CHARACTERIZATION OF SQUAMOUS CELL CARCINOMAS DURING HIGH AND LOW GLUCOSE TREATMENTS

by

Haleigh Gerold

APPROVED BY SUPERVISORY COMMITTEE:

_____
Jung-whan Kim, Chair

_____
John Burr

_____
Nikki Delk

CHARACTERIZATION OF SQUAMOUS CELL CARCINOMAS DURING HIGH AND LOW GLUCOSE TREATMENTS

by

HALEIGH GEROLD, BS

DISSERTATION

Presented to the Faculty of

The University of Texas at Dallas

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN

MOLECULAR AND CELL BIOLOGY

THE UNIVERSITY OF TEXAS AT DALLAS

December 2019

ACKNOWLEDGMENTS

I would like to begin by thanking the members of the Department of Cell and Molecular Biology at The University of Texas at Dallas. Without the exceptional education and background provided to first year students, I could not have achieved the level of research I did while a master's student at UTD.

I next would like to thank Dr. Ruben Ramirez for his encouragement and opportunities provided. Through opportunities he extended, I learned extensive amounts of knowledge that benefited my research career. I also was able to develop as a better teacher and group speaker while participating as a Teaching Assistant.

Lastly, and most importantly, I would like to thank Dr. Jung-whan (Jay) Kim for the opportunity to participate as a student researcher in his lab. Without Dr. Kim's financial support, I would not have had the same opportunity to discover my passion for cancer research. Through students in his lab, I had the opportunity to learn many research techniques that I will utilize for the remainder of my research career. I would like to specifically thank Meng-hsiung Hsieh for all of his unconditional help with learning research techniques. Without him, I would not be the scientist I am today.

August 2019

CHARACTERIZATION OF SQUAMOUS CELL CARCINOMAS DURING HIGH AND LOW GLUCOSE TREATMENTS

Haleigh Gerold, MS
The University of Texas at Dallas, 2019

Supervising Professor: Jung-whan (Jay) Kim

Lung squamous cell carcinoma (LSqCC) is one of the many subtypes of lung cancer, the first leading cause of cancer-related deaths in the United States. Despite many years of research effort, not much has been accomplished as far as improving treatment and patient 5-year survival rates for lung cancer. Through metabolic analysis, much research has been published describing LSqCC and squamous carcinoma cells (SqCC), in general, that describes potentially targetable phenotypes. Research published out of our lab showed LSqCC is highly dependent on glucose for survival, a targetable observation. In addition, results implicated transcription factors p63 and SOX2 are important for glucose uptake. Here, I perform a brief study of SqCC regarding the effect of the TAp63 protein isoform on glucose addiction and their ability to adapt to low glucose environments. This study reveals the non-importance of TAp63 in glucose uptake. Moreover, I demonstrate the ability for multiple SqCC to adapt and survive in low glucose environments. In addition, I provide preliminary insight into differential protein expression of low glucose adapted cells based on proteins previously studied in

our lab. In summary, this study provides further insight into the upregulated glycolytic flux seen in SqCCs.

TABLE OF CONTENTS

ACKNOWLEDGMENTS……...……..…iv

ABSTRACT……..……v

LIST OF FIGURES……..……..viii

LIST OF TABLES……....ix

LIST OF ABBREVIATIONS……..……x

INTRODUCTION…….....……..……...1

HYPOTHESIS…….....…….3

METHODS…….…….....……....3

RESULTS…….....……7

DISCUSSION…….…….....……12

APPENDIX A……...……15

FIGURES……..……...15

APPENDIX B……..……21

TABLES……21

REFERENCES…….……..22

BIOGRAPHICAL SKETCH……..…….24

CURRICULUM VITAE

LIST OF FIGURES

Figure 1. TAp63 overexpression has no measurable effect on glycolytic activity and ROS handling in glucose-addicted Squamous Cell Carcinoma.....15

Figure 2. TAp63 knockdown has no measurable effect on glycolytic activity and ROS handling in glucose-addicted Squamous Cell Carcinoma.....16

Figure 3. Squamous Cell Carcinomas can adapt and survive under glucose restriction.....17

Figure 4. SqCCs switch from using glucose to glutamine as a major energy source during glucose-restriction.....18

Figure 5. SqCCs p-ACC, p-AMPK, ΔNp63α and GLUT1 levels alter in low-glucose adapted cells.....19

Figure 6. Low-glucose adapted and parental cell subcutaneous xenograft showed no difference in growth.....19

Figure 7. Orthotopic lung injection model successful in initiating lung squamous cell carcinoma confined to the lungs.....20

LIST OF TABLES

Table 1. Normal chow and ketogenic diet formulas.....21

LIST OF ABBREVIATIONS

LSqCC – lung squamous cell carcinoma

SqCC – squamous cell carcinoma

ADC - adenocarcinoma

GLUT1 – glucose transporter 1

DCFDA - 2′,7′-Dichlorodihydrofluorescein diacetate

2-NBDG – 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)

ROS – reactive oxygen species

GFP – green fluorescent protein

Introduction

Lung cancer remains one of the leading causes of cancer deaths, despite years of research efforts that have seen treatment improvements for many other types of cancer [1,2,9]. Of the multiple types, the two most prominent are Non-small cell (NSCLC) subtypes adenocarcinoma (ADC) and squamous cell carcinoma (SqCC), the latter of which is accountable for 25-30% of all lung cancer cases [2]. While either type could arise from smoking, lung squamous cell carcinoma (LSqCC) is most frequently associated with current and previous smokers [2,3]. One major difference between these 2 types is the anatomical location in which the tumors usually arise. LSqCC tumors usually arise in the proximal lungs and bronchi while ADC tumors are usually found in the distal part of lungs [1,2,3]. Another difference is that squamous cell carcinoma tumors are usually varying in degree of keratinization [2,4]. When keratinization is not present or distinguishable, immunohistochemical marker p63 may be used to distinguish SCC from ADC [2,4].

Despite years of research, treatment for lung squamous cell carcinomas has not improved significantly. With the current trend of cancer treatment focusing on Immunotherapy, only one new treatment option has been presented for a limited number of LqSCC patients. Patients with >50% of LSqCC tumors expressing programmed death ligand 1 (PD-L1) may receive Pembrolizumab, a PD-L1-blocking antibody [5]. This high level of expression only occurs in ~23-30% of advanced non-small cell lung cancer (NSCLC) patients, meaning most LSqCC patients will not benefit from this treatment [5]. The front-line treatment then for the majority of LSqCC patients

is still platinum-based doublet chemotherapy that targets all highly proliferative cells, a method that only prolongs advanced NSCLC patient survival ~8-11 months [5,6]. More treatment options are quickly needed for LSqCC, contributing to this becoming a focus of research in our lab Research completed in our lab up to this point shows a heavy reliance on glucose for survival in multiple SqCC cell lines. The SqCC lines are much more susceptible to glucose deprivation than adenocarcinomas [7]. These squamous cancer cells use transcription factors ΔNp63 and SOX2 to enhance the transcription of the glucose transporter GLUT1, therefore increasing glycolytic flux [7]. When glucose uptake is inhibited via ketogenic diet or WZB117, squamous cell carcinoma cell death increases and tumor growth decreases versus adenocarcinoma [7]. As is the case with many cancers, we observed that SqCC can eventually become adapted to this glucose deprivation, metabolically switching to use of some other carbon source. This observation needs to be investigated further and is one of the main areas of research presented here.

Hypothesis

The TAp63 protein has no role in the induction of the GLUT1 transporter in squamous cell carcinomas.

Methods

Mice. FSP-cre $Tom^{flox/flox}$ $GLUT1^{flox/flox}$ mice and FSP-cre $Tom^{flox/flox}$ $PDH^{flox/flox}$ C57BL/6 mice were purchased from the Jackson Laboratory. Lung cancer was induced by orthotopic injection of Lewis Lung Carcinoma cells. NOD/SCID mice were purchased from the Jackson Laboratory and used for subcutaneous xenografts. All experimental procedures were approved by the IACUC and The University of Texas at Dallas.

Cell culture. All SCC cell lines used in this study were obtained from the University of Texas Southwestern Medical Center. Parental cells were cultured in 10 mM or 25 mM glucose DMEM (Sigma) with 5% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Sigma) and 1% non-essential amino acids (Sigma) at 37oC in 5% CO_2 humidified chamber. Adapted cells were cultured 0 mM glucose DMEM (Gibco) with 5% FBS (Sigma), 1% Pen/Strep (Sigma), 1% NEAA (sigma), and brought up to 1 mM or 0.5 mM glucose with glucose solution (Sigma). Cell lines expressing shRNA are cultured in media with puromycin for selection. Cells with over expression of genes are cultured in media containing blasticidin or hygromycin for selection.

Proliferation and Viability. Cells were seeded at $5x10^4$ in 12-well plates and counted every 24 hours. Cells are detached via Trypsin () and collected, spun down, and resuspended in a determined volume. 10ul samples were then loaded into Hemocytometers and counted. For viability, cells are suspended in 50% Trypan Blue () and 50% media. 10ul samples are loaded on hemocytometer and positive, viable cells and negative, unviable cells are counted.

Glutamine deprivation experiments. The cells were plated in 12-well plates and treated with media containing glutamine and media without glutamine. Proliferation and viability were then measured according to methods. For CB-839 (Selleckchem) treatment, cells were plated in a 12-well plate and then treated next day with .5 uM-2 uM CB-839 or DMSO (Sigma). Proliferation and viability were measured every 24 hours after that via the previous methods listed.

In vivo xenograft tumor experiment. 5×10^6 cells were subcutaneously implanted in the back of NOD/SCID mice (Jackson Lab) between 4 to 6 weeks old. Before injection, cells were collected and resuspended in 50% Matrigel (Sigma) and 50% Hank's Balanced Salt Solution (Sigma). In total, 12 mice were used for the xenograft experiment. 6 mice were fed normal chow and 6 mice were fed ketogenic diet. Normal chow and ketogenic diet were purchased from 4 Research Diets Institute. Tumor volume, blood glucose, and weight were measured weekly or at indicated times using electronic calipers, blood glucose meter, and scale, respectively.

In vivo LLC injection. FSP-cre Tom$^{flox/flox}$ GLUT1$^{flox/flox}$ and FSP-cre Tom$^{flox/flox}$ PDH$^{flox/flox}$ C57BL/6 mice were obtained from Jackson Laboratories. Orthotopic lung injections involved directly injecting Lewis Lung Carcinoma-luciferase cells into left lateral lung lobe through the middle axillary line. LLC-luciferase cells allowed for bioluminescent imaging of the tumor via subcutaneous luciferin injection in the neck. The luciferin (Sigma) is administered at 150 mg/kg. Bioluminescence imaging was performed via IVIS Lumina III imager (PerkinElmer). Bioluminoscore was quantified via Living Image 4.5V.

Blood glucose measurement. The mice were fasted for 6 hours before measuring weekly blood glucose. After a tail snip is made, a glucometer (OneTouchUltra) is used to measure glucose level.

Immunoblot of proteins. RIPA lysis buffer with added protease inhibitor (Roche) was used to lyse the cells. After, 20% amplitude sonication was completed for 10 seconds and the lysates cleared by 14,000 rpm centrifugation for 10 minutes at 4°C. Protein lysates were separated via SDS-PAGE and transferred to PVDF membrane (Fisher). After blocking in 5% milk in TBST, primary antibody was added for overnight. Secondary antibody conjugated with HRP was added at room temperature for one hour. SuperSignal West Pico or Femto (ThermoFisher) was used to visualize protein bands. Primary antibodies used: p63 (1:1,000; Biocare Medical CM163A), GLUT1 (1:1,000; Alpha Diagnostics GT11-A), V5-tag (1:1,000; Cell Signaling Technology #13202),

TAp63(1:500, 10ul in 5ml dilution; BioLegend #618902), p-ACC (1:1,000; Cell Signaling Technology #3661S), p-AMPK (1:1,000; Cell Signaling Technology #2531S), NRF2 (1:1000; Abcam ab137550), and β-actin (1:5,000; Sigma A5441).

mRNA Quantification. RNA was isolated from cells using Trizol (Sigma) and then Direct-zol RNA MiniPrep kit (Zymo Research). 1-step RT-PCR was performed using KAPA SYBR FAST One-Step qRT-PCR Master Mix(2x) kit (Kapa Biosystems). Quantitative PCR was performed using the CFX-96 real-time PCR System (BioRad).

***In Vitro* Metabolic Assays.** Glucose uptake was measured using the Glucose Uptake Cell-Based Assay kit (Cayman) with apigenin as a negative control. Cells were washed with Phosphate Buffered Saline (PBS, Sigma) and then treated with 2-NBDG for 1 hour before imaging. Emission at 535 nm was measured using a fluorescent confocal microscope (Nikon Eclipse Ni-U) and fluorescent intensity quantified in Fiji (NIH). Intracellular ROS levels were measured via DCFDA assay (Cayman) with H_2O_2 as a positive control. Cells were washed with PBS and treated for 1 hour with DCFDA. Emission at 535 nm was measured using a fluorescent confocal microscope (Nikon Eclipse Ni-U) and fluorescent intensity quantified in Fiji (NIH).

Results

TAp63 has no measurable effect on glycolytic activity and ROS handling in glucose-addicted Squamous Cell Carcinoma. To determine whether the TAp63

protein plays the same role as the DNp63 protein in glycolytic activity and ROS handling, TAp63 was overexpressed using a viral vector in LSqCC line HCC2814. The TAp63 protein was confirmed to be overexpressed (Figures 1a and 1b, RT and WB). TAp63 protein overexpression seems to have no effect on GLUT1 levels when compared to GFP-expressing cells, unlike the DNp63 protein (Fig. 1a and 1b). TAp63 over expression has no effect on the proliferation rate of cells, indicating that TAp63 does not increase GLUT1 transcription (Fig 1c). To confirm, a glucose uptake assay showed there was no difference in the amount of 2-NBDG taken up by TAp63 over expressing cells compared to parental cells (Fig 1d). Since glucose can be shunted to Pentose Phosphate Pathway and used for ROS handling, I also measured ROS levels to confirm there are no differences in glucose intake. The level of reactive oxygen species (ROS), measured using DCFDA, showed no difference between TAp63 over expressing cells and parental cells, indicating no increase in glucose uptake (Fig 1e). These data taken together indicate that the TAp63 protein does not increase GLUT1 transcription, glycolytic activity, or the ability to handle ROS.

The TAp63 protein was then targeted for knockdown in 2 different squamous cell lines, HCC2814 and KYSE70. As a control, the same cell lines were transduced with a scramble RNA (scr). The knockdown of TAp63 was confirmed in both cell lines via RT-PCR (Fig. 2b). Consistent with findings in the overexpression line, knockdown of TAp63 has no effect on GLUT1 mRNA or protein levels in either SqCC line (Fig. 2a and 2b). Proliferation curves show no difference in growth for TA knockdown cells when compared to scr cells, indicating that TAp63 knockdown has no effect on glucose

transport (2c). To confirm these findings, glucose uptake assays showed no difference in the amount of 2-NBDG taken up by the TAp63 knockdown cells compared to scr cells (2d). As in the overexpression, ROS levels were measured in TAp63 knockdown cells to confirm that there is no increase in glucose uptake, and therefore better ability to compensate for ROS. TAp63 knockdown cells showed no differences in ROS levels compared to scr cells. These data taken together indicate that the protein isoform TAp63 has no effect on GLUT1 transcription, unlike the ΔNp63 isoform.

Squamous Cell Carcinomas can adapt and survive under glucose restriction and also produce spheroids under stress. Previous research published in our lab showed SqCC is highly addicted to glucose compared to ADC [7]. We next wanted to investigate whether SqCCs can adapt and survive under low glucose conditions. Lines HCC95 and KYSE70 were able to adapt, over time (how long??), to a media glucose concentration of 1 mM. Line HCC2814 was able to adapt to a media glucose concentration of 0.5 mM. Adaptation was measured and confirmed via proliferation assays compared to parental cells in 10 mM or 25 mM glucose media (Fig 3a-d).

All cancer cell lines adapted (that went through adaptation process) produced spheroids that were released into the media when switched to 1 mM and 0.5 mM glucose concentration. The spheroids seem to be a byproduct of stress and are continually released into the media. These spheroids were collected for subcutaneous xenografts.

SqCCs switch from using glucose to glutamine as a major energy source during glucose-restriction. After adapting SqCCs to low glucose concentrations, I next investigated possible carbon sources that could compensate for glucose and be utilized as an energy source by the cells. Current published research indicates that some cancers are highly dependent on glutamine as their major energy source [11]. Due to this knowledge, I investigated whether glutamine was an important factor for survival of the adapted SqCCs. When glutamine is not added to the growth media, a significant decrease in cell proliferation and viability was seen in all 3 squamous cancer lines (Fig 4a). To confirm the finding that glutamine is now the major energy source, cells were treated with CB-839 (Selleckchem), a glutaminase inhibitor. Both cell proliferation and viability were significantly decreased in cells treated with CB-839 after 72 hours of treatment. (Fig. 4b). These data together indicate that squamous cell carcinomas exhibit metabolic plasticity when exposed to glucose restriction, switching to glutamine as the main carbon source.

SqCCs ACC, AMPK, ΔNp63α and GLUT1 levels alter in low-glucose adapted cells. LSqCCs usually experience a high number of mutations with alterations possible in many pathways [9,12]. The PI3K/AKT pathway is aberrantly activated in some SqCCs but the functionality of this is not yet known [9,12]. It has been shown that AMPK, a stress-sensing kinase, could be a regulator of PI3K/AKT, a pathway commonly mutated in LSqCC [12].

The AMPK signaling pathway is an energy-sensing pathway that is activated under stressful conditions. In this pathway, AMPK is phosphorylated under stress, resulting in phosphorylation of Acetyl CoA Carboxylase (phospho-ACC) [12]. Due to the fact that the LSqCC are undergoing stress to adapt to glucose deprivation, I decided to investigate whether this stress alters the AMPK signaling pathway. Adapted cells have significantly reduced protein levels of phospho-ACC compared to parental cells (Fig. 5). Induction of phospho-ACC seems to be inhibited in a time-sensitive manner when parental cells are exposed to glucose restriction. These data taken together indicate that phospho-ACC induction may be inhibited, or the protein degraded when cells are exposed to a low glucose environment, a finding that is unusual in a high stress environment. The AMPK signaling pathway is altered under stressful conditions and has been shown to regulate Akt [13]. I would next investigate further the functionality of the relationship between AMPK and PI3K/AKT activation in LSqCC.

Research published in our lab up to this point has shown the involvement of ΔNp63α and SOX2 in enhancing the transcription of GLUT1, increasing the glycolytic activity of squamous cell carcinomas [7,10]. I then wanted to investigate whether levels of ΔNp63α or GLUT1 were altered in cells adapted to low glucose. Adapted cells showed much higher levels of GLUT1 induction than parental cells in normal conditions (Fig. 5). Parental cells treated with acute glucose deprivation induced higher GLUT1 levels in a time-dependent manner compared to parental cells in normal conditions (Fig. 5). Furthermore, adapted cells show little to no induction of ΔNp63α, instead inducing ΔNp63γ isoform (Fig. 5). Parental cells exposed to acute glucose deprivation showed a

decrease of ΔNp63α and an increase of ΔNp63γ in a time-dependent manner (Fig. 5). These data taken together imply that during glucose starvation, SCC lines upregulate GLUT1 potentially by expressing a different ΔNp63 isoform, ΔNp63γ. The reason for this switch in isoform expression is unexplained and needs to be investigated further.

Low-glucose adapted and parental cell subcutaneous xenograft showed no difference in growth. Next, I wanted to understand whether adapted cell line HCC2814 would experience any difference in growth than parental cell line *in vivo.* I also wanted to know whether ketogenic diet would have any effect on the growth of the adapted cell lines, as it does in the parental. Both cell lines were injected subcutaneously into SCID mice and tumors were measured once per week. Half of the mice in each group n=6 (parental or adapted) received normal chow, while the other received ketogenic diet. The experiment was ended due to issues with the mice and blood glucose. Data is shown in Fig. 6.

Orthotopic lung injection model successful in initiating lung squamous cell carcinoma confined to the lungs. Excluding tumor cells, fibroblasts are the main cell type present in the tumor microenvironment [14]. With our lab's focus on lung cancer metabolism, we decided to extend our research into the metabolism of fibroblasts (in the tumor microenvironment of? Better way?) associated with lung cancer. In order to study this, a reliable orthotopic lung injection model is needed. I developed an orthotopic lung injection protocol that comprises of injecting Lewis Lung Carcinoma (LLC) cells into

C57BL/6 mice. These LLC cells have been modified to carry Luciferase gene for bioluminescent imaging and visualization of the tumor. After the left thoracic area is shaven and the animal anesthetized, cells are injected with 50% Matrigel directly at the middle axillary line into the lungs. After two weeks, mice can be shaved and injected with Luciferin to visualize the tumor using a bioluminescent imager. Due to the aggressiveness of the LLC cell line, the experiment must be ended 4 weeks post-injection when $1x10^5$ cells are orthotopically implanted.

After orthotopic lung injection was performed, mice were checked for health daily. After 1 week, the tumors were not visible via bioluminescent imaging. After 2 weeks post-injections, tumors in all mice injected were visualized and majority confined to the lungs (Fig. 7). A minority (n=1) of the group had cancer cells that spread inside the pleural cavity.

Discussion

As previously stated, lung cancer is the first leading cause of cancer-related death in the US. It is of particular importance due not only to high level of incidence, but also because of its affiliation with smoking cigarettes as a major risk factor [6,9]. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases, and within that category exists lung squamous cell carcinoma, accounting for ~30% of all cases (LSqCC) [6]. While lung adenocarcinoma (ADC) is the most common type of lung cancer, LSqCC is the type most affiliated with current and previous smokers.

There has not been as much improvement in identifying biomarkers for LSqCC as for ADC. Common targetable mutations such as in the EGFR and ALK genes have been identified in ADC and other non-squamous cancers, expanding treatment options and resulting in improved patient survival [6]. Recent work has shown that PIK3CA gene has a high copy number in a small percentage of LSqCC cases [12]. The purpose for this seems to be to increase PI3K/Akt signaling for growth although not fully understood [12]. Further investigation in the ability to target these high copy number genes is needed to improve treatment for patients with LSqCC.

The AMPK signaling pathway is activated during times of nutrient stress and its function is to regulate energy homeostasis. During low nutrient conditions, AMPK is phosphorylated which in turn phosphorylates ACC [15]. There is little to no induction of p-ACC in the glucose deprived cells (Fig. 5), a result that needs further investigation to understand.

The transcription factor ΔNp63α has been implicated in the enhancement of GLUT1 gene transcription in SqCCs [10]. After parental cells were adapted to low glucose conditions, protein expression showed a switch from the induction of ΔNp63α to ΔNp63γ isoform. The reason for this switch in isoform and the function of the ΔNp63γ protein in this context has yet to be investigated.

Many molecular components must be involved in order for cells to make the switch from glucose to glutamine utilization. Somehow, SqCC is able to overcome the stress faced during deprivation and eventually adapt and grow at the same rate as parental cells. Mechanisms allowing upregulation of Pentose Phosphate Pathway and therefore

improved reactive oxygen species handling may lead to the ability to adapt. Further investigation into the transcription factors and enzymes making glutamine utilization possible is necessary to understand the paths this carbon source is contributing to. RNA sequencing could be used for initial investigation into differentially expressed genes in parental versus low glucose adapted SqCCs.

Attempt to target and treat the low glucose adapted carcinoma based on findings (continue to start with whether ketogenic diet has any differences in effect on low vs high glucose adapted tumor).

APPENDIX A

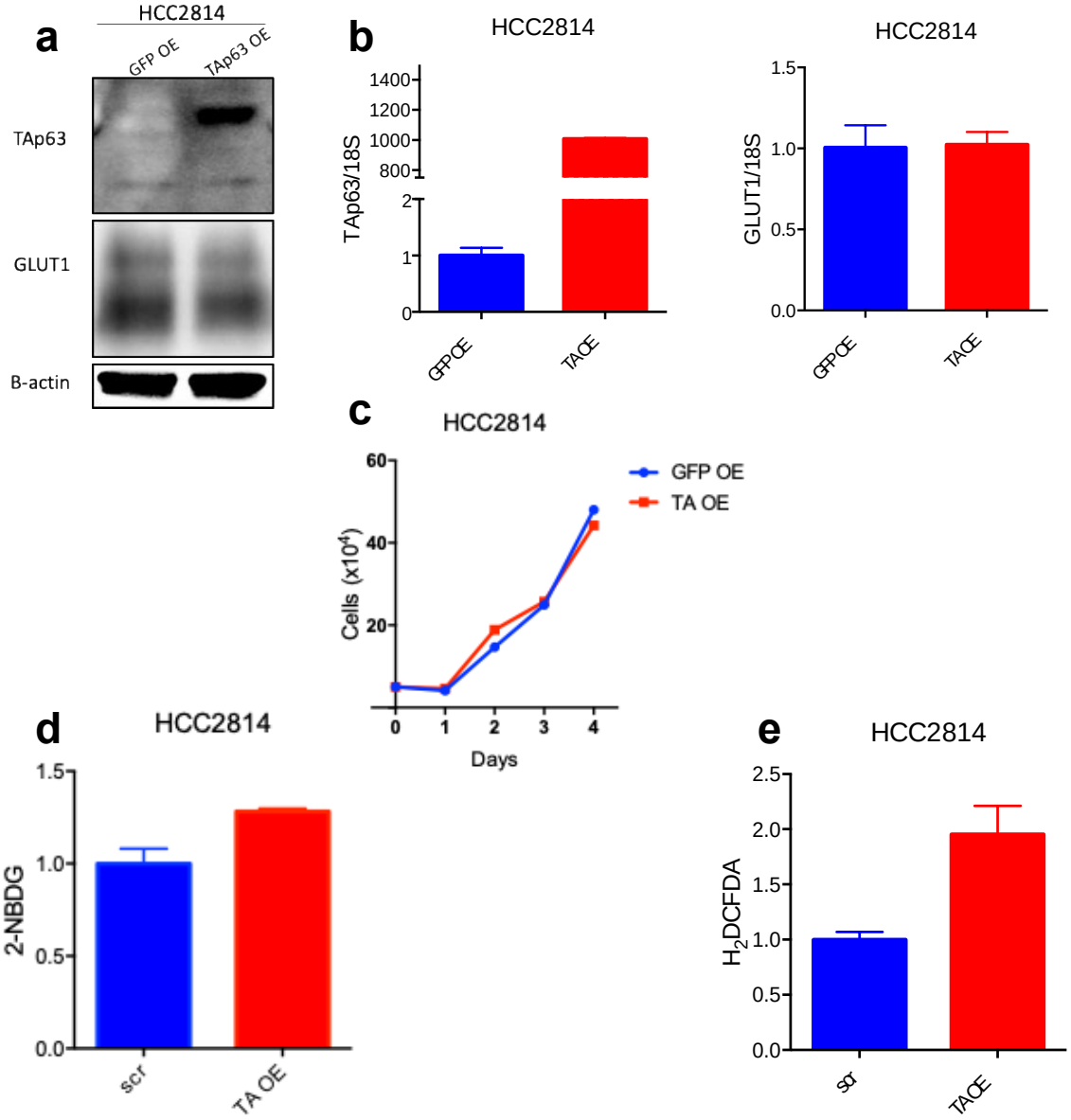

Figure 1 | TAp63 has no measurable effect on glycolytic activity and ROS handling in glucose-addicted Squamous Cell Carcinoma. (a) Western blot showing TA overexpression results in no difference in GLUT1 levels in TAp63 over expressing cells. **(b)** RT-PCR confirming that TA is overexpressed and that it has no effect on GLUT1 levels. **(c)** Proliferation curve showing no difference in growth rate. **(d)** Glucose uptake assay showing no difference in the amount of 2-NBDG taken up. **(e)** ROS levels measured via DCFDA showing that ROS is only slightly increased in TAp63 overexpressing cells.

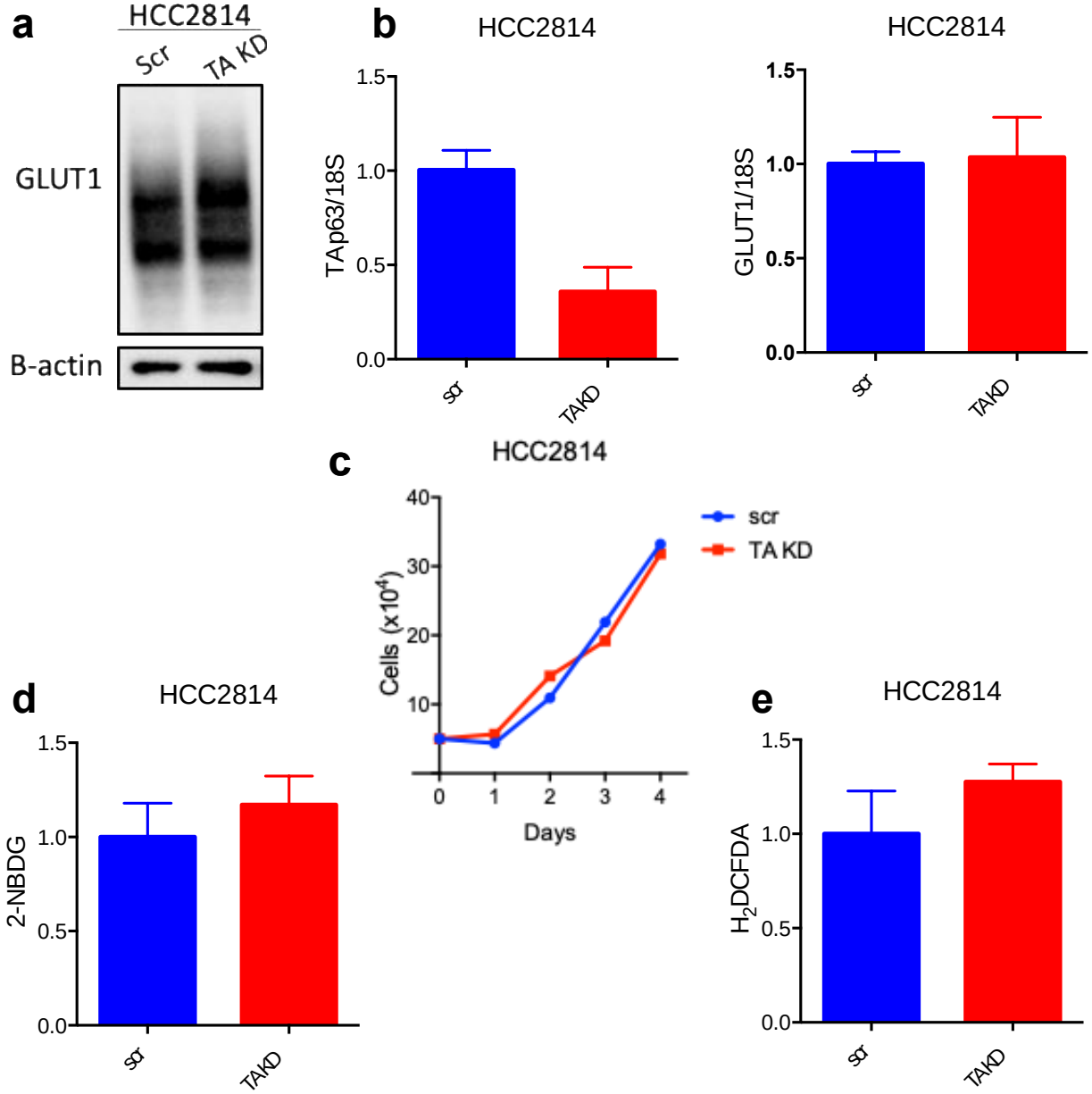

Figure 2 | TAp63 knockdown has no measurable effect on glycolytic activity and ROS handling in glucose-addicted Squamous Cell Carcinoma. **(a)** Western blot confirming that TA knockdown has no effect on GLUT1 protein levels. **(b)** RT-PCR confirming TA knockdown and that TA knockdown has no effect on GLUT1 levels in HCC2814. **(c)** Proliferation curves show that TA knockdown has no effect on growth in HCC2814. **(d)** Glucose uptake assays showing there is no difference in 2-NBDG taken up in TA knockdown cells. **(e)** ROS measured via DCFDA show that ROS levels are unaffected by TA knockdown in both cell lines.

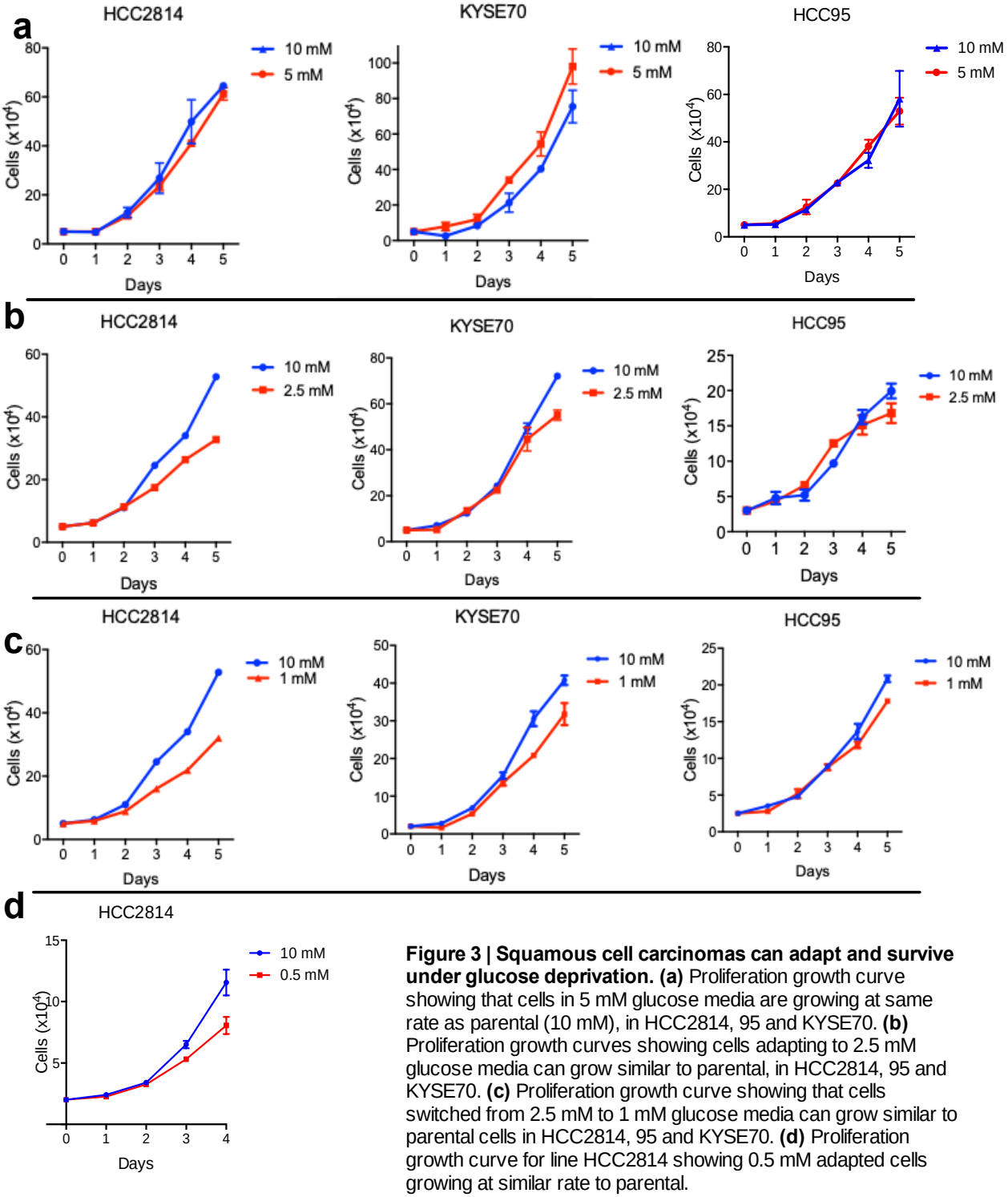

Figure 3 | Squamous cell carcinomas can adapt and survive under glucose deprivation. (a) Proliferation growth curve showing that cells in 5 mM glucose media are growing at same rate as parental (10 mM), in HCC2814, 95 and KYSE70. **(b)** Proliferation growth curves showing cells adapting to 2.5 mM glucose media can grow similar to parental, in HCC2814, 95 and KYSE70. **(c)** Proliferation growth curve showing that cells switched from 2.5 mM to 1 mM glucose media can grow similar to parental cells in HCC2814, 95 and KYSE70. **(d)** Proliferation growth curve for line HCC2814 showing 0.5 mM adapted cells growing at similar rate to parental.

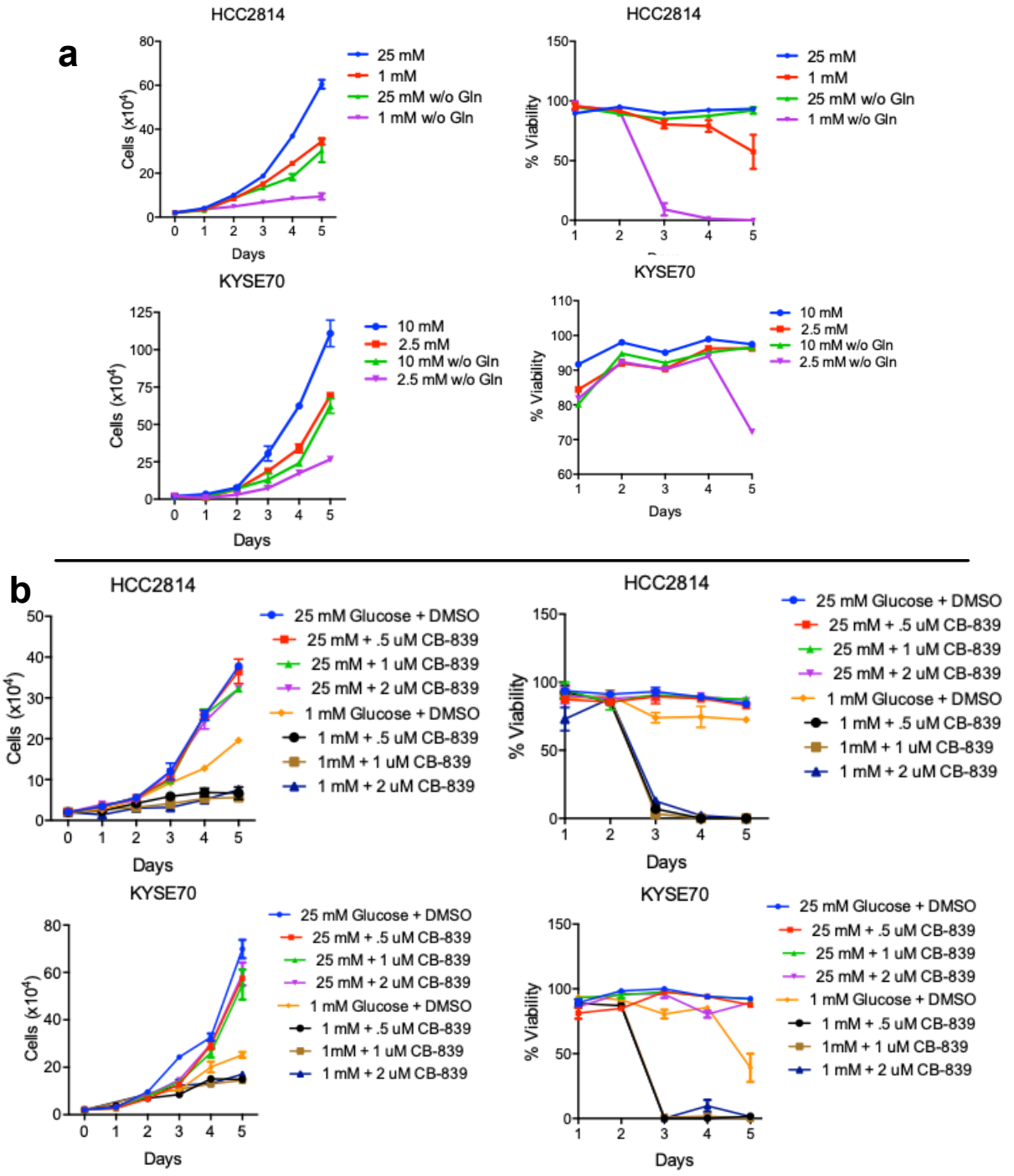

Figure 4 | SCCs switch from using glucose to glutamine as a major energy source during glucose-restriction. (a) Viability and proliferation measured over 5 days. Parental (25 mM) vs adapted (1 mM) with and without glutamine added to the media. **(b)** Viability and proliferation measured over 5 days. Parental (25 mM) vs adapted (1 mM) with .5 uM- 2 uM CB-839 or DMSO (control) added 24 hours after plating.

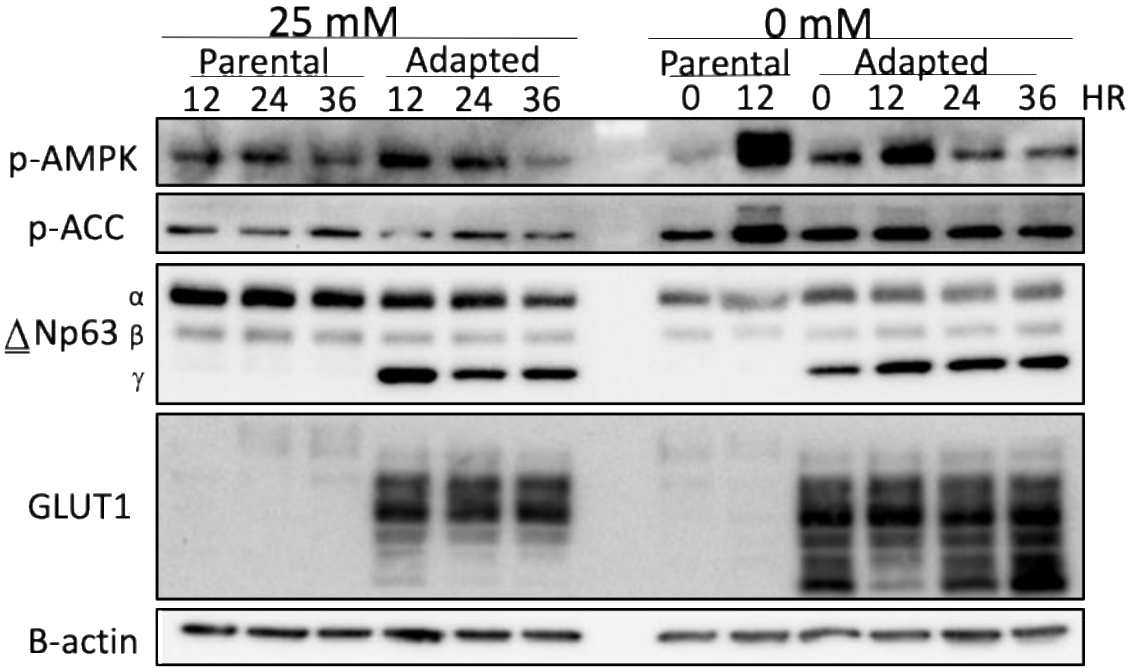

Figure 5 | SqCCs ACC, AMPK, Np63 and GLUT1 levels alter in low-glucose adapted cells. Western blot of lysates from parental and adapted cells exposed to acute glucose deprivation (0 mM) and high glucose (25 mM).

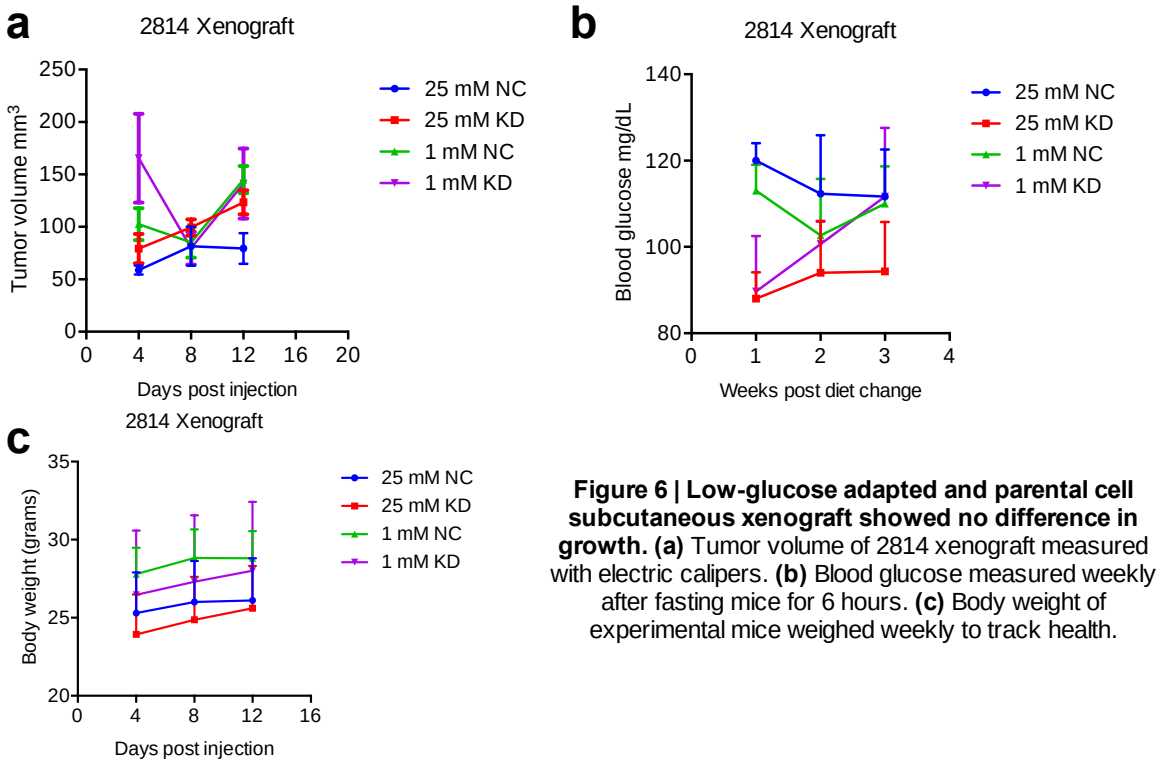

Figure 6 | Low-glucose adapted and parental cell subcutaneous xenograft showed no difference in growth. (a) Tumor volume of 2814 xenograft measured with electric calipers. **(b)** Blood glucose measured weekly after fasting mice for 6 hours. **(c)** Body weight of experimental mice weighed weekly to track health.

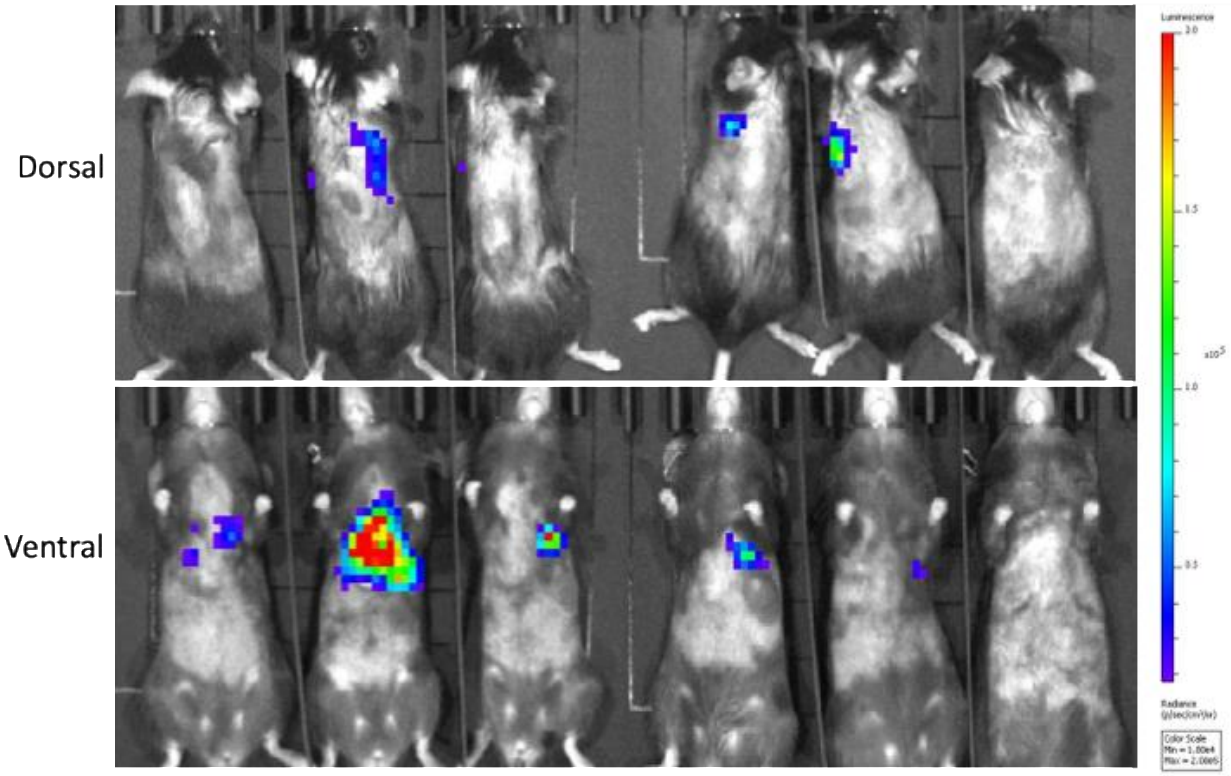

Figure 7 | Orthotopic lung injection model successful in initiating lung squamous cell carcinoma confined to the lungs. Bioluminescence imaging and quantification of shaved C57BL/6 FSP+ mice after 2 weeks post-orthotopic lung injection.

APPENDIX B

Table 1 | Normal chow and ketogenic diet formulas.

	Normal Chaw		*Ketogenic Diet*	
	gm%	kcal%	gm%	kcal%
Protein	21	20	17	10
Carbohydrate	57	55	0.2	0.1
Fat	12	25	67	89.9
Total		100		100
kcal/gm	4.2		6.7	
Ingredient	gm	kcal	gm	Kcal
Casein, 80 Mesh	200	800	100	400
L-Cystine	3	12	1.5	6
Corn Starch	397.7	1591	0	0
Maltodextrin 10	150	600	0	0
Sucrose	0	0	0	0
Cellulose, BW00	50	0	50	0
Soybean oil	25	225	25	225
Lard	87.7	789	380.2	3421.8
Mineral Mix, S10026	10	0	10	0
DiCalcium Phospate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	0	0
Vitamin Mix, V10001c	0	0	1	4
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0.025	0	0	0
FD&C Red Dye #40	0	0	0.05	0
Total	970.45	4057	604.75	4057

REFERENCES

1. Youlden, D.R., Cramb, S.M., and Baade, P.D. The International Epidemiology of Lung Cancer: geographical distribution and secular trends. *J Thorac Oncol*. 2008; 3: 819–831

2. Hirsch, F.R., Spreafico, A., Novello, S., Wood, M.D., Simms, L., and Papotti, M. The prognostic and predictive role of histology in advanced non-small cell lung cancer: a literature review. *J Thorac Oncol*. 2008; 3: 1468–1481

3. American Cancer Society. What is non-small cell lung cancer? http://www.cancer.org/cancer/lungcancer-non-smallcell/detailedguide/non-small-cell-lung-cancer-what-is-non-small-cell-lung-cancer. Accessed July 15, 2019.

4. Travis, W.D., Brambilla, E., Nicholson, A.G. et al., The 2015 World Health Organization Classification of Lung Tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. *J Thorac Oncol*. 2015; 10: 1243–1260

5. Socinski, Mark A. et al., Current and Emergent Therapy Options for Advanced Squamous Cell Lung Cancer. *J Thorac Oncol*. 2018; 13(2): 165-183

6. Socinski, M.A., Obasaju, C., Gandara, D. et al. Clinicopathologic features of advanced squamous NSCLC. *J Thorac Oncol*. 2016; 11: 1411–1422

7. Goodwin, J., et al., The distinct metabolic phenotype of lung squamous cell carcinoma defines selective vulnerability to glycolytic inhibition. *Nat Commun*. 2017. 8: p. 15503.

8. Siegel, R.L., K.D. Miller, and A. Jemal, Cancer statistics, 2018. *CA Cancer J Clin*, 2018. 68(1): p. 7-30.

9. Cancer Genome Atlas Research, N., Comprehensive genomic characterization of squamous cell lung cancers. *Nature*, 2012. 489(7417): p. 519-25

10. Hsieh M., et al., p63 and SOX2 dictate glucose reliance and metabolic vulnerabilities in squamous cell carcinomas. *Cell Reports*, 2019; 28: p. 1860-1878

11. Vanhove K, Derveaux E, Graulus GJ, et al. Glutamine Addiction and Therapeutic Strategies in Lung Cancer. *Int J Mol Sci*. 2019;20(2):252.

12. Yamamoto, H., et al., PIK3CA mutations and copy number gains in human lung cancers. *Cancer Res*, 2008; 68(17): p. 6913-21.

13. Han F, Li CF, Cai Z, et al. The critical role of AMPK in driving Akt activation under stress, tumorigenesis and drug resistance. *Nat Commun*. 2018;9(1):4728.

14. Alkasalias T, Moyano-Galceran L, Arsenian-Henriksson M, Lehti K. Fibroblasts in the Tumor Microenvironment: Shield or Spear?. *Int J Mol Sci*. 2018;19(5):1532.

15. Park S.H., et al. Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. *J of Applied Physiology*. 2002; 92(6): p. 2475-2482.

BIOGRAPHICAL SKETCH

Haleigh Elizabeth Gerold was born and has always lived in Dallas, Texas. She completed her Bachelor of Science in Biology in 2016 at Texas Tech University in Lubbock, Texas. While studying there, she was an undergraduate researcher in the lab of Dr. Michael San Francisco, under instruction of Dr. Shalika Silva. Her projects entailed studying biofilm formation of the chytrid fungus, *Batrachochytrium dendrobatidis*, and studying alternative therapeutic agents to target and treat this fungus. Due to her experience in this lab, she decided to pursue a career in research. In 2017, Haleigh was accepted to the Cell and Molecular graduate program at The University of Texas at Dallas.

CURRICULUM VITAE

Haleigh Gerold

3930 McKinney Ave. Apt. 254, Dallas, Tx 75204

haleighgerold12@gmail.com Tel: 972-369-9073

Statement of Purpose: I am currently finishing the Thesis portion of a M.S. in Cell and Molecular Biology and seeking a position in a research laboratory. Due to my previous experience, my goal is to find a position in cancer research so I can continue to gain experience and publications in this field before applying to MD and PhD/MD programs.

EDUCATION

The University of Texas at Dallas (UTD), Dallas, Texas 08/2019
M.S., Cell and Molecular Biology *GPA 3.59/4.0*

Texas Tech University (TTU), Lubbock, Texas 12/2016
B.S., Biological Sciences *Core courses GPA 3.4/4.0*

RESEARCH EXPERIENCE

Department of Molecular and Cell Biology, UTD, Dallas, Texas
Master's Student at Laboratory of Dr. Jung-whan(Jay) Kim *03/2018 – 06/2019*

Project 1: p63 and SOX2 Dictate Glucose Reliance and Metabolic Vulnerabilities in Squamous Cell Carcinomas (SCC).

- Examine the interaction between transcriptional factor p63 for GLUT1 induction in SCC.
- Apply GEMM and PDX model to study pharmacologically and dietary targeting SCC's glucose reliance.

Project 2: SCC metabolic plasticity under glucose restriction and spheroid formation. (Project leader)

- Produce adapted SCC cell lines from glucose restricted media.
- Generate spheroid structure *in vitro* and establish spheroid PDX *in vivo*.
- Assess the effects of ketogenic diet on adapted and parental SCC.
- Show glutamine is the major carbon source for SCC adapted to glucose restriction.
- Investigate molecular mechanisms involved in the switch from glucose to glutamine.

Project 3: The relationship between p63 and NRF2 in LSCC. (Project leader)

- Produce p63-deficient SCC to study NRF2 expression and reactive oxygen species levels.
- Investigate how changes in NRF2 expression effect PPP and glutathione-synthesizing enzymes.

Project 4: Targeting GLUT1 and Pyruvate Dehydrogenase/Pyruvate Dehydrogenase Kinase 1 axis in fibroblasts to assess anti-tumor effects. (Project leader)

- Establish and apply orthotopic mouse lung injection protocol.
- Establish and maintain multiple lines of GEMMs with fibroblast metabolic alterations.
- Investigate how altering the metabolism of fibroblasts affects tumor growth by utilizing orthotopic allograph on GEMM.

Department of Biological Sciences, TTU, Lubbock, Texas
Student Researcher at Laboratory of Dr. San Francisco *04/2016 – 08/2017*

B. dendrobatidis is a chytrid fungus responsible for a significant decline in amphibian populations.

Project 1: Anti-fungal effects of alternative therapeutics on *B. dendrobatidis*

- Investigate how alternative therapeutics affect biofilm formation

Project 2: Mechanisms of infection of amphibians by *B. dendrobatidis*: early fungal-frog interaction

- Study chemotaxis of zoospores with compounds produced by amphibian skin

PUBLICATION

p63 and SOX2 Dictate Glucose Reliance and Metabolic Vulnerabilities in Squamous Cell Carcinomas.

Meng-Hsiung Hsieh, Yoon Jung Kim, Joshua H. Choe, ***Haleigh Gerold****, Chance Nowak, Min Kyu Kang, Ji Yun Jeong, Shin Yup Lee, Brandon Faubert, Zhenyu Xuan, E. Dale Abel, Claudio Scafoglio, David B. Shackelford, John D. Minna, Pankaj K. Singh, Vladimir Shulaev, Leonidas Bleris, Kenneth Hoyt, James Kim, Masahiro Inoue, Ralph J. DeBerardinis, Tae Hoon Kim, Jung-whan Kim.*

Under review by ***Cell Reports***

RESEARCH TECHNIQUES

Mice Work:

- GEMM and immunocompromised mouse breeding and keeping
- Genotyping
- Bioluminescence imaging
- Subcutaneous xenograft and patient derived xenograft
- Intraperitoneal injection

- Orthotopic lung injections
- Mouse lung and subcutaneous tumor harvesting

Tissue Culture:

- Tissue cell culture for up to twenty different cancer cell lines
- Mouse lung primary cell culture
- Lentiviral particle production for gene modification
- Doxycycline-inducible stable cell line generation
- Flow cytometry for cell sorting

Molecular and Biochemistry:

- Western blot
- qPCR
- Immunohistochemistry, sectioning and staining
- Immunocytochemistry
- Luciferase reporter assay
- Intracellular ROS detection via DCFDA, DHE, BODIPY, 4HNE, γH2.AX, and MitoSox Red
- Intracellular glucose consumption detection via Glucose Uptake Assay

Programming:

- Beginner's experience with Python for biological sciences purposes
    - Built a very simple program to analyze qPCR results (calculate 2-fold change for expression levels)

TEACHING EXPERIENCE

The University of Texas at Dallas (UTD), Dallas, Texas
Graduate Teaching Assistant
Undergraduate courses:

- **Anatomy and Physiology I**
  08/2017 - 06/2018
    - Assist students in learning all histology and anatomical models introduced in the lab.
    - Create and administer quizzes to help students prepare for lab practicals.
    - Hold open labs before each practical exam to allow students to freely review the models and ask questions.
    - Proctor and grade lecture exams and lab practical exams.
    - Examples of subjects taught: identification of many tissue types (histology), identification of almost entire muscular, skeletal, and nervous system anatomy, special senses (hearing, gustation, eye sight), physiology of the brain using EEG, etc.

- **Eukaryotic Cell and Molecular Biology**
  08/2018 - 12/2018
  - Responsible for building material for and holding 1 workshop per week to cover material that was taught by professor during lectures.
  - Hold an additional review workshop before each exam to cover all material and questions students have.
  - Proctor and grade quizzes and lecture exams.

Graduate course:

- **Biochemistry**
  01/2019 – 05/2019
  - Answer all questions students have regarding the material taught by the professor.
  - Grade weekly Article Reviews in which the students are to critically analyze the results of an assigned scientific journal, as if it was a manuscript review.
  - Grade group presentations. Students are assigned a scientific journal and must present the major results of this journal to their peers.
  - Proctor and grade exams and quizzes.

RELEVANT COURSE WORK

Graduate level:

- Cell Biology, Molecular Biology, Quantitative Biology, Biochemistry, Oncogenes, Into to Programming

Undergraduate level:

- Cell and Molecular Biology, Biochemistry, Microbiology, Organic Evolution, Genetics, Latin and Greek Terminology, Biology I&II, Medicinal Botany, Pathophysiology, Anatomy, Physiology, Chemistry and Organic Chemistry I&II, Environmental Plant Physiology

REFERENCES

Dr. Meenakshi Maitra, Senior Lecturer**,** The University of Texas at Dallas (UTD)
Email: Meenakshi.Maitra@utdallas.edu
Phone: 972-883-4588

Dr. Nicole De Nisco, Assistant Professor, The University of Texas at Dallas (UTD)
Email: Nicole.DeNisco@UTDallas.edu

Dr. Michael San Francisco, Dean of Honors College, Texas Tech University (TTU)
Email: Michael.SanFrancisco@ttu.edu