at 37ºC for 10 days.
Each individual spheroid formed in the 96 well ULA plate was imaged The spheroids were evaluated for their size, as well as tightness. The images were analyzed for the area of the spheroids by using the edge-detection/thresholding method on ImageJ software.

3.9 Immunocytochemistry Assay

To determine the subcellular localization of the junctional proteins E-Cadherin and β-Catenin in SW480 and SW620 cells treated with metformin (10mM) in the presence or absence of L-glutamine as well as with CB-839 (1μM) or a combination of metformin and CB839, an immunocytochemistry (ICC) assay was conducted. 500,000 cells were counted and seeded onto glass bottom confocal dishes (SPL, Korea). After 24 hours of culture, cells were treated with the various drugs and media as indicated in the figure for 24h. Next, the cells were washed twice with PBS and fixed with 4% PFA solution for 15 minutes at room temperature. After the fixation, the cells were washed twice with PBS and incubated with 1% Triton X-100 for 30 minutes at 37°C. After this step, the plates were incubated with the primary antibodies (Table 3) at 4°C overnight. The subsequent steps were conducted in the dark. The next day, the cells were washed twice with PBS and then incubated with the secondary antibodies for 1 hour at 37°C. Then, the cells were washed twice with PBS and incubated with Alexa Flour 647 Phalloidin (Thermo Fisher Scientific, USA) for 1 hour at 37°C. After a final wash with PBS twice, the cells were incubated with 300 nM DAPI for 5 minutes at room temperature. Finally, cells were washed twice with PBS

and imaged by using confocal microscopy (Zeiss LSM 800 Laser Scanning Microscope (Germany)) at 20x magnification.

3.10 Bioinformatics Analyses

Raw microarray signals from GSE67342 dataset for 24 h metformin treatment of LoVo colon cancer cells and vehicle control were downloaded from Gene Expression Omnibus (GEO) and normalized by Robust Multichip Average (RMA) method (Gautier vd., 2004; Irizarry vd., 2003). Afterwards, differential expression analysis between metformin treated and controls was carried out using limma package and significance was determined by moderate t-test adjusted for multiple hypothesis testing according to Benjamini and Hochberg procedure false discovery rate (FDR) and minimum 1 log-fold change (LFC) difference between the conditions. All probes were converted into ensemble IDs, and for genes with multiple probes, the most significant one was kept for further analysis.

Three different approaches were used to further evaluate the differentially expressed genes GSE67342:

Approach 1: Raw microarray signals from GSE89523 Human Gene 2.0 ST microarray dataset which compares gene expression differences between SW480 and SW620 when grown in spheroid culture were downloaded from Gene Expression Omnibus (GEO) and normalized by Robust Multichip Average (RMA) method (Gautier vd., 2004; Irizarry vd., 2003). During signal extraction and normalization, the argument “core” was used to measure gene-centric transcript abundance, not the exon-centric one. Afterwards, differential expression analysis between the two cell lines was carried out using limma package and genes’ significance level was determined by moderate t-test adjusted for multiple hypothesis testing according to Benjamini and Hochberg procedure false discovery rate (FDR) and minimum 1 log-fold change (LFC) difference. Probe annotation into gene symbol and entrez ID was carried out using hugene20sttranscriptcluster.db package. a list of 1160 EMT genes from the dbEMT 2.0 database (M. Zhao vd., 2019). Significant EMT genes were selected according to FDR < 0.05 and 1LFC criteria.

Approach 2: A ground breaking study in 2019 identified 16 cell membrane receptors and 24 transcription factors that belong to KRAS, Wnt and Notch signaling pathways and genes that are involved in the process of EMT (McFaline-Figueroa vd., 2019). All significant genes in cells knocked out for EGFR, MET, ITGAV, ITGB1 and FGFR1 for both spontaneous and TGFβ-induced EMT from the publicly-available results of this study (McFaline-Figueroa vd., 2019) and matched them with genes significantly up- and downregulated with at least 1LFC after 24 hours of metformin treatment. The data available in GSE114687 (McFaline-Figueroa vd., 2019) were available as pre-processed normalized data and the genes were selected accordıng to sıgnıfıcantly enrıched score (FDR < 0.05). After identifying genes with significant differences for each condition, we then concentrated on the common genes showing significant differences in spontaneous and TGFβ-induced EMT that were at the same time significantly up- and downregulated by metformin treatment. All analyses were carried out in R 4.x statistical package, Linux or Windows operating system.

Approach 3: L-glutamine was identified in the course of the experiments to affect the stemness behavior of SW480 and SW620 cells treated with metformin. A gene expression signature that was shown to predict stemness (Nio vd., 2017) as a function of GLS1 expression and Wnt/β-catenin activity in liver cancer patients (Li vd., 2019) was evaluated in the metformin treated/untreated dataset.

All analyses were carried out in R 4.x statistical package, Linux or Windows operating system.

3.11 Statistical Analyses

Each experiment was repeated at least 2 times independently with at least 3 technical replicates for each biological replicate. The data were analyzed and graphs were generated with GraphPad Prism 6 (La Jolla, CA, USA). Statistical significance was evaluated with t-test unless otherwise indicated. $p \leq 0.05$ was considered to be significant.

4. RESULTS

Preliminary data reported in the proposal indicated that the proliferation of SW480 and SW620 cells treated with metformin in complete medium using the Xcelligence system indicated that SW620 cells were more resistant to metformin compared to SW480 cells. To further validate the reported data and to determine whether the withdrawal of L-glutamine affected the functional characteristics as well as signaling pathways in these cell lines, we carried out a series of experiments as described below.

4.1 Proliferation of SW480 and SW620 cells with metformin treatment and under glutamine restriction

SW480 and SW620 cells (10,000 cells per well) were plated in separate 96 well plates for 24h and then treated with 5 and 10 mM metformin in either absence or presence of 2mM L-glutamine. After 72 hours, proliferation of these cells were evaluated by using an MTT assay (Figure 1).

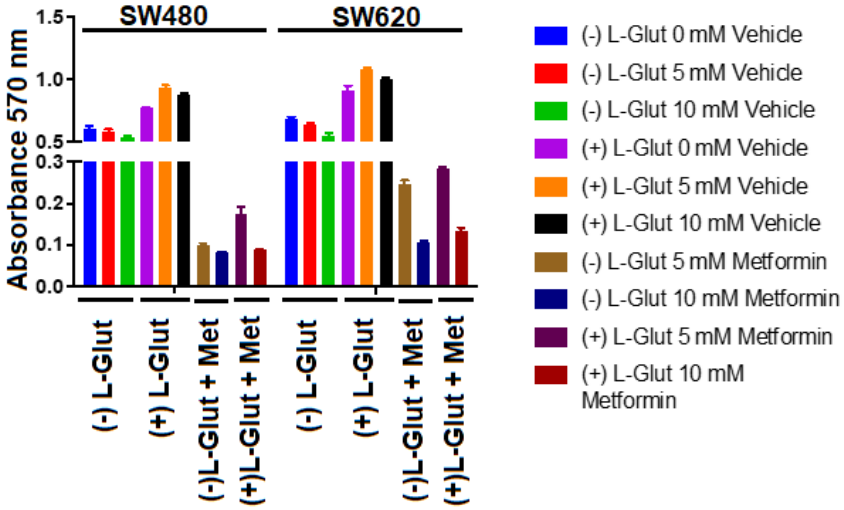


Figure 1. Proliferation of SW480 and SW620 cells in the presence of 5mM and 10mM Metformin in the presence or absence of L-glutamine.

Cells were cultured with 0, 5 or 10 mM Metformin and in the absence and presence of L-glutamine (L-Glut) for 72 h followed by an MTT assay. Metformin was dissolved in water (vehicle) prior to use. Two independent biological replicates were conducted.

The absence of L-glutamine decreased the viability of both cells lines, and addition of 5mM and 10mM Metformin further reduced the viability in a dose dependent manner. Of note, SW480 cells showed greater vulnerability to metformin when grown in the absence of L-glutamine, compared to the presence of L-glutamine [(bars 7 (light brown) and 9 (dark brown)]. Supporting previous studies (P. Zhang vd., 2020) and our own preliminary data, SW620 cells showed an increased resistance to metformin in a dose dependent manner irrespective of the presence of L-glutamine.

To mimic L-glutamine withdrawal, we used the GLS1 inhibitor CB-839 (Telaglenastat), which is a potent and selective inhibitor of multiple splice variants of glutaminase (Gross vd., 2014). With the combination of metformin and CB-839, both Complex-I and GLS1 are expected to be inhibited, which creates a significant blockade in the mitochondrial ATP production in these cells resulting in the suppression of growth and proliferation.

4.2 Colony formation assay of SW480 and SW620 cells treated with metformin and CB839

A long term proliferation over a period of 14 days was carried out with a colony formation assay (Figure 2).

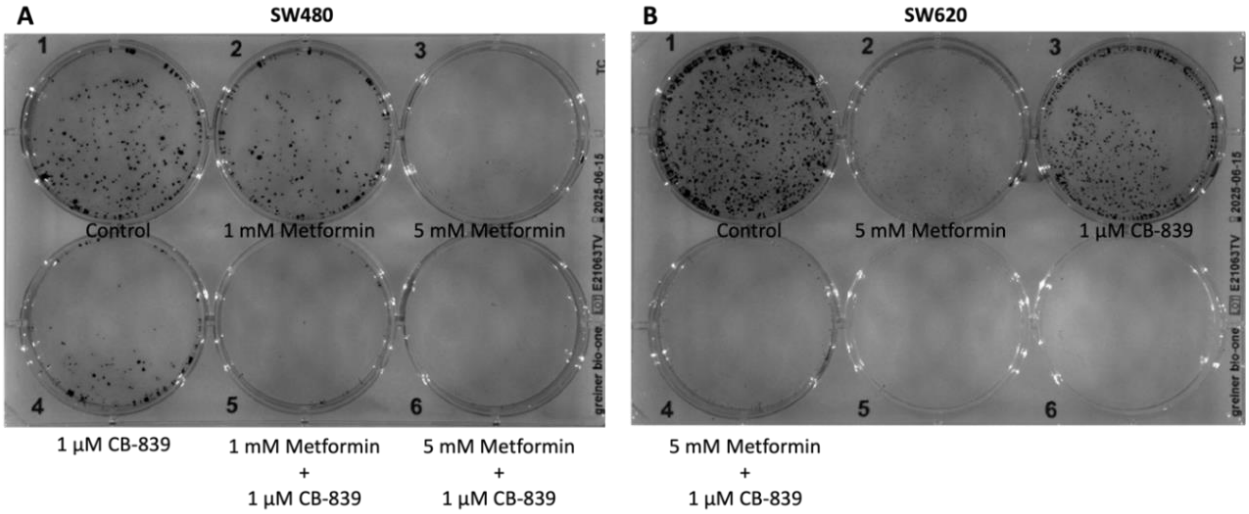


Figure 2. Colony formation assay.

SW480 (A) and SW620 (B) cells (1000 cells per well) were cultured in the presence of different doses (1 or 5 mM) of metformin and 1 µM CB-839 over a period of 14 days. The plates were then fixed with methanol followed by staining with crystal violet. Two independent biological replicates were used. Wells 5 and 6 of the 6-well plate were empty.

We observed a decrease in the colony formation capacities of both cell lines when treated with metformin (Figure 2). SW480 cells showed a more robust decrease in colony numbers at a lower concentration of metformin (1mM) compared to SW620 cells. With 5mM metformin, no colonies were visible with SW480 cells, whereas smaller but numerous colonies were seen with SW620 cells (Figure 2A well 3 versus Figure 2B well 2). Additionally, SW620 cells were seen to be more resistant to glutaminase inhibition compared to SW480 cells. This differential vulnerability of SW620 and SW480 cells may arise via two different mechanisms:

- *Activation of nutrient sensing pathways.*
- *Evaluation of epithelial or mesenchymal characteristics of SW480 and SW620 cells*

Both of these mechanisms were next evaluated.

4.3 Activation of nutrient sensing pathways

SW480 and SW620 cells were treated with either water (vehicle) or 10mM metformin for 6h and the cells were collected for protein isolation and Western blot using standard techniques (Figure 3). A very short duration of treatment (6 hours) was selected for this experiment since nutrient sensing pathways are known to react within minutes to hours to energy deficiency (Wojnacki vd., 2021). The activation (phosphorylation) of nutrient sensing proteins AMP Kinase (AMPK), ribosomal protein S6 (S6) and Glycogen Synthase Kinase 3 beta (GSK-3β) as well as the autophagy related protein Beclin-1 were evaluated (Figure 3). Since metformin inhibits mitochondrial respiration, it is also known to activate AMPK via phosphorylation at T172 (Qu vd., 2014).

SW480 cells treated with metformin in complete medium showed an increase p-AMPK compared to vehicle treated cells, as expected (Figure 3). SW480 cells grown in the absence of L-glutamine also showed an increase in p-AMPK compared to cells grown in the presence of L-glutamine (lanes labeled as vehicle Figure 3), suggesting that in the absence of L-glutamine ATP generation in reduced in these cells. With metformin treatment in L-glutamine deprived cells, a further increase in p-AMPK was seen, suggesting that both electron transport chain (inhibited by metformin) and TCA cycle anaplerosis (mediated by L-glutamine) contributed to energy generation in these cells. The same pattern was observed in SW620 cells as well.

GSK-3β is a promiscuous kinase that can phosphorylate serine 652, 648, 644 and 640 in the enzyme glycogen synthase. Several different nutrient sensing kinases can regulate the activity of GSK-3β itself via an inhibitory phosphorylation at Serine 9 including Akt, protein kinase A (PKA), protein kinase C, p70S6 kinase, among others (Frame vd., 2001). We therefore examined whether GSK-3β was differentially inhibited in the presence of metformin.

With metformin treatment in both SW480 and SW620 cells grown in complete medium, a robust increase in the phosphorylation of GSK-3β was observed, suggesting that reduced energy levels in cells was associated with reduced glycogen synthesis, an expected outcome. However, in SW480 cells, metformin treatment in the absence of L-glutamine resulted no enhancement in the inhibitory phosphorylation of GSK-3β. This suggests that the combined treatment either re-activates glycogen synthesis (which is difficult to envisage as most of the energy generating

pathways have been inhibited in these cells), or GSK-3β activates one of its many other substrates, such as those involved in the activation of Wnt signaling (please see data described under Work package 2 for further evaluation of this hypothesis).

In SW620 cells, while L-glutamine deprivation increased the phosphorylation of GSK-3β, treatment with metformin increased the phosphorylation further. This was completely contrary to the data we observed in SW480 cells, suggesting major divergence in GSK-3β mediated signaling in these cells under combination treatment.

We also evaluated the activation of the mTOR pathway by determining the phosphorylation of the mTOR target ribosomal protein S6. This pathway is active when nutrients are available, enabling the cells to grow and proliferate. When AMPK is active, the mTOR pathway is expected to be inhibited. We observed that in SW480 cells, metformin treatment or L-glutamine withdrawal did not lead to a decrease in pS6 phosphorylation despite an increase in p-AMPK. In SW620 cells, however, L-glutamine withdrawal led to a decrease in phosphorylation of S6. A similar decrease was seen in SW620 cells treated with metformin in complete medium; however, the combined condition of L-glutamine withdrawal and metformin treatment did not lead to a further decrease in phosphorylation of S6. Further examination of the activation of autophagy related pathways indicate that in SW620 cells (but not SW480 cells) metformin treatment in complete medium, L-glutamine withdrawal and combination led to an increase in autophagy pathways as shown by an increase in Beclin 1 in these cells. Overall, our data suggest that SW620 cells, but not SW480 cells, are capable of activation autophagy related pathways when major nutrient sources are depleted (via inhibition of electron transport chain and L-glutamine anaplerosis into the TCA cycle). Considering that SW620 cells survive better with the combination of metformin and glutamine withdrawal/CB839 treatment, we suggest that the activation of autophagy in these cells may provide the ATP necessary for survival. The lack of autophagy activation in SW480 cells may lead to their greater vulnerability to the treatments.

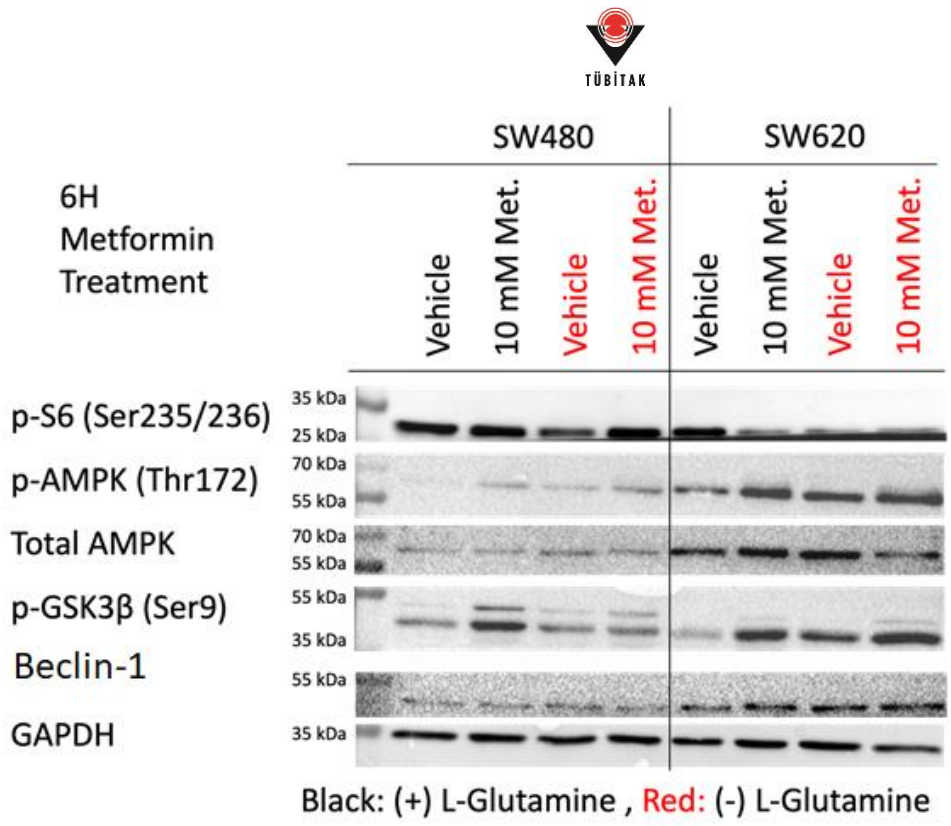


Figure 3. Western blot analysis of nutrient sensing pathways.

SW480 and SW620 cells were treated with 10mM metformin in the presence (black labels) or absence (red labels) of L-glutamine for 6 hours. The cells were then processed for total protein isolation. GAPDH was used as the loading control.

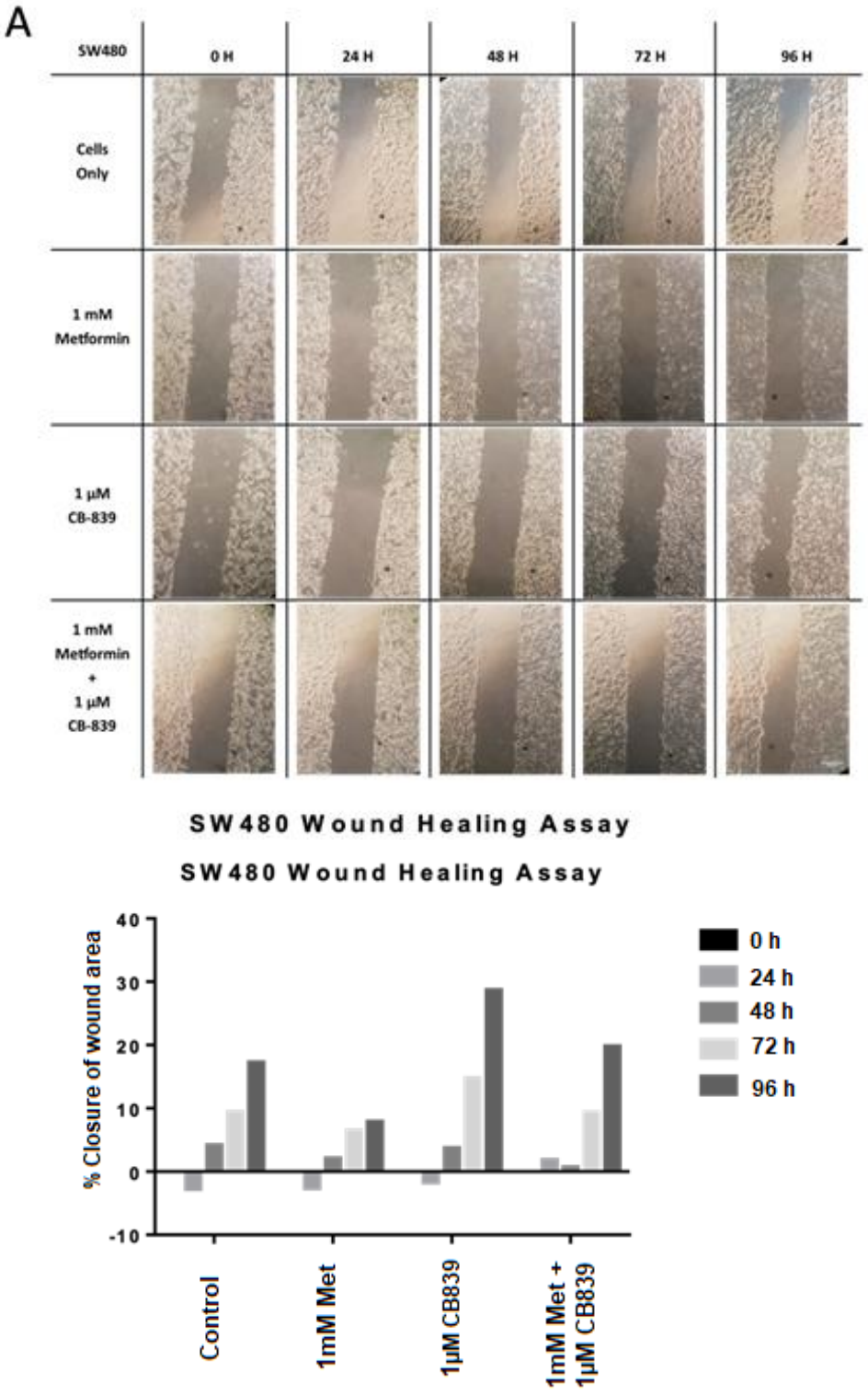
In the light of these observations, we suggest that SW480 cells are more vulnerable to cell death when treated with a combination of metformin and L-glutamine withdrawal compared to SW620 cells; the latter may be more resistant to this combination via the activation of autophagic signaling.

4.4 Evaluation of epithelial or mesenchymal characteristics of SW480 and SW620 cells

4.4.1 Motility of SW480 and SW620 cells treated with metformin

Previous studies have shown that 2mM metformin treatment of SW480 cell resulted in slower closure of a scratch wound assay over a duration of 96h compared to untreated cells (Amable vd., 2020). We have hypothesized a differential effect of metformin on the motility of SW480 and

SW620 cells. In order to assess the motility rate of these cells, a wound healing assay was carried out in the presence and absence of L-glutamine (Figure 4).



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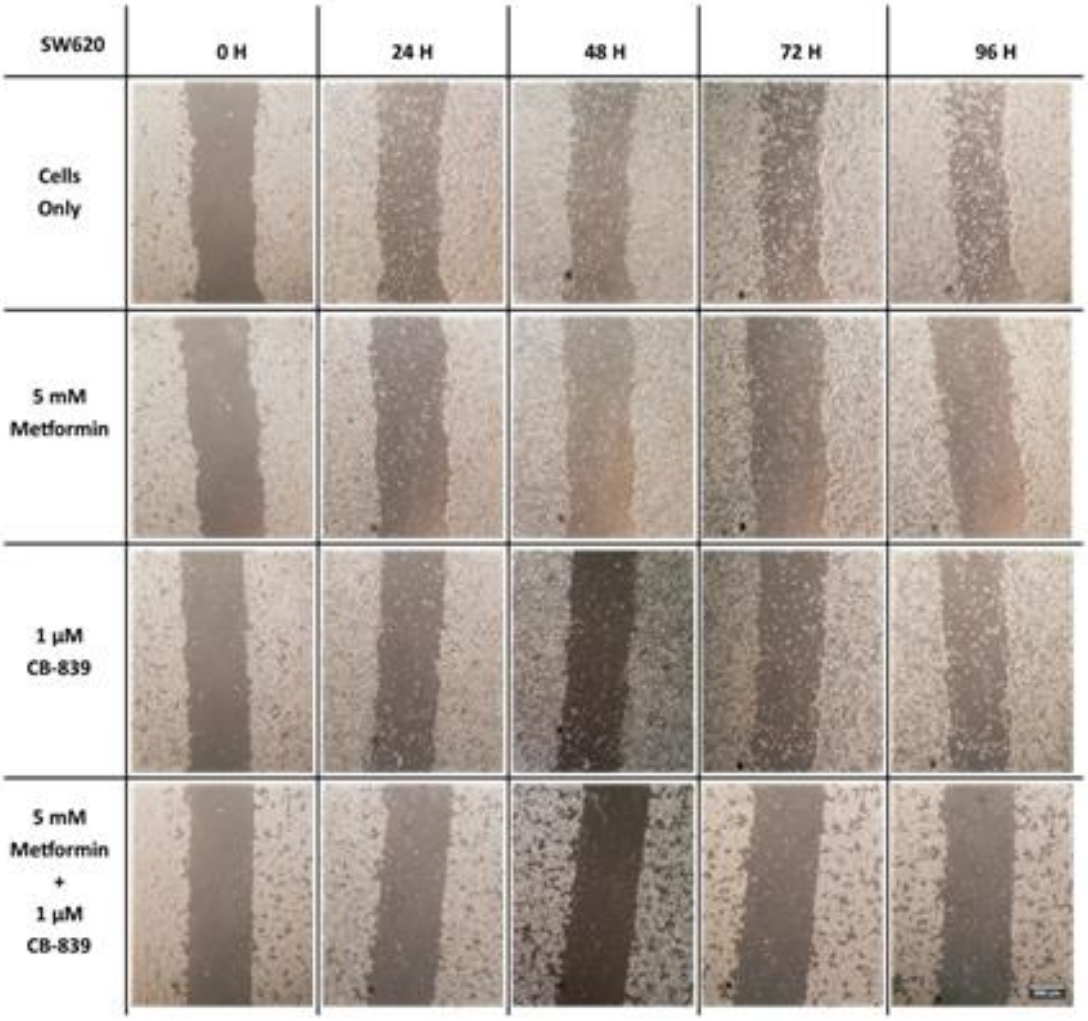


SW620 Wound Healing Assay

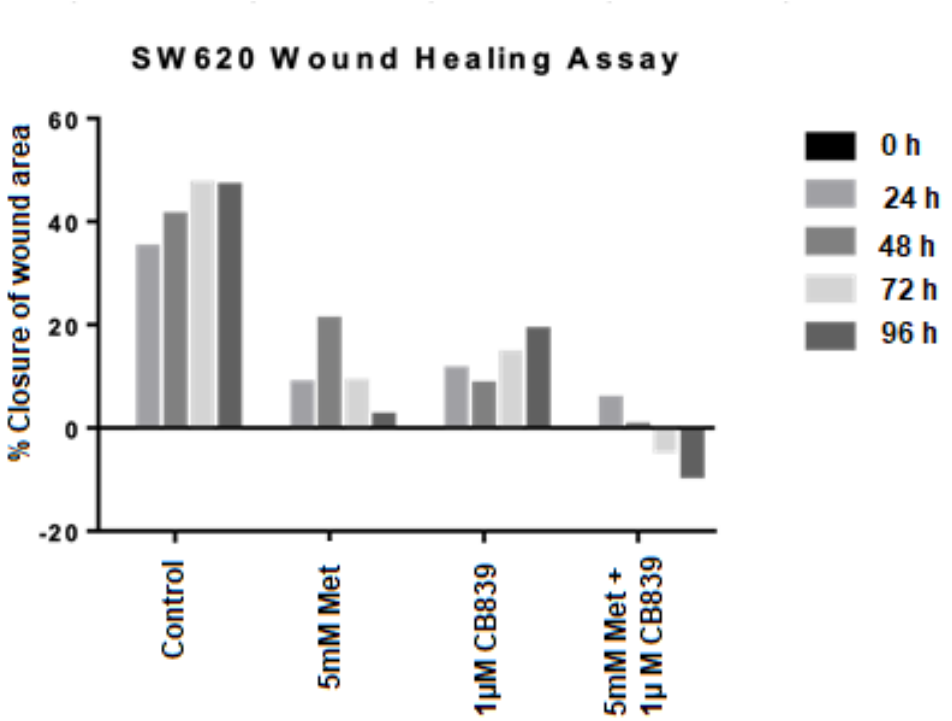


Figure 4. The differential effect of metformin in the presence and absence of L-glutamine to the motility of SW480 and SW620 cells.

The motility of both cell lines were determined by using a scratch wound healing assay in the presence of 1 mM Metformin (SW480), 5 mM (SW620) Metformin and 1 µM CB-839. The cells were also treated with 0.1mM mitomycin C to ensure that the wound closure was not due to cell proliferation.

Different concentrations of metformin were used for each cell line, since optimization studies and colony formation assay showed extensive death with 5 mM metformin in SW480 cells (Figure 2A). CB-839 was used to mimic L-glutamine starvation. We observed a modest decrease in the motility of SW480 cells in the presence of metformin (Figure 4A); the motility of SW620 cells was more robustly decreased with metformin treatment (Figure 4B). Interestingly, L-glutamine starvation also showed a differential response between the two cell lines. According to our observations, the SW480 cell line closed the scratch wound with CB839 treatment (GLS1 inhibition) (Figure 4A) better than untreated cells, suggesting better cell survival and movement in these cells. SW620 cells were less efficient in closing the wound in the presence of CB839 compared to untreated cells (Figure 4B). Treatment of SW620 cells with both agents was additive showing a stronger inhibition of motility with both GLS1 and Complex 1 inhibition in these cells. These data support

previous findings showing that L-glutamine withdrawal can enhance the sensitivity of these cells to metformin (Kim vd., 2018). Our observations may have resulted from greater reliance of SW480 cells on glycolysis (which would be the major energy generating pathway in the absence of glutamate (Robinson vd., 1992)). SW620 cells, being more reliant on OXPHOS, which was effectively abolished with metformin and CG839 treatment, were unable to close the scratch wound as effectively.

4.5 Effect of metformin on stemness behaviour of SW480 and SW620 cells

Previous studies have shown that one of the key regulators of stemness in CRC is the Wnt pathway (Ben-Ze'ev vd., 2016). Supporting this, we observed a differential response in the phosphorylation of GSK-3β between SW480 and SW620 cell lines when these cells were treated with 10 mM metformin in the presence or absence of L-glutamine (Figure 3). GSK3 (found as two isoforms α and β) is a key regulatory protein which stands at the crossroad of many essential pathways including gluconeogenesis and Wnt signaling. GSK-3β is known to be basally active and unlike other kinases; phosphorylation at Ser-9 or Ser-21 via extracellular stimulation inhibits its kinase activity (Lee ve Kim, 2007). Phosphorylation of S-9 in GSK3β causes the N-terminal tail of GSK-3β to act as a pre-phosphorylated substrate, or pseudosubstrate (Frame vd., 2001). This phosphorylated S-9 tail self-associates in the primed-substrate binding pocket, hindering the binding of actual primed substrates, and thus diminishing primed-substrate phosphorylation by GSK-3β. In its active form, GSK-3β can phosphorylate β-catenin after a priming phosphorylation by casein kinase 1; this event targets β-catenin to the proteasomal degradation machinery. Wnt ligands induce disruption of the complex, which prevents GSK3 from phosphorylating β-catenin, allowing the accumulation and nuclear import of β-catenin (Frame vd., 2001).

In SW620 cells, metformin treatment in the absence of L-glutamine showed an increase in the Ser-9 phosphorylation of GSK-3β (Figure 3), suggesting an increase in Wnt signaling and β-catenin activation. However, in SW480 cells, increase in phosphorylated GSK-3β with metformin treatment required the presence of L-glutamine. Since these cells are originated from the same patient and are genetically identical, we hypothesized that the presence or absence of L-glutamine may lead to the differential activation of the Wnt signaling pathway.

To further support our hypothesis, mRNA expression of downstream target of the Wnt pathway c-MYC (T. C. He vd., 1998) was evaluated by using qRT-PCR (Figure 5). SW480 and SW620 cells were treated for 24h with 10mM metformin in the presence or absence of L-glutamine. The cells were then processed for RNA isolation, cDNA synthesis and RT-qPCR as per standard protocols (Taskoparan vd., 2017).

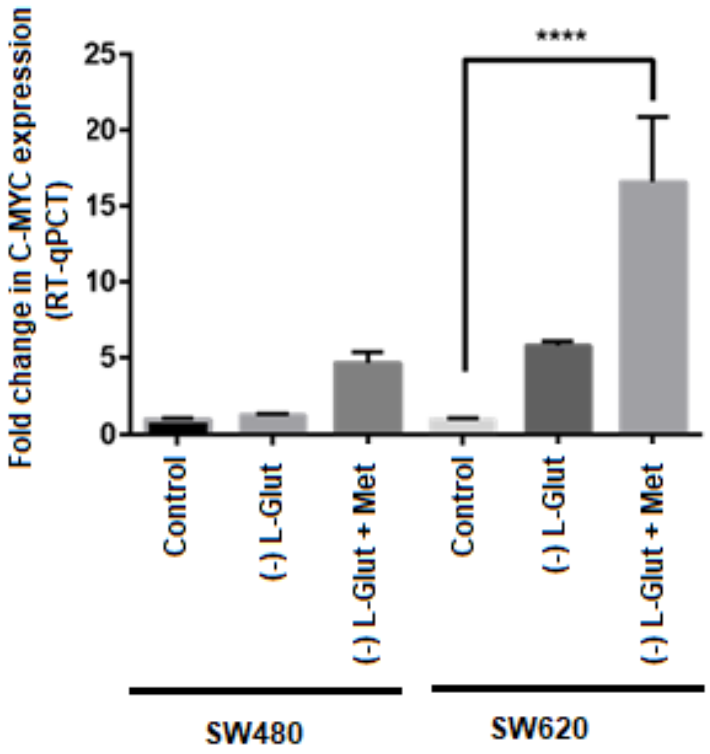


Figure 5. Expression of C-MYC in metformin +/- L-glutamine treated SW480 and SW620 cells.

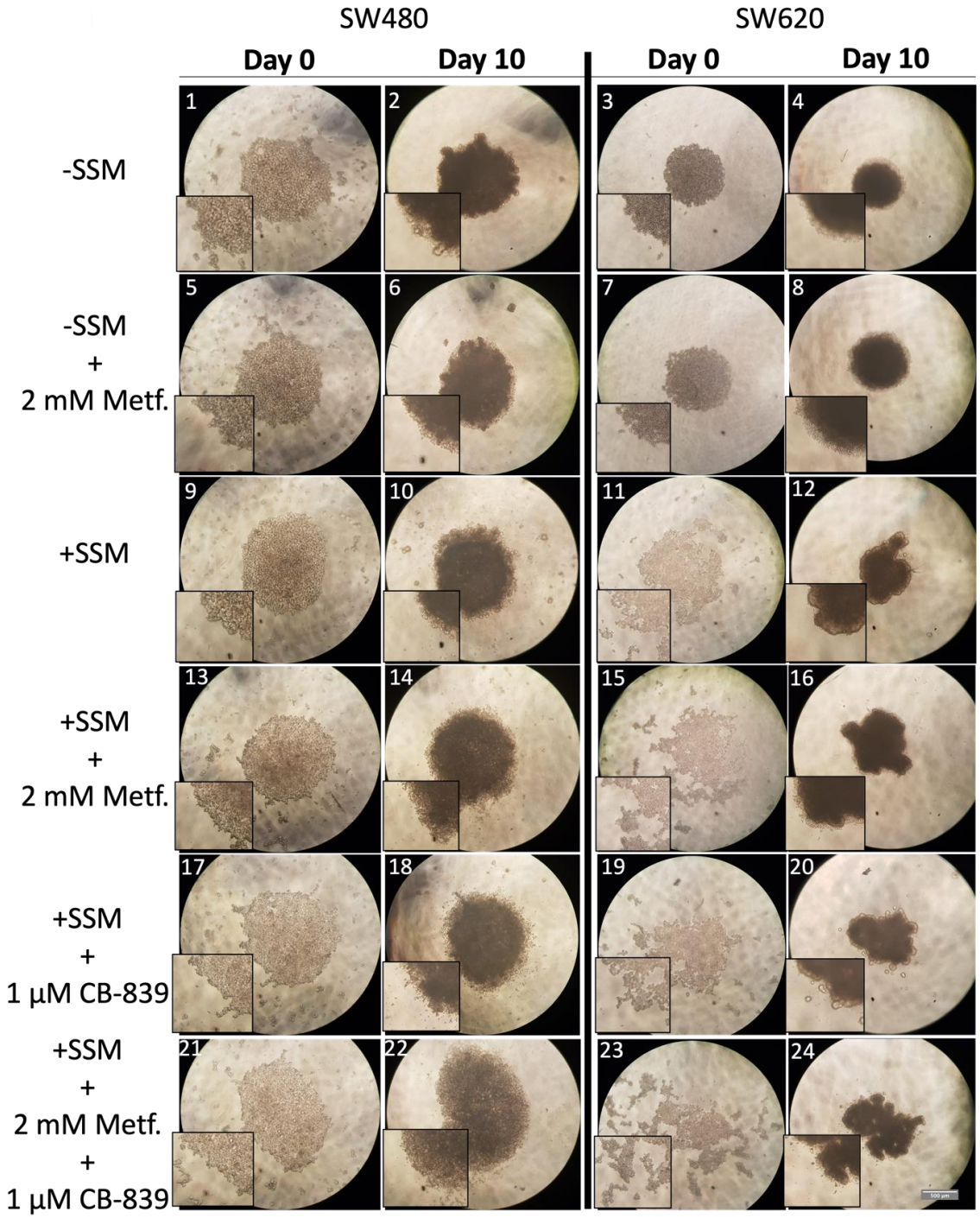
SW480 and SW620 cells were treated with 10 mM metformin in the absence and presence of 2mM L-glutamine. The expression of c-MYC was evaluated by using qRT-PCR. β-actin was used for normalization. The data are presented as fold change with respect to the expression in each cell line grown in complete medium. Data from two independent biological replicates is shown. ($p<0.0001$, t-test).

In SW620 cells, c-MYC levels were significantly upregulated with the combination of L-glutamine withdrawal and metformin treatment. This reinforces our hypothesis that enhanced survival of SW620 cells observed with the combination of metformin treatment and L-glutamine withdrawal could be via enhanced Wnt signaling. This is supported by the high GSK-3β phosphorylation observed in these cells with the combination; Ser-9 phosphorylation of GSK-3β results in the accumulation of β-catenin and its nuclear translocation (Frame vd., 2001). SW480 cells undergoing the combined treatment did not show a similar increase in c-MYC levels, neither did these cells show an increase in Ser-9 phosphorylation of GSK-3β (Figure 3), suggesting that increased Wnt signaling may not be seen in these cells. These cells also showed very poor survival with the combination of L-glutamine withdrawal and metformin treatment (Figure 1).

The Wnt pathway is known to regulate stemness behavior (Fodde ve Brabletz, 2007). In order to assess the effect of metformin on stemness behavior of SW480 and SW620 cells, a spheroid formation assay in stem cell enriched medium was carried out (Figure 6).

4.5.1 Spheroid formation assay

SW480 and SW620 cells were seeded into a 96-well ultra-low-attachment plate (ULA) at a 50,000 cells/well density in DMEM/F12 complete medium. The stem cell selection medium was prepared as described in Materials and Methods. When the serum free DMEM/F-12(HAM)1:1 medium was supplemented with 10% FBS only (complete medium), it was designated as (-) stem cell selection medium (-SSM). When the medium was supplemented with stem cell selection medium, it was designated as (+) stem cell selection medium (+SSM). 24 hours after seeding on ULA plates in either (–)SSM or (+)SSM medium, treatments were applied and the cells were allowed to form spheroids (first day of treatment was considered as Day0). The (–)SSM cells were either untreated, or treated with 2mM metformin. The (+)SSM cells were treated with 2mM metformin, 1μM CB839 or a combination of the two at the same concentrations. For 10 days after the start of treatment, each well was imaged every day and the treatments were refreshed every 2 days. The images and analyses for day 0 and day 10 are shown in Figure 6. Parts of the images were magnified further (shown as insets) for better understanding of the alterations in the morphology of the spheroids.


SW480
SW620
Day 0
Day 10
Day 0
Day 10
-SSM
-SSM + 2 mM Metf.
+SSM
+SSM + 2 mM Metf.
+SSM + 1 µM CB-839
+SSM + 2 mM Metf. + 1 µM CB-839
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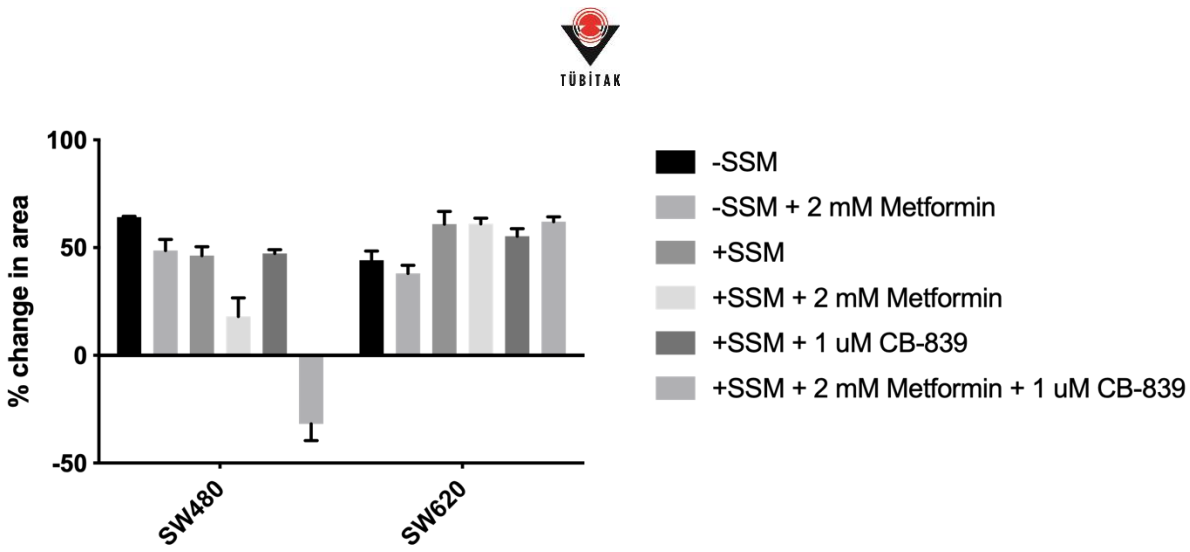


Figure 6. Spheroid formation assay.

SW480 and SW620 cells were seeded as 50,000 cells/well density into ultra-low attachment plates and cultured in the presence of 2 mM Metformin or 1 μM CB-839 or a combination of the two for 10 days. Cells grown in in complete medium with or without metformin treatment are designated as –SSM and –SSM + 2mM Metformin. The images were analyzed for their area with ImageJ. SSM: Stem cell selection medium, Metf: Metformin. Data shown are representative of two independent biological replicates.

In general, SW620 cells formed tighter spheroids in complete medium compared to SW480 cells, irrespective of the presence of metformin. A tighter spheroid is usually characterized by stronger cell-cell junctions. In the presence of the stem cell selection medium for 10 days, the SW480 spheroids had more diffuse borders compared to the (-)SSM spheroids. The same phenotype was observed when these spheroids were generated in the presence of either metformin or CB839. Treatment with CB839 resulted in less dense, relatively pale spheroids. However, the combined treatment of SW480 cells with metformin and CB839 for 10 days in the presence of SSM resulted in very large pale spheroids (Figure 6-22) suggesting a loss of cell to cell adhesion. Nonetheless, the size of the spheroids formed from SW480 cells cultured in stem cell selection medium (Figure 6-10) did not show a statistically significant difference in area compared to the spheroids cultured in complete medium (Figure 6-2), suggesting that the overall stem cell characteristics of these cells were low.

SW620 cells cultured in stem cell selection medium for 24h (Day 0) showed remarkably reduced cell-cell contact compared to the spheroids grown in complete medium (Figure 6-11 versus Figure 6-3). This indicates reduced cell – cell contact, suggesting the presence of more stem cells in SW620 cell compared to SW480 cells. The spheroids grown for 10 days in SSM did not show any difference in the overall area, but the shape of the spheroids were showed remarkable difference. With treatment, the spheroids acquired a lobular appearance, which observed to the greatest extent in spheroids that were treated with the combination of metformin and CB839. Recent studies suggest that lobular budding observed in spheroid morphologies was connected to the reduced E-cadherin expression and subsequent loose of cell-to-cell adhesion (Wang vd., 2021). Overall, these differential responses observed in the spheroid formation capacity might arise from the cancer stem cell phenotype of the SW620 cells, which is further exacerbated when treated with metformin in combination with CB839.

4.5.2 Expression of stemness markers in 2D culture and spheroids

To support our observations with the qualitative appearance of spheroids with stem cell enrichment and treatments, we evaluated the expression of the stemness marker EpCAM (epithelial cell adhesion molecule) in the spheroids using RT-qPCR. EpCam is a cell surface molecule that is known to be highly expressed in colorectal cancer stem cells (Dalerba vd., 2007). For this, individual spheroids from 10 independent wells of the ULA plates undergoing the same treatment were pooled and total RNA was isolated, followed by cDNA synthesis and RT-qPCR. Supporting the spheroid formation assay, SW620 spheroids cultured in SSM showed an increase in the expression of EpCAM compared to SW620 spheroids generated in complete medium. SW480 spheroids grown in the presence of SSM did not show any increase in the expression of EpCAM compared to spheroids grown without SSM; in fact a decrease in expression was observed [Figure 7, (-)SSM versus (+)SSM for SW480 cells]. This data suggests a stronger selection of stem cells from SW620 cell compared to SW480 cells when grown as spheroids in SSM.

SW480 spheroids treated with 2mM metformin showed a decrease in EpCAM expression in both complete medium and SSM. Metformin was shown to reduce stem cell characteristics of spheroids

generated from HCT116 cells in a dose dependent manner (C. Zhang ve Wang, 2019). Therefore, the reduction in EpCAM expression in SW480 spheroids with metformin treatment was expected. With SW620 spheroids however, metformin treatment did not lead to a reduction in the expression of EpCAM; on the contrary, an increase in expression was observed in both (-)SSM and (+)SSM media. Moreover, a modest reduction in EpCAM expression was observed in (+)SSM spheroids treated with CB839, which was increased when co-treatment with metformin was carried out. This data supports differential responses of SW480 and SW620 cells on the basis of their cancer stem cell behavior. SW620 cells overall were seen to have a higher potential to form cancer stem cells, which was further exacerbated when treated with metformin and a combination with CB839. These data also support our observations of spheroid shape and density.

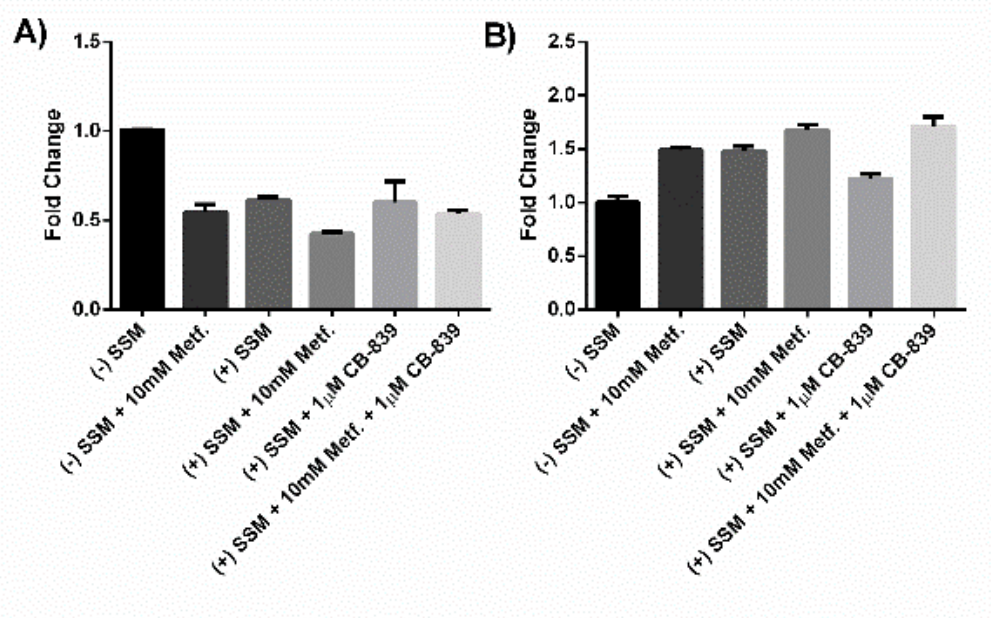


Figure 7. Determination of EpCam mRNA expession in SW480 (A) and SW620 (B) spheroids in response to 2mM metformin and 1μM CB-839 treatment.

Stem cell selection medium enhanced the expression of EpCAM in SW620 cells, but not it SW480 cells. Additionally, EpCAM mRNA expression was increased upon treatment with 2mM metformin in both (-) SSM and (+) SSM spheroids. SSM: stem cell selection medium Metf.: metformin. β-actin was used for normalization. Data shown are average of two independent replicates.

Since the role of metformin in reducing stemness markers has been widely reported (Jaromy ve Miller, 2021), we also evaluated whether the expression of the stemness marker EpCAM was

altered when the cells were grown in regular tissue culture plates in their ATCC recommended medium (2D culture). We observed that the mRNA expression of the stemness marker was reduced quite dramatically in SW480 and SW620 cells treated with metformin. A combination of L-glutamine withdrawal and metformin treatment reduced the expression of the stemness marker further in SW620 cells. These data are highly reminiscent of a previous study where withdrawal of glutamine was shown to sensitize pre-sorted EpCAM high SW620 cancer stem cells to metformin (Kim vd. 2018). However, it needs to be emphasized here that culturing cells in tissue culture plates in the absence of SSM is highly unlikely to generate any stem cell enrichment. In SW620 cells CB839 treatment reduced EpCAM expression whereas L-glutamine withdrawal did not, suggesting that GLS1 inhibition and L-glutamine withdrawal did not result in identical outcomes at least in this cell line.

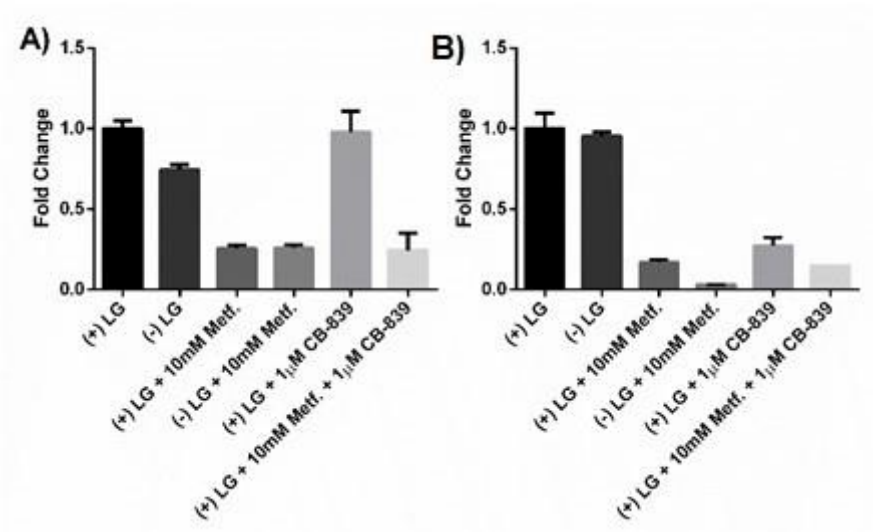


Figure 8. Determination of the change in colorectal cancer stem cell marker EpCam in response to L-glutamine withdrawal, metformin and CB-839 (mimicking L-glutamine starvation) treatment in SW480 (A) and SW620 (B) cells.

Withdrawal of L-glutamine slightly decreased the expression of EpCAM in both cell lines, but CB-839 treatment significantly decreased the expression only in SW620 cells. In both cell lines metformin treatment dramatically decreased the expression of EpCam. LG: L-Glutamine, Metf: Metformin

Our expectation in the current proposal was that metformin treatment would lead to the formation of smaller spheroids with lower stem cell like characteristics in SW480 and SW620 cells. This

expectation was fulfilled with SW480 cells, which overall did not show much enrichment of stem cell like characteristics to begin with. SW620 cells; however, showed an enrichment of stem cell characteristics, which was enhanced further with metformin and also with a combination of CB839 and metformin. This is the first report to our knowledge suggesting that the combination of GLS1 inhibition and metformin treatment may enhance stemness behavior of metastatic and to a lesser extent, non-metastatic cells.

4.6 Effect of metformin on Epithelial to Mesenchymal Transition (EMT)

To evaluate whether inhibition of mitochondrial respiration via metformin in the presence or absence of L-glutamine mediated anaplerosis can differentially affect the EMT characteristics of SW480 and SW620 cells, EMT markers were evaluated by using RT-qPCR (Figure 9) Western blot (Figure 10) and immunocytochemistry (Figure 11) assays.

4.6.1 RT-qPCR to evaluate the expression of EMT markers in cells grown in 2D culture.

The mRNA expression of EMT markers E-cadherin (*CDH1*) and vimentin (*VIM*) was evaluated in both SW480 and SW620 cells in response to L-glutamine withdrawal, as well as treatment with metformin or CB-839 (Figure 9). The expression of E-cadherin showed an increase in response to L-glutamine withdrawal in both cell lines while the expression was decreased upon treatment with 10mM metformin in both cell lines (Figure 9A and C). The expression of vimentin in SW480 cells did not show any significant difference in response to treatment with metformin, CB839 or L-glutamine withdrawal. The decrease in E-cadherin expression in response to metformin treatment indicates a deregulation of cell adhesion in these cells. However E-cadherin is a protein highly regulated via post-translational mechanisms, therefore protein level expression of E-cadherin is also determined (Geng vd., 2012).

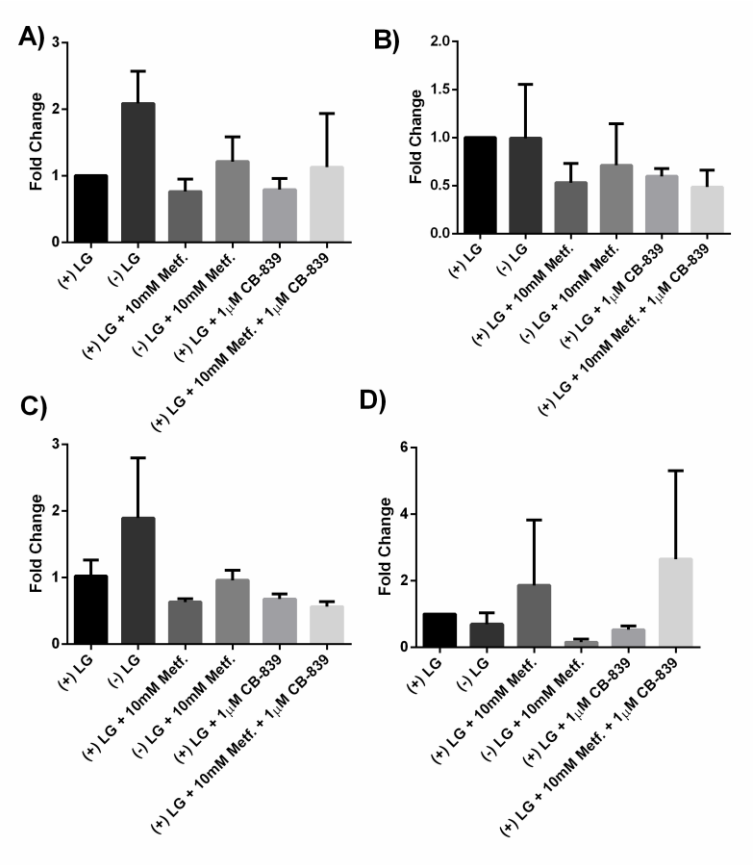


Figure 9. mRNA expression of EMT markers.

mRNA levels of EMT markers E-cadherin and vimentin were determined in SW480 (A: E-cadherin, B: Vimentin) and SW620 (C: E-cadherin, D: Vimentin) cells. E-cadherin expression increased in response to L-glutamine withdrawal and decreased in response to metformin treatment in both cell lines. The combined treatment did not show any change in expression. Vimentin expression did not show any significant change in response to L-glutamine withdrawal or treatments. LG: L-Glutamine, Metf: Metformin

4.6.2. Western blot to evaluate the expression of EMT markers in cells grown in 2D culture.

SW620 and SW480 cells were treated with 10mM metformin in the presence or absence of L-glutamine. Additionally, the cells were treated with 1μM CB-839 alone or in combination with 10mM metformin for 24h The cells were then collected and processed for whole cell lysate isolation and western blot according to standard protocols (Figure 10).

SW620 cells treated with metformin showed a decrease in the E-cadherin protein levels compared to vehicle (water) treated control cells (Figure 10 right panel). The combination of metformin with either L-glutamine deprivation or treatment with CB839 decreased the E-cadherin amount drastically. The same cells also showed a decrease in β-catenin levels suggesting major deregulation of cell-cell adhesion. No difference in vimentin levels were observed with any of the treatments.

We did not observe any bands for E-cadherin at 130 kD in SW480 cells with two different brands of antibodies and three independent replicates. We are currently unable to explain why we were unable to observe any bands for E-cadherin since this cell line is known to express the protein (Qin vd., 2016). β-catenin levels showed a slight decrease in SW480 cells treated with metformin compared to the vehicle (water) control cells. However, removal of L-glutamine or treatment with CB-839 increased the protein levels of beta-catenin irrespective of the presence of metformin. The expression of vimentin was seen to increase with all treatments compared to untreated control SW480 cells.

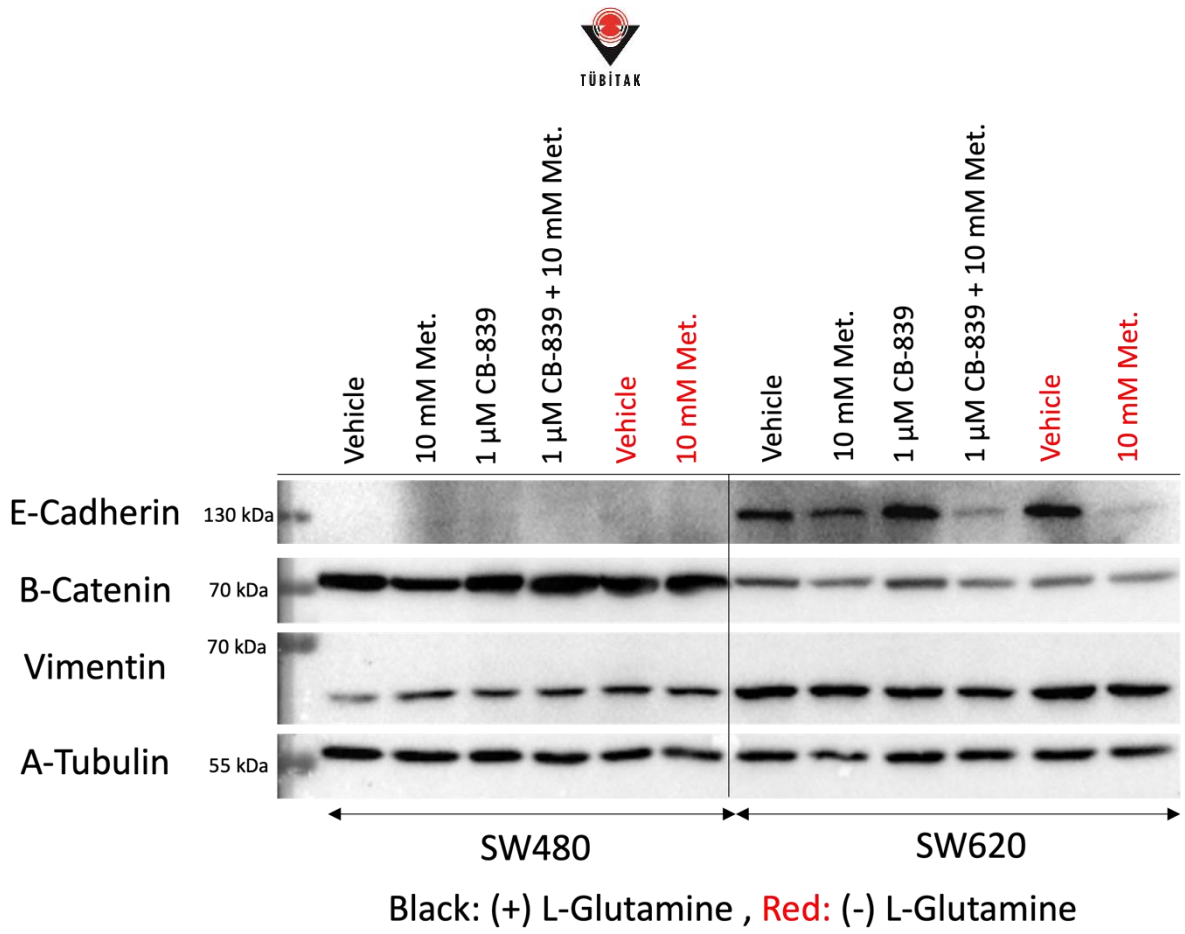
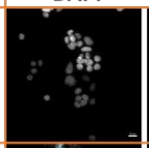
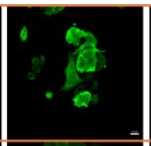
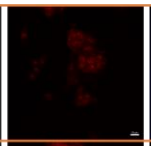
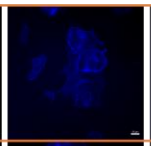
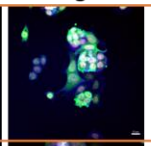
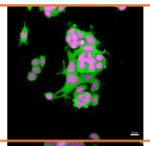
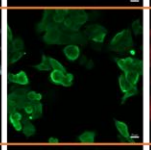
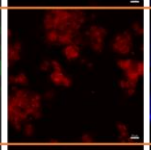
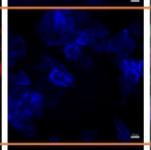
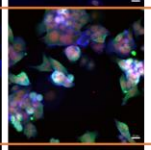
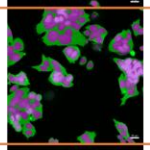
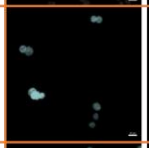
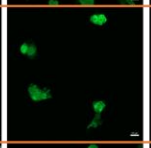
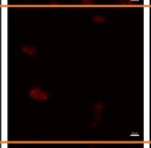
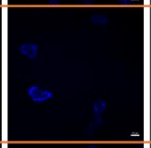
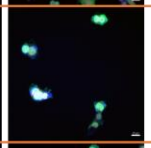
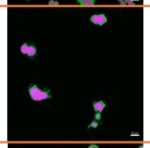
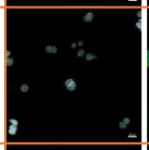
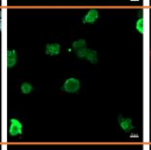
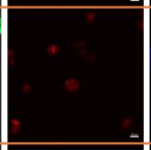
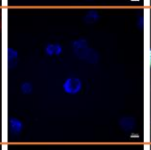
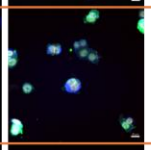
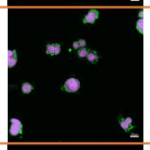
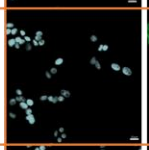
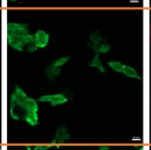
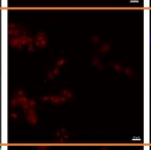
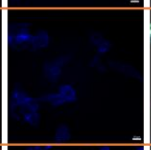
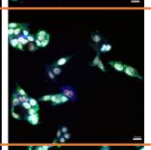
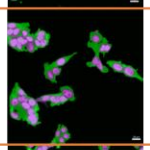
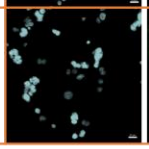
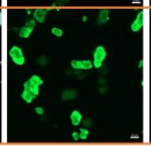
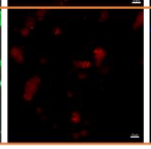
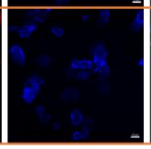
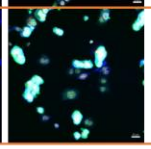
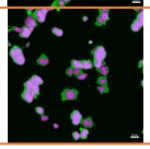


Figure 10. Western blot analysis of epithelial and mesenchymal markers.

SW480 and SW620 cells were treated with metformin (10mM) in the presence and absence of L-glutamine for 24 hours or with CB839 (1µM) or a combination of CB839 and metformin in complete medium. Membranes were blotted with α-Tubulin as a loading control. Representative figure from two independent biological replicates is shown.

4.6.3. Immunocytochemistry to evaluate the expression of cell-cell adhesion proteins in cells grown in 2D culture.

A western blot with total protein lysate shows overall protein levels but not their location, and the specific cellular location of junctional proteins is important for their function. Therefore, we carried out an ICC assay using SW480 and SW620 cells treated with metformin in the presence or absence of L-glutamine, treated with CB839 or a combination of CB-839 and metformin (Figure 11). The location of E-cadherin and β-catenin were evaluated. The nuclei were stained with DAPI and the cytoskeletal structures were stained with phalloidin.

SW480	DAPI	B-Catenin	E-Cadherin	Phalloidin	Merge	B-Catenin & DAPI Colocalization
+L-Glutamine						
-L-Glutamine						
+L-Glutamine +10 mM Metformin						
-L-Glutamine +10 mM Metformin						
+L-Glutamine +1 uM CB-839						
+L-Glutamine +1 uM CB-839 +10 mM Metformin						

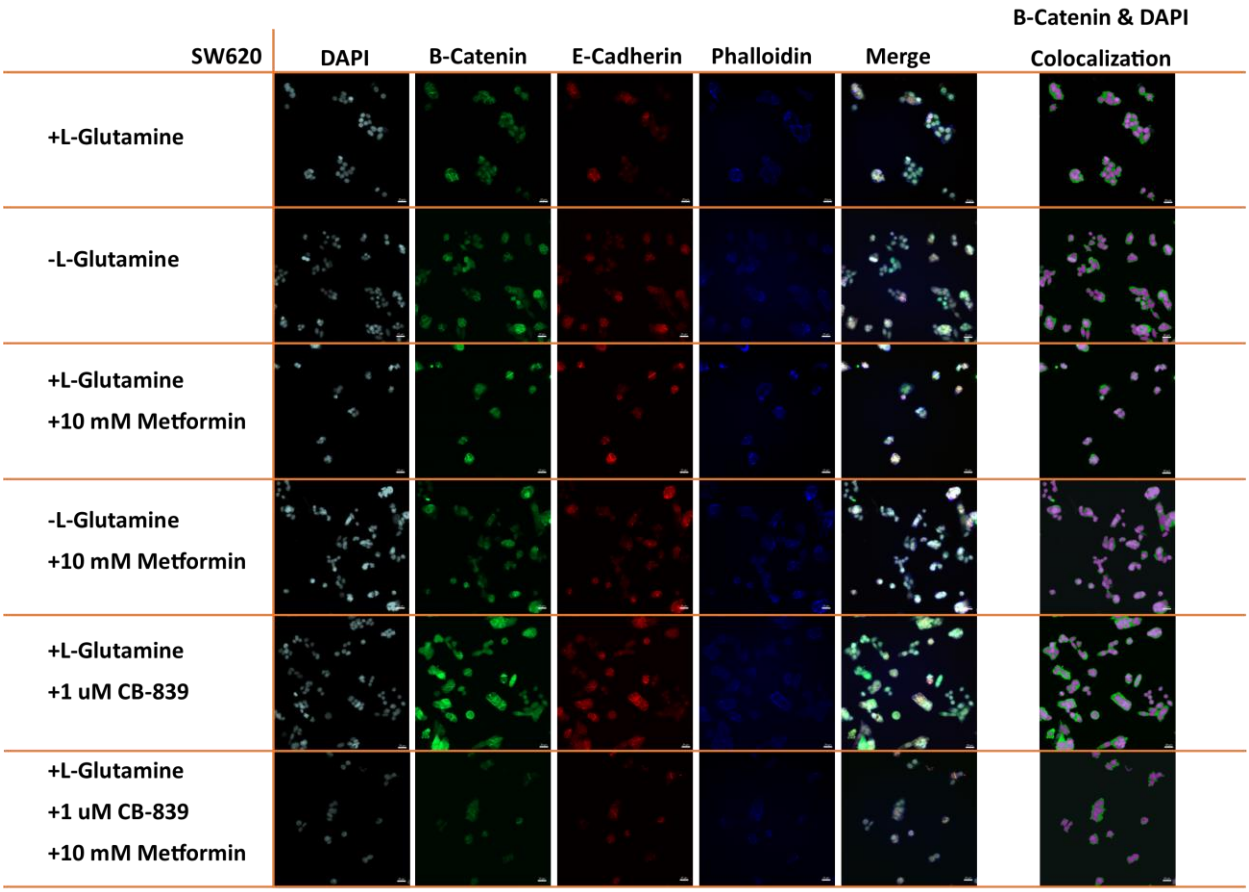


Figure 11. Immunocytochemistry analysis of EMT markers.

SW480 and SW620 cells were treated with 10 mM Metformin in the presence and absence of L-glutamine as well as 1 μM CB-839 or a combination of metformin and CB839 in complete medium for 24 hours. Samples were stained with DAPI for nuclear visualization, phalloidin for cytoskeletal visualization, β-catenin and E-cadherin antibodies to evaluate their subcellular localization.

Previous studies from the literature suggest that in CRC, E-cadherin expression is not always absent or reduced, but the protein is frequently redistributed from the cell membrane to the cytoplasm (Jiang ve Hiscox, 1997). In many in-vitro and in-vivo tumor model systems, progressive loss of E-cadherin expression has been reported to increase the aggressiveness of carcinomas and worsening of prognosis, whereas ectopic expression of E-cadherin can reduce tumor initiation and invasion. Various studies have shown that nuclear E-cadherin could negatively (Su vd., 2015)

or positively (Kam ve Quaranta, 2009) regulate Wnt/ß-catenin-induced exacerbation of cancer stem cell phenotype.

In SW480 cells grown in complete medium (with L-glutamine), nuclear E-cadherin was observed. With L-glutamine starvation, E-cadherin was observed to be equally distributed between the nucleus and cytoplasm. With metformin treatment, E-cadherin was more cytosolic irrespective of the presence or absence of L-glutamine supporting many previous studies on the enhancement of epithelial characteristics of cells with metformin treatment. The localization of β-catenin, however, changed considerably with treatments. Thus, SW480 cells showed high cytosolic β-catenin grown in the presence or absence of L-glutamine. When the cells were treated with metformin in the presence of L-glutamine, beta-catenin was more nuclear. Treatment with metformin in the absence of L-glutamine showed an even distribution between the nucleus and cytosol. As clearly evident from the co-localization of DAPI and β-catenin, treatment of SW480 cells with CB839 resulted in cytosolic beta-catenin (as also seen with cells grown in the absence of L-glutamine). Combination of metformin with CB839 resulted in a dramatic increase in nuclear beta-catenin. In general, nuclear beta catenin is associated with activation of Wnt signaling and higher stemness characteristics (Fodde ve Brabletz, 2007). The 5-fold increase in the mRNA expression of c-MYC in observed with SW480 cells treated with metformin in the absence of L-glutamine corroborates these data (Figure 5).

SW620 in general showed a membrane localization of E-cadherin irrespective of all treatments carried out, although western blot showed a robust decrease in E-cadherin expression with metformin, metformin + L-glutamine withdrawal and metformin + CB839 (Figure 10). With β-catenin, however, the location was primarily nuclear in the control cells (as expected from a metastatic cell line); upon L-glutamine deprivation, the β-catenin was seen to be enriched in the cytosol. Treatment of SW620 cells with metformin in the presence of L-glutamine showed a redistribution of the protein to the cytosol (compared to untreated cells) supporting previous studies on a reduction in stemness/metastatic properties of cancer cells treated with metformin (Amable vd., 2020). However, cells treated with metformin in the absence of L-glutamine showed increased nuclear beta-catenin, suggesting that a combination of L-glutamine deprivation and metformin can increase the stemness of these cells. This was also supported by a 17-fold increase

in C-MYC expression (Figure 5). SW620 cells grown with CB839 alone showed mostly membrane localized β-catenin, while a combination of metformin and CB839 resulted in a redistribution of beta-catenin from the membrane to the nucleus (as clearly evident from the co-localization of DAPI and β-catenin).

Immunohistochemistry assay clearly showed that, combined treatment of metformin and CB-839 (mimicking L-glutamine starvation) might cause a shift in the EMT character and stemness of the more mesenchymal cell line SW620. In the SW480 cells, the combination also results in an increase in stemness characteristics; however, to a lesser extent than SW620 cells. This may be related to the higher susceptibility of SW480 cells (compared to SW620 cells) to cell death by metformin treatment as observed by the MTT assay (Figure 1), colony formation assay (Figure 2) and spheroid formation assay (Figure 6).

4.6 *In silico* analysis of publicly available datasets to identify novel EMT/Stemness candidates

Metformin is known to alter EMT characteristics in cancer cells. Since we observed modest alterations in the mRNA expression of CDH1 and VIM, we aimed to evaluate whether other markers of EMT may be differentially expressed as a result of metformin treatment that can be further tested in our model. The primary data set that these analyses were based on is GSE67342 (J. He vd., 2015) in which the LoVo colon cancer cell line was treated with metformin or vehicle for 8 or 24h and the differential expression of genes was reported. Since we have treated our cells with metformin for 24h, we have used the 24h treatment data for all analyses.

Our analyses relied on three different approaches:

1. Differentially expressed EMT related genes were identified between SW480 and SW620 cells (presented as preliminary data in the proposal). We determined whether any of these genes were also differentially expressed in the metformin treated and untreated cells. To

help us address the mechanism behind differential response of SW480 and SW620 cells to metformin.

2. A ground breaking study in 2019 identified 16 cell membrane receptors and 24 transcription factors that belong to KRAS, Wnt and Notch signaling pathways and genes that are involved in the process of EMT (McFaline-Figueroa vd., 2019). We have carried out an *in silico* analysis to examine whether genes that are differentially expressed in the cells knocked out for EGFR, MET, ITGAV, ITGB1 and FGFR1 [GSE114687 (McFaline-Figueroa vd., 2019)] are also differentially expressed in the metformin treated LoVo cells [GSE67342 (J. He vd., 2015)].
3. L-glutamine was identified in the course of the experiments to affect the stemness behavior of SW480 and SW620 cells treated with metformin. A gene expression signature that was shown to predict stemness (Nio vd., 2017) as a function of GLS1 expression and Wnt/β-catenin activity in liver cancer patients (Li vd., 2019) was evaluated in the metformin treated/untreated dataset.

4.6.1. Comparison of differentially expressed genes (DEG) between metformin treated cells and EMT related genes in SW480 and SW620 cells

929 genes in the metformin treatment dataset (GSE67342) and 1155 genes in SW480 vs. SW620 spheroids dataset (GSE89523) were differentially expressed (FDR < 0.05, LFC < -1 or LFC > 1). The genes that were differentially expressed with metformin treatment obtained from GSE67342 were compared with the EMT-related genes we obtained in our preliminary analysis of GSE79523. Only 4 showed significant differential expression between cells treated with metformin and control (FDR < 0.05, LFC > 1), and only JUN was upregulated by metformin treatment (Table 4).

Table 4. Common differentially expressed genes in GSE89523 and GSE67342. Differentially expressed genes in two datasets were compared and common EMT genes are shown. Identified genes were filtered with FDR < 0.05 and LFC < -1 or LFC > 1

Gene	Metformin Treatment	SW480 vs SW620
CXCR4	Downregulated	High in SW480
JUN	Upregulated	High in SW620
FOXQ1	Downregulated	High in SW620
LGR5	**Downregulated**	**High in SW620**

4.6.2. Comparison of DEGs between metformin treated cells with the EMT signature

A breakthrough study using state of the art approaches of CRISPR-Cas9 to knock down 40 epithelial-to-mesenchymal transition (EMT) transcription factors and pooled single cell RNA-Sequencing showed that EMT rather than being a discrete process as it had been considered for so many years, is a continuous path involving the activation and suppression of multiple gene regulatory networks in the cells (McFaline-Figueroa vd., 2019). A gene list with EMT related genes that were significantly upregulated either spontaneously or with TGBβ treatment when EGFR, MET, ITGAV, ITGB1 and FGFR1 were knocked out with CRISPR Cas-9 system was generated. Based on the methodology described above, a gene list (Table 5) showing common genes that are related to EMT (common to spontaneous and TGFβ induced) and were up or down regulated with metformin treatment was generated that can be evaluated in further studies.

Table 5. EMT related genes (spontaneous, TGFβ induced and common) that are up or down regulated with metformin treatment.

UPREGULATED WITH METFORMIN TREATMENT		
Spontaneous EMT	***TGFβ induced EMT***	***Common Genes***
WRNIP1, CCNL1, RHOV, DGKE, NR4A1, ATF3	CHORDC1, MT1G, NFRKB, COBLL1, EIF1, AVPI1, RPS16, PTK2, RPL18, PSMA3, GNB5, NFKBIZ, OTUD1, SRRM2, RPL5, HNRNPA1,	HIST1H1C, CXCL2, FOS, SOD2, RPS27A, JUN, RBPMS, GADD45A, CXCL3,

	CDC42, HNRNPC, SIK1, SNX5, THBS1, PDE4D, ISG15, GADD45B, CCL20, RP11-328M4.2, CTTN, SRSF11, KLHL24, NDUFA10, ZNRD1, HSPA1B, MYL6, HNRNPM, RPL35A, RBM8A, CTGF, NACA.	MT1X, SQSTM1, RPL13, HES1, FBXO32, KLF6, TM4SF1, ID3, RPL7, DUSP1, HMGB2.
DOWNREGULATED WITH METFORMIN TREATMENT		
PNISR, TUBD1, FAM217B, NDRG1, DSG3, PUM2, TM2D3	REV3L, PNMA1, MT-ND6, ARID5B, ZNF827, DLG1, MIR22HG, CSNK2A1, SFXN1, ASAP2, BNIP3L, INHBA, TRMT10C, GRHL3, HNRNPH3, FLRT3, TMEM2, PHLDB3, PMEPA1, SPC25, RFWD2, CIRBP, CPSF6, CXCR4, CASC7, BNIP3, PDCD6, VMP1, ATAD2, CDK5R1, EHF, HAS3, KIF5B, INSIG1, REL, ID1, TRIO, RAB27A, SLC25A13, SRSF1, SETD5-AS1, PTGS2, RBM39, FAM162A, EIF5, H2AFJ, NSMCE4A, LLPH, LIPG, TMEM150C, C2orf49, PAG1, FGFR1OP, SBNO1, ZEB1, HNRNPA0, ITGA2, SYNCRIP, GPRC5A, WRB, CHD9, MAST4, ETF1, TMEM219, APPL2, MIR210HG, TUG1, UBE2I, SMURF2, CERK, DDX28, PRDM1, RBFOX2, SLC38A1, C1orf116, USP9X, GAN, NEDD9, THNSL1, ATPAF1, CASP8, POLR3B, RTN4IP1, DDX3X, NT5DC2.	DUSP6, IGFBP3, TARSL2, NEAT1, CLIC3, HTRA1, SERPINB5, EGR1, S100A6, TRA2B, HMGA2, TIMM21, TSEN2, H2AFX, S100A10, SLC25A32

4.6.3. Comparison of DEGs between metformin treated cells and stemness signature according to GLS1 expression

A stemness signature consisting of 19 specific genes was described for liver cancer (Nio vd., 2017). The expression of these genes could be used to classify liver cancer patients into low, medium or high stemness categories. The expression of GLS1 was determined in each category and was found to increase as the stemness characteristics increased. Since treatment with CB839 or L-glutamine withdrawal in combination with metformin treatment was seen to enhance stemness characteristics of SW620 cells, we determined whether any of the stemness marker genes were differentially expressed in the metformin treated/untreated LoVo data set (GSE67342). The expression of LGR5 was the only stemness marker that we found to be

significantly reduced in expression (>1 LFC, FDR < 0.05) in the metformin treated cells. Two different probes of LGR5 (210393_at and 213883_at) were found to be significantly ($p<0.05$, adjusted p value) reduced by 1.29 and 1.04 fold in metformin treated cells.

Although stemness signatures for different cancer types can vary and the stemness signature used in the current analysis was for liver cancer, the fact that Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) expression was positively correlated with GLS1 expression and the gene was also significantly differentially expressed in metformin treated cells suggests that LGR5 may be an interesting marker for future studies. LGR5 is an “orphan” receptor belonging to the G protein-coupled receptor (GPCR) family of proteins (Xu vd., 2019). In a seminal manuscript from Hans Clevers’ lab in 2011, Lgr5 was shown to modulates canonical Wnt signaling strength through binding to its ligand R-spondin in intestinal crypts (de Lau vd., 2011). Since then, LGR5 has been extensively reported to potentiate Wnt/β-catenin signaling pathway, thereby stimulating cancer stem cell proliferation and self-renewal, as well as tumor formation, EMT and cell mobility (Xu vd., 2019).

Our initial attempts at evaluating the mRNA expression of LGR5 under different treatment conditions in both SW480 and SW620 cells failed due to lack of any amplification in RT-qPCR (data not shown), although both cell lines are known to express the gene (Hirsch vd., 2014). We will re-design the primers that we have used (sequence given in Table 1) and evaluate the expression of LGR5 as well as other genes that have come up with approach 2.

5. DISCUSSION

Clinical trials with metformin as a chemopreventive agent are usually short term in their design for ethical reasons. One of the first clinical trials to examine the chemoprevention effect of low-dose (250 mg/day) metformin for 1 year on metachronous colorectal adenoma/polyp formation was a multicenter, double-blind, placebo-controlled, randomized phase 3 trial in non-diabetic patients at high risk to develop adenoma (Higurashi vd., 2016). The outcomes of this trial suggested that metformin could significantly suppress adenoma formation compared to the placebo control group. This study had several limitations, including a short follow-up time of just 1 year. Chemopreventive trials are generally held for 3-5 years. Several more clinical trials are either ongoing or have been completed with the use of metformin in combination with other chemotherapy drugs. A small single-center, single-arm phase 2 clinical trial with 50 patients with measurable and progressing metastatic disease were enrolled to receive metformin at 800mg 2 times a day plus 5-FU 425 mg/m2 and leucovorin 50 mg intravenously weekly for 8 weeks. Of these 50 patients, 11 met the end point criteria. The combination of 5-FU and metformin showed prolonged median survival only in the obese patients in the cohort (Miranda vd., 2016). A follow up trial with obese patients (BMI >31) who were non diabetic and received 1000mg metformin twice a day for 12 weeks did not show any change in the activation of ribosomal protein S6 or the proliferation marker Ki-67 in rectal biopsies (Zell vd., 2020). These data suggest that the exact role of metformin as a chemotherapeutic agent needs to be evaluated further with pre-clinical trials.

The initial design of the current study focused on understanding whether SW480 and SW620 cells derived from the primary colorectal tumor and lymph node metastasis respectively from the same patient responded differentially in EMT and stemness characteristics to metformin. The expectation was to observe SW620 cells showing a stronger reduction in EMT and stemness characteristics with metformin compared to SW480 cells.

Our initial experiments suggested that SW620 cells were more resistant to metformin, as also indicated in the literature (Kim vd., 2018). However, the same study also showed that glutamine withdrawal could enhance the sensitivity of SW620 cells to metformin. Similarly, the combined

treatment of SW480 as well as SW620 cells with metformin and cisplatin was shown to enhance cell death via apoptosis in both cell lines to the compared to individual treatments (P. Zhang vd., 2020). However, these studies have a common characteristic of culturing the SW480 and SW620 cells in high glucose DMEM, which is not the ATCC defined medium for these cell lines. SW480 and SW620 cells are known to rely on mitochondrial respiration and should be grown in Leibovitz L-15 medium containing galactose, rather than glucose and incubated in 100% air rather than 5% CO2. These differences in culture conditions can significantly alter the metabolic characteristics of cells and therefore their response to drugs.

Our overall findings suggest that both metformin and L-glutamine withdrawal resulted in reduced proliferation in both cell lines; however, SW620 cells were comparatively less vulnerable to the combination compared to SW480 cells. Growth of the cells as spheroids in stem cell enrichment medium in the presence of metformin, the GLS1 inhibitor CB839 and the combination showed remarkably divergent appearance of the spheroids compared to the spheroids obtained in complete medium (non stem cell enriching) (Figure 6). These data suggest strongly that the two cell lines are likely to differ considerably in their stem cell characteristics, as well as response of the stem cell enriched population to metformin and/or glutamine withdrawal. A previous study carried out with FACS sorted stem cells from SW620 cells indicated that the stem cell population from these cells were highly vulnerable to metformin when glutamine was withdrawn (Kim vd., 2018). The latter study did not evaluate the effect of treatment in SW480 cells. Our data strongly suggest that the stem cell characteristics of SW620 cells grown in tissue culture plates (not enriched for stem cells) were reduced with metformin and CB839 or L-glutamine withdrawal, but not the cells that were enriched for stem cells as indicated by the expression of EpCAM (Figure 8).

Based on the expression of c-Myc, β-catenin translocation to the nucleus and the appearance of spheroids grown in in stem cell enriched medium, our data strongly suggest that metformin alone can reduce stemness characteristics of SW480 and SW620 cells. However, the combination of metformin and CB839, both of which are orally available, safe drugs may lead to the enrichment of stem cell like characteristics in the surviving cell population, particularly in more metastatic cells.

Our experiments indicated the importance of pre-clinical studies in better evaluation of the effects of metformin on cancer cells, particularly in combination with other agents that can modify bioenergetics. However, further studies with FACS sorted stem cells that can evaluated with robust stemness markers are needed to establish firmly the effect of the combination of metformin and CB839 cancer stem cells.