

4-25-2021

The Effect of Antibiotic Selective Pressure on MDR1 and ASCT2 Expression in Human Hepatocellular Carcinoma

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Guizzetti, Isabella G.; Bode, Barrie P.; and Bothwell, Paige Jay, "The Effect of Antibiotic Selective Pressure on MDR1 and ASCT2 Expression in Human Hepatocellular Carcinoma" (2021). *Honors Capstones*. 1140. <https://huskiecommons.lib.niu.edu/studentengagement-honorscapstones/1140>

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NORTHERN ILLINOIS UNIVERSITY

"The Effect of Antibiotic Selective Pressure on MDR1 and ASCT2 Expression in Human Hepatocellular Carcinoma"

A Capstone Submitted to the

University Honors Program

In Partial Fulfillment of the

Requirements of the Baccalaureate Degree

With Honors

Department Of

Biological Sciences

By

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DeKalb, Illinois

April 25, 2021

University Honors Program
Capstone Faculty Approval Page

Capstone Title (print or type)

"The Effect of Antibiotic Selective Pressure on MDR1 and ASCT2 Expression in Human Hepatocellular Carcinoma"

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Department of (print or type)

Biological Sciences

Date of Approval (print or type)

April 25, 2021

Date and Venue of Presentation

CURE April 19-23, 2021

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HONORS CAPSTONE ABSTRACT

Our laboratory has used the CRISPR-Cas9 gene editing system to eliminate the expression of the *SLC1A5* gene, which encodes the ASCT2 amino acid transporter, which is implicated in driving the glutamine-dependent growth of several cancers. At last year's URAD event, the isolation of clones confirmed for knockout (KO) of ASCT2 in two human hepatocellular carcinoma (HCC) cell lines – Huh7 and SK-Hep1 - was described. As these ASCT2 knockout (ASCT2^{KO}) cell lines are currently being deployed in studies assessing the role of this transporter in tumorigenesis, we asked two fundamental questions: 1) Does the removal of antibiotic selective pressure – under which ASCT2^{KO} lines are selected – result in the re-appearance of ASCT2 in populations or selected clones of Huh7 and SK-Hep1? 2) Does the continuous exposure of populations or isolated clones of Huh7 or SK-Hep1 to lethal antibiotics used in selection of ASCT2^{KO} lines lead to increased expression of the Multi-Drug Resistance Transporter MDR1? These two questions are fundamental to the interpretation of tumorigenesis results. ASCT2 and MDR1 expression was assessed by western blot analysis, and growth of the lines were measured by the colorimetric MTT assay. The results indicate that the SK-Hep1 cell lines targeted for ASCT2^{KO} exhibited no transporter expression in the absence or presence of antibiotic selective pressure. Conversely, Huh7 populations targeted for ASCT2^{KO} unexpectedly exhibited enhanced ASCT2 expression under antibiotic selection. Huh7 ASCT2^{KO} clones in contrast showed no re-emergence of ASCT2 expression, suggesting that each was successfully “knocked out” for this transporter. Likewise, SK-Hep1 exhibited no MDR1 expression, but Huh7 significantly expressed this drug resistance transporter; its abundance was increased by Puromycin, the antibiotic used for selection of CRISPR-Cas9-mediated ASCT2^{KO} lines. This drug-induced expression of MDR1 was blunted in Huh7 ASCT2^{KO} lines, suggesting that ASCT2 might play a role in drug resistance in cancerous cells. Finally, studies were done to test the growth rate of Huh7 clonal cell lines compared to the population, as well as study the growth of the Huh7 and Sk-Hep ASCT2^{KO} populations when grown in the presence of two drug therapies-Metformin and Sorafenib. It was found that the clonal cell population initially showed a slower growth rate, however the rate increased after 72 hours to become consistent with the population and control. It was also observed that Metformin and Sorafenib slightly slowed the growth of Huh7 cells, but neither drug had an effect on Sk-Hep cells overall. These results suggest that while ASCT2 elimination does not affect growth, it may render targeted cancer cells more vulnerable to certain chemotherapies. Further studies can be designed to test this hypothesis.

Introduction/ Overview

Human hepatocellular carcinoma (HCC) is the most common type of liver cancer tumor in adults, and is the third leading cause of cancer deaths in the world (NIH, 2019). Current 5-year survival rates are just above 20%, and it is estimated that over 40,000 people will be diagnosed in 2021 (NIH, 2021). Hepatitis C and Hepatitis B virus are a major risk factor for developing HCC, as is heavy alcohol consumption and liver cirrhosis, and patients that develop liver cancer are most commonly men over the age of 50 (Dimitroulis *et al*, 2017). The most preferred treatment for HCC is resection, or liver transplantation, as the removal of tumors leads to a much higher 5-year survival rate over 50% (Dimitroulis *et al*, 2017). However, this treatment option is only possible in approximately 20% of diagnosed patients due to the size and location of tumors and the function of the liver (Dimitroulis *et al*, 2017). Due to these factors, interventional therapies, such as chemotherapy, is often the best remaining choice for treatment.

The most common drug therapy for HCC is Sorafenib, a tyrosine kinase inhibitor, that can be used alone or in combination with other therapies to treat tumor growth (Cabral *et al*, 2020). Sorafenib works by targeting growth factors and RAF kinases in tumor cells to block the signals that the cells need to continue growing and dividing (NIH, 2019). However, the effect of this treatment is often not enough to fully treat the growth of the tumor, as many patients ultimately develop resistance to the drug (Cabral *et al*, 2020). Sorafenib resistance has been found to be common among HCC patients, as the HCC cells are heterogenous in nature, and it is therefore easier for some cells to develop resistance (Cabral *et al*, 2020). These cells are then able to more easily survive and proliferate, rendering the treatment ineffective (Cabral *et al*, 2020).

Our project focuses on two cell lines of HCC that are grown *in vitro* and have been modified using the CRISPER-Cas9 system to knockout the glutamine transporter Alanine-Serine-Cystine transporter 2 (ASCT2), which acts as a glutamine antiporter in conjunction with L-Type amino acid transporter 1 (LAT1) (Bothwell *et al*, 2018). These two transporters were found to be upregulated in human cancer cell lines, and were the focus of previous studies in the lab (Fuchs and Bode, 2005). The permanent dysfunctional mutations of ASCT2 were induced done in both an epithelial cell line (Huh7), as well as a mesenchymal cell line (Sk-hep) and puromycin resistance was also induced in the cell lines to allow for antibiotic selecting during cell culture. (Bothwell *et al*, 2018). The necessity of antibiotic selective pressure was the initial question of this study, as it has implications in tumorigenesis studies that were going to be conducted, where antibiotics would not be present during growth due to the lethality of the levels that would be required.

Previous screening of these cell lines was done using populations of cells that had not been clonally selected, and therefore were a mixture of mutational variants (Bothwell *et al*, 2018). The population cells were also used during the initial study of antibiotic selective pressure, however unexpected results determined that clonal isolated populations of cells would be required for further studies. The Sk-hep cell line is mesenchymal and grows relatively homogenously. Huh7 epithelial cell lines are much more heterogenous, and can exhibit variations among individual cells in a population, which is why clonal isolation became important for studying this cell line.

Also included in both initial and further screening of these cells for ASCT2, the levels of multidrug resistance gene 1 (MDR1) was also observed. MDR1 is a gene often found in drug resistant cancers, and it is suggested that it may play an important role in drug resistance in HCC (Gao *et al*, 2015). MDR1 levels in each cell line has implications for how they will respond

when growing the presence or absence of antibiotics, as well as how the cells may respond to drug treatments like Sorafenib. Both of these factors were studied in cells that had been screened for MDR1 and ASCT2 to assess if there is a correlation between the levels of these proteins, and the ability of the cells to become resistant to drug treatments.

Materials and Methods

Cell Culture

This study used two human hepatocellular carcinoma cell lines, HUH7 and SK-Hep, which represent an epithelial/differentiated phenotype, and a mesenchymal /undifferentiated phenotype, respectively (Bothwell *et al*, 2018). Likewise, HUH7 is a model for a primary HCC cell, whereas SKH-Hep models a motile, metastatic HCC cell. Also used in this study were HUH7 A3 and SK Hep A3, derivatives of HUH7 and SK-Hep that were established in previous work by transfection with a CRISPR-Cas9 plasmid that targeted and eliminated the expression of an amino acid transporter called ASCT2 (Bothwell *et al*, 2018). The plasmid also contained a puromycin resistance gene that was used to select for cells that had a successful knock-out of ASCT2 by growing them in media that contained puromycin.

The cells in this study were thawed from cryo-preserved stocks, and grown in six-well plates containing either normal culture medium (4.5 mg/ml D-glucose, 10% triple 0.1 μ m filtered fetal bovine serum (FBS), 2 mM L-glutamine, and 1% antibiotic/anti-mycotic solution) or that same medium with an addition of puromycin antibiotic (3.0 μ g/ml for HUH7 A3 and 0.5 μ g/ml for SK Hep A3). All cells were grown at 37°C in a humidified atmosphere of 5% CO₂-95% air.

Clonal Isolation and ASCT2 Knockout Verification:

Two human hepatocellular carcinoma cell lines, HUH7 A3 and SK Hep A3, were established in previous work by transfection of HUH7 and SKHep parent lines with a CRISPR-Cas9 plasmid. That plasmid targeted and eliminated the functional expression of the SLC1A5 gene, which encodes an amino acid transporter called ASCT2. The plasmid also contained a puromycin resistance gene that was used to select for cells that had a successful knock-out of ASCT2 by growing them in media that contained puromycin.

Clonal selection was performed by growing ASCT2 knock out cell lines (SK Hep A3 and HUH7 A3 cells) at a very low plating density (2×10^3) in a 150mm plate. After 3-4 days, when the cells formed observable colonies, twelve) unique colonies were isolated with sterile cloning cylinders, a petroleum-based seal, trypsinization, harvested, and each clonal colony transferred to a separate well of a 12-well plate. Each well was subsequently harvested, passed to 100 mm culture plates, and harvested for protein. The clonal populations were then screened using the Western blot and Protein Harvest protocol to determine the level of reemerging ASCT2.

Protein Harvest and Western Blot:

For initial studies of the necessity of antibiotic selective pressure, total populations of HUH7 A3 cells and SK-Hep A3 cells were passed for one month before protein samples were harvested once a week for three consecutive weeks. Cells for clonal isolation screening were prepared as stated above. For the second study of the necessity of antibiotic selective pressure to sustain ASCT2 transporter knockout, HUH7 A3 population cells, two of the six confirmed ASCT2^{KO} clones - HUH7 A3 clone B cells, and HUH7 A3 clone E cells were passed for three weeks following the cell culture protocol before protein samples were collected. Total cellular

protein samples were prepared using Laemmli lysis buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.8], and 1% protease inhibitor cocktail). The protein samples were homogenized by sonication and quantified by measuring absorbance at 280 nm using nanodrop spectrophotometry.

Western blots were performed by size separation of all samples by vertical SDS-polyacrylamide gel electrophoresis, transfer from gel to PVDF membranes by liquid tank transfer, and detection of target protein expression by overnight incubation with solutions of anti-ASCT2 or anti-MDR-1 primary antibodies. While equal amounts of cellular protein in sample buffer were added to each lane, as a control for loading, all blots were additionally incubated with anti- β -actin antibody or H3 HRP antibody (loading control antibodies – against abundant proteins whose expression remains relatively constant under a variety of conditions), depending on the molecular weight of the target protein. Targets were visualized by 1 hour incubations with anti-rabbit, HRP-linked antibodies, followed by brief 1 min incubations in a LumiGLO chemiluminescent substrate solution and imaging performed with a G:Box chemiluminescence imager and GeneSnap image capture.

MTT Growth Assay

For methylthiazol tetrazolium (MTT) analysis, four Huh7 cell lines that had been continually grown in 3.0 μ g/ml puromycin media (NS, Population, Clone B, Clone E) were plated at an initial density of 4×10^3 cells/well in 96-well plates. After 48 hours, the culture medium was removed, all cells were washed twice with 1X PBS, and normal DMEM with 3.0 μ g/ml puromycin was added. MTT analysis was performed on one 96-well plate at the following time points: 48 hr (T1), 72 hr (T2), and 96 hr (T3). Cells were washed once with 1X PBS and then incubated for three hours in a solution of MTT (0.5 mg/mL) in phenol red-free DMEM, at

37°C. After incubation, the formazan crystals in each well were solubilized with an acidic solution of 0.04 M HCl in absolute isopropanol, and 570 and 650 nm absorbance measurements were taken using an Epoch microplate spectrophotometer. Background absorbance was subtracted from each measurement (570 – 650 nm), and the final values were calculated as the average \pm SD of at least four separate determinations.

Results

Effect of Antibiotic Selective Pressure

Initial studies of the effect of selective pressure with CRISPR-Cas9 treated HUH7 and SK-Hep cell lines showed varying results between the two cell line types (Figure 1). When analysed with a Western Blot there were no traces of ASCT2 re-emergence in SK-Hep population cells lines no matter if they were grown in the presences or absence of puromycin. However, in Huh7 populations, there was a slight trace of ASCT2 found in the “knockout” population grown without puromycin, and a stronger re-emergence of ASCT2 was paradoxically seen in population grown with puromycin. To gain further insight on these result, additional Western Blot screening was done to study the levels of MDR1 in all of the tested cell populations and similar results were found, with little MDR1 expression in SK-Hep cells, and greater expression in Huh7 cells. Additionally, Huh7 cells growth in the presence of Puromycin showed a greater level of the protein, which could be an adaptive response to the antibiotic (drug) in the media.

First Trial (Population Only)

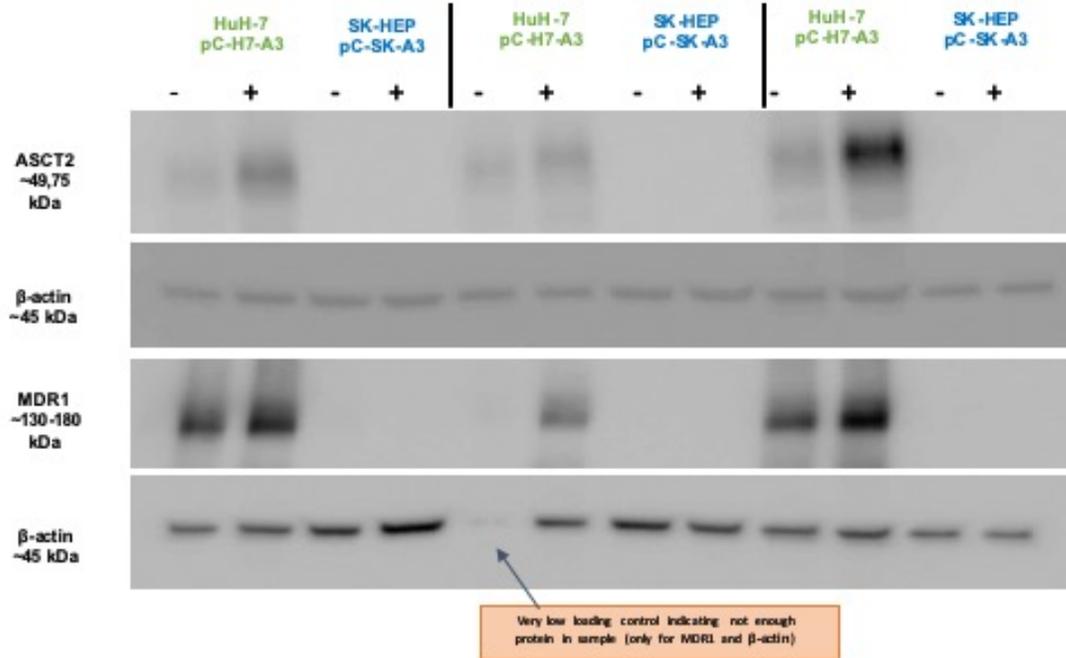


Figure 1. ASCT2 and MDR1 in Huh7 and Sk-hep ASCT2^{KO} population cells lines measured using Western blot analysis as described in Materials and Methods. The chemiluminescent bands for both targets (ASCT2 and MDR1) are shown in comparison to β-actin as a loading control.

Clonal Isolation and ASCT2 Knockout Verification

To eliminate heterogenous cell population effects, work was done to isolate clonal cell populations (originating from a single cell) of SK-Hep and Huh7 so that any cells with traces of ASCT2 could be avoided in further studies (Figure 2). All twelve of the tested SK-Hep clonal cell isolates showed no re-emergence of ASCT2, which was consistent with the initial experiment result, and it was determined that further study of the cell line was no longer

necessary. Two of the eight clonal populations (A-H) of Huh7 (Clones D and F) showed traces of ASCT2. The other six did not have any visible trace of ASCT2, and clones B and E were chosen to continue the studies.

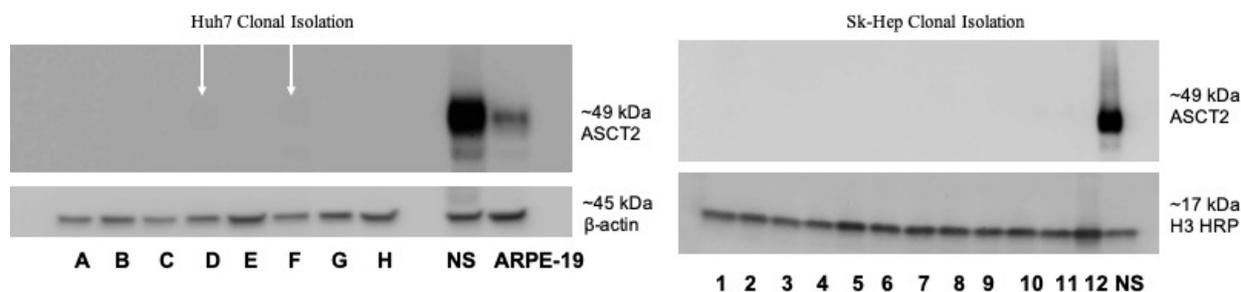


Figure 2. ASCT2 in Huh7 and Sk-hep in clonally isolated population cell lines measured using Western blot analysis as described in Materials and Methods. The chemiluminescent bands for both targets (ASCT2 and MDR1) are shown in comparison to β -actin as a loading control.

Effect of Antibiotic Selective Pressure (continued)

The initial experiment studying the effects of antibiotic selective pressure were repeated, this time with only four variants of the Huh7 cell line: a nonsense control (retains antibiotic resistance but not ASCT2 targeting for CRISPR-Cas9), the original ASCT2^{KO} population of cells, clonal population B ASCT2^{KO}, and clonal population E ASCT2^{KO}. Once again, the population of cells exhibited re-emergence of ASCT2, with a higher level being seen in the cells grown in the presence of puromycin. Neither of the isolated ASCT2^{KO} clonal cell lines showed any trace of ASCT2 when grown in either condition, confirming that the ASCT2^{KO} population of cells used in the first round of studies had some cells with ASCT2 re-emergence, and some (e.g.,

clones B and E) without (Figure 3). This also suggests that antibiotic selective pressure may not be necessary when growing ASCT2^{KO} cell in vitro, which has implications for growing cells *in vivo* for tumorigenesis studies. It can also be seen that MDR1 is again upregulated in the control and the population of cells grown in the presence of puromycin, which could be in response to the addition of the antibiotic drug in the media. Neither clone showed an upregulation of MDR1 in the presence or absence of puromycin

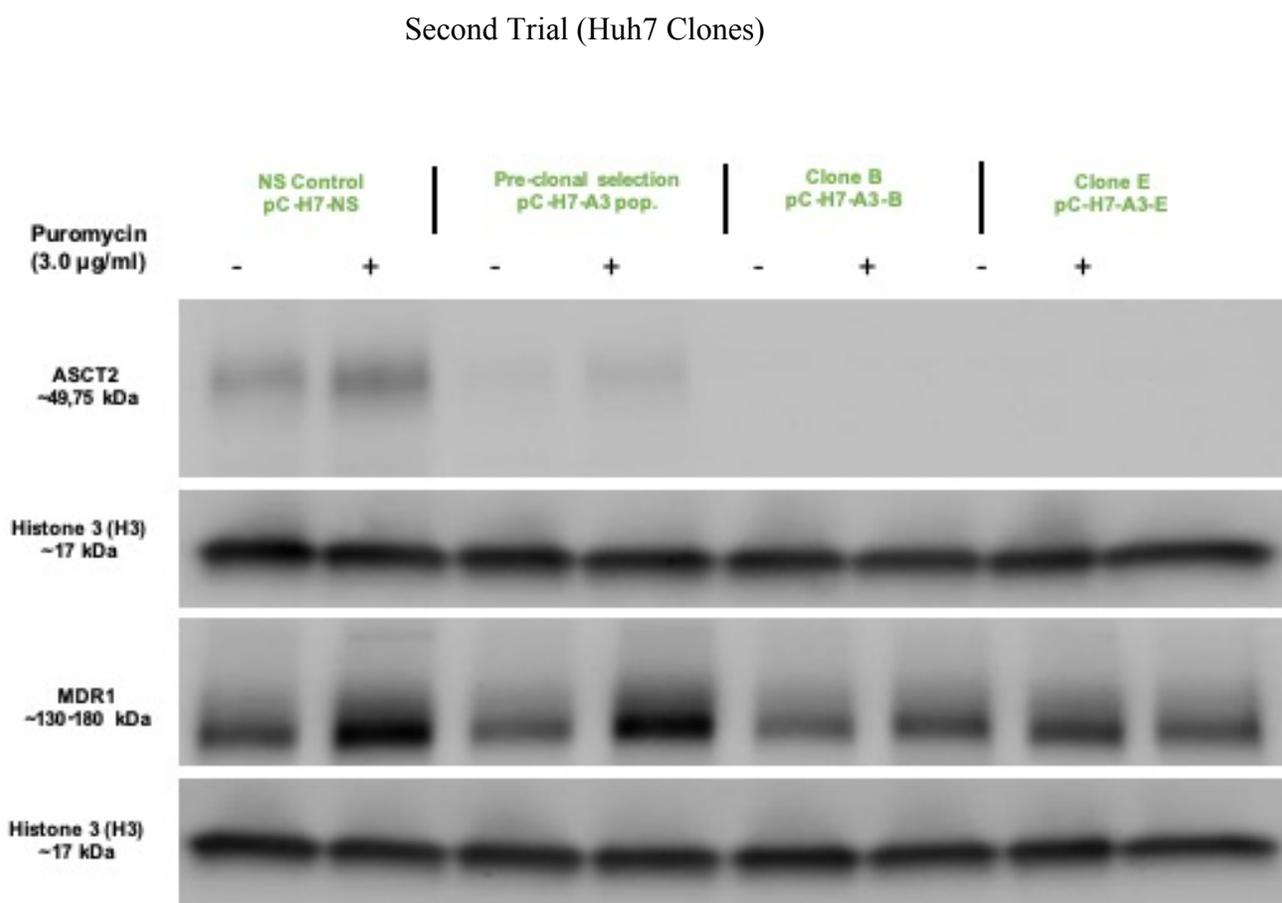


Figure 3. ASCT2 and MDR1 in Huh7 ASCT2^{KO} population and clonal cells lines measured using Western blot analysis as described in Materials and Methods. The chemiluminescent bands for both targets (ASCT2 and MDR1) are shown in comparison to Histone 3 as a loading control.

HUH7 A3 Growth MTT Assay

The MTT Assay was done to study of the rate of cell growth of the clonal populations compared to NS and the population of cells. It was found that the clone B isolates grew at a slower rate than the nonsense control, population or Clone E up until 72 hours. Following this, the growth rate of these clones increased to match the other three cell populations by 96 hours (Figure 4). This was consistent with what was observed during cell culture, where clonal populations would tend to initially grow at a slower rate than the other cells populations, causing increased time between confluence.

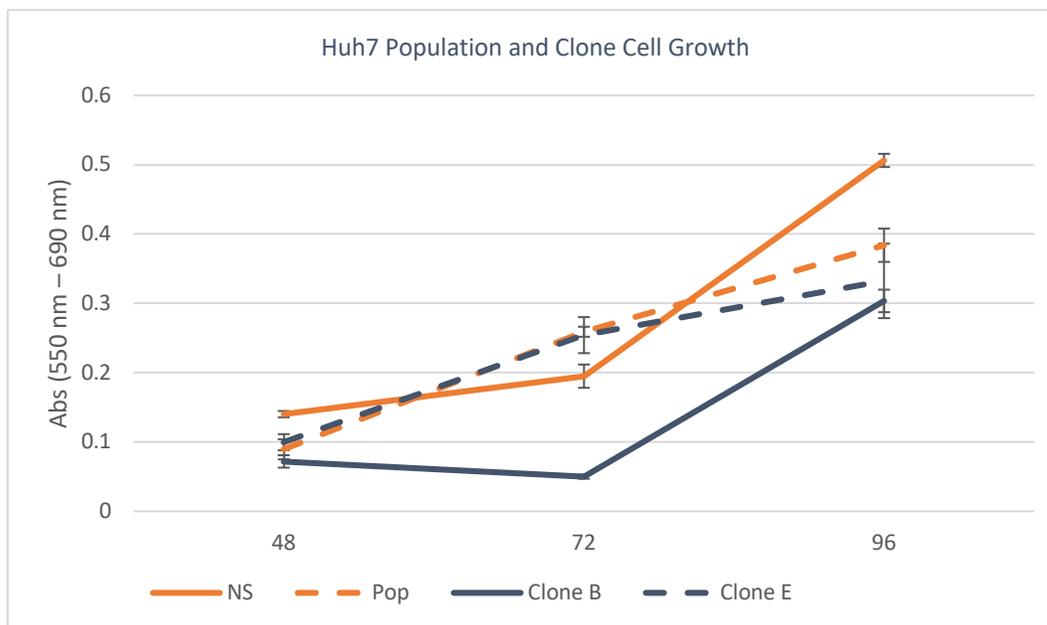


Figure 4. MTT analysis of cell growth of Huh7 ASCT2^{KO} population and clonal cell lines following three weeks of growth in puromycin media analyzed using MTT assay as explained in materials and methods

Additional MTT Growth Assays of Huh7 and SK-Hep in Other Drug Treatments

Metformin is a common medication for the treatment of type II diabetes, but has also been found to slow down tumour growth. Sorafenib is the current most common treatment for liver cancer clinically. Therefore, these two treatments were tested in conjunction with ASCT2^{KO} in cells to determine if the combination had an effect on slowing cell growth rate. During this additional MTT assay, it was observed that Metformin had a small effect of the growth rate on all three cells lines of Huh7, while the treatment did not have a significant effect on any of the SK Hep lines. The SK Hep knockout clones also exhibited the largest rate of growth of any of the cell lines. It was also observed that Sorafenib had an effect on the Huh 7 cell lines, also slowing the growth rate of the treated cells in comparison to the cells grown in the DMSO control (Figure 5). The data suggests that Sorafenib may have temporarily slowed the growth of the SK-Hep cell lines, however, they all showed increased growth from 48 to 72 hours. Overall, it can be concluded that the Huh 7 cell lines tested were more susceptible to both treatments, and the knockout clones showed the slowest growth rate of any cell lines. It can also be concluded that the SK Hep cell lines grew at a faster rate, and the Knockout clones showed the highest growth rate of any cell line tested.

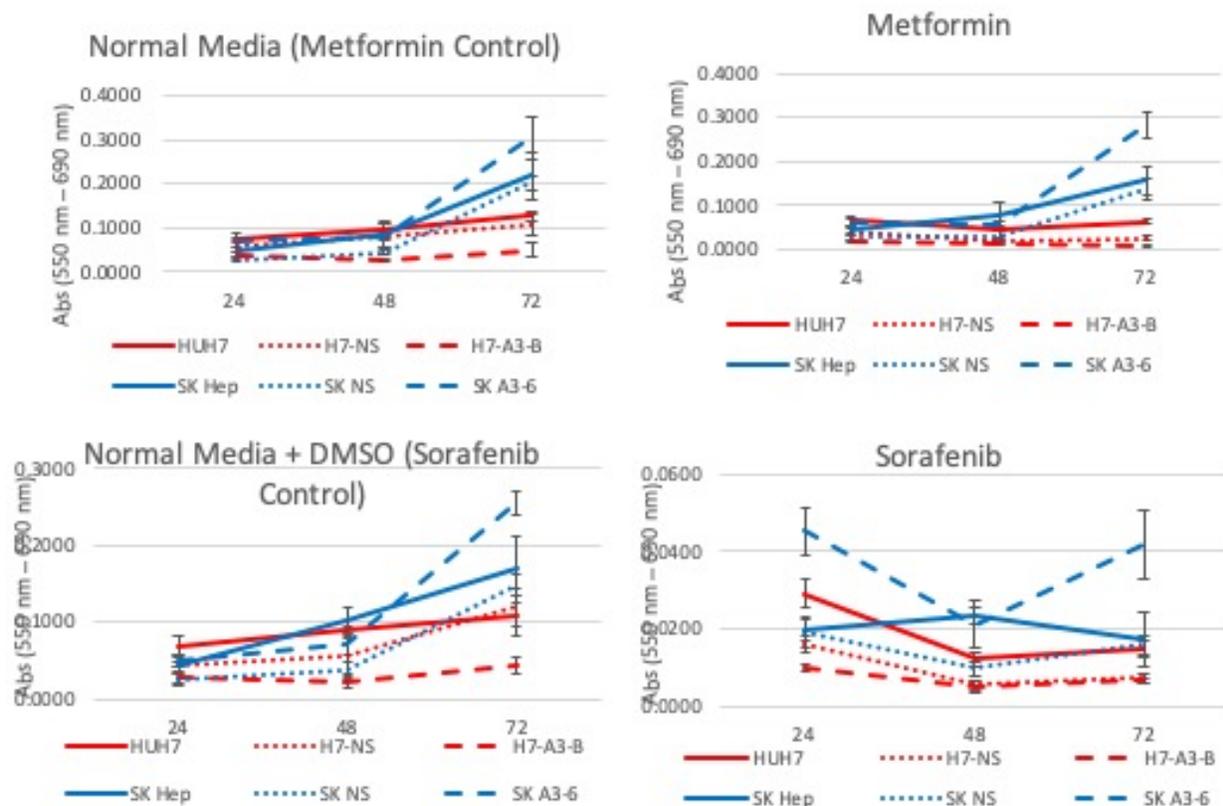


Figure 5. MTT growth assay of Huh7 and Sk-Hep ASCT2^{KO} population and clonal cell lines in media containing no additions, metformin, DMSO, or sorafenib and analyzed using MTT assay as explained in materials and methods

Discussion

While some results were consistent with what was expected, some were also unexpected, and lead to further investigations within the project. During initial experiments of ASCT2 Knockout Huh7 and Sk-Hep population cell lines, it was found that ASCT2 was reemerging in Huh7 cells that were previously thought to be complete ASCT2^{KO} grown both with and without antibiotic selective pressure but not in Sk-Hep ASCT2^{KO} cells grown in either condition (Figure

1). What was most surprising about these results was the observation that ASCT2 seemed to be expressed even more in the cells grown in the presence of puromycin than those grown without it, which is why the samples were also screened for MDR1 expression to see if drug transportation within the cells was also playing a role (Figure 1). Huh7 was then seen to also have a higher expression of MDR1 when grown in the presence of puromycin, which corresponds to the histology of these cells, as they very similar to hepatocytes that have high levels of drug transporters. This also suggested the MDR1 may be expresses in response to the presence of an antibiotic in the media, and its upregulation may suggest a correlation with ASCT2 expression. These questions require further study.

Additional work was then done to isolate clonal populations of both Huh7 ASCT2^{KO} cells and Sk-Hep ASCT2^{KO} cells that could then be screened to see if the reemergence of ASCT2 was in all cells of just single cells spread throughout the population (Figure 2). All screened SK-Hep clonal populations were complete knockouts for ASCT2, as seen in the original study. This was expected, as Sk-Hep cells are relatively homogenous, and individual cells are less likely to show unique expression. In the Huh7 clonal populations, it was seen that two of the eight clonal lines had slight expression of ASCT2. This also made sense as Huh7 cells tend to be heterogenous in population. This is important for future studies, as the definitive ASCT2^{KO} clonal cell lines could be used to ensure that some cells in the population that may have slight reemergence of ASCT2 would not cause variance in results, as seen in initial experiments with only populations of each cell line growth with and without antibiotics. This also has implications in tumorigenesis studies done with populations of cells, rather than the clonally isolated lines, as the populations may not be complete knockouts.

As seen in the second trial of Huh7 ASCT2^{KO} cells including both the population of cells, as well as the clonally isolated populations, when ASCT2 was present, there was also an increase in the levels of MDR1 expression (Figure 3). Both the control line, and the Huh7 ASCT2^{KO} population cells had ASCT2 expression after three weeks of growth both with and without puromycin. In both of these types of cells, ASCT2 was expressed at a higher level when grown in puromycin media, as was MDR1. In both clonal Huh7 cell lines, there was not a reemergence of ASCT2, and not upregulation of MDR1 in the cells grown with puromycin. As previously stated, these results suggest a connection between ASCT2 and MDR1, as the knockout of ASCT2 lead to lower expression of MDR1. This data suggests that ASCT2 might be required in part for MDR1 to become active in response to drugs in the environment, which has broader implications on drug-resistant cancers where ASCT2 is also upregulated.

Conclusions

All studies with Sk-Hep population and clonal isolate ASCT2^{KO} cells line remained consistent with no reemergence of ASCT2 in any condition or among any of the clonal cell lines and no MDR1 expression in the population. The results reflect the nature of these cells as homogenous and mesenchymal, and it can be concluded that if grown *in vivo* for tumorigenesis studies, the absence of antibiotic selective pressure should not affect the outcome. The Huh7 cells showed an increase in ASCT2 and MDR1 in population, but when cells were clonally isolated, it could be confirmed that ASCT2 was completely knockout out, and it did not reemerge in the presence or absence of antibiotics. The clonal cell lines also did not show an upregulation of MDR1 when in the presence of puromycin, which suggests a connection between

ASCT2 and drug transporters in the HCC cell. Further studies would be required for this hypothesis however. When the growth rate of cell types was studied, it was found that SK-hep population and clone cells grew at a greater pace than Huh7 population and clones, and were less susceptible to additional drug treatments. In the first trial Huh7 cell growth was affected by both metformin and sorafenib treatment, however, the assay would need to be repeated to make a definitive conclusion on how greatly the growth rate was affected, and how it may be correlated with the expression of ASCT2 and MDR1 in the cells. Finally, when the growth of different Huh7 cell lines were assessed, there was an initial difference in the growth rate of the confirmed ASCT2^{KO} clonal populations compared to the total population, but this difference dissipated as cells reached higher culture densities. Thus, the observations made during cell culture that the ASCT2^{KO} clones grow more slowly than the ASCT2^{KO} population is confirmed, but only during the initial phases of culture growth, which are subsequently overcome.

In conclusion, the question of whether continuous antibiotic pressure is necessary to sustain CRISPR-Cas9-mediated ASCT2 knockout has a complex answer: No, if the cells have confirmed total initial ASCT2 knockout as a population (SK-Hep) or in clones isolated from complex, incomplete knockout populations. Investigation of the latter population (HUH7) yielded the paradoxical answer that antibiotic selective pressure actually enhanced (selected for) the re-expression of the intended target in heterogenous cell populations. The initial question was posed because the role of these transporters in tumorigenesis is being actively investigated, and once explanted into host animals, antibiotic selective pressure is lost. These studies confirm what was suspected: that permanent gene edits (knockouts) made in homogenous cell populations no longer require the continuous presence of the selective antibiotic initially used to isolate the ASCT2^{KO} cell populations.

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