STARVING CANCER: A STUDY OF GLUTAMINE DEPENDENCY IN CANCER CELLS

G. E. Bingham
Breck School, Minneapolis, MN

This study examined glutamine dependency in cancer cells, where glutamine serves as the primary mitochondrial energy source. Effects of the protein cyclin D1 on glutamine metabolism were examined because cyclin D1 is a major driver of cancer-cell proliferation. Specific goals were 1) to find human cancer-cell lines that are glutamine dependent, 2) to knock down glutamine and overexpress cyclin D1 in each glutamine-dependent cell line, and 3) to determine effects of overexpression of cyclin D1 on viability of glutamine-dependent cells in absence of glutamine. Three cancer-cell lines were tested for glutamine dependency: SkHep liver-cancer cells, LnCap prostate-cancer cells, and HUH7 liver-cancer cells. Cell culturing, viability tests, protein assays, and Western blots were used to identify which of these cell lines are glutamine dependent and to determine effects of cyclin D1 overexpression on this dependency. Viability tests showed that both healthy AML12 mouse cells (control) and SkHep cancer cells are glutamine dependent, whereas LnCap and HUH7 are not. Results suggest that viability of glutamine-starved SkHep cancer cells significantly decreases ($p = 0.0019$) when cyclin D1 is overexpressed and significantly increases when cyclin D1 is knocked down ($p = 0.05$). On the other hand, AML12 cell viability significantly increases when cyclin D1 is overexpressed ($p = 0.0018$) and significantly decreases ($p = 0.0407$) when cyclin D1 is knocked down. These results show an interesting difference between glutamine-dependent cancer cells and healthy cells that may be exploited to target glutamine-dependent cancer cells.

Keywords: glutamine-dependent cancer cells, cyclin D1, abnormal cancer-cell metabolism

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Abbreviations: TCA: Tricarboxylic acid; ATP: Adenosine triphosphate; FBS: Fetal Bovine Serum; TTBS: Tris Buffered Saline 0.05% Tween 20; RT-PCR: Reverse transcriptase-Polymerase Chain Reaction

INTRODUCTION

Cancer is one of the leading causes of death in the world, resulting in 7.6 million deaths each year[1]. Cancer treatments, such as chemotherapy and radiation, lessen mortality rates, but these treatments are brutally damaging to the body because they not only kill cancer cells but also harm healthy cells. A great deal of research has been done to find less invasive cancer treatments, which has included extensive work on the role of glucose in cancer-cell metabolism, but less research has been done on the role of glutamine in abnormal cancer-cell metabolism. The purpose of this study was to investigate the role of glutamine in abnormal cancer-cell metabolism to develop less invasive cancer treatments.

Glutamine is the most abundant amino acid in the human body and serves as both a building block of proteins and as an important source of nitrogen for both healthy and cancerous cells since it contains both an amine and an amide side-chain that are used for nucleotide and amino acid biosynthesis[2]. Glutamine was important to this work because it is essential for cancer-cell proliferation, growth, and survival.

Healthy cells depend on glycolysis of glucose to pyruvate that produces acetyl-coA, which enters the TCA cycle to generate ATP though a process of oxidative phosphorylation in the mitochondria. Because oxygen is required to metabolize glucose, this process only occurs in healthy cells when conditions are aerobic. When conditions are anaerobic in healthy cells, glycolysis of pyruvate leads to fermentation where lactate is produced[3]. However, in cancerous cells, glycolysis of pyruvate leads to fermentation where lactate is produced even when oxygen is present—a process that is prevented in normal cells in an aerobic environment[3,4]. This abnormal metabolic process is known as aerobic glycolysis, also know as the Warburg effect.

A group of cancer-cell lines that are glutamine dependent, which are cells that cannot proliferate or maintain their viability without glutamine, were used. Metabolism of glutamine-dependent cancer cells is different than in metabolism in cancerous cells that are not glutamine dependent[8]. While glutamine-dependent cancerous cells undergo the same inefficient abnormal aerobic glycolysis (Warburg effect) that non-glutamine-dependent cancer cells exhibit, in glutamine-dependent cancer cells, glutamine serves as the primary mitochondrial energy source[2]. In these cells, glutamine is broken down by glutaminase, an enzyme that requires high phosphate concentrations to be fully active. The product catalyzed by glutaminase is glutamate that is a precursor in the production of α-ketoglutarate, which is an intermediate in the TCA cycle. High concentrations of phosphate found in the mitochondria of glutamine-dependent cancerous cells may explain the connection between glutaminase activity and the extent of proliferation of glutamine-dependent cancerous cells[2]. Because of this, glutamine may be a key to new treatments for cancers where the cancer cells are glutamine dependent[2,5].

The effects of the protein cyclin D1 on glutamine metabolism were examined because cyclin D1 is a primary driver of cancer-cell proliferation, which is also when Warburg effect tends to be high[6]. Cyclin D1 was overexpressed in glutamine-dependent cancer cells based on studies by Mullany *et al.*[6] and Bode *et al.*[7] that suggested glutamine-responsive cells are susceptible to overexpression of cyclin D1. This susceptibility of glutamine-responsive cells to overexpression of cyclin D1 occurs because overexpression of cyclin D1 inhibits glycolysis in the mitochondria where glutamine serves as the primary source of energy in glutamine-dependent cells[8,9]. Based on the study by Mullany, it was hypothesized that when glutamine-dependent cancer cells are deprived of glutamine, overexpression of cyclin D1 would result in decreased viability of glutamine-dependent cancer cells[6].

Specific goals were: 1) to find human cancer-cell lines that are glutamine dependent, 2) to knock down glutamine and overexpress cyclin D1 in each glutamine-dependent cell line, and 3) to determine effects of overexpression of cyclin D1 on viability of glutamine-dependent cells in absence of glutamine. Three cancer-cell lines were selected to test for glutamine dependency: SkHep liver-cancer cells, LnCap prostate-cancer cells, and HUH7 liver-cancer cells. SkHep cells were used because the study by Bode suggested that SkHep cells might be glutamine dependent[7]. LnCap prostate-cancer cells were studied because overexpression of cyclin D1 is known to play a key role in tumorgenesis and metastases of prostate-cancer cells[9]. A second liver-cancer line, HUH7, was used because Bode suggested that the SkHep liver-cancer cells might be glutamine dependent[7]. Based on studies by Albrecht (unpublished, used with permission) that showed the healthy mouse cell line AML12 is glutamine dependent, AML12 were used as a control.

MATERIALS

SKhep liver-cancer cells, LnCap prostate-cancer cells, HUH7 liver-cancer cells, and healthy AML12 mouse cells were purchased from American Type Culture Collection (Manassas, Virginia). Primers were purchased from Integrated DNA Technologies. Cyclin D1 antibodies were purchased from Sigma, (St. Louis, Missouri), Santa Cruz Biotechnology, (Dallas, Texas), and Millipore, (Billerica, Massachusetts), and anti-rabbit and anti-mouse secondary antibodies were purchased from Chemicon, (Billerica, Massachusetts).

METHODS

Cell Culture

Cell lines SkHep, LnCap, HUH7, and AML12 were incubated in fetal bovine serum-enriched (FBS) media [500 mL of 1X Dulbecco's Modified Eagle Medium (DMEM), 45.0 mL of fetal bovine serum, 5.0 mL of Pen-Strep (10,000 units/mL penicillin and 10,000 µg/mL streptomycin), and 20 µL of 500 ng/mL dexamethasone] at 37.0 ˚C in a 5% CO_2 environment. Cells were plated, allowed to sit for three hours, and then media was removed and fresh FBS media was added. Next, amounts of 2.0 nM short-interfering RNA (siRNA) were added to cells (Table 1).

Table 1. Calculated amounts of siRNA for siRNA transfection

Number of wells	Volume of complexed siRNA	Volume of DMEM media
6 Well	400 µL	1600 µL
12 Well	200 µL	800 µL
24 Well	100 µL	400 µL
96 Well	20 µL	80 µL

At 24 hours, controls, siD1, and ADV-D1 media (Table 2) were added to cells in a cell plate in triplicate. DMEM media was removed 48 hours after siRNA was added, and cells were washed in phosphate-buffered saline (PBS) [80 g of NaCl, 2 g of KCl, 11.5 g of $Na_2HPO_4(H_2O)_7$, 2 g of KH_2PO_4].

Glutamine Dependency of Cell lines

Viability Test: At 24 hours after SiRNA was added, 20 µL of Promega CellTiter-Blue were added to each well of a 96-well plate. The plate was incubated for four hours and read using a Bio-tek PowerWave at 570 nm and cell viability rates were recorded. Results were graphed using Microsoft Excel. Because there was no variability in cell death between the positive and negative controls at 24 hours, the viability procedure, was repeated and culturing time was extended to 48 hours.

Cyclin D1 and Overexpression Verification

Harvesting Protein: Media was removed from serum-starved and glutamine-starved cells, and cells were washed in 200 µL of cold PBS. Cells were lysed with tween-lysis buffer. Bottoms of well plates were scraped, and cell suspensions were pipetted into fresh sample tubes. To lyse cells, samples were sonicated for 10-15 seconds and then centrifuged at 14,000 rpm for ten minutes at 4 ˚C. Protein supernatants were transferred to fresh 2-mL screw-cap tubes. Western blot aliquots were prepared by adding 50 µL of each protein sample to 50 µL of 2x sodium dodecyl sulfate in fresh 2-mL screw-cap tubes. Protein samples were vortexed, boiled for five minutes, and stored at -20 ˚C.

Protein assay: Harvested protein samples were diluted with dH_2O at a 1:4 (v:v), vortexed and then were added to a 96-well plate in triplicate. Bovine serum albumin (BSA) stock solution was made to concentrations of 0, 0.25, 0.50, 0.75, and 1.0 mg/mL, and 25 µL of BSA stock solution were added to 2.5 µL of lysis buffer, 25 µL of Bio-Rad Reagent A, and 200 µL of Bio-Rad Reagent B. The mixture was allowed to stand for ten minutes, and concentrations of protein samples were determined using a Bio-tek PowerWave XS spectrophotometer at 760 nm. A line of best fit was run in order to see variability in results.

Western Blotting: Samples were aliquoted with 2X sample buffer [4 mL of H_2O, 2 mL of 0.125 M tris, 6 mL of 10% SDS, 1.5 mL of glycerol, 30 mg of bromophenol blue, and 0.6 mL of β-mercaptoethanol]. Next, 12% acrylamide gel [7.5 mL of 1.5 *M* tris/HCl, 2.5 mL of 0.5 *M* tris/HCl, 12 mL of acrylamide, 20 µL of tetramethyl-ethylenediamine, 400 µL of ammonium persulfate, and 10.4 mL of ddH_2O] was added to gel plates and left to solidify. Gels were run at 120 V in 1X SDS running buffer and then transferred to a polyvinylidene fluoride membrane overnight at 30 V at 4.0 ˚C. Membranes were blocked in 2.5% non-fat dry milk for one hour and then washed in 2.0 mL of tris-buffered saline and Tween 20 three times each for ten minutes.

Sigma and Santa Cruz primary cyclin D1 antibodies were chosen because a study by Mullany *et al.*[6] showed they effectively bond to cyclin D1. The primary cyclin D1 antibody was added at a rate of 1:500 (v:v) with BSA. Each membrane was washed in wash buffer [500.0 mL of TTBS, 1 gram of I-block,

Table 2. Media preps for cell lines.

Abbreviation	Description	Media
-Serum	Negative control 1	DMEM media without fetal bovine serum
+Serum	Positive control 1	Fetal bovine serum-enriched DMEM media
+siD1	Blocked cyclin D1	Fetal bovine serum-enriched DMEM media with siRNA
+siC	Positive control 2	Fetal bovine serum-enriched DMEM media with siRNA
ADV-D1	Overexpressed cyclin D1	DMEM media without fetal bovine serum transfected with cyclin D1
ADV-GFP	Negative control 2	DMEM media without fetal bovine serum transfected with GFP

250.0 mL of tween] three times for ten minutes. The secondary antibody (anti-rabbit or anti-mouse) was washed in 1:5000 (v:v) wash buffer for one hour. Membranes were washed three times for ten minutes in wash buffer, twice in 1X assay buffer [100.0 mL of 1M tris and 5.0 mL of 1.0 M $MgCl_2$, and ddH_20 to 500.0 mL] for two minutes each, and then in 2 mL of Tropix CDP-Star (St. Louis, Missouri) for five minutes. Membranes were developed with a Kodak ImageStation 2000R, using two exposures, and analyzed with Kodak 1D software.

Because neither the Santa Cruz nor the Sigma cyclin D1 antibody worked, the procedure was run with a third primary antibody cyclin D1 rabbit from Millipore, which the study by Mullany[6] had shown to be an effective cyclin D1 antibody.

RNA Isolation: RNA was isolated using the following Ambion (Grand Island, New York) manufacturer instructions. To begin, 1.0 mL of trizol reagent was added to each well of a six-well plate to homogenize cells. Then, the cell suspension was transferred to micro-centrifuge tubes and incubated at room temperature for ten minutes. Next, samples were centrifuged at 7,800 rpm for ten minutes at 4.0 ˚C. RNA pellets were washed with 1 mL of 75% ethanol, votexed, and centrifuged again at 7,500 rpm for ten minutes at 4.0 ˚C. Finally, the RNA pellet was resuspended in RNAse-free water and stored at -70.0 ˚C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): RNA samples were quantified using a NanoDrop spectrophotometer to determine concentration of samples. RNA samples were then diluted to given concentrations, and 5 µg of diluted RNA samples were added to 5 µL of 10X DNAse buffer and 2 µL of DNAse. After a 30-minute incubation at 37.0 ˚C, 5 µL of DNAse inactivation reagent were added, samples were rocked for five minutes, and then samples were centrifuged for one minute. Next, 5 µL of PCR mix [11 µL 10X reverse-transcriptase buffer, 20 µL $MgCl_2$, 20 µL dNTP, 5 µL random hexamers, 2 µL RNAsin, 35 µL RNA, and 2 µL reverse-transcriptase enzyme with 15 µL ddH_2O] were added to each sample. RT-PCR was run for 15 min at 25 ˚C for denaturing, 30 min at 42 ˚C for annealing, and five min at 95 ˚C for elongation. Then, 5 µL of cDNA were added to each well of a 96-well plate along with 5 µL of master mix [SYBR green enzyme, primers—GLS1, GLUT2, or GLUL—and ddH_2O]. The well plate was spun at 500 rpm and then loaded into a Roche LightCycler 480 II, which quantified numbers of genes present in each well. Results were analyzed using Microsoft Excel.

Analysis: Cell viability of cyclin D1 (+siD1) and the positive control (+siC) were compared using two-sample Student's *t*-tests, with significance set at $p \leq 0.05$. Results for overexpression of cyclin D1 (ADV-D1) and overexpression of the negative control (ADV-GFP) were also compared using a two-sample Student's *t*-test, with significance set at $p \leq 0.05$. Standard deviations from the mean were calculated using Microsoft Excel.

RESULTS

Figure 1 shows that cancerous SkHep cell line is glutamine dependent, seen by the significant increase

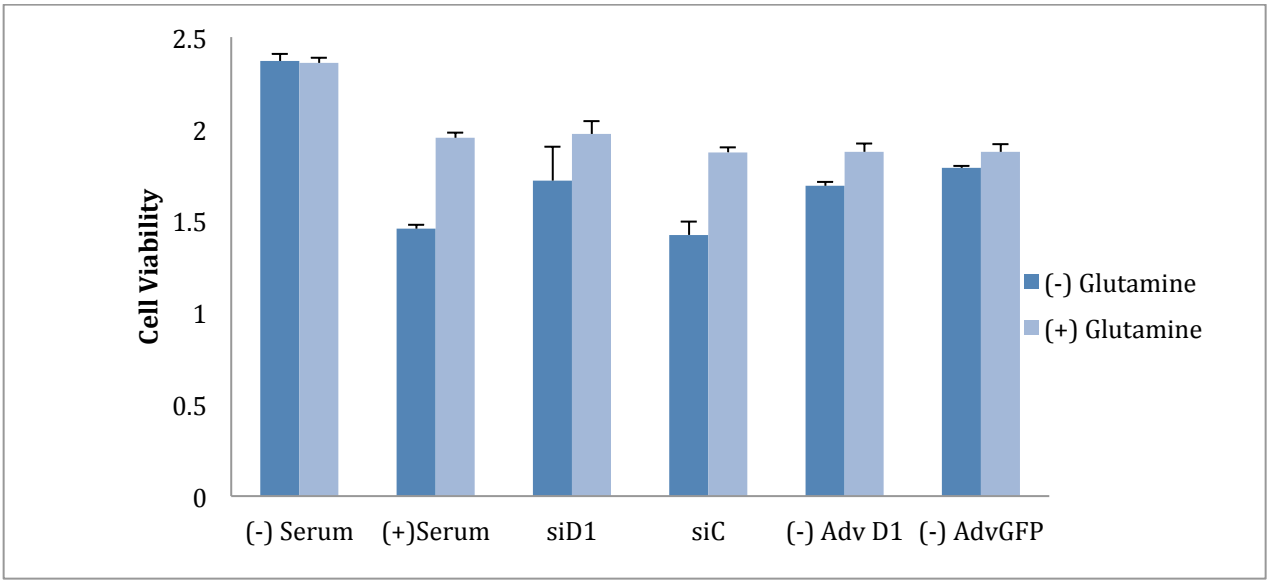

Figure 1. SkHep viability at 48 hours after siRNA transfection. Standard deviation bars show confidence levels. Two-sample *t*-tests were run to determine statistical significance, with significance set at $p < 0.05$.

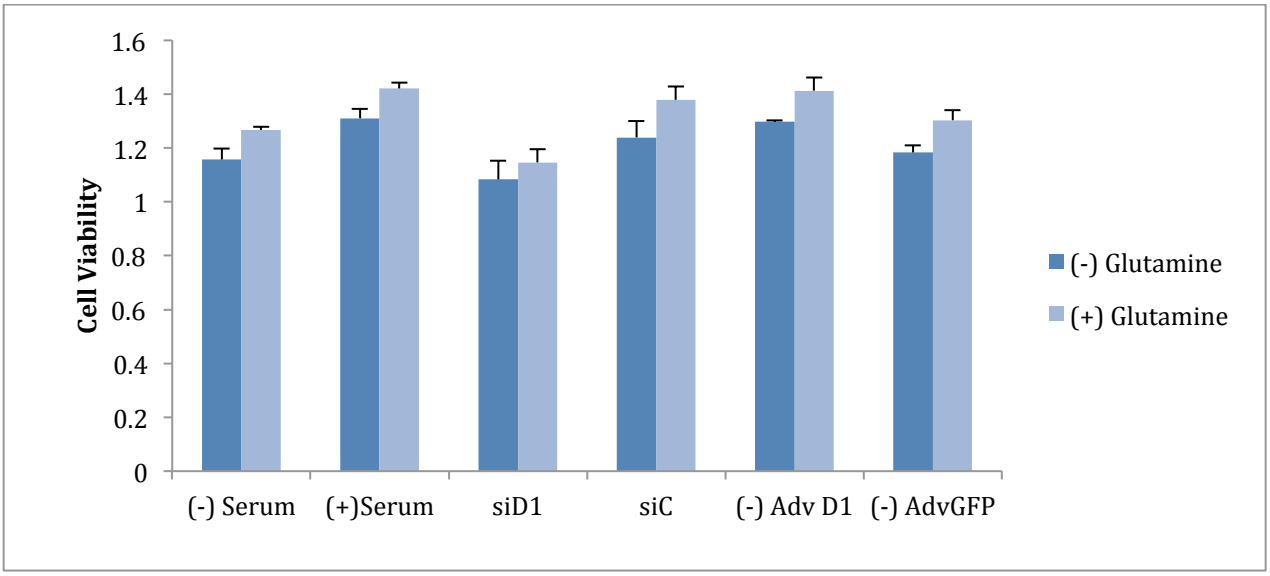

Figure 2. AML12 viability at 48 hours after siRNA transfection. Standard deviation bars show confidence levels. Two-sample t-tests were run to determine statistical significance, with significance set at $p < 0.05$.

in cell viability between cells starved of glutamine (-Glutamine) and cells given glutamine (+Glutamine) ($p = 0.00002$). Figure 2 also shows that when the liver-cancer cell line SkHep was starved of glutamine, overexpression of cyclin D1 (ADV-D1) significantly decreased cell viability compared to the negative control (ADV-GFP) ($p = 0.0019$). When these cells were starved of glutamine and cyclin D1 was knocked down (siD1), cell viability significantly increased compared to the positive control (siC) ($p = 0.05$).

Figure 2 shows that healthy AML12 control line is glutamine dependent, seen by the significant increase

in cell viability of cells given glutamine (+Glutamine) compared to cells that were starved of glutamine (-Glutamine) ($p = 0.01$). Figure 2 also shows that when AML12 was starved of glutamine, overexpression of cyclin D1 (ADV-D1) significantly increased cell viability compared to the negative control (ADV-GFP) ($p = 0.0018$). When AML12 cells were starved of glutamine and cyclin D1 was knocked down (siD1), cell viability significantly decreased compared to the positive control (siC) ($p = 0.04$).

Figures 3 and 4 show results of Western blots done to confirm expression of cyclin D1. Results confirm expression in the negative control (-Serum) and in both positive controls (+Serum and +SiC) in the cancerous SkHep cell-line (Fig. 3) and in the healthy AML12 cell-line (Fig 4). Lack of a band at +siD1 shows cyclin D1 was knocked down. The darker band at ADV-D1 shows overexpression of cyclin D1, verified by the positive control (ADV-GPF).

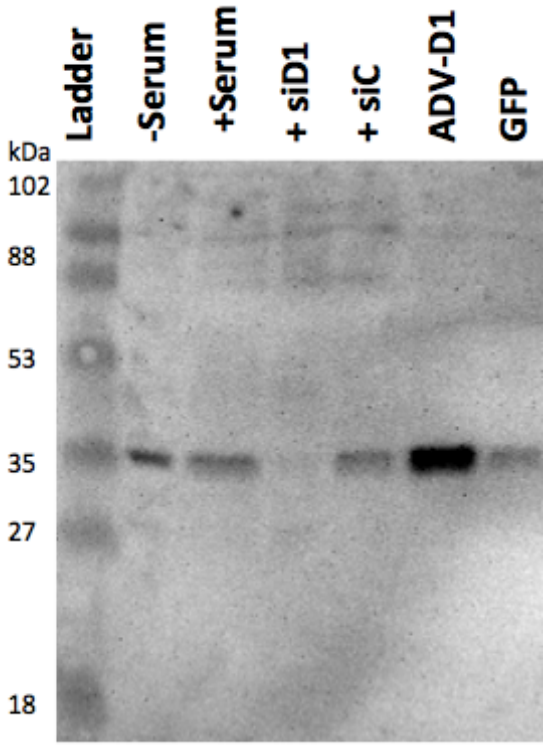

Figure 3. SkHep lysates were probed with cyclin D1 confirming that cyclin D1 was blocked (siD1) and cyclin D1 was overexpressed at ADV-D1.

Figures 5 and 6 show results from viability tests for the human-prostate LnCap cell line and the liver-cancer HUH7 cell line. There was no significant increase in cell viability for either cell line in the presence of glutamine (+Glutamine) compared to cells that were starved of glutamine (-Glutamine) ($p = 0.735$ and $p = 0.616$, respectively). Figures 7 and 8 show results of Western blots done to confirm expression of cyclin D1 in viability tests for LnCap and HUH7.

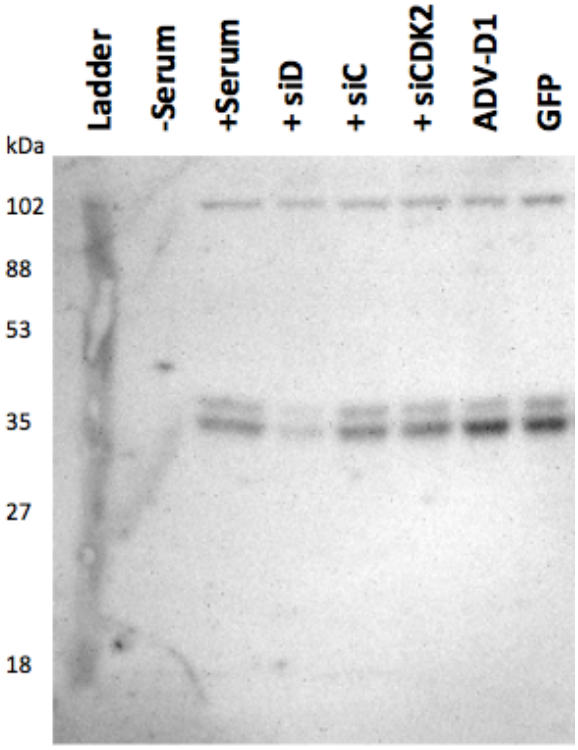

Figure 4. AML12 lysates were probed with cyclin D1 confirming that cyclin D1 was blocked (siD1) and cyclin D1 was overexpressed at ADV-D1.

DISCUSSION

The first goal of this study was to find human cancer-cell lines that are sensitive to glutamine deprivation. Results suggested that both the SkHep liver-cancer cell line and the healthy AML12 mouse cell line are glutamine dependent. Viability tests for the SkHep liver-cancer cell line and the healthy AML12 mouse-cell line showed significant increases in cell viability in the presence of glutamine ($p = 0.00002$ and $p = 0.01$, respectively). However, viability tests

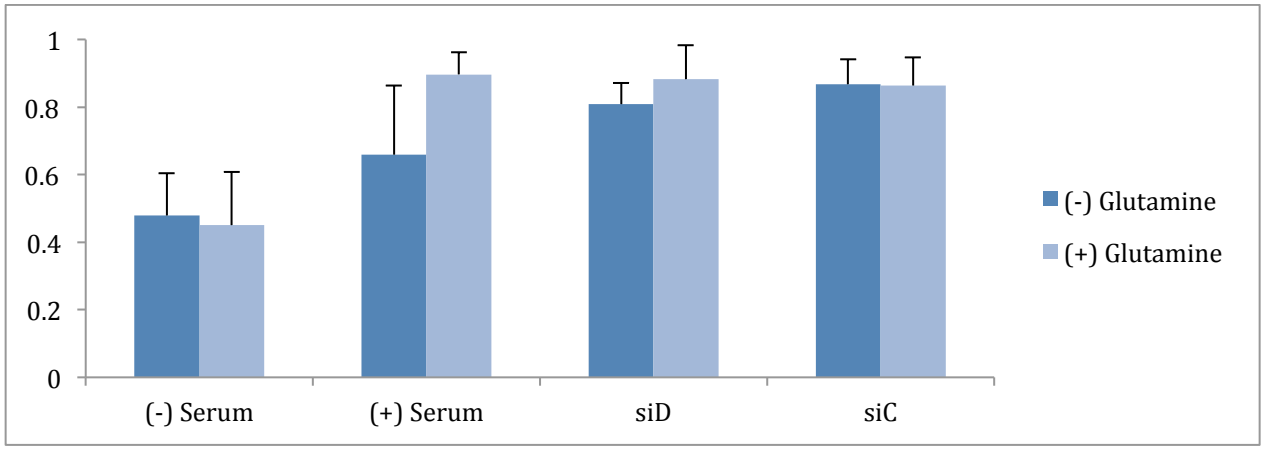

Figure 5. LnCap viability at 48 hours after siRNA transfection. Error bars show standard deviations. Two-sample *t*-tests were run to determine statistical significance. There was no significant increase in viability without glutamine ($p = 0.735$).

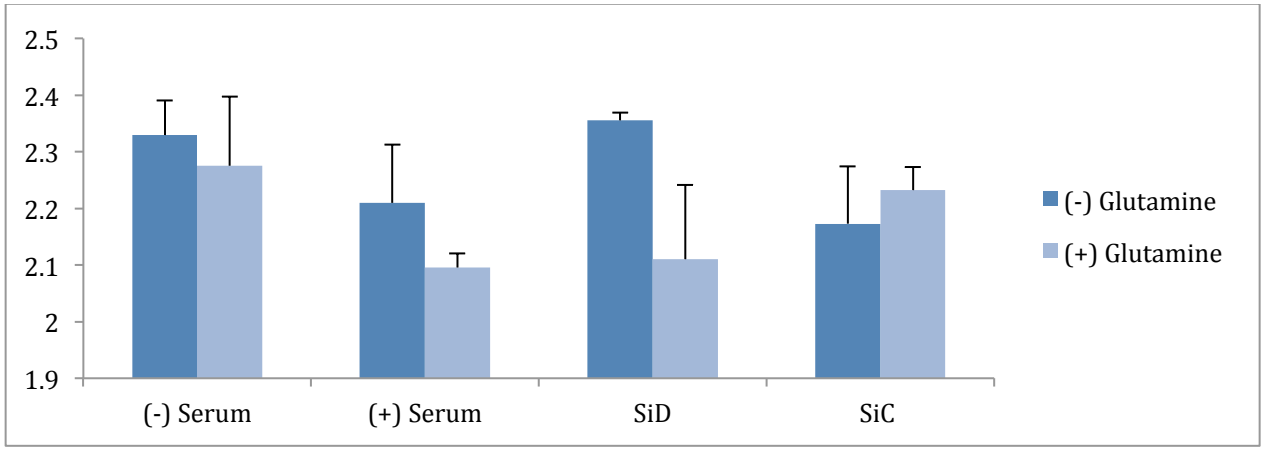

Figure 6. HUH7 viability at 48 hours after siRNA transfection. Error bars show standard deviations. Two-sample t-tests were run to determine statistical significance. There was no significant increase in viability without glutamine ($p = 0.616$).

suggested that LnCap human-prostate cells and HUH7 liver-cancer cells are not glutamine dependent ($p = 0.735$ and $p = 0.616$, respectively). The second goal of this study was to determine effects of overexpression of cyclin D1 on cell viability in absence of glutamine. Results supported the hypothesis that when glutamine-dependent cancer cells are deprived of glutamine, overexpression of cyclin D1 would cause a decrease in cell viability. In glutamine-dependent SkHep liver-cancer cells, cell viability significantly decreased when cells were starved of glutamine and cyclin D1 was overexpressed ($p = 0.0019$); however, when cyclin D1 was knocked down, SkHep cell viability significantly increased compared to controls ($p = 0.05$). These findings support the work by Mullany[6] that showed glutamine-responsive cells are susceptible to overexpression of cyclin D1. On the other hand, viability of healthy AML12 cells significantly increased when cells were starved of glutamine and cyclin D1 was overexpressed ($p = 0.0018$) and significantly decreased when cyclin D1 was knocked down compared to controls ($p = 0.04$).

CONCLUSION

This study suggests that overexpression of cyclin D1 decreases cell viability in glutamine-dependent SkHep liver-cancer cells that are starved of glutamine. However, results suggest that this is not the case in glutamine-dependent healthy AML12 mouse cells. These results support the idea that glutamine plays a unique role in glutamine-dependent cancer cells where it serves as the primary mitochondrial energy source.

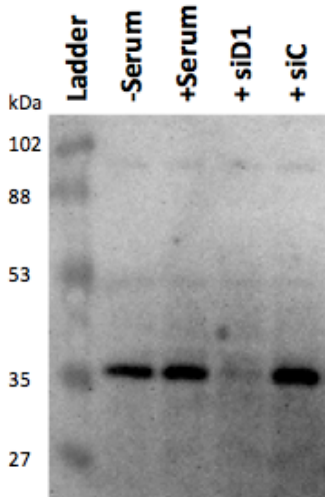

Figure 7. LnCap lysates were probed with cyclin D1, confirming that cyclin D1 was blocked (siD1).

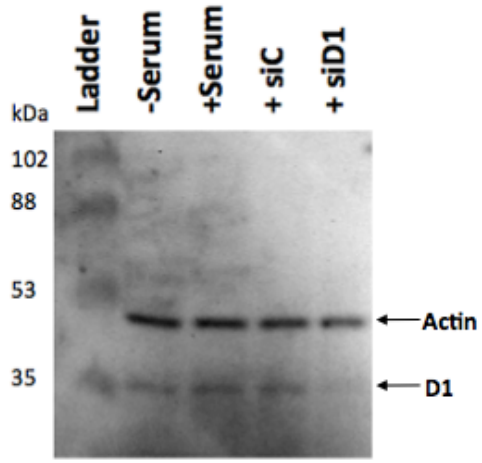

Figure 8. HUH7 lysates were probed with cyclin D1, confirming that cyclin D1 was blocked (siD1) and with actin to confirm samples contained protein.

Future work should be done to identify other glutamine-dependent cancer-cell lines to determine if susceptibility to overexpression of cyclin D1 holds for these cancer-cell lines. Additionally, effects of cyclin D1 overexpression on cancerous cells that are not glutamine dependent should be determined with and without glutamine to verify that susceptibility to cyclin D1 expression is exclusively characteristic of glutamine-dependent cells. Then, work should be done to find a way to target glutamine-dependent cancer cells.

A possible first step in targeting glutamine-dependent cancer cells may involve reprogramming the unique glutamine-dependent metabolic system. Since glutamine serves as the primary mitochondrial energy source in cancer cells that depend on *glutamine,* reprogramming glutamine-dependent mitochondria to produce anabolic precursors from glutamine that inhibit the mitochondrial respiratory path may slow growth of cancer cells. To study ways which anabolic precursors may be safely targeted without shutting down healthy mitochondrial respiratory paths, a review by Wise and Thompson[2] suggests that positron emission tomography (FDG-PET) may be used in a way that is similar to studies that tracked glucose metabolism in cancer tumors using fluorodeoxyglucose. Using FDG-PET may lead to ways to safely inhibit of one or more specific components of glutamine metabolism without wholesale inhibition of glutamine metabolism[2].

A possible second step to target glutamine-dependent cancer cells may involve lowering blood glutamine levels. Since cancer cells take up and metabolize glutamine to a degree that far exceeds their needs, lowering blood glutamine levels is another possibility. Preliminary studies have shown that L-asparaginase depletes levels of glutamine in the blood, but these studies have suggested this treatment may be toxic. Phenylbutyrate lead is another possibility because it conjugates with glutamine levels in the blood and is then excreted in the urine[10]. If successful, the abnormal metabolism of glutamine-dependent cancer cells may be the key to developing new treatments for these types of cancers.

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