CURRICULUM VITAE

David J. Wisniewski, Ph.D. Candidate
Wisniewski.david.j@gmail.com

EDUCATION

2013- 2018	University of Maryland, Baltimore Graduate Program in Life Sciences Ph.D. candidate in Molecular Medicine - Cancer Biology track Thesis Advisor: Abraham Schneider D.D.S., Ph.D. Anticipated graduation date: December 21, 2018 - GPA: 3.82
2009-2013	Gettysburg College B.S. in Health Sciences, Minor in Biology

RESEARCH EXPERIENCE

2013-2018	University of Maryland, Baltimore Ph.D. Dissertation Research *"Elucidating the Role of Fatty Acid Synthase in Oral Carcinogenesis and Potential Therapeutics"* Advisor: Abraham Schneider D.D.S., Ph.D.
June-July 2012	Wistar Institute- Philadelphia, Pennsylvania Summer Research Internship "*Determine the differences between p53 R72 and P72 polymorphisms in response to AMPK activation"* Advisor: Dr. Maureen Murphy

PUBLICATIONS

Peer-reviewed journal articles

Wisniewski DJ, Ma T, Schneider A. Nicotine induces oral dysplastic keratinocyte migration via Fatty Acid Synthase-dependent Epidermal Growth Factor Receptor activation. Experimental Cell Research 370(2): 343-352, 2018.

Ma T*, **Wisniewski DJ***, Irhama H, Guo D, Shu Y, Schneider A. Loss of organic cation transporters impairs anti-neoplastic effects of metformin, but not phenformin, in oral squamous cell carcinoma cells. *Co-first authors. (**Manuscript in preparation**)

Wisniewski DJ, Ma T, Schneider A. High Glucose Increases Oral Dysplastic Keratinocyte Growth and Migration via Fatty Acid Synthase dependent Epidermal Growth Factor Receptor Activation (**Manuscript in preparation**)

Wisniewski DJ, Ma T, Schneider A. FASN Inhibition with TVB-3166 Induces Apoptosis, and Reduces Migration and EGFR Signaling in OSCC Cells (**Manuscript in preparation**)

Invited reviews

Wisniewski DJ, Ma T, Schneider A. Advances in the Chemopreventive Targeting of Oral Carcinogenesis. Current Oral Health Reports 2(2): 63-72, 2015.

RELEVANT SKILLS AND QUALIFICATIONS

- Adept at hypothesis conception, experimental design and execution targeted at the hypothesis, and subsequent data interpretation and analysis
- Highly experienced with *in vitro* cell culture and assays including: immunoblotting, immunofluorescence, proliferation, migration and invasion based assays, transfection (overexpression, siRNA, etc.)
- Experience with immunohistochemistry, qPCR, immunoprecipitation
- Excellent communicator, collaborator and writer
- Strong willingness and desire to learn about new topics of research, as well as learn and improve upon laboratory techniques relevant to any topic of study required
- Proficient in presenting data at seminars and conferences, as well as departmental journal club and data club
- Adept at manuscript preparation and writing as primary author
- Highly experienced with data analysis using ImageJ, GraphPad and Microsoft Excel

CONFERENCE AND SEMINAR PRESENTATIONS

Oral Presentations

April 2018 **DJ Wisniewski***, T Ma, H Irhama, D Guo, Y Shu and A Schneider. *Loss of organic cation transporters impairs anti-neoplastic effects of metformin but not phenformin in oral squamous cell carcinoma cells*. 9th Annual Cancer Biology Research Retreat. Sponsored by: University of Maryland Greenebaum Cancer Center, University of Maryland BioPark and the Graduate Program in Life Sciences. University of Maryland, Baltimore, MD.

March 2018 **DJ Wisniewski***, T Ma, and A Schneider. *High glucose drives FASN/EGFR mediated malignant-like shift in oral precancer*. American Association for Dental Research Annual Meeting, Fort Lauderdale, FL.

October 2017 **DJ Wisniewski*,** T Ma, and A Schneider. *Nicotine and hyperglycemia: potential activators of a fatty acid synthase/EGFR signaling axis in oral precancer progression*. University of Maryland, Program in Molecular Medicine, Student Seminar, Baltimore, MD

March 2017 **DJ Wisniewski*,** T Ma, JW Jones, MA Kane and A Schneider. *Nicotine-induced de novo lipogenesis promotes malignant-like behavior in oral precancer*. International Association for Dental Research Annual Meeting, San Francisco, CA.

Posters

May 2017 **DJ Wisniewski***, T Ma and A Schneider. *Potential role of nicotine-induced Fatty Acid Synthase in EGFR signaling activation in oral precancer progression*. 8th Annual Cancer Biology Research Retreat. Sponsored by: University of Maryland Greenebaum Cancer Center, University of Maryland BioPark and the Graduate Program in Life Sciences. University of Maryland, Baltimore, MD.

October 2016 **DJ Wisniewski*,** T Ma and A Schneider. *The role of nicotine induced de novo lipogenesis in oral precancer*. 1st Annual Molecular Medicine Research Retreat. University of Maryland, Baltimore, MD

May 2016 **DJ Wisniewski***, T Ma and A Schneider. *The role of nicotine-induced de novo lipogenesis in oral precancer*. 7th Annual Cancer Biology Research Retreat. Sponsored by: University of Maryland Greenebaum Cancer Center, University of Maryland BioPark and the Graduate Program in Life Sciences. University of Maryland, Baltimore, MD. **(Award-winning poster**

TEACHING TRAINING

Spring 2018 *CIRTL: An introduction to evidence based undergraduate STEM learning*

Online course with weekly meetings focused on learning and improving upon teaching methods for STEM-based courses, primarily focusing on active learning.

Spring 2018 *Tools for Securing Faculty Positions: Teaching Panel: What to know about teaching at different types of institutions*

Attended a teaching panel discussing various teaching and research responsibilities required depending on institution type and size, as well as information regarding application for teaching positions and writing teaching statements

INSTITUTIONAL SERVICE

Spring 2017 *University of Maryland Cancer Biology Retreat Planning Committee*

Responsibilities included planning, setting up and running the annual research retreat

Fall 2016 *University of Maryland Molecular Medicine Retreat Planning Committee*

Responsibilities included planning, setting up and running the research retreat

Spring 2016 *University of Maryland Molecular Medicine Mock Qualifying Exam Committee*

Acted as a committee member for pre-candidacy students to perform a mock qualifying exam and helped provide feedback prior to their real exam

PROFESSIONAL SOCIETY MEMBERSHIPS

2017 - present American Association for Dental Research
2017 - present International Association for Dental Research

REFERENCES

Abraham Schneider DDS, Ph.D.
Associate Professor
University of Maryland School of Dentistry
Department of Oncology and Diagnostic Sciences
Baltimore, MD
Phone: 410-706-6856
Email: Schneider66@umaryland.edu

Tao Ma Ph.D.
Research Associate
University of Maryland School of Dentistry
Department of Oncology and Diagnostic Sciences
Baltimore, MD
Phone: 410-706-1655
Email: Tma@umaryland.edu

Renty Franklin, Ph.D.
Professor and Interim Chair
University of Maryland School of Dentistry
Department of Oncology and Diagnostic Sciences
Baltimore, MD
Phone: 410-706-7259
Email: RFranklin@umaryland.edu

Abstract

Title of Dissertation: Elucidating the Role of Fatty Acid Synthase in Oral Carcinogenesis and Potential Therapeutics

David J. Wisniewski, Doctor of Philosophy, 2018

Dissertation Directed by: Abraham Schneider, Associate Professor, University of Maryland School of Dentistry, Department of Oncology and Diagnostic Sciences

The 5-year overall survival rate in oral squamous cell carcinoma (OSCC) has remained relatively unchanged over decades, due to late stage diagnosis and high recurrence rates. This work investigates two potential contributing risk factors associated with OSCC development: nicotine, present in traditional combustible tobacco cigarettes and electronic nicotine delivery systems, and high glucose as associated with Type II diabetes and hyperglycemia. A novel therapeutic, TVB-3166, for OSCC treatment was also studied through *in vitro* experiments, which may help improve clinical treatments for fully developed, often late-stage OSCC. Through cell viability and growth assays, scratch assays to mimic *in vitro* migration, and western blotting, we determined that both nicotine and high glucose caused oral dysplastic keratinocytes to exhibit an increase in malignant-like behavior. High expression levels of fatty acid synthase (FASN), a key *de novo* lipogenic enzyme, have been implicated in OSCC, and this work presents the first evidence that both nicotine and high glucose markedly increase oral dysplastic keratinocyte FASN expression, which drives epidermal growth factor receptor (EGFR) signaling, a key pro-oncogenic signaling pathway commonly associated with oral carcinogenesis. We also demonstrate that TVB-3166, a novel selective FASN inhibitor, induces apoptosis and reduces *in vitro* OSCC cell migration. Moreover, TVB-3166 inhibits basal EGFR activity

and several other oncogenic signaling proteins. This further establishes a potential role for FASN and EGFR not only in the progression of oral epithelial dysplastic pre-malignant lesions, but in fully-developed OSCC tumors. Overall, this work suggests that both nicotine and high glucose play a role in OSCC progression, specifically as it relates to FASN-dependent EGFR activation. Further, the novel drug TVB-3166 should be investigated in future pre-clinical animal models as a potential adjunct to OSCC therapeutics. Through an improved understanding of risk factors for OSCC development, as well as determination of novel therapeutic strategies, this work aims to improve overall patient survival through prevention of OSCC development, as well as discovery of new adjunctive treatments for fully established tumors.

Elucidating the Role of Fatty Acid Synthase in Oral Carcinogenesis and Potential Therapeutics

by
David J. Wisniewski

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2018

Dedication

To my cancer surviving mother, June Wisniewski.

To the memory of my late father, Kenneth Wisniewski.

Your strength drives me.

Acknowledgements

I would like to express my sincere gratitude to several individuals who have encouraged and supported me, as well as contributed to my doctoral studies in the Molecular Medicine program at the University of Maryland. I first would like to sincerely thank, and express my gratitude to my mentor and dissertation committee chair, Dr. Abraham Schneider, for his immeasurable help and guidance in both science and life. Thank you for showing me how to not only conduct excellent scientific research, but also for providing me with career guidance and being an excellent example of how a proper mentor should act. I cannot thank you enough, and look forward to staying in touch and collaborating together in the future.

I also would like to thank the rest of my dissertation committee, Drs. Renty Franklin, Michele Vitolo, Paul Shapiro, John Basile, and Maureen Kane. You were always easy to schedule meetings with, and provided insight into my work that provided perspective that I otherwise would not have had.

I would like to express my sincere gratitude to lab members who helped contribute to this work, through experimental assistance, collaboration, and/or technical support in experiments. First off, I would like to thank Dr. Tao Ma, who helped train me in all of the work that I performed as part of my graduate studies, and was always willing to assist me and offer advice whenever I needed it. Thank you so much for everything you have helped me with. I would also like to recognize Dr. Deepak Menon, who helped me with determination of certain experimental approaches, and provided countless assistance regarding technical aspects of certain experiments.

Last but not least, I would like to thank my family for all of their encouragement and support throughout my academic life. Thank you to my beautiful wife Emily, who was always supportive and understanding, even when I was inaccessible due to heavy workloads or stress; your presence alone in my life gives me the drive to do what I love. Thank you to my mother, June, who has always been by my side, encouraging me to do what makes me happy. Thank you to my father, Kenneth, who at a very young age instilled the importance of education in me. Thank you to my brother, James, for never understanding my topic of research but still offering support and acting like you understood. Thank you to my parent in-laws, Bonnie and Dave, who have always re-affirmed my strengths, even when sometimes I felt that I didn't have any. I would also like to give my thanks to everyone in the extended Wisniewski, Matson, Aiello, and Bathurst families, who are too many to name, but each at one point or another made me feel like what I am doing is significant and worthwhile.

The work presented in this dissertation was supported by the National Institutes of Health R01 DE023578, and University of Maryland School of Dentistry funds (to A.S.).

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Chapter One

Introduction

Problem Statement

Cancer of the oral cavity has an estimated 51,540 diagnosis in the United States in 2018, with an estimated 10,030 deaths [1]. The majority of these are oral squamous cell carcinoma (OSCC), and the traditional risk factors for OSCC development include heavy tobacco use in western countries and betel and areca nut chewing in south and south-east Asia [2, 3]. OSCC develops in the form of multistep carcinogenesis, as similar genetic alterations are observed in oral dysplastic, potentially malignant and fully established OSCC's, typically as the normal oral mucosa is exposed to carcinogens present in combustible tobacco smoke over a long period of time [4, 5].

Within the past decade, there has been a concerted effort to improve imaging for diagnosis, surgical techniques, and targeted therapies with the hope of improving patient outcomes, as 5-year survival was previously 50-60% for several decades. Traditionally, surgical resection is the standard of care for tumors (~30%), and surgical techniques have improved in recent years. Further, adjuvant radiotherapy (~50%), chemoradiotherapy (~18%) or adjuvant cetuximab treatment (~2%) in EGFR positive tumors has been carried out in order to improve patient outcomes [6-8].

A recent study compared patient outcomes from 1990-2000 and 2001-2011 to determine whether the improvements in treatment options affected patient survival rates. Indeed, this retrospective study found that overall 5-year survival improved from 59% to 70%; however, there was a noticeable increase in distant metastasis and more advanced tumor grade in patients from 2001-2011. Further, when stratified by primary tumor size,

lymph node involvement, and distant metastasis, 5-year survival drops from 81% (Stage I) to 63% (Stage II), to 55%, and 41% in Stages III and IV, respectively [7]. Therefore, to increase patient survival there is a need to improve preventive measures as well as treatment strategies for later stage OSCC.

It is common for patients to present with advanced disease, as 52% of patients present with a TNM stage of IV [7, 8]. This may be due to the fact that patients delay in going for initial diagnosis, which may contribute to the high incidence of advanced disease [9]. Further, recurrence of disease is common, as one study found 20% of patients presenting with recurring disease following treatment [10]. Considering the large population of OSCC patients with late-stage disease with poor survival, as well as tumor recurrence following treatment, indicating a need for improved treatment options, this dissertation work set out to target this clinical problem in two ways. First, it has been previously established that gaining a better understanding of risk factors, specifically lifestyle risk factors, will improve outcomes by preventing initial disease development [8]. Second, when OSCC is already developed, we must improve drug therapy options, specifically for more aggressive tumors. This leads to the focus of this dissertation: determining risk factors and molecular pathways that affect OSCC initiation and formation, as well as novel treatments which eventually may improve overall patient outcome.

Oncogenic Signaling Pathways

Cell cycle progression, metabolism, motility, survival, and angiogenesis play a significant role in the malignant behavior of cancer cells, as these processes are perturbed by deregulated signal transduction pathways. Gaining insight into these oncogenic cascades is critical, as understanding of their aberrant role within cancer cells will allow

for specific chemopreventive and therapeutic targeting, which will ultimately help control tumor development and progression, including in OSCC [11, 12].

The OSCC oncogenome has recently been characterized through analysis of tumors and cell lines in order to determine mutations, gene expression changes, epigenetic alterations, and aberrant signaling which may contribute to the malignant phenotype of OSCC [13-15]. However, it is important to determine which pathways are key "driver" pathways, and which may not critically affect tumor malignancy and response to treatment, also known as "passenger" pathways [13, 14, 16, 17].

Epidermal Growth Factor Receptor in OSCC

The majority of OSCC tumors are dependent on uncontrolled activation of the epidermal growth factor receptor (EGFR) and the phosphatidylinositol 3'-kinase (PI3K)-Akt-mammalian Target of Rapamycin (mTOR) pathway, which are commonly associated with tumor growth, survival, and motility [14, 15, 18-20]. EGFR gene amplification is common in high-grade dysplasias, and is associated with an increased risk of tumor progression [21, 22]. Amplification of the wild-type EGFR is associated with poor clinical outcomes, and the 5-year survival has been found to be only 9% in patients with EGFR amplification, compared to a survival of 71% in patients with normal copy numbers [22-27].

As a result, small molecule inhibitors and monoclonal antibodies aimed at EGFR have been synthesized in order to control this oncogenic signaling [20, 28]. Unfortunately, there is still a lack of therapeutic response to EGFR targeted drugs. This is exemplified in OSCC, where cetuximab, a monoclonal antibody against EGFR, has limited clinical

efficacy even though EGFR and its associated ligands are overexpressed in 50-100% of cases [29, 30]. Furthermore, the constitutively active truncated mutant EGFR, EGFR variant III, has been observed in approximately 40% of OSCC cases, and is associated with cetuximab resistance [31-33]. Cetuximab is currently the only "standard of care" targeted therapy that is FDA approved for head and neck squamous cell carcinomas. Cetuximab is used in adjuvant to radiation therapy, following recurrence after platinum based chemotherapy, or with platinum based chemotherapy in recurrent or metastatic disease. There are some small molecule tyrosine kinase inhibitors (TKI) and other anti-EGFR antibodies in various phases of clinical trials, however there is still an obvious lack of efficacy in terms of targeted therapy in OSCC [34]. Emerging research is being conducted by studying the role of immunotherapy in OSCC, however there is currently little evidence suggesting that this will improve overall survival [35].

Considering the clinical problems pertaining to both the current lack of successful targeted therapies in OSCC, as well as the poor late stage survival rate, this work focuses on elucidating novel pathways which may contribute to both tumor formation and progression, and may be more successfully targeted in oral premalignant lesions as well as fully established OSCCs.

Metabolic Reprogramming in Cancer

In the 1920's, Otto Warburg published a series of articles which described tumor cells as highly glycolytic, meaning increased glucose uptake and increased lactic acid production in cancer as compared to normal cells. Further, he indicated that this glycolytic phenotype in tumor cells occurred in the presence of oxygen, which is noteworthy because

normal cells utilize oxidative phosphorylation in the presence of oxygen. Oxidative phosphorylation generates energy at a more efficient rate than glycolysis, which normally occurs only in the absence of oxygen [36-38]. This phenotype published by Warburg almost 100 years ago was termed the "Warburg effect", and is still relevant in cancer research today. Warburg incorrectly hypothesized that this effect was due to dysfunctional mitochondria, as it is now known that mitochondria typically function normally in tumor cells. In fact, this altered cancer metabolism has less to do with glycolysis specifically, and more to do with the tumor cells necessity for various intermediate products involved in the glycolytic pathway [39, 40].

The Warburg effect is of great interest in cancer research, as the understanding of differences between tumor cells and normal cells will lead to specific targeting of tumor cells while not affecting normal cells, limiting the toxic side effects associated with chemotherapy, radiation therapy, and even targeted therapies. Further definition of the altered metabolism in cancer cells first described by Warburg will potentially lead to the implementation of drugs specifically targeting cancer metabolism. Although there are a number of pathways associated with altered cancer cell metabolism, the work presented in this dissertation particularly focuses on a pathway that was discovered in 1994, and it is frequently upregulated in cancer: the *de novo* lipogenesis (DNL) pathway [41].

De novo Lipogenesis and Fatty Acid Synthase in Cancer

DNL is the process by which carbohydrates are converted to free fatty acids, typically in the liver and adipose tissue (Figure 1.1) [42]. Glucose is converted to pyruvate through glycolysis, and then pyruvate is converted to citrate in the mitochondria through

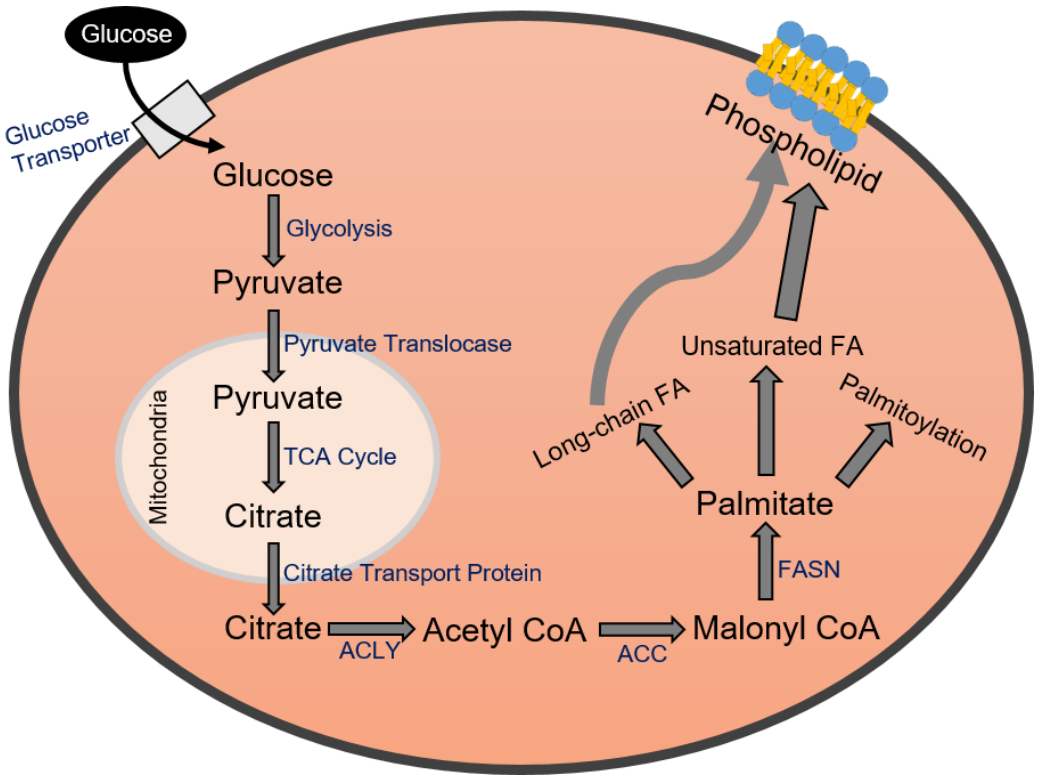

Figure 1.1. *De novo* lipogenesis.

Glucose enters the cell through glucose transporters, and is ultimately converted to pyruvate through glycolysis. Pyruvate is shuttled into the mitochondria, converted to citrate through the tricarboxylic acid cycle (TCA cycle), and shuttled back into the cytosol, where it is converted to acetyl CoA by ATP citrate lyase (ACLY). Acetyl CoA is converted to malonyl CoA by acetyl CoA carboxylase (ACC), which is ultimately processed by fatty acid synthase (FASN) to palmitate, a 16-carbon saturated fatty acid. Palmitate can be added to proteins post-translationally, a process called palmitoylation, or can be elongated and/or desaturated to long-chain fatty acids (FA) or unsaturated FA. Combinations of saturated and unsaturated fatty acids of varying lengths can be ultimately processed to phospholipids, affecting the composition of the cell membrane. This figure was adapted from Ameer *et al*., see reference [42].

the tricarboxylic acid cycle (TCA cycle). In the cytosol, a series of enzymatic reactions occur starting with ATP-citrate lyase converting citrate to acetyl CoA. Then acetyl CoA carboxylase converts acetyl CoA to malonyl CoA, and malonyl CoA is later processed by fatty acid synthase (FASN) to palmitate, a 16-carbon saturated fatty acid [42, 43]. DNL has been recently considered a process which is upregulated in cancer, and therefore presents as a targetable pathway which should not affect normal, non-hepatic cells, since the body relies very little on DNL for serum lipid homeostasis [42].

The rate-limiting, final enzyme in DNL, FASN, was characterized in breast cancer in 1994, and subsequently found to be upregulated in various cancer types, including OSCC [41, 44, 45]. Cancer cells tend to be autonomous, in that they prefer to synthesize their own lipids as opposed to relying on dietary lipids [46]. Despite the prognostic value FASN provides, the exact purpose that elevated FASN provided to cancer cells had remained elusive for years. Recently, FASN has been implicated in membrane biogenesis for rapidly proliferating cancer cells [47, 48]. FASN affects membrane biosynthesis and physiology, as the lipids synthesized by FASN, and subsequently elongated and desaturated, can be processed into various phospholipid species [49]. These changes in membrane fluidity and deformability in turn affects localization, dimerization, and even recycling of trans-membrane receptors [50]. FASN has been implicated in lipid raft formation, as well as invadopodia formation for cell migration and invasion [51, 52].

Considering the importance of several trans-membrane signaling receptors in both cancer development and progression to metastatic disease, we set out to determine whether stimulation of FASN by potential risk factors strongly associated with oral carcinogenesis

could affect oncogenic signaling in oral dysplastic keratinocytes. In addition, we wanted to elucidate whether FASN inhibitors could potentially perturb growth factor signaling, specifically EGFR signaling, considering the importance of this receptor in OSCC and the lack of effective treatments targeting this oncogenic signaling network.

The Relationship Between FASN and EGFR in Cancer

Recently, it has been reported that FASN expression in cancer can regulate EGFR activity. Since EGFR plays such an important role in oral carcinogenesis, the majority of this work focuses on determining whether FASN can indeed induce and regulate EGFR activation. Palmitoylation, the process by which the FASN end-product palmitate is covalently attached to proteins post-translationally, can induce ligand independent EGFR activation through dimerization of EGFR within the cell membrane in cancer [53, 54]. Although the specific relationship between FASN-dependent palmitoylation of EGFR is interesting, further work needs to be done in order to fully characterize these mechanisms. Our study relies solely on the effect of FASN expression on EGFR activation, as observed by phosphorylation, and brings up future directions which may ultimately define how FASN regulates EGFR activity.

FASN inhibition has been shown to inhibit EGFR activity in cancer. Yet, there is a major gap in knowledge in the field of oral oncology, where it has yet to be investigated [55, 56]. FASN has also been shown to be down-stream of EGFR, indicating a potential feed-back loop between the two proteins [57]. FASN inhibitors increase the efficacy of EGFR inhibitors in cancer, which is hypothesized to be due to the membrane altering effects of FASN inhibitors on lipid rafts and other membrane fluidity changes [56, 58].

For example, DNL increases the saturation of the lipid bilayer, altering potential biophysical interactions and fluidity in the membrane [49]. Although previous studies suggest a close relationship between FASN and EGFR in cancer, investigating this relationship further in oral carcinogenesis is paramount to elucidate pathways underlying disease development and progression, and to identify potential, more effective therapeutic approaches.

Potential Drivers and Inhibitors of FASN Expression

Due to the aforementioned evidence that FASN overexpression plays a role in cancer, this dissertation focuses on the potential up-regulation of FASN by specific risk factors associated with OSCC development, and the targeting of FASN with specific inhibitors in OSCC. Nicotine has been considered safe by the general public, as it is only thought to be the addictive component of tobacco smoke and other nicotine-containing products. However, nicotine has been recently found to induce oncogenic signaling in cancer, and has further been found to induce hepatic FASN and DNL in a rat model [59-66]. Although not currently considered a risk factor for OSCC development, taking into account the large population using electronic nicotine delivery systems including electronic cigarettes (e-cigarettes) who may have occult oral epithelial dysplasias developed due to prior heavy tobacco smoking, this dissertation work investigates whether nicotine could have pro-oncogenic effects in oral dysplastic keratinocytes, specifically as it relates to a potential upregulation of FASN expression, and whether it should be considered a future risk factor [67].

Other than nicotine, the present study investigates whether FASN could be stimulated by high glucose or hyperglycemia, a recently discovered risk factor for OSCC

development [68-70]. High glucose is a known inducer of hepatic FASN, however this relationship has not yet been investigated in oral carcinogenesis [71, 72]. Evidence supports that tobacco smokers, who are likely to develop oral dysplastic lesions, are more likely to develop hyperglycemia as it relates to Type II Diabetes Mellitus (DM). Therefore, elucidating the effect of high glucose on FASN and a potential driving of a malignant-like phenotype in non-malignant dysplastic cells is very important, especially considering the rising incidence of both diagnosed and undiagnosed Type II DM in the United States [73, 74]. Investigation of the effects of both nicotine and high glucose on FASN induction will help determine and define a potential link between DNL with two risk factors frequently associated with OSCC development.

Potential targeted therapeutics to inhibit FASN when it is highly overexpressed in cancer is crucial. Only recently has one FASN inhibitor, TVB-2640, entered phase II clinical trials in breast cancer, due to off-target toxicities associated with other FASN inhibitors [75]. Although TVB-2640 is not commercially available, we were able to acquire the commercially available drug TVB-3166, which is developed by the same company, and demonstrated to be non-toxic in *in vivo* pre-clinical animal models [76]. Determination of the effects of this drug in OSCC will help potentially characterize a novel therapeutic for this type of cancer, which is promising due to the previously described relationship of FASN with EGFR signaling in cancer. Considering the limited availability of non-chemotherapeutic drugs to treat OSCC tumors, studying novel potential drugs that may eventually improve response rates and lower toxicities on normal tissues is of utmost importance.

This work will test a general hypothesis through three specific aims:

General Hypothesis

FASN is a key mediator of EGFR activation in oral carcinogenesis, positioning itself as a unique metabolic therapeutic target in OSCC tumors

Specific Aims

Specific Aim 1. ***Elucidate whether exposure to nicotine affects FASN protein expression in oral dysplastic keratinocytes, and the role this plays in oral dysplastic keratinocyte migration***

Hypothesis: Oral dysplastic keratinocytes exposed to nicotine will acquire a migratory phenotype through FASN-dependent EGFR activation.

Approach: Through *in vitro* experimental approaches, nicotine addition to oral dysplastic keratinocytes and spontaneously immortalized normal oral keratinocytes will determine whether nicotine has any effects on FASN protein expression and EGFR activation (Y1173 phosphorylation) by western blotting. Further, the effects of nicotine-induced FASN on EGFR activation will be evaluated through both FASN chemical inhibition and genetic silencing. Also, the effects of nicotine induced FASN and EGFR on cell migration will be determined through chemical inhibition of FASN and/or EGFR. Potential upstream signaling mediators affecting the effects of nicotine on FASN and EGFR will be elucidated with various chemical inhibitors known to affect FASN expression in cancer, namely PI3K (LY294002), EGFR (cetuximab, AG1478), and mTOR (rapamycin). Lastly, generation of

a stable FASN over-expressing oral dysplastic keratinocyte cell line will elucidate whether FASN alone can activate EGFR, and whether it can induce cell migration in an EGFR dependent manner, through the use of chemical inhibition of EGFR.

Specific Aim 2. ***Determine whether high glucose induces FASN protein expression in oral dysplastic keratinocytes, and whether this affects oral dysplastic keratinocyte viability and migration***

Hypothesis: High glucose induces an increase in oral dysplastic cell viability and migration through FASN-dependent EGFR activation.

Approach: The *in vitro* effects of high glucose (20 mM) on FASN protein expression and EGFR activation (Y1173 phosphorylation) in oral dysplastic keratinocytes and spontaneously immortalized normal oral keratinocytes will be elucidated through western blotting. Further, through cell viability assays, the effects of high glucose on oral dysplastic keratinocyte viability over time, in the presence and absence of FASN and EGFR inhibitors, will define whether changes in cell viability are dependent on high glucose driven FASN and/or EGFR pathways. Cell migration changes in response to high glucose in oral dysplastic keratinocytes will be determined through scratch assays, and FASN and EGFR inhibitors will also be used in order to elucidate whether these changes are FASN and/or EGFR dependent. FASN inhibitors will be used to further elucidate whether high glucose induced EGFR activation, observed by western blot, is affected by FASN. Lastly, FASN-overexpressing dysplastic oral keratinocyte cell lines will be established. The effects of FASN overexpression on cell viability will be determined, and EGFR inhibition

will be performed in order to determine if these changes in cell viability are EGFR dependent.

Specific Aim 3. ***Examine the effects of the selective FASN inhibitor TVB-3166 on OSCC cell growth and migration.***

Hypothesis: Selective FASN inhibition with TVB-3166 inhibits EGFR activation, and will affect *in vitro* OSCC cell growth and migration.

Approach: The effects of TVB-3166 on *in vitro* OSCC cell and normal oral keratinocyte derived from human gingiva cell viability, cell number and migration will be characterized, along with the effects of TVB-3166 on EGFR activation (Y1173 phosphorylation) and apoptosis through PARP cleavage by western blotting. The effect of FASN inhibition on EGFR signaling will be further studied by stimulating cell migration with recombinant EGF after pre-treatment with TVB-3166 to determine whether FASN inhibition abrogates EGF-induced cell migration. The effect of TVB-3166 on EGFR activation by EGF will be also monitored by western blot. Lastly, TVB-3166 effects on known oncogenic protein expression will be evaluated by the proteome profiler human XL oncology array kit™.

Organization of Thesis Contents

In **Chapter 2 (**specific aim 1), we show that nicotine increased FASN protein expression in oral dysplastic keratinocytes. Considering the importance of FASN in OSCC and other cancers, this study provides cautionary evidence that although nicotine is considered relatively safe, it is indeed able to increase protein expression of an enzyme that has been shown to support cancer growth and metastasis. Further, through *in vitro* cell migration assays, we show that nicotine increases oral dysplastic keratinocyte migration,

which is a well-established hallmark of cancer. This increase in cell migration was due to FASN, as pharmacological inhibition of FASN ablated the pro-migratory effect of nicotine. Nicotine increased EGFR activation through an observed increase in phosphorylation of Y1173, and this activation of EGFR was FASN-dependent, as both pharmacological inhibition and genetic silencing of FASN reduced this effect, indicating that both nicotine and FASN play a significant role in EGFR signaling activation. Further, nicotine induced EGFR activation had a pro-migratory effect, since EGFR pharmacological inhibition ablated the pro-migratory effects of nicotine. Lastly, stable overexpression of FASN in the dysplastic oral keratinocyte (DOK) cell line, independent of nicotine, activated EGFR, and induced cell migration in an EGFR-dependent manner, again implicating FASN in having pro-migratory effects on oral dysplastic keratinocytes in an EGFR-dependent manner. Although these effects were observed in oral dysplastic keratinocytes, we did not observe these cellular changes in spontaneously immortalized normal oral keratinocytes, implying that these effects may be specific to cells already exhibiting dysplastic changes.

Taken together, these data indicate that nicotine may have pro-oncogenic effects on oral dysplastic keratinocytes, and patients with oral dysplastic lesions should be strongly advised to stop using any product containing nicotine. Further, this study shows that FASN plays a significant role in EGFR signaling activation. Given the importance of EGFR in OSCC growth, survival, and metastasis, our promising findings provide translationally relevant evidence that FASN inhibition may be a novel treatment to control aberrant EGFR signaling in both oral dysplasias and OSCC.

In **Chapter 3** (specific aim 2), we present novel evidence that high glucose, as an *in vitro* cellular model mimicking Type II DM and associated hyperglycemia, affects

FASN protein expression, as well as increases oral dysplastic keratinocyte viability and migration. This study also examines whether FASN upregulation leads to EGFR activation, and aims at providing further evidence of the close mechanistic relationship between FASN and EGFR as described in **Chapter 2**. We discovered that high glucose induced FASN activated EGFR through Y1173 phosphorylation, and that this again was a FASN specific phenomenon, as pharmacological inhibition of FASN blocked EGFR activation. Further, the increase in oral dysplastic keratinocyte cell viability and migration were due to both FASN and EGFR, as pharmacological inhibition abrogated these responses. Lastly, the FASN overexpressing DOK cell line exhibited a glucose-independent increase in cell number, plausibly through enhanced cell proliferation, which was again abrogated by EGFR pharmacological inhibition. This study provides novel insight that high glucose can make oral dysplastic keratinocytes act more malignant-like through increased cell proliferation and migration. Further, this study confirms the observed relationship between FASN and EGFR activation, as stimulation of FASN with high glucose, instead of nicotine, also results in FASN-dependent EGFR activation. Overall, these data demonstrate that high glucose triggers a pro-oncogenic effect on oral dysplastic keratinocytes, and implies that the glycemic status in patients should be always monitored in parallel with the potential development of oral dysplastic lesions.

Considering the evidence of the relationship between FASN and EGFR in oral dysplastic keratinocytes following a stimulus with nicotine or high glucose, we next elucidated in **Chapter 4** (specific aim 3) whether this event was also exemplified in OSCC cell lines with endogenously high FASN and/or EGFR expression. Using TVB-3166, a potent and selective FASN inhibitor which does not exhibit toxicity in mice, we found that

FASN inhibition reduced phosphorylated EGFR in OSCC cell lines with basal EGFR activation, and also induced apoptosis. Interestingly, TVB-3166 reduced cell migration in a cell line with high basal EGFR activation, and a cell line without, indicating that the effect of TVB-3166 on reducing OSCC migration may also be EGFR independent. However, when stimulating a non-migratory OSCC cell line with EGF to induce EGFR-dependent cell migration, TVB-3166 partially reduced the EGF-induced migratory response. Collectively, our findings indicate that EGFR plays an important role in OSCC cell growth and migration inhibition in response to the FASN inhibitor TVB-3166, although this may not be the only factor contributing to TVB-3166 drug sensitivity. We then decided to perform an antibody protein array to determine whether other intervening oncogenic factors were affected following treatment with TVB-3166 in OSCC *in vitro*, and indeed found several promising targets which can be studied in the future. Overall, this study shows that TVB-3166 induces apoptosis and reduces *in vitro* migration in OSCC cell lines, and also affects EGFR activation. This indicates that EGFR activation, as well as other oncogenic signaling markers found in the protein array, may function as potential biomarkers indicating anti-neoplastic responses triggered by FASN inhibition via TVB-3166 in OSCC.

Finally, **Chapter 5** presents a summary of the major conclusions of this dissertation work, and suggests potential future directions for this research.

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Chapter Two

Nicotine Induces Oral Dysplastic Keratinocyte Migration via Fatty Acid Synthase dependent Epidermal Growth Factor Receptor Activation[1]

Abstract

Despite advances in diagnostic and therapeutic management, oral squamous cell carcinoma (OSCC) patient survival rates have remained relatively unchanged. Thus, identifying early triggers of malignant progression is critical to prevent OSCC development. Traditionally, OSCC initiation is elicited by the frequent and direct exposure to multiple tobacco-derived carcinogens, and not by the nicotine contained in tobacco products. However, other nicotine-containing products, especially the increasingly popular electronic cigarettes (e-cigs), have unknown effects on the progression of undiagnosed tobacco-induced oral premalignant lesions, specifically in regard to the effects of nicotine. Overexpression of fatty acid synthase (FASN), a key hepatic *de novo* lipogenic enzyme, is linked to poor OSCC patient survival. Nicotine upregulates hepatic FASN, but whether this response occurs in oral dysplastic keratinocytes is unknown. We hypothesized that in oral dysplastic keratinocytes, nicotine triggers a migratory phenotype through FASN-dependent epidermal growth factor receptor (EGFR) activation, a common pro-oncogenic event supporting oral carcinogenesis. We report that in oral dysplastic cells, nicotine markedly upregulates FASN leading to FASN-dependent EGFR activation and increased cell migration. These results raise potential concerns about e-cig safety, especially when used by former tobacco smokers with occult oral premalignant lesions where nicotine could trigger oncogenic signals commonly associated with malignant progression.

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Introduction

Approximately 410,000 new cases of oral cancer, largely oral squamous cell carcinoma (OSCC), are diagnosed each year worldwide with an estimated 146,000 deaths [77]. In the United States, 51,540 newly diagnosed OSCC tumors and 10,030 deaths are expected to occur in 2018 [1]. Although treatments have unquestionably improved in recent years, the 5-year OSCC survival rate remains at around 50% [77]. Heavy tobacco use continues to be the major risk factor for OSCC development, and the poor survival can be attributed to late diagnosis along with high recurrence rates following treatment [78].

Typically, the direct and frequent exposure of healthy oral mucosa to a myriad of tobacco carcinogens, present in combustible as well as smokeless tobacco products, may lead to the formation of potentially malignant lesions that histologically exhibit different degrees of epithelial dysplastic changes [78]. In fact, it is difficult to predict the transformation rate of oral epithelial dysplasias to OSCC, as it has been reported to be between 0.13-17.5% [79]. Thus, defining determinants of neoplastic progression is critical to the field of oral carcinogenesis.

The use of electronic nicotine delivery systems, also called vaping devices or electronic cigarettes (e-cigs), is increasing at an alarming rate around the world [67]. Although nicotine is the main trigger for addiction to tobacco and tobacco-derived products, it is not considered a carcinogenic chemical *per se* [66]. Surprisingly, the effects of nicotine in the oral cavity have been largely understudied. Indeed, approximately 50% of current and former cigarette smokers, including individuals who are possibly developing potentially malignant oral dysplastic lesions, have started vaping nicotine-containing e-cigs [67]. To this end, there is a critical need to determine whether nicotine induces any

pathobiological effect on oral dysplastic keratinocytes, especially if former heavy users of combustible and/or smokeless tobacco start using a vaporized form of nicotine as part of a smoking cessation intervention.

It has been recently reported that in OSCC nicotine affects tumor cell gene expression, apoptosis evasion, migration, proliferation and chemoresistance [59-64]; yet there is little evidence available on the role of nicotine in oral precancer. One study indicates that nicotine increases markers for early cancer progression in human oral keratinocytes, while another shows nicotine is capable of suppressing apoptosis in oral premalignant cells [80, 81]. Considering the potentially large population of individuals with occult, undiagnosed oral premalignant lesions who are using nicotine-containing e-cig devices, and the gap that exists on the basic understanding of the effects of nicotine on oral dysplastic keratinocytes, we designed this study to gain mechanistic insight into the role of nicotine in oral precancerous cells.

Interestingly, nicotine increases hepatic fatty acid synthase (FASN) expression and activity [65]. FASN is responsible for the final step of *de novo* lipogenesis, and is typically not expressed in non-hepatic tissues [82]. However, in the particular case of the oral mucosa, FASN is overexpressed in oral hyperkeratosis, dysplasia, and OSCC, and is associated with histological grade and risk of recurrence [82, 83]. Moreover, FASN pharmacological inhibition reduces proliferation and migration by 50% in an *in vivo* OSCC model, implicating a role for FASN in OSCC cell behavior [84]. Furthermore, FASN appears to be very relevant to cancer since it has been linked to Epidermal Growth Factor Receptor (EGFR) activation [53, 54, 85]. In OSCC, EGFR overexpression and its aberrant pro-oncogenic signaling are strongly associated with tumor progression, advanced clinical

stages and worse survival rate outcomes [78, 86]. In fact, nicotine exposure causes EGFR-dependent growth and migratory signals in cancer cells [87-90]. Due to the significant pro-oncogenic relationship that FASN and EGFR signaling might play in oral carcinogenesis as it relates to the effects of nicotine both in hepatic and non-hepatic tissues, we hypothesized that oral dysplastic keratinocytes exposed to nicotine may acquire a migratory phenotype through FASN-dependent EGFR activation. Our data show that exposure of oral dysplastic keratinocytes to nicotine led to a marked increase in FASN protein expression and FASN-dependent EGFR phosphorylation and pathway activation. Furthermore, nicotine-stimulated FASN significantly increased oral dysplastic keratinocyte migration, a common hallmark of malignant cells. Collectively, this study provides novel insight into the role of nicotine on FASN/EGFR signaling and cell migration in oral precancerous cells.

Materials and Methods

Cell culture and reagents

Human-derived dysplastic oral keratinocytes (DOK; Millipore Sigma, St. Louis, MO) were grown and maintained in high glucose Dulbecco's modified Eagle's media (DMEM, Millipore Sigma) supplemented with 10% fetal bovine serum (FBS, Millipore Sigma), 1% L-glutamine (Gibco, Grand Island, NY), 0.05% Hydrocortisone (Millipore Sigma), 1% antibiotic/antimycotic (Millipore Sigma). DOKs were starved in low-glucose serum-free DMEM (Gibco) overnight prior to experiments, followed by continued treatment in serum-free low-glucose medium. Human-derived Leuk-1 cells (kindly provided by Dr. Hening Ren, University of Maryland, Baltimore) were grown and

maintained in Keratinocyte Serum Free medium with growth factor supplement (Gibco) and 1% antibiotic/antimycotic. Leuk-1 cells were growth factor starved overnight, and treated continuously in the growth factor free medium. Human-derived spontaneously immortalized normal oral keratinocytes (NOK-SI) (kindly provided by Dr. Silvio Gutkind, University of California, San Diego) served as a normal, non-dysplastic oral keratinocyte control cell line. NOK-SI were grown, maintained, and treated under the same conditions as Leuk-1. Cells were cultured at 37°C and 5% CO_2.

In specific experiments, cells were pre-treated for 2 hours with inhibitors LY294002 (Cell Signaling, Danvers, MA), TVB-3166 (Millipore Sigma), cetuximab (obtained from the University of Maryland School of Medicine, Greenebaum Comprehensive Cancer Center Pharmacy, Baltimore, MD), AG1478 (Millipore Sigma), or rapamycin (Cell Signaling), followed by treatment with liquid (-)-nicotine (Millipore Sigma) for the indicated times.

FASN RNA interference

Short interfering RNA (siRNA) experiments were performed as previously described [91]. Briefly, DOK and Leuk-1 cells were plated at a density of 15,000 cells/cm^2. Cells were transfected with either 50 nM control, non-targeted scramble siRNA (Qiagen #1027280, Germantown, MD) or 100 nM FASN siRNA (Cell Signaling, #12613S) diluted in HiPerfect transfection reagent (Qiagen) following the manufacturer's recommendations. After 48 hours, cells were cultured free of serum or growth factor overnight, and then treated with nicotine for 24 hours.

Western blotting

After the indicated experiments, cells were lysed with M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred onto polyvinylidene difluoride membranes, and immunodetection was performed by incubating membranes overnight at 4^{o}C with indicated antibodies as previously described [92]. The following Cell Signaling primary antibodies were used: rabbit monoclonal against FASN (#3180, 1:1000), rabbit polyclonal against phospho-Akt S473 (#9271, 1:1000), rabbit polyclonal against total Akt (#9272, 1:1000), rabbit monoclonal against phospho-EGFR Y1173 (#4407, 1:500), rabbit polyclonal against phospho-S6 S240/S244 (#2215, 1:1000), and rabbit monoclonal against total S6 (#2217, 1:1000). Rabbit polyclonal against total EGFR (#sc-03, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal against β-Actin (#A5441, 1:20000, Millipore Sigma) were also used as primary antibodies. Peroxidase labeled goat anti-rabbit IgG (#5220-0458, 1:10000) and goat anti-mouse IgG (#5450-0011, 1:20000) were purchased from Seracare (Milford, MA) and used as secondary antibodies.

Wound closure migration assay

Cells were plated onto six-well plates in complete media at a density of 100,000 cells/cm^2 in order to reach near total confluency. The following day, cells were serum or growth factor starved overnight. This was followed by a 2-hour pre-treatment with the indicated inhibitors. Next, the cell monolayer was uniformly scratched with a 200 uL pipet

tip, followed by PBS washing thrice to remove cell debris. Treatment with either nicotine and/or continued specific inhibitor was then initiated for the indicated time period. Migrating cells toward the acellular area of the wells were photographed at a 10X magnification with a digital camera attached to an Axiovert 100 A microscope. The acellular gap was measured by area with ImageJ software (National Institutes of Health, Bethesda, MD) at the time of scratching (time 0) and at the final time point. Percentage of gap closure was calculated as ([Initial Area-Final Area]/Initial Area) x100.

FASN overexpression

To assess the effects of FASN in oral dysplastic keratinocytes, DOK cells were co-transfected with plasmids encoding human FASN (pCMV-SPORT6 vector containing FASN cDNA, catalog #MHS6278-202759913, Dharmacon, Lafayette, CO) and a puromycin resistance vector to select for FASN stably transformed cells (pPUR vector, catalog #631601, Takara, Mountain View, CA). Transfections were conducted by nucleofection according to the protocol provided by the manufacturer (Amaxa Biosystems, Köln, Germany). FASN stable overexpression was confirmed by clone selection of puromycin-resistant cells that showed high FASN expression by western blotting. As negative controls we used untransfected parental DOK cells and DOK cells transfected with pPUR vector only.

MTS cell viability assay

The effect of nicotine on cell viability was evaluated through a colorimetric assay by using the CellTiter 96® AQueous One Solution (MTS) reagent following the manufacturer's protocol (Promega, Madison, WI). Oral dysplastic keratinocytes were plated overnight in triplicate in 96-well plates at a density of 7,000 cells per well in complete media. The following day, cells were serum or growth factor starved overnight, and then treated with 10 μM nicotine. After 24 hours. 10 μl MTS reagent was added directly to each well, and incubated at 37°C for 1-4 hours. Absorbance was read at 490 nm with a Biotek Epoch spectrophotometer.

Statistics

Statistical analyses were performed on experiments in triplicate by using the Prism 6.0 biostatistics program (GraphPad Software). All data were expressed as the mean value ± standard error of the mean. Results were considered statistically significant when *$p<0.05$, **$p<0.01$ or ***$p<0.001$ following one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test, unless otherwise specified. Data from experiments containing only two groups were analyzed with an unpaired, two-tailed Student's *t* test.

Results

Nicotine induces oral dysplastic keratinocyte migration.

To determine the effect of nicotine on oral dysplastic keratinocyte migration *in vitro*, we performed wound closure cell migration assays as described in the Materials and Methods section, and calculated the closure of the scratch-induced acellular gap over time. In Leuk-1 cells, which were originally derived from a dysplastic leukoplakia adjacent to an early invasive tongue SCC in a 47-year-old woman with a history of heavy smoking, exposure to nicotine led to a statistically significant acellular gap closure at 8 and 24 hours when compared to unexposed controls (Figure 2.1A and 2.1C) [93]. As shown in Figure 2.1B and 2.1D, DOK cells, which were originally isolated from a dysplastic tongue leukoplakia in a 57-year-old man who was a heavy smoker, also responded to nicotine by increasing the closure of the acellular gap area [94]. In DOK cells, however, statistical significance ($p<0.01$) was only observed at 24 hours following nicotine treatment. These findings indicate that nicotine is capable of acting as a strong inducer of cellular migration in two distinct lines of oral dysplastic keratinocytes derived from tongue leukoplakia, the most common premalignant lesion in the oral cavity [78]. Of note, nicotine did not affect normal oral keratinocyte migration (Figures 2.2A and 2.2B).

Nicotine increases FASN protein expression levels in oral dysplastic keratinocytes.

Since we found that nicotine could induce oral dysplastic keratinocyte migration *in vitro*, we decided to investigate a potential signaling pathway associated with this response. Considering the recent discovery that nicotine stimulates hepatic FASN expression, we next conducted experiments to elucidate whether this effect occurs in non-hepatic cells

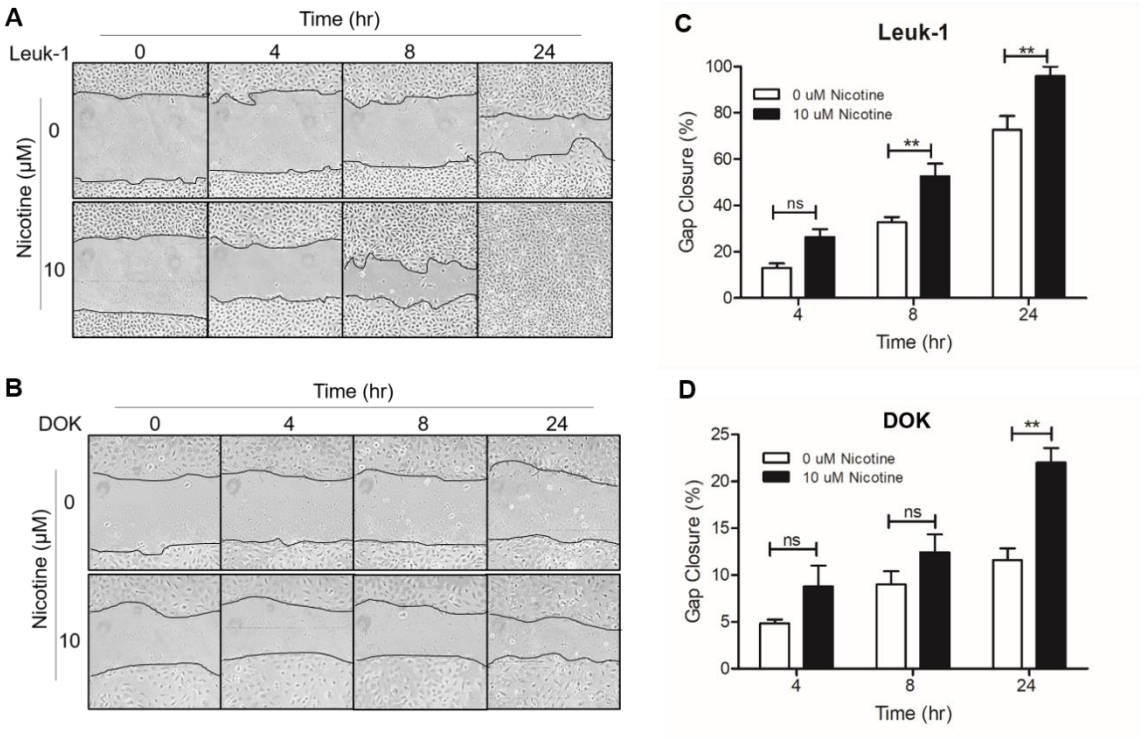

Figure 2.1. Nicotine induces oral dysplastic keratinocyte migration.

(A) Leuk-1 and (B) DOK cells were plated to confluency overnight. The next day cell monolayers were scratched with a pipet tip, followed by treatment with 10 μM nicotine or vehicle control for the indicated times. Quantification of percentage acellular gap closure is shown for Leuk-1 (C) and DOK (D) cells. Data represent mean ± S.E.M. **p<0.01, when compared to untreated control.

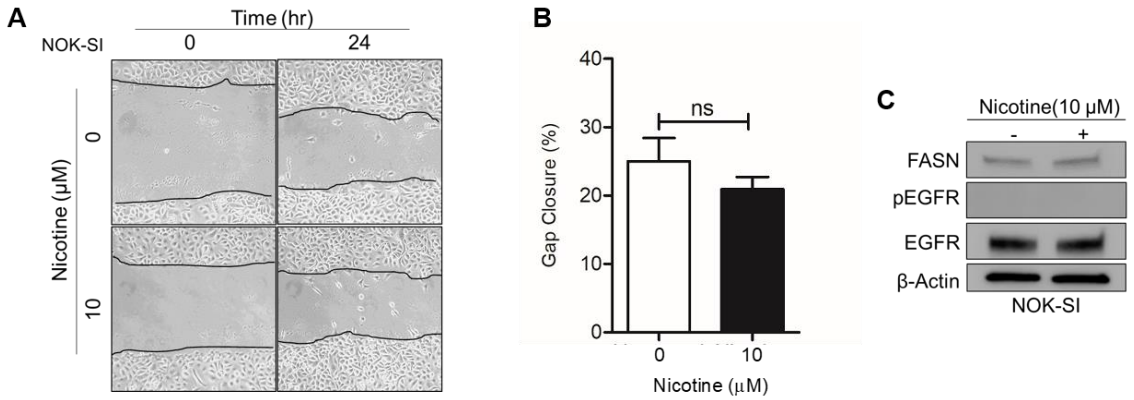

Figure 2.2. Nicotine does not alter FASN/EGFR signaling or cell migration in NOK-SI cells.

(A) NOK-SI cells were plated overnight to confluency, the cell monolayers were scratched with a pipet tip, and cells were allowed to migrate while being treated with vehicle control or 10 μM nicotine for 24 hours. Quantification of percentage acellular gap closure is shown for imaged (B) NOK-SI cells. Data represent mean ± S.E.M. "ns" indicates groups were not statistically significant when compared with unpaired, two-tailed students *t* test ($p>0.05$). (C) NOK-SI cells were treated with vehicle control or 10 μM nicotine for 24 hours. Following treatment, whole cell lysates were collected and subjected to western blotting for FASN, p-EGFR (Y1173), total EGFR, and β-Actin (loading control).

such as Leuk-1 and DOK cells [65]. Interestingly, we found that nicotine is capable of increasing FASN protein expression at time points that coincided with the stimulation of cellular migration (Figure 2.3A and 2.3B). In Leuk-1 cells, FASN upregulation was evident as early as 2 hours after nicotine treatment, and up to 24 hours. Likewise, nicotine-induced FASN in DOK cells increased as early as 1 hour, and up to 24 hours. FASN expression in normal oral keratinocytes was not affected by nicotine treatment (Figure 2.2C).

Nicotine stimulates oral dysplastic keratinocyte migration in a FASN-dependent manner.

Next, we sought to explore whether the nicotine-stimulated increase in FASN expression played a major role on the enhanced migratory phenotype of oral epithelial dysplastic cells. To this end, we performed scratch assays in both Leuk-1 and DOK cells. Before scratching the cell monolayer, cells were pre-treated with TVB-3166 (10 μM), a specific and potent FASN inhibitor, followed by nicotine exposure for 24 hours. Our data demonstrate that nicotine was capable of stimulating a statistically significant migratory response, which was markedly blunted by pre-treatment with TVB-3166 in Leuk-1 (Figure 2.3C and 2.3D) and DOK (Figure 2.3E and 2.3F), respectively. Although nicotine-induced cell migration was reduced in a statistically significant manner in both Leuk-1 and DOK cells, our findings suggest that Leuk-1 cells are more responsive to the pro-migratory effects of nicotine; therefore, the inhibitory action of TVB-3166 is more noticeable. Collectively, these results indicate that nicotine-induced oral dysplastic keratinocyte migration is in part mediated by the upregulation of FASN in response to nicotine exposure.

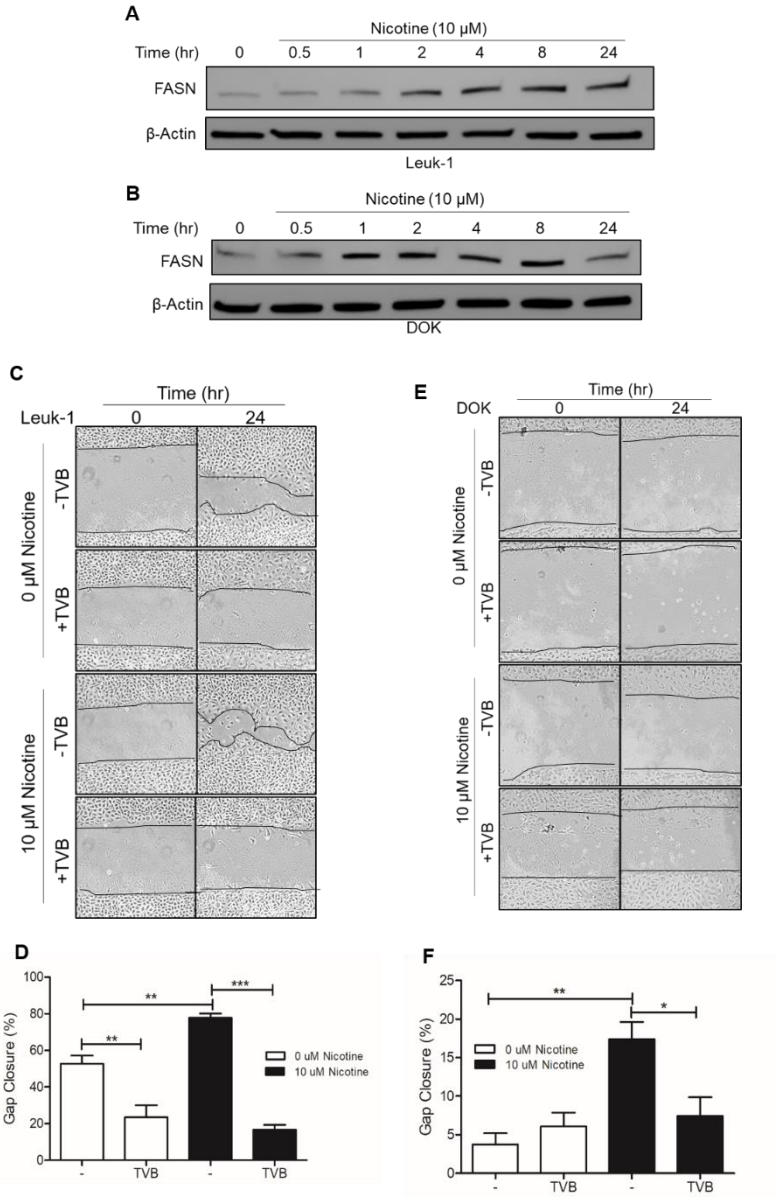

Figure 2.3. Nicotine induces FASN-dependent oral dysplastic keratinocyte migration.

(A) Leuk-1 and (B) DOK cells were treated with nicotine for the indicated times. Following treatment, whole cell lysates were collected and subjected to western blotting for FASN. β-Actin served as loading control. (C) Leuk-1 and (E) DOK cells were plated to confluency overnight, followed by pre-treatment for 2 hours with the FASN inhibitor TVB-3166 (10 μM). The cell monolayers were next scratched with a pipet tip. Following scratching, cells were left untreated or treated with 10 μM nicotine for 24 hours. Quantification of percentage acellular gap closure is shown for Leuk-1 (D) and DOK (F) cells. Data represent mean ± S.E.M. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Nicotine upregulates FASN in an EGFR/PI3K/mTOR independent manner.

Following the observation that FASN plays an important role in driving oral dysplastic keratinocyte migration, we subsequently sought to gain insight into the potential upstream signaling mediators underlying nicotine-induced FASN upregulation. We investigated the impact of the EGFR/PI3K/mTOR pathway, which is among the most well-known regulators of FASN expression in cancer cells [57, 95-97].

As depicted in Figure 2.4A and 2.4B, when both Leuk-1 and DOK cells were pre-treated with cetuximab or AG1478 (EGFR inhibitors), LY294002 (PI3K inhibitor) or rapamycin (mTOR inhibitor) prior to a 2-hour nicotine stimulation, no changes were noticed in nicotine-induced FASN expression. Noteworthy, the chemical inhibitors successfully downregulated their known downstream targets including the phosphorylated forms of Akt (PI3K target) and S6 (mTOR target), respectively. Therefore, these data suggest that the increased FASN expression in response to nicotine occurs in an EGFR/PI3K/mTOR independent manner.

Nicotine activates EGFR in a FASN-dependent manner.

Recent studies suggest that FASN may affect EGFR signaling in lung and prostate cancer cells [54]. Thus, we determined whether nicotine could act as a stimulus of EGFR activation in oral dysplastic keratinocytes. Although nicotine did not affect the phosphorylated status of EGFR after 2 hours of treatment as shown in Figure 2.4, nicotine did stimulate EGFR phosphorylation (Y1173) after a 24-hour exposure in both Leuk-1 and DOK cells (Figure 2.5A and 2.5B).

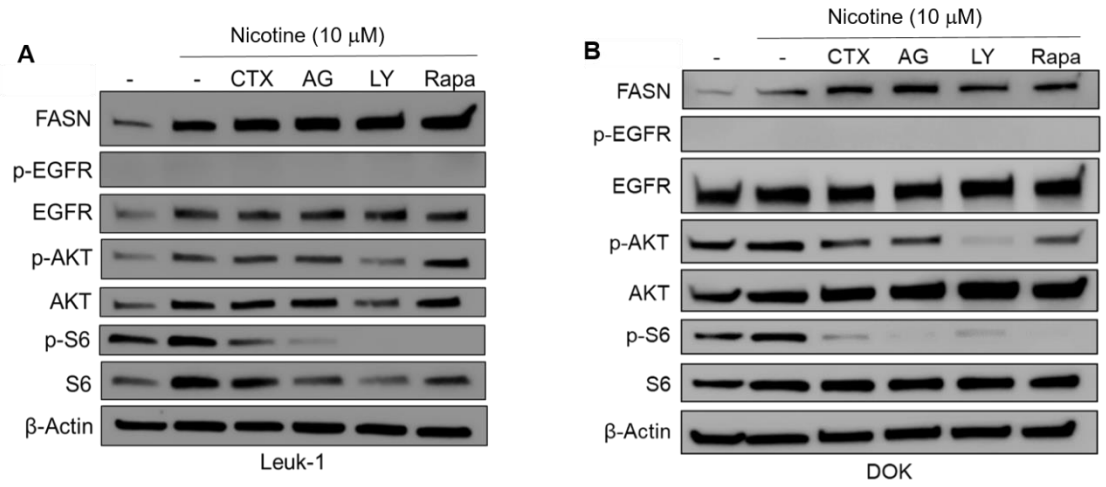

Figure 2.4. Nicotine upregulates FASN in an EGFR/PI3K/mTOR independent manner.

(A) Leuk-1 and (B) DOK cells were pre-treated for 2 hours with 10 μg/mL cetuximab (CTX), 10 μM AG1478 (AG), 10 μM LY294002 (LY), or 10 nM rapamycin (Rapa) prior to treatment with nicotine for an additional 2 hours. Then, whole cell lysates were collected and subjected to western blotting for FASN, p-EGFR (Y1173), total EGFR, p-AKT (S473), total AKT, p-S6 (S240/244), total S6 and β-Actin (as loading control).

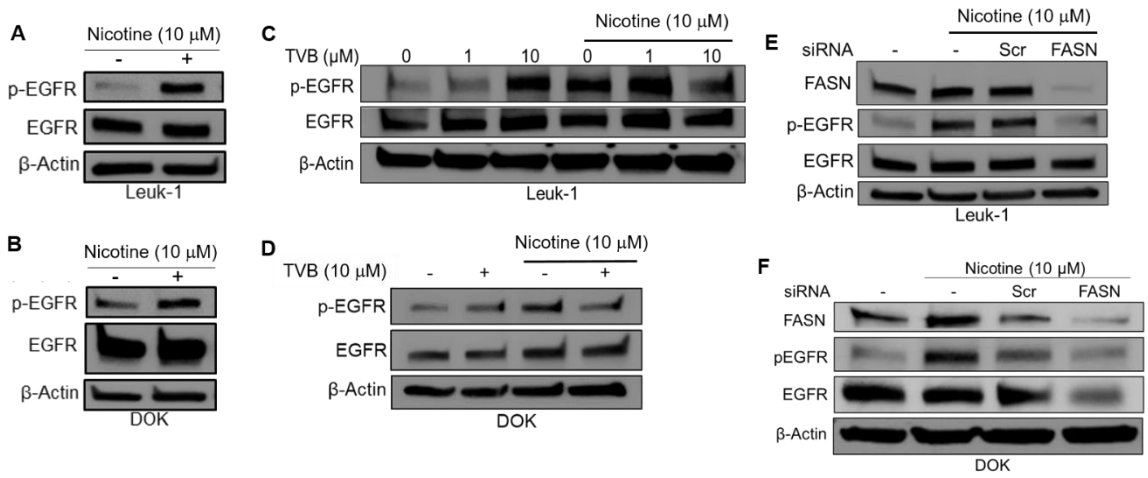

Figure 2.5. Nicotine activates EGFR in a FASN-dependent manner.

(A) Leuk-1 and (B) DOK cells were stimulated with nicotine for 24 hours. Following treatment, whole cell lysates were collected and subjected to western blotting for p-EGFR (Y1173), total EGFR and β-Actin (as loading control). (C) Leuk-1 and (D) DOK cells were pre-treated with TVB-3166, a FASN inhibitor, for 2 hours. Then, without removing the inhibitor, 10 μM nicotine was added to the media for 24 hours. Cells were then collected, lysed, and immunoblotted for p-EGFR (Y1173), total EGFR and β-Actin (loading control). (E) Leuk-1 and (F) DOK cells were transfected with either 50 nM of control scramble siRNA or 100 nM FASN siRNA followed by a 24-hour nicotine stimulation. Cells were then lysed and western blotting was performed to assess protein expression levels of FASN, p-EGFR (Y1173), total EGFR and β-Actin (loading control).

Nicotine exposure, however, did not lead to EGFR activation in normal oral keratinocytes (Figure 2.2C). To confirm that nicotine-induced FASN was indeed responsible for increasing EGFR activation, we pre-treated Leuk-1 as well as DOK cells with TVB-3166 prior to nicotine stimulation, and found that a considerable reduction in phosphorylated EGFR levels occurred when cells were treated with nicotine in the presence of the FASN inhibitor (Figure 2.5C and 2.5D).

These results were confirmed by transfecting a FASN siRNA duplex into Leuk-1 and DOK cells prior to nicotine exposure. In contrast to what was observed in untransfected or scrambled siRNA-transfected cells, FASN knock down prevented EGFR activation in response to nicotine (Figure 2.5E and 2.5F). Of note, in DOK cells, FASN knockdown also resulted in a significant decrease in total EGFR levels (Figure 2.5F), whereas this response was less evident in Leuk-1 cells (Figure 2.5E).

EGFR tyrosine kinase inhibition prevents FASN-mediated oral dysplastic keratinocyte migration in response to nicotine.

Next, we conducted experiments to define whether nicotine-induced FASN upregulation and the downstream activation of EGFR was responsible for the observed increase in cell migration. We stimulated Leuk-1 and DOK cell migration with a 24-hour exposure to nicotine in the presence or absence of the EGFR tyrosine kinase inhibitor AG1478. Our results indicate that EGFR tyrosine kinase inhibition significantly reduced nicotine-induced migration in both cell lines (Figure 2.6).

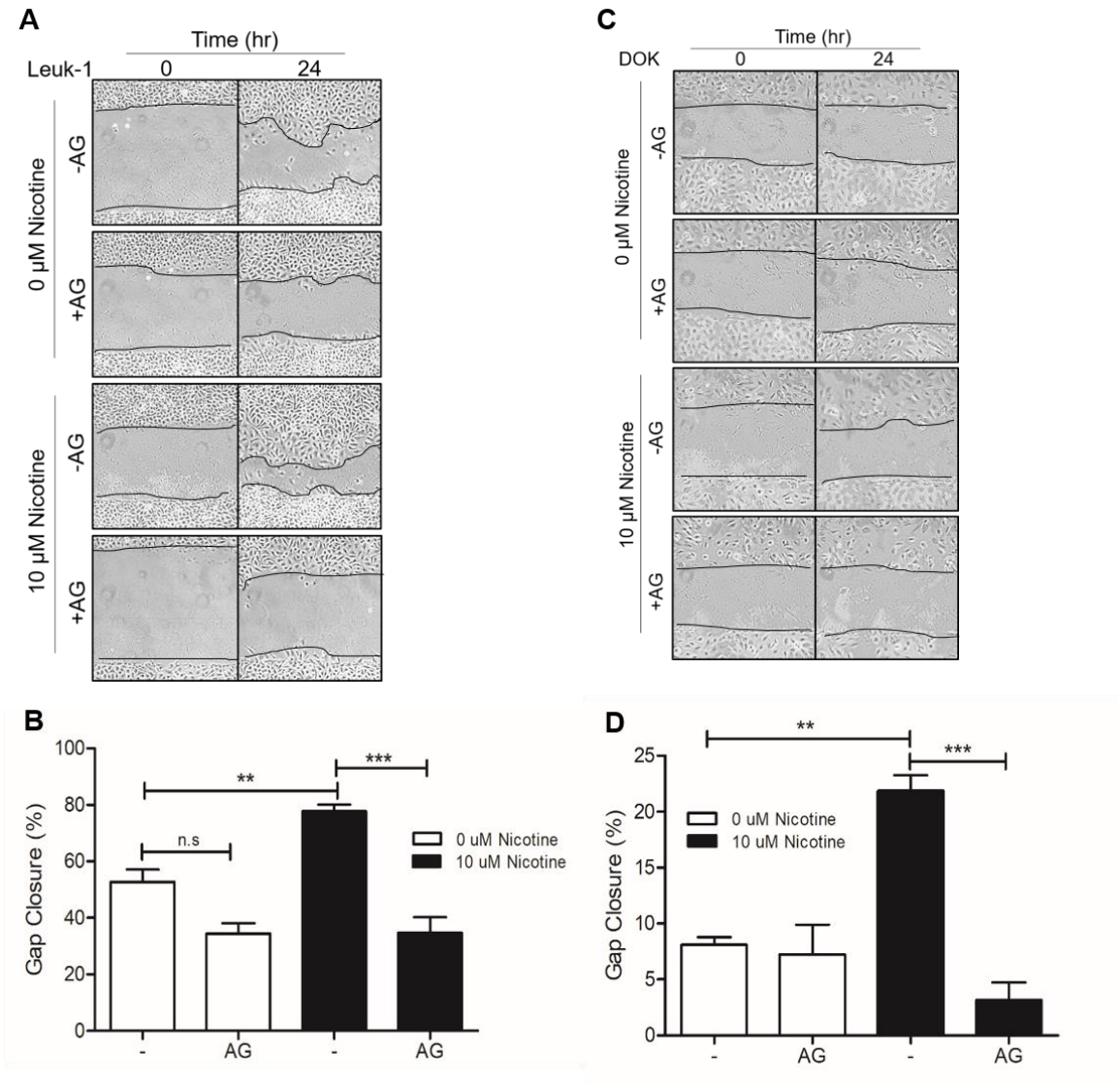

Figure 2.6. EGFR kinase inhibition prevents FASN-mediated oral dysplastic keratinocyte migration in response to nicotine.

(A) Leuk-1 and (C) DOK cells were plated overnight to confluency and pre-treated for 2 hours with 1 µM AG1478, an EGFR tyrosine kinase inhibitor. Then, cell monolayers were scratched with a pipet tip, and cells allowed to migrate with continued AG1478 treatment for 24 hours. Quantification of percentage acellular gap closure is shown for imaged Leuk-1 (B) and DOK cells (D). Data represent mean ± S.E.M. **p<0.01 and ***p<0.001.

EGFR tyrosine kinase inhibition markedly reduces cell migration in FASN-overexpressing oral dysplastic keratinocytes.

To validate these findings we stably transfected DOK cells with a FASN plasmid construct. FASN overexpression led to higher levels of phosphorylated EGFR expression when compared to parental or pPUR-transfected cells, confirming that increased FASN expression is alone capable of activating EGFR (Figure 2.7A). Further, when compared to parental cells and pPUR-transfected cells, FASN overexpression led to a marked increase in DOK cell migration (Figure 2.7B and 2.7C). Interestingly, we observed a significant reduction in cell migration when FASN-overexpressing DOK cells were pre-treated with AG1478 (Figure 2.7D and 2.7E). Overall, the data presented in this study provides new evidence demonstrating a role of nicotine on FASN-mediated EGFR activation, and the contribution of these signaling mediators in promoting a migratory phenotype in oral dysplastic keratinocytes.

Discussion

There is very little known regarding the effect of nicotine on oral soft tissues despite the popular use of nicotine-containing combustible and smokeless tobacco products, and more recently the increasing use of devices such as e-cigs. A number of studies have shown that nicotine, although not a carcinogenic product *per se*, may act as an inducer of proliferative and migratory responses in OSCC cells [59-64]. To the best of our knowledge, our study provides the first evidence that nicotine is capable of promoting a migratory phenotype, a cellular hallmark of malignancy, in oral dysplastic keratinocytes. Since nicotine containing e-cigs are increasingly being used by current and former smokers, who

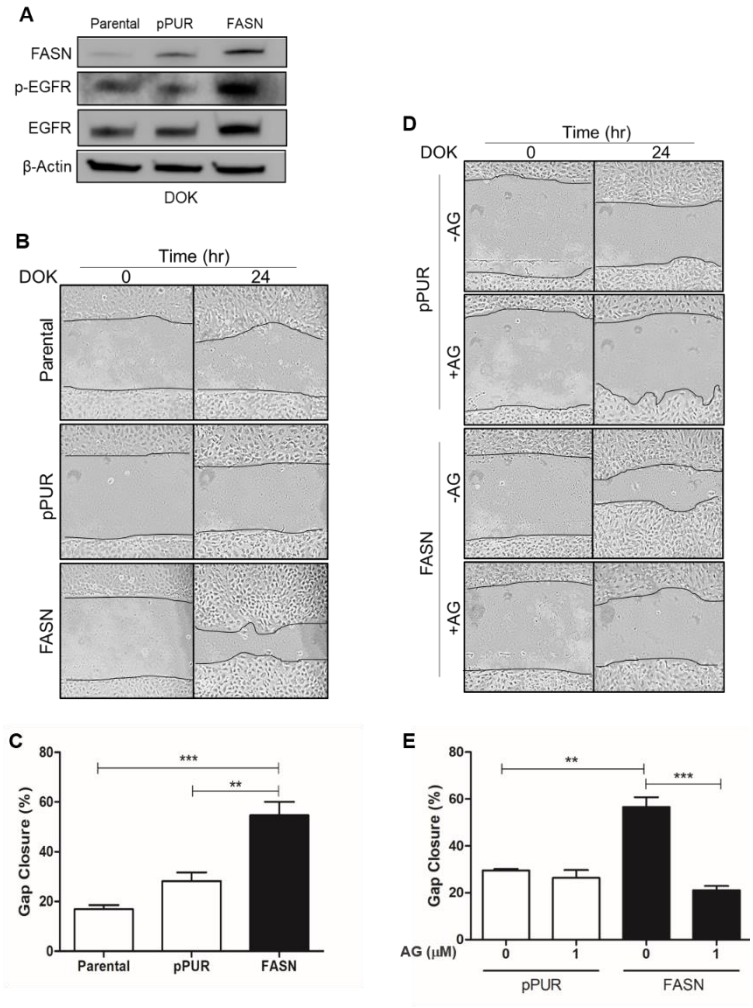

Figure 2.7. EGFR tyrosine kinase inhibition markedly reduces cell migration in FASN-overexpressing oral dysplastic keratinocytes.

(A) Parental DOK, DOK expressing pPUR vector, and FASN-overexpressing DOK cells were all cultured in serum free media for 24 hours. Then, whole cell lysates were collected and immunoblotted for FASN, p-EGFR (Y1173), total EGFR and β-Actin (as loading control). (B) Stably FASN overexpressing DOK cells were plated to confluency overnight, along with DOK parental cells (non-transfected) and DOK transfected with a puromycin-resistance vector (pPUR) as controls. Next, cell monolayers were scratched with a pipet tip, and cell migration assessed after 24 hours. (C) Quantification of percentage acellular gap closure is shown for imaged parental, pPUR-transfected and FASN-transfected DOK cells. (D) pPUR and FASN-overexpressing DOK cells were pre-treated with 1 μM AG1478 for 2 hours, the cell monolayer was scratched, and cells were allowed to migrate for 24 hours with continuous AG1478 treatment. (E) Quantification of percentage acellular gap closure is shown for imaged cells. Data represent mean ± S.E.M. **$p<0.01$ and ***$p<0.001$.

may have developed oral premalignant lesions following frequent contact with combustible tobacco-derived carcinogens, it is in fact worrisome that exposure of these lesions to vaporized nicotine may potentially trigger progression to OSCC tumors.

Most *in vitro* studies investigating nicotine in cancer use a range of 1-10 μM nicotine [98-100]. In this study, the lowest dose observed to have migratory and protein expression effects was 10 μM (data not shown), which correlates with other studies. It is worth noting that in the particular case of e-cigs, the concentration of nicotine in the e-cig vapor typically falls within the 1-10 μM range [101]. Therefore, we decided to use this concentration range, which would potentially come in direct contact with the oral mucosa. Studies elucidating the nicotine content in e-cig vapor are scarce and vary studies exploring the potential nicotine concentration exposed to the oral cavity, as well as systemically, are required to improve future studies on the effect of nicotine in the body. Regardless, we are confident that our nicotine doses are biologically relevant to e-cig vapor as well as other *in vitro* experiments [98-100].

This study clearly demonstrates that nicotine is capable of increasing FASN expression in non-hepatic cells, specifically oral dysplastic keratinocytes. As the enzyme controlling the final step of *de novo* lipogenesis, FASN contributes to membrane biosynthesis, energy storage and lipid signaling necessary for malignant cell growth. FASN synthesizes palmitate from acetyl-CoA and malonyl-CoA, which can then be processed into various other lipid types. These lipids can form membranes and lipid rafts, modify proteins for signaling and localization, and further localize growth factor receptors. Considering that most non-hepatic cell types rely on free, and not *de novo* fatty acids, this implicates FASN as a potential target for cancer treatment [102]. Interestingly, FASN has

been recently shown to affect OSCC proliferation and migration [84]. Considering this role of FASN in cancer, our findings on the effect of nicotine on FASN unravels a potential pathway for nicotine promoting pro-oncogenic effects in oral dysplastic keratinocytes.

In addition, FASN has the potential to activate EGFR, and this study presents the first evidence that nicotine triggers FASN expression, which subsequently leads to increased EGFR activation. FASN is known to activate EGFR, although not through nicotine treatment [53, 54, 85]. Since FASN has been implicated in lipid raft structure, as well as growth factor receptor dimerization, it is logical to hypothesize that nicotine is activating FASN/EGFR via cell membrane dynamics; however, current evidence of this is lacking. Palmitoylation of EGFR, the addition of palmitate to intracellular EGFR domains, is one possible mechanism for this activation, as evidenced by a recent study by Bollu *et al.* [54]. However, another study indicates that palmitoylation inhibits EGFR signaling [103]. Interestingly, when FASN was knocked down with siRNA in DOK cells (Figure 2.5F), phosphorylated and total EGFR protein levels decreased, indicating a potential effect of FASN on the regulation of EGFR stability. However, this was not observed with FASN inhibitors or in Leuk-1 cells. This underlines the fact that further understanding of the effects of FASN on EGFR biology and activity are required, specifically nicotine-induced FASN/EGFR signaling and potential effects of FASN on EGFR stability and recycling.

It is apparent from our results that the close interaction between FASN and EGFR induced by nicotine exposure is responsible for enhancing oral dysplastic keratinocyte migration. Of note, nicotine was incapable of increasing cell migration in normal oral keratinocytes (Figure 2.2A). Since the majority of OSCC tumors express high EGFR levels and a subset of those tumors rely on EGFR signaling, these findings point to nicotine as a

potential promoter of oral dysplastic keratinocyte malignant progression [78, 104]. FASN has been considered more of a cancer-associated protein as opposed to a cancer-causing protein [102]; however, based on our results FASN may play a role in the early stages of oral carcinogenesis, since even in premalignant cells, FASN is capable of activating EGFR-dependent cell migration. Although cell proliferation is another step in early carcinogenesis, we mainly focused on cell migration since nicotine treatment, as well as FASN overexpression, did not significantly affect oral premalignant cell growth over 24 hours (Figure 2.8). This confirms that the observed increase in cell migration in response to nicotine or FASN overexpression is indeed due to pro-migratory cell functions and not cell growth, which is a common cause of error in scratch assays. This does not rule out that higher doses of nicotine may induce oral dysplastic keratinocyte proliferation. Thus, future experiments must elucidate whether nicotine is capable of affecting cell growth with potentially higher doses over longer periods of time.

It remains unclear what upstream signaling mediators are responsible for nicotine-induced FASN expression and future work is warranted to gain insight into this process as we provide evidence that these effects are in part EGFR/PI3K/mTOR independent. We observed that nicotine affects not only phosphorylated EGFR status, but also slightly increases the phosphorylated forms of Akt and S6. Yet, the activation of these proteins seem to not play a key role in nicotine-induced FASN as evidenced by the lack of responses to cetuximab, AG1478, LY294002 and rapamycin. Further, nicotine may also increase total EGFR, Akt and S6 as shown in Figures 2.4 and 2.5. This, along with the rapid (<2 hours) increase in FASN protein, indicates that nicotine may potentially be affecting protein translation or degradation.

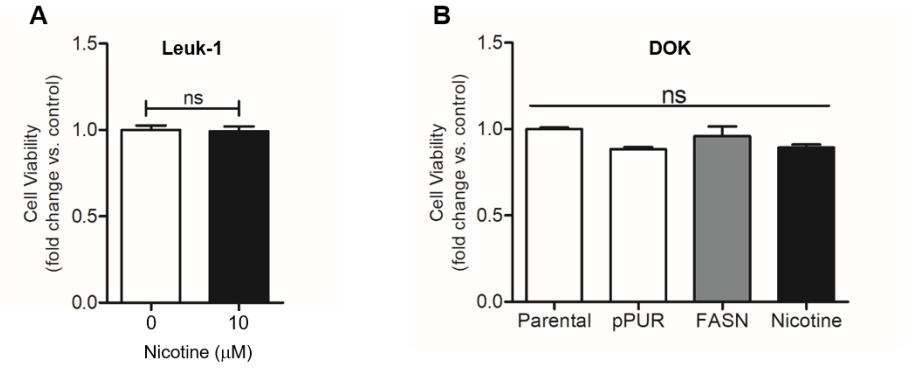

Figure 2.8. Nicotine and FASN overexpression do not affect oral dysplastic keratinocyte cell viability in 24 hours.

(A) Leuk-1 cells were treated with vehicle control or 10 μM nicotine for 24 hours. (B) Parental, pPUR and FASN DOK cells were nicotine-free, and parental DOK cells were also treated with 10 μM nicotine for 24 hours. Following treatment, MTS reagent was added, and absorbance readings at 490 nm determined changes in cell viability. Data represent mean ± S.E.M. "ns" indicates all groups are not statistically significant when compared ($p>0.05$).

In OSCC, FASN expression correlates with USP2a, a de-ubiquitinating enzyme, however this is a correlation based on immunohistochemical, and not functional, analysis [44]. In prostate cancer, USP2a stabilizes FASN expression by de-ubiquitinating FASN, therefore blocking it from proteasomal degradation [105]. Nicotine may be increasing USP2a expression, which would stabilize FASN in oral premalignant cells. Further investigation is required in order to determine if the increase in FASN expression is USP2a dependent, or if it is potentially stabilized through other mechanisms. Future studies will also determine whether nicotine affects protein translation as a potential mechanism of the observed effects in total protein changes.

Overall, our study raises concerns about the role of nicotine on the cellular behavior of oral dysplastic keratinocytes, and whether nicotine, which is present in tobacco products as well as the increasingly more popular e-cigs, acts as a promoter of malignant progression. We clearly recognize that liquid nicotine, as used in this study, might not replicate cellular responses to vaporized nicotine derived from e-cigs. However, future studies must also take into consideration the development of novel technologies for exposing oral premalignant cells, and eventually lesions, to nicotine derived from liquids used in electronic smoking devices.

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Chapter Three

High Glucose Increases Oral Dysplastic Keratinocyte Growth and Migration via Fatty Acid Synthase dependent Epidermal Growth Factor Receptor Activation

Abstract

Type II Diabetes Mellitus (DM) related hyperglycemia has recently been described as a risk factor for oral squamous cell carcinoma (OSCC) development. Further, tobacco smoking, which is known to cause oral dysplastic lesion development as well as fully established OSCC's, has recently been linked to the onset of Type II DM. Therefore, investigation into the effects of hyperglycemia on oral dysplastic lesion development and progression to OSCC is warranted. We set out to determine whether *in vitro* high glucose levels were capable of inducing a malignant-like phenotype in oral dysplastic cells by significantly increasing cell growth and migration. We recently provided the first evidence that fatty acid synthase (FASN) activates epidermal growth factor receptor (EGFR) in oral dysplastic cells. This led us to investigate whether high glucose was also capable of increasing malignancy through a FASN/EGFR signaling axis, and we found that high glucose indeed upregulates FASN protein expression. High glucose induced FASN also activated EGFR signaling, and enhanced oral dysplastic cell growth and migration in a FASN and EGFR-dependent manner. Taken together, these data imply that hyperglycemia has the potential to drive progression from oral epithelial dysplasia to OSCC through FASN mediated EGFR signaling, providing both a targetable pathway as well as two potential biomarkers for OSCC progression.

Introduction

As stated in **Chapter 2**, elucidation of potential risk factors and signaling pathways contributing to OSCC development will potentially help prevent or halt tumor development, which in turn will help improve patient outcomes [78]. Tobacco smoke is a well-established risk factor for OSCC development, as carcinogens in the smoke cause dysplastic changes within epithelial cells, leading to the formation of potentially malignant dysplasias [78]. A more recently discovered risk factor for OSCC development is hyperglycemia, or high blood glucose, as it relates to type II diabetes mellitus (DM) [68-70].

The prevalence of DM, in particular adult-onset or type II, continues to rise in the United States. Approximately 30 million people in the United States (9.3% of the population) have diabetes, with the majority being type II. Furthermore, approximately 8 million Americans remain undiagnosed, with systemic and uncontrolled hyperglycemia [73]. According to the 2014 U.S. Surgeon General's Report on Smoking and Health, new evidence indicates that smoking, the traditional risk factor in oral carcinogenesis, is also causally linked to the onset of type II DM [74]. Therefore, there is a potentially large population of individuals with developing oral dysplastic lesions who are more likely to develop hyperglycemia, of which a large sub-population may go undiagnosed and untreated.

Although the role of diabetic hyperglycemic levels (≥11 mM) in cancer development is less clear when compared to other factors such as insulin, insulin growth factors or pro-inflammatory cytokines, the independent effects of hyperglycemia in cancer development and progression cannot be ruled out [106-110]. For instance, hyperglycemic

patients, regardless of age, gender and smoking habits, were more likely to develop oral pre-malignant lesions [111]. Further, type II DM and hyperglycemia is associated with an elevated risk for OSCC development, along with decreased overall survival, and increased metastasis [68-70]. This underlines the importance of delineating a further understanding between hyperglycemia and OSCC development and progression, specifically as it relates to hyperglycemic effects on oral dysplastic lesions.

Hyperglycemia is a known stimulus for proliferation, increased growth factor signaling and chemoresistance in various cancer types [108-110, 112, 113]. However, very little investigation into the effects of hyperglycemia on dysplastic, potentially malignant lesions has been undertaken [114-116]. To our knowledge, the effect of hyperglycemia on oral dysplasias has yet to be investigated, therefore this gap in knowledge presented an interesting opportunity considering that the prevalence of DM continues to rise, and hyperglycemia correlates with oral dysplastic lesion and OSCC development. We set out to determine if hyperglycemia is capable of promoting oral dysplastic cells to behave more malignant-like, through increased cell growth and migration. Since patients with undiagnosed or even periodically monitored oral pre-cancerous lesions might eventually develop DM-associated hyperglycemia, this study will provide insight into a clinically relevant issue.

This study was also conducted to determine potential mechanistic pathways which may be responsible for disease progression in response to hyperglycemia. We previously reported that fatty acid synthase (FASN) protein expression was increased by nicotine, another potential driver of oral premalignant lesion progression, and led to an increased migratory phenotype in oral dysplastic keratinocytes [117]. Hyperglycemia has been

reported to affect FASN protein expression, as hepatic FASN was increased in a hyperglycemic rat model, and hyperglycemia induced FASN-dependent chemoresistance in breast cancer cells [71, 72].

FASN, the final key enzyme in the *de novo* lipogenic pathway, is an interesting factor to investigate for premalignant lesion progression, as it is typically overexpressed in dysplasias and OSCC, and correlates with histological grade and rate of recurrence, although it typically exhibits minimal expression in non-hepatic tissues [82, 83]. Further, FASN inhibition *in vivo* reduced OSCC proliferation and migration [84]. FASN has also been linked to Epidermal Growth Factor Receptor (EGFR) activation, which is associated with tumor progression and poor clinical outcome in OSCC, providing a potential pro-oncogenic pathway for oral dysplastic cell progression in response to hyperglycemia [53, 54, 78, 85, 86]. Emerging evidence also demonstrates that EGFR has a major role in activating pathways that mediate DM-induced systemic complications, such as kidney failure and cardiac dysfunction, further underlining that hyperglycemia may have a pro-oncogenic effect on the EGFR pathway in oral dyplasias [118-122].

Taken together, this led us to our hypothesis, that high glucose induces an increase in oral dysplastic cell viability and migration through FASN-dependent EGFR activation. Our data show that a high concentration of glucose (20 mM) significantly increases oral dysplastic cell viability and migration. Further, we demonstrated that these responses were strongly associated with FASN-dependent EGFR activation. This novel study provides insight on the role hyperglycemia has on oral dysplastic cell viability and migration, two key hallmarks of malignancy, and indicates that blood glucose levels should be monitored

in parallel with oral dysplastic lesion development, as it may play a role in the progression of oral dysplastic lesions into fully established OSCCs.

Materials and Methods

Cell culture and reagents

Leuk-1, DOK and NOK-SI cells were grown and maintained as previously described in **Chapter 2 (page 27)**. Prior to experimentation, cells were serum starved with low-glucose serum-free DMEM (Gibco) overnight. In experiments with inhibitor treatment, cells were pre-treated for 2 hours with inhibitors cerulenin, TVB-3166, AG1478 (Millipore Sigma), or cetuximab (obtained from the University of Maryland School of Medicine, Greenebaum Comprehensive Cancer Center Pharmacy, Baltimore, MD). Then, cells were continuously treated with either normal glucose (5 mM) or high glucose (20 mM) DMEM supplemented with 1% FBS, along with continued inhibitor treatment. 5 and 20 mM glucose DMEM were made by adding D-(+)-Glucose (Sigma) to glucose-free, serum-free DMEM (Gibco).

Western blotting

Western blotting was performed as previously described in **Chapter 2 (page 29)**. Cell Signaling primary antibodies were used: rabbit monoclonal against FASN (#3180, 1:1000), rabbit monoclonal against phospho-EGFR Y1173 (#4407, 1:500). Rabbit polyclonal against total EGFR (#sc-03, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal against β-Actin (#A5441, 1:20000, Millipore Sigma) were also used as primary antibodies. Secondary antibodies included peroxidase labeled goat

anti-rabbit IgG (#5220-0458, 1:10000, Seracare, Milford, MA) and goat anti-mouse IgG (#5450, 0011, 1:20000, Seracare).

Wound closure migration assay

Cells were plated to confluency, then serum starved overnight, followed by a 2 hour pre-treatment with the indicated inhibitors. Next, a uniform "wound" was formed by scratching the cell monolayer with a 200 uL pipet tip. Cell debris was removed by PBS washing. Cells were then treated with either normal glucose (5 mM) or high glucose (20 mM) DMEM with 1% FBS for the indicated time period, along with continued inhibitor treatment as indicated. Cell migration was analyzed in a similar manner as described in **Chapter 2 (page 29)**.

Oris™ cell migration assay

Oris™ cell migration (Platypus Technologies, Madison, WI) was carried out according to the manufacturer's protocol. Briefly, 50,000 cells were seeded around the cell stopper in 96 well plates. Cells were pre-treated with 10 µg/mL cetuximab for 2 hours, then the stopper was removed, creating an acellular gap in the center of each well. Cells were cultured in normal (5 mM) or high (20 mM) glucose DMEM with 1% FBS and continued inhibitor treatment for 18 hours. Cells were imaged, with time 0 control images being taken immediately following stopper removal. Migration was quantified as previously described in **Chapter 2 (page 29)**.

FASN overexpression

DOK cells were co-transfected with plasmids encoding FASN and a puromycin resistance vector (pPUR) as described in **Chapter 2 (page 30)**.

MTS cell viability assay

1,500 DOK cells or 5,000 Leuk-1 cells were plated per well in a 96 well plate in complete media and subsequently serum starved overnight. Cells were pre-treated for 2 hours with inhibitors as indicated, and then treated with normal glucose (5 mM) or high glucose (20 mM) DMEM with 1% FBS and continued inhibitor treatment, for 5 days. MTS readings were acquired as described in **Chapter 2 (page 31)**.

Cell Counting

5,000 cells per well were plated in a 96 well plate in complete media and then serum starved overnight. Following plating, DOK FASN overexpressing or DOK pPUR expressing (control) cells were cultured in normal glucose (5 mM) DMEM supplemented with 1% FBS for 5 days. Images of individual wells were taken with the Biotek Cytation 5 imaging system at the beginning of treatment (Day 0) and the end of treatment (Day 5) with a high-contrast bright field 4x objective. Cell counting analysis was performed by using a protocol for label-free cell counting provided by Biotek. In summary, images were taken slightly out of focus. Then, images underwent background flattening, and conversion to a black background. Using the provided Biotek software, cells were counted with the cellular analysis function. Change in cell number for each individual well between 0 and

5 days was calculated, and normalized relative to vehicle control in the pPUR DOK cell line.

Statistics

Experiments were carried out in triplicate, and statistical analyses were performed as described in **Chapter 2 (page 31).**

Results

High glucose increases FASN protein expression in normal and dysplastic oral keratinocytes.

Considering the increasing incidence of hyperglycemia, as well as the role of FASN in cancer, we first determined whether high glucose affected FASN protein expression in both normal and dysplastic oral keratinocytes. Leuk-1 and DOK cells, which are both oral dysplastic cell lines, were treated with high glucose (20 mM) for 2 to 24 hours, and normal glucose (5mM) for 24 hours in order to elucidate the effects of high glucose on FASN protein expression (Figure 3.1A and 3.1B). In both Leuk-1 and DOK cells, high glucose increased total FASN protein expression as compared to normal glucose at 24 hours, showing that hyperglycemia increases FASN in oral dysplastic keratinocytes *in vitro*. Interestingly, there was a slight increase in FASN expression under high glucose treatment conditions over 24 hours in spontaneously immortalized normal oral keratinocytes (NOK-SI) cells (Figure 3.1C). This indicates that the effect of high glucose on FASN protein expression is not exclusive to oral dysplastic keratinocytes.

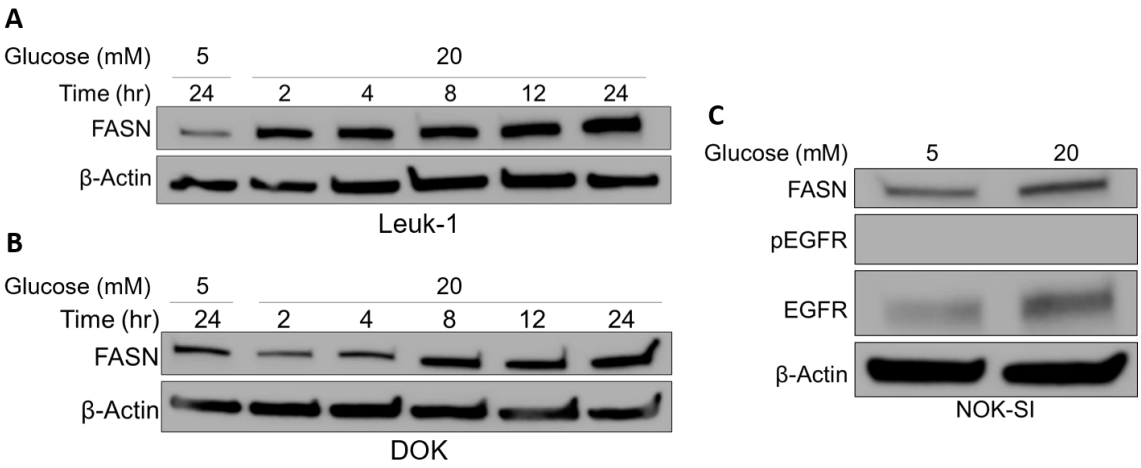


Figure 3.1. High glucose increases FASN protein expression in normal and dysplastic oral keratinocytes.

(A) Leuk-1 and (B) DOK cells were serum starved overnight, then cultured in normal (5 mM) or high (20 mM) glucose DMEM containing 1% FBS for the indicated time points. Whole cell lysates were collected and subjected to western blotting for FASN. β-Actin served as loading control. (C) NOK-SI cells were cultured similarly, except they were only exposed to normal or high glucose for 24 hours, lysed, and subjected to western blotting for FASN, pEGFR (Y1173), total EGFR, and β-Actin (loading control).

High glucose increases oral dysplastic keratinocyte cell viability over time in a FASN-dependent manner.

Following the discovery that high glucose increases FASN protein expression in normal and dysplastic oral keratinocytes *in vitro*, we evaluated whether high glucose could increase cell viability over time, a measurement likely reflective of increases in cell number. FASN has previously been associated with OSCC cell proliferation, therefore we hypothesized that the observed FASN protein expression increase by high glucose in Figure 3.1 may have an effect on cell growth. We observed statistically significant increases in cell viability in both Leuk-1 (Figure 3.2A) and DOK (Figure 3.2B) over a 5-day period in response to high glucose, indicating that there was an increase in total viable cell number as compared to normal glucose conditions ($p<0.001$). Further, in both oral dysplastic keratinocyte cell lines, FASN inhibition (by cerulenin or TVB-3166) completely abrogated the effects of high glucose on cell viability ($p<0.001$), while not affecting cell viability under normal glucose conditions. These data show that high glucose increases cell proliferation, as measured by MTS cell viability changes over time, in a manner that is dependent on the increase in FASN protein expression by high glucose, as exhibited in Figure 3.1. Interestingly, high glucose reduced NOK-SI cell viability over 3 days (Figure 3.2C), which implies that this response in oral dysplastic cells may not occur in normal oral keratinocytes. Considering there was an observed slight increase in FASN protein expression in NOK-SI cells (Figure 3.1C), this change does not seem to be significant enough to increase cell viability, as evidenced by the results in Figure 3.2C.

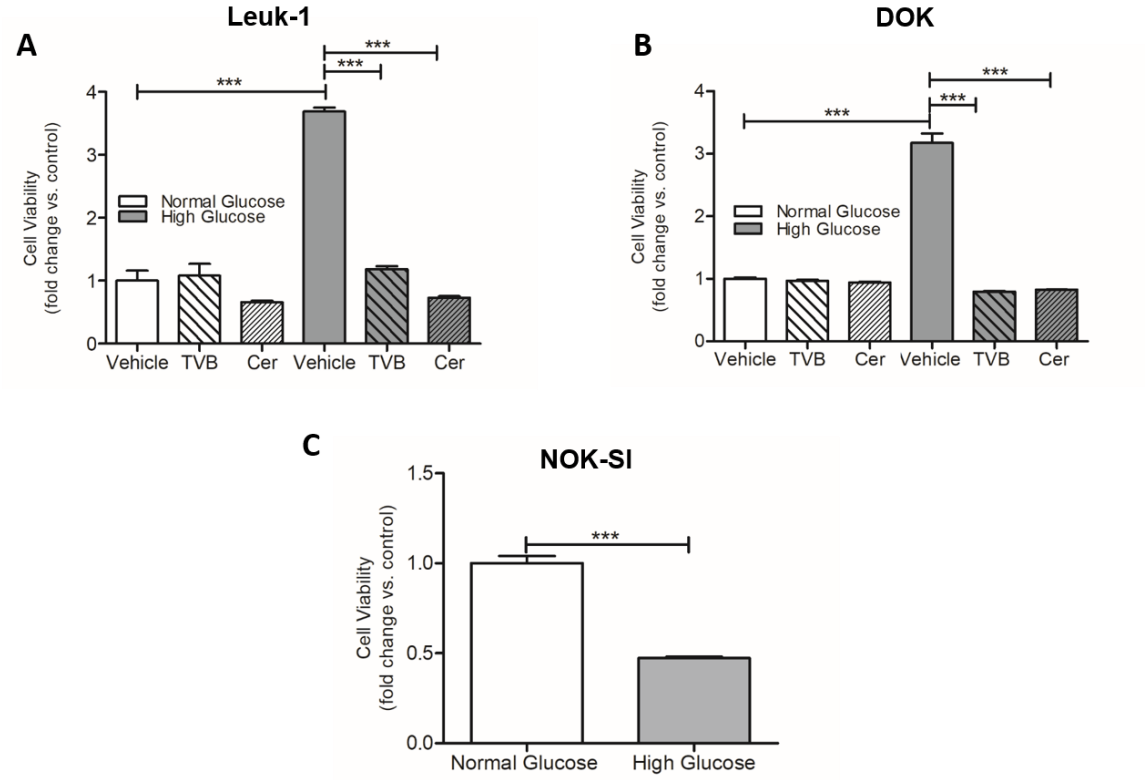


Figure 3.2. High glucose increases oral dysplastic keratinocyte cell viability over time in a FASN-dependent manner.

(A) Leuk-1 and (B) DOK cells were plated, serum starved overnight, and then pre-treated with 10 μM Cerulenin (Cer) or TVB-3166 (TVB) for 2 hours. Following inhibitor pre-treatment, cells were cultured in normal (5 mM) or high (20 mM) glucose DMEM containing 1% FBS, with continued inhibitor treatment, for 5 days. (C) NOK-SI cells were plated, serum starved overnight, and treated with normal (5 mM) or high (20 mM) glucose DMEM containing 1% FBS for 3 days. Cells were then incubated with MTS reagent and absorbance was read at 490 nm to determine cell viability. Data represent mean ± S.E.M. ***$p<0.001$ when compared to indicated treatment following one-way ANOVA and Tukey post test.

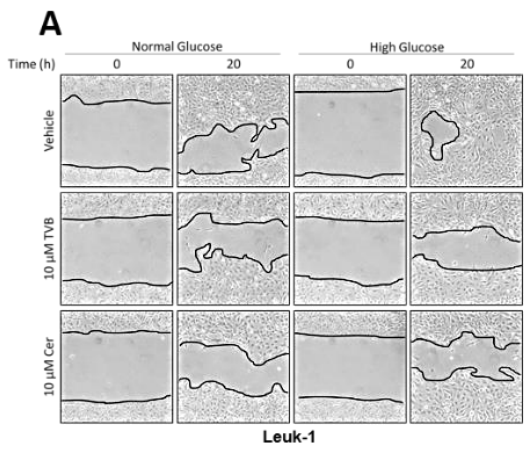
High glucose stimulates oral dysplastic keratinocyte migration in a FASN-dependent manner.

FASN has also been implicated in cancer cell migration, which led us to elucidate whether the high glucose induced FASN expression also affected the motility of oral dysplastic keratinocytes. Over 20 hours, Leuk-1 (Figure 3.3A, B) and DOK (Figure 3.3D, E) cells were cultured under normal and high glucose concentrations, in the presence of FASN inhibitors (TVB-3166 and cerulenin), and their cell migration was observed.

Not only did high glucose increase oral dysplastic cell migration *in vitro* in a statistically significant manner (Leuk-1 $p<0.05$, DOK $p<0.01$), but FASN inhibition also abrogated the high glucose induced cell migration in a statistically significant manner in both Leuk-1 ($p<0.01$) and DOK (TVB-3166 $p<0.05$, Cerulenin $p<0.01$). Taken in concert with the previous experiments in this study, high glucose increases FASN protein expression in both Leuk-1 and DOK oral dysplastic keratinocytes, and this increase in FASN increases both cell viability (over five days) and cell migration (over 20 hours). High glucose did not increase cell viability over 24 hours in Leuk-1 (Figure 3.3C) or DOK (Figure 3.3F) cells, confirming that the observed migration at 20 hours is not due to cell doubling. Further, high glucose did not stimulate cell migration in NOK-SI cells (Figure 3.3G, H), which shows that this effect of glucose may be specific to dysplastic cells and not normal oral keratinocytes.

Figure 3.3. High glucose stimulates oral dysplastic keratinocyte cell migration in a FASN-dependent manner.

(A) Leuk-1 and (D) DOK cells were plated to confluency, serum starved overnight, and pre-treated with 10 μM TVB-3166 (TVB) or Cerulenin (Cer) for 2 hours. Following pre-treatment, the cell monolayer was scratched with a pipet tip, and cultured with either normal (5mM) or high (20 mM) glucose DMEM containing 1% FBS, with continued inhibitor treatment, for 20 hours. (G) NOK-SI cells were plated to confluency, serum starved, scratched, and treated with normal (5 mM) or high (20 mM) glucose DMEM containing 1% FBS for 24 hours. Photos were acquired at the time of scratching (time 0) and end point, and gap closure was quantified as the change in acellular area for (B) Leuk-1, (E) DOK, and (H) NOK-SI cells. Leuk-1 (C) and DOK (F) cells were plated, serum starved overnight, and treated with normal (5 mM) or high (20 mM) glucose DMEM containing 1% FBS for 24 hours, then incubated with MTS reagent to determine cell viability. Absorbance at 490 nm was taken at Time 0 and 24 hours. Fold change compared to Day 0 was calculated in order to determine total change in viability over 24 hours. Data represent mean ± S.E.M. *$p<0.05$ and **$p<0.01$ when comparing 3 or more groups, one-way ANOVA and Tukey post test was carried out. An unpaired students *t* test was used to compare 2 groups, with n.s indicating $p>0.05$.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Normal Glucose
High Glucose
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20
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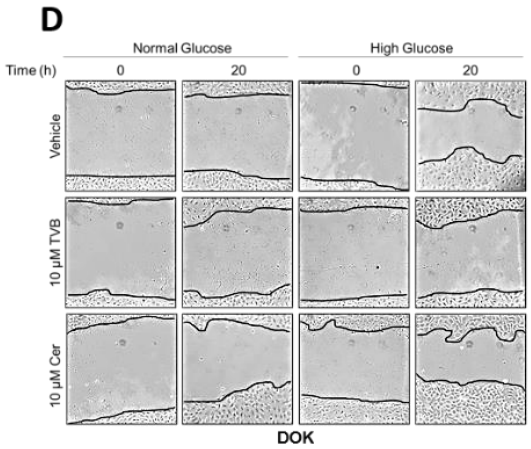
10 μM Cer
Leuk-1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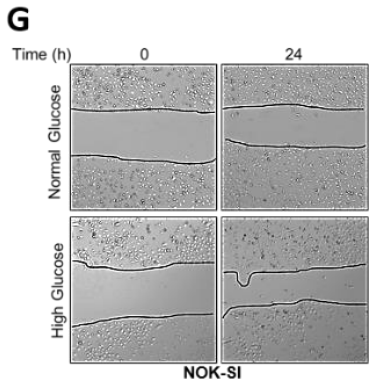
G
Time (h)
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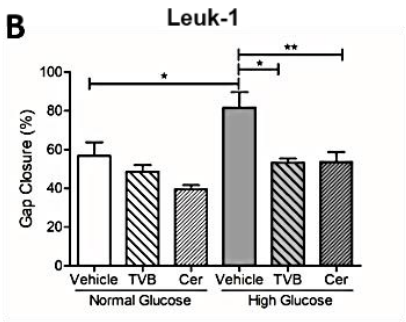
24
Normal Glucose
High Glucose
NOK-SI


B
Leuk-1
Gap Closure (%)
Vehicle
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TVB
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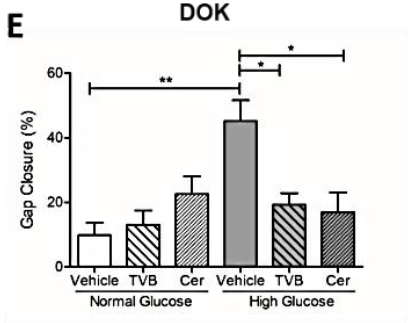
Normal Glucose
High Glucose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Gap Closure (%)
Vehicle
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Vehicle
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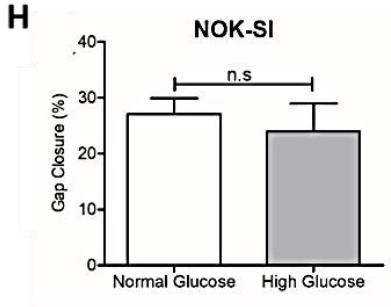
Normal Glucose
High Glucose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Gap Closure (%)
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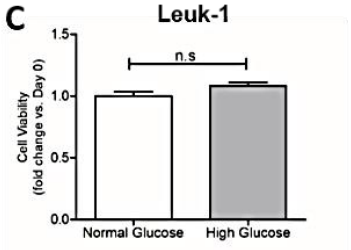
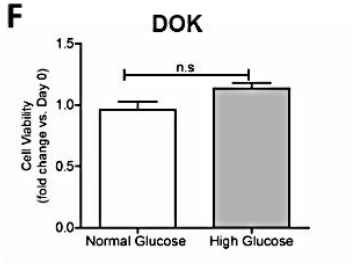
High Glucose


C
Leuk-1
n.s
Cell Viability
(fold change vs. Day 0)
Normal Glucose
High Glucose


F
DOK
n.s
Cell Viability
(fold change vs. Day 0)
Normal Glucose
High Glucose

High glucose activates EGFR in oral dysplastic keratinocytes in a FASN-dependent manner.

Since FASN has been associated with EGFR activity, and EGFR can be linked to both cell growth and migration, we wanted to determine whether high glucose induced FASN expression had any effect on EGFR activation. Considering that EGFR is phosphorylated upon activation, we used phosphorylated EGFR (Y1173) as a marker for EGFR activation. We discovered that over 24 hours of high glucose treatment, EGFR was activated in both Leuk-1 (Figure 3.4A) and DOK (Figure 3.4B) cells. Further, in both oral dysplastic cell lines we observed that FASN inhibitors Cerulenin and TVB-3166 were capable of reducing high glucose induced EGFR phosphorylation.

Therefore, high glucose activates EGFR through enhanced EGFR phosphorylation, which is dependent on high glucose induced FASN upregulation. Of note, high glucose was not capable of inducing EGFR activation in NOK-SI cells (Figure 3.1C), indicating that this response occurs in dysplastic cells but not normal oral keratinocytes. Interestingly, total EGFR levels increased in NOK-SI cells in response to high glucose.

High glucose increases oral dysplastic keratinocyte cell viability over time in an EGFR-dependent manner.

To determine if EGFR activation contributes to the previously observed increase in cell viability over time by high glucose, we treated Leuk-1 (Figure 3.5A) and DOK (Figure 3.5B) cells with EGFR inhibitors cetuximab and AG1478, and observed the effects of these inhibitors on high glucose induced cell viability over time. Both EGFR inhibitors completely abrogated high glucose induced dysplastic cell viability changes over 5 days in both cell lines in a statistically significant manner ($p<0.001$), while having no effect on cell

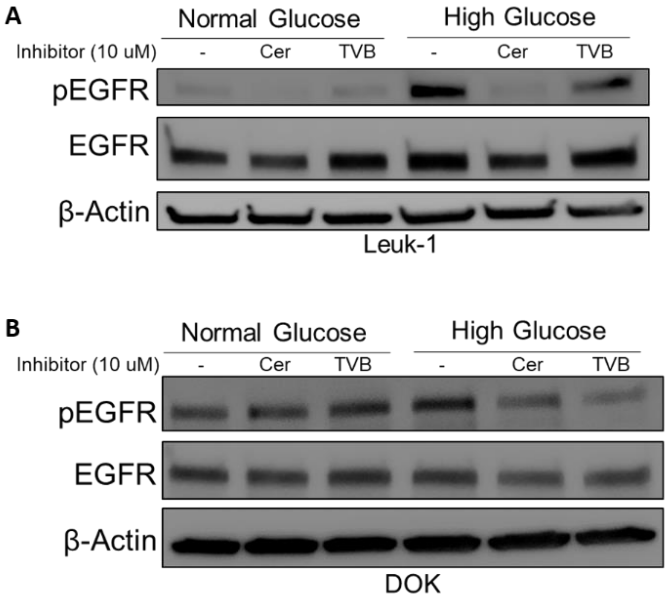


Figure 3.4. High glucose activates EGFR in oral dysplastic keratinocytes in a FASN-dependent manner.

(A) Leuk-1 and (B) DOK cells were serum starved overnight, followed by pre-treatment with 10 μM TVB-3166 (TVB) or Cerulenin (Cer) for 2 hours. Cells were then cultured with either normal (5mM) or high (20 mM) glucose DMEM containing 1% FBS, with continued inhibitor treatment, for 24 hours. Whole cell lysates were collected and subjected to western blotting for pEGFR (Y1173) and total EGFR. β-Actin served as loading control.

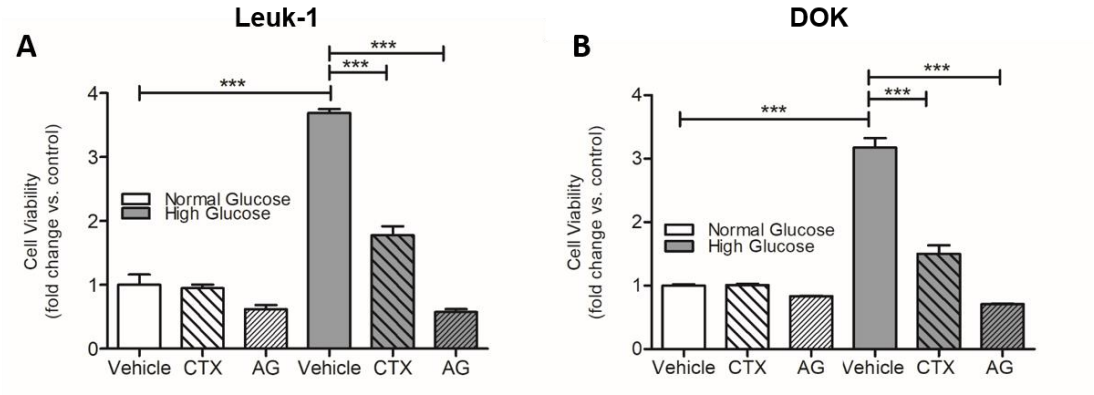


Figure 3.5. ***High glucose increases oral dysplastic keratinocyte cell viability over time in an EGFR-dependent manner.***

(A) Leuk-1 and (B) DOK cells were plated, serum starved, and pre-treated with either 10 µg/mL cetuximab (CTX) or 10 µM AG1478 (AG) for 2 hours. Following inhibitor pre-treatment, cells were cultured in normal (5 mM) or high (20 mM) glucose DMEM containing 1% FBS, with continued inhibitor treatment, for 5 days. Cells were then incubated with MTS reagent and absorbance was read at 490 nm to determine cell viability. Data represent mean ± S.E.M. ***$p<0.001$ when compared to indicated treatment following one-way ANOVA and Tukey post test.

viability in normal glucose conditions. This confirms that the increase in cell viability we previously observed to be affected by FASN is also affected by active EGFR signaling.

EGFR inhibition reduces high glucose induced oral dysplastic keratinocyte migration.

Taking into account that EGFR activity also affects cell migration, we wanted to determine if high glucose induced oral dysplastic keratinocyte migration was also due to EGFR activation. Therefore, we carried out a wound healing assay similar to Figure 3.3, however we treated Leuk-1 (Figure 3.6A) and DOK (Figure 3.6C) cells with the EGFR inhibitors cetuximab and AG1478 in both normal glucose and high glucose.

Through quantification of gap closure, we observed that Leuk-1 (Figure 3.6B) and DOK (Figure 3.6D) both had high glucose induced migration reduced by EGFR inhibition in a statistically significant manner ($p<0.001$). These data suggest that EGFR activity is necessary for the observed increases in dysplastic oral keratinocyte migration in response to high glucose.

FASN induces oral dysplastic keratinocyte proliferation in an EGFR-dependent manner.

Next, we set out to determine whether FASN overexpression alone, independent of glucose concentration, affected oral dysplastic keratinocyte cell growth in an EGFR dependent manner. We previously created a stable FASN overexpressing DOK cell line, in which we observed that FASN overexpression alone was capable of inducing cell migration in an EGFR dependent manner [117]. Here, we planned to observe whether that same FASN overexpressing DOK cell line was capable of a higher cell viability over time, indicating an increase in cell growth, and whether that was driven by EGFR activation.

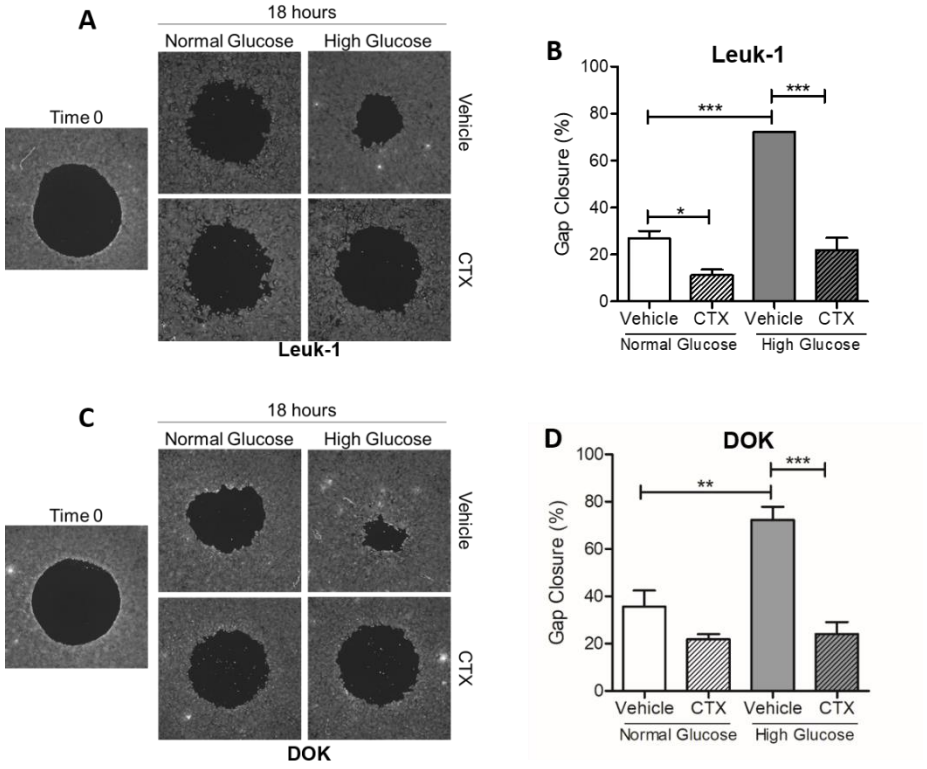


Figure 3.6. High glucose stimulates oral dysplastic keratinocyte cell migration in an EGFR-dependent manner.

(A) Leuk-1 and (C) DOK cells were plated to confluency in an Oris™ cell migration 96 well plate, serum starved overnight, and then pre-treated with 10 µg/mL cetuximab (CTX) for 2 hours. Following pre-treatment, the cell stopper insert was removed and cells were cultured with either normal (5mM) or high (20 mM) glucose DMEM containing 1% FBS, with continued inhibitor treatment, for 18 hours. Gap closure was quantified as the change in acellular area for both (B) Leuk-1 and (D) DOK cells. Data represent mean ± S.E.M. *p<0.05, **p<0.01 and ***p<0.001 when compared to indicated treatment following one-way ANOVA and Tukey post test.

Over a 5-day period, FASN overexpressing cells had higher cell viability as compared to both untransfected parental DOK cells and pPUR control cells in an MTS assay (Figure 3.7A). Cell viability was determined at Day 0 and Day 5 to control for the fact that FASN overexpressing cells may have an altered basal MTS reading. To confirm the role of EGFR in this increase in cell viability, as well as to determine if these changes were indeed due to increases in cell number, we imaged and subsequently counted total cell number in both the pPUR control cell line and the FASN overexpressing cell line over time (Figure 3.7B). Indeed, total cell number over 5 days increased in the FASN DOK cells, and this increase in cell number was abrogated in a statistically significant manner when treated with cetuximab ($p<0.001$). This confirms that higher protein expression levels of FASN alone in DOK cells increased cell proliferation in an EGFR activation-dependent fashion.

Discussion

Since hyperglycemia has recently become an established risk factor for OSCC development, along with the recent evidence that smoking tobacco, a known carcinogen, is linked to the onset of type II DM, we sought to determine whether the potential premalignant lesions developing from tobacco smoking might be detrimentally affected by hyperglycemia [68-79, 111]. To the best of our knowledge, we present the first evidence that high glucose markedly drives oral dysplastic cell growth and migration *in vitro*.

Oral premalignant lesions may develop as a result of frequent exposure of oral mucosa to carcinogens in tobacco smoke, but also in association with hyperglycemia. Considering that a large population of Americans with hyperglycemia are undiagnosed and

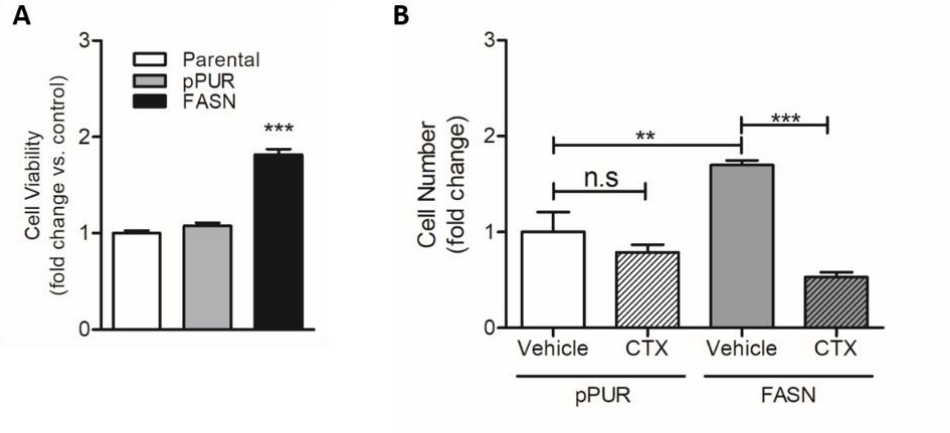


Figure 3.7. FASN overexpression stimulates oral dysplastic keratinocyte cell growth over time in an EGFR-dependent manner.

(A) Parental DOK cells, pPUR transfected DOK cells (serving as empty vector) and FASN transfected DOK cells were plated, serum starved overnight, and cultured in normal glucose (5mM) DMEM containing 1% FBS for 5 days. Cells were then incubated with MTS reagent and absorbance was read at 490 nm to determine cell viability. MTS readings from the end-point were compared with MTS readings from the time of plating (time 0) in order to normalize viability increases within each individual cell line. Data represent mean ± S.E.M. ***$p<0.001$ when compared to indicated treatment following one-way ANOVA and Tukey post test. (B) pPUR transfected DOK cells (serving as empty vector) and FASN transfected DOK cells were plated equally, serum starved overnight, then pre-treated with 10 µg/mL cetuximab (CTX) for 2 hours. Cells were then incubated in normal glucose (5 mM) DMEM with 1% FBS and continued inhibitor treatment for 5 days. Images were acquired with the Biotek Cytation 5 imaging system after 5 days, and cell counting was carried out for individual wells. Data represent mean ± S.E.M, with **$p<0.01$ and ***$p<0.001$ when compared to indicated treatment following one-way ANOVA and Tukey post test. N.S. indicates data not significant ($p>0.05$).

therefore untreated, these data indicate that both oral dysplastic lesions as well as blood glucose levels should be closely monitored in current and former smokers.

Typically, 5 mM glucose is considered a "normal" glucose concentration, as this is within the blood glucose concentration range in healthy, non-diabetic adults. Although the majority of *in vitro* studies investigating hyperglycemia and cancer have used 25 mM glucose, we used 20 mM, as it is rare for uncontrolled hyperglycemia to rise above 15-20 mM [108-110, 112, 113]. A plasma glucose level >11 mM is required for a diagnosis of diabetes, and this concentration can rise up to 20 mM in severe uncontrolled cases [123]. Further, mice which are homozygous for an obese spontaneous mutation (ob/ob) which serve as experimental pre-clinical models for hyperglycemia exhibited blood glucose of between 200-400 mg/dl, or approximately 11-22 mM [124]. Therefore, we decided to use 20 mM as our high glucose treatment, as it is in the upper range of known *in vivo* blood glucose concentrations.

Although high glucose has been reported to stimulate FASN expression, to our knowledge it has yet to be investigated in oral dysplastic cells or OSCC [71, 72]. We wanted to determine whether this pathway was also contributing to our observed effects of high glucose on oral dysplastic cell growth and migration. Indeed, we discovered that high glucose in oral dysplastic keratinocytes increased FASN, and that FASN upregulation was responsible for potentiating oral dysplastic cell growth and migration. FASN has previously been discussed as a targetable enzyme in established solid tumors, however the role of FASN in disease initiation has only recently been evaluated, specifically in breast epithelial cells [85]. We recently confirmed the potentially oncogenic role of FASN in oral epithelial dysplasias, as FASN overexpression alone in dysplastic oral keratinocytes

stimulated cell migration, and in this current study we showed that FASN overexpression also increased cell growth [117]. Considering the mounting evidence that FASN contributes to the malignancy of several cancer types, this study provides evidence that FASN, as stimulated by high glucose, is capable of promoting malignant characteristics of non-malignant oral epithelial dysplastic cells.

It has been hypothesized that FASN is necessary for localization and modification of growth factor receptors in the cell membrane [125]. FASN has recently been implicated in EGFR activation, as evidence supports that the addition of palmitate, the lipid synthesized by FASN, to the intracellular domain of EGFR causes dimerization and activation of downstream EGFR signaling [53, 54]. These recent findings in non-small cell lung cancer and prostate cancer cell lines led us to investigate whether the increase in FASN in response to high glucose could potentially activate EGFR, which is typically associated with malignant growth and migration in cancer cells. We discovered that high glucose increased EGFR activation as observed by phosphorylation of Y1173. Further, inhibition of FASN reduced this activation of EGFR, indicating that FASN plays a role in activating EGFR in response to high glucose in oral dysplastic cells. Moreover, EGFR activation contributed to high glucose induced oral dysplastic cell growth and migration, as EGFR inhibitors blocked high glucose driven cell growth and migration. Considering we have previously shown that nicotine also stimulates EGFR activity in response to FASN stimulation in oral dysplastic keratinocytes, and the gap in knowledge regarding the relationship between FASN and EGFR activation, specifically in oral dysplasia and OSCC, future directions should be focused on elucidating the exact mechanisms by which FASN acts upon EGFR intracellular signaling [117]. Since FASN also affects the lipid

composition of the cell membrane, it is possible that FASN is modifying cell membrane dynamics by altering lipid rafts or other structures [125]. Interestingly, high glucose increased FASN and total EGFR, but not pEGFR, in normal oral keratinocytes (NOK-SI). Considering the role of EGFR amplification in OSCC development, future studies may elucidate if this increase in total EGFR in normal oral keratinocytes is capable of inducing malignancy over longer time points [29, 30].

Although we found that high glucose-induced FASN upregulation leads to EGFR activation, and this results in increased oral dysplastic keratinocyte growth and migration, we did not investigate which upstream factors mediate high glucose induced FASN. It is likely that these observed effects of high glucose on oral dysplastic keratinocytes is downstream of glucose transporter 1 (Glut1), the most widely distributed and heavily implicated glucose transporter in cancer [126]. Future experiments should explore whether these effects of glucose are dependent on Glut1 function. Likewise, elucidation of underlying mechanisms regulating FASN function should be considered in future studies. FASN has been shown to be regulated by a de-ubiquitinating enzyme USP2a, as well as transcriptionally controlled by sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate-responsive element binding protein (ChREBP) [105, 127]. Our study did not specifically study whether FASN is being upregulated in response to high glucose through transcriptional control, proteosomal degradation, or other potential regulatory pathways. Further understanding of the response of dysplastic cells to high glucose, as well as how FASN and EGFR are intertwined in malignant processes, will help identify future targetable pathways as they relate to cancer development and progression. In fact, if FASN

is affecting EGFR activity, especially through membrane dynamics, it is possible FASN is altering other signaling pathways as well, and this requires further investigation.

The role of hyperglycemia in oral dysplasia progression as it relates to FASN/EGFR signaling can be further investigated through *in vivo* mouse models. There are several viable hyperglycemic mouse models, including the aforementioned ob/ob model, as well as the Akita mouse and the non-obese diabetic mouse (NOD), among others [124]. 4-Nitroquinoline 1-oxide (4-NQO) is a known orally administered carcinogen that induces OSCC development when introduced in drinking water for rodents [128]. A potentially novel, oral-specific model would use hyperglycemic and normal mice with or without 4-NQO treatment to determine if this had any effect on dysplastic and OSCC tumor development in mice. Further, we could determine if FASN expression was affected through gene and protein expression analysis of these lesions. Potential dosing with both cetuximab and the FASN inhibitor TVB-3166 in normoglycemic and hyperglycemic mice could further elucidate whether these two pathways play a key role in chemically-induced OSCC development in response to 4-NQO administration.

Overall, this study raises concerns regarding hyperglycemia and oral carcinogenesis, as high glucose appears to act as a strong driver of oral dysplastic keratinocyte growth and migration in a FASN/EGFR dependent manner. Notably, this study implicates FASN as a potential key metabolic mediator capable of inducing oncogenic signaling through EGFR activation. Considering that tobacco smoking is associated with hyperglycemia, this study provides evidence that premalignant lesion development and progression should be closely monitored with blood glucose levels, as

potentially undiagnosed and untreated hyperglycemia could play a role in oral dysplastic lesions progressing to fully established OSCC's.

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Chapter Four

FASN Inhibition with TVB-3166 Induces Apoptosis, and Reduces Migration and EGFR Signaling in OSCC Cells

Abstract

Novel therapeutics for oral squamous cell carcinoma (OSCC), especially in advanced cases, are critically needed in order to significantly improve overall 5-year patient survival. Fatty acid synthase (FASN) expression has recently been linked to OSCC cell growth and metastasis. Considering the key role that the epidermal growth factor receptor (EGFR) plays in OSCC development and progression, and recent findings that FASN may enhance EGFR activity, this study was designed to investigate the effects of TVB-3166, a novel FASN inhibitor, in OSCC cell growth and migration. Preclinical studies in mice have shown that TVB-3166 lacks side effects, a promising development since use of early generation FASN inhibitors resulted in severe anorexia and weight loss. Here, we hypothesized that FASN inhibition with TVB-3166 markedly reduces OSCC cell growth and migration by negatively affecting EGFR activation. We found that TVB-3166 induced apoptotic cell death in a number of OSCC cell lines, but did not affect the growth of normal oral keratinocytes (NOK). Interestingly, TVB-3166 significantly reduced OSCC cell migration, downregulated basal EGFR activation, and prevented EGF-induced EGFR activation and cell migration. Notably, we observed through an antibody array that the expression of several cancer-associated proteins was clearly altered in response to TVB-3166 treatment, suggesting that other oncogenic pathways might also be affected by targeting FASN. These findings provide the basis for future studies aimed at elucidating the effects of FASN inhibition, by TVB-3166 or similar formulations, on specific oncogenic pathways associated with OSCC progression. Moreover, these promising results

present exciting possibilities for exploring the therapeutic effects of novel, non-toxic FASN inhibitors in pre-clinical models of oral carcinogenesis.

Introduction

Following our discovery that both nicotine and high glucose induced fatty acid synthase (FASN) protein expression played a role in epidermal growth factor receptor (EGFR) activation in oral dysplastic keratinocytes, we sought to examine whether this phenomenon was also identified in oral squamous cell carcinoma (OSCC) cell lines, more specifically in OSCC cells with basal EGFR activation [117]. FASN-dependent EGFR palmitoylation has been recently implicated in ligand-independent EGFR activation, and subsequent downstream signaling in lung and pancreatic cancer [53, 54]. In addition, FASN inhibition may alter cell membrane structures such as lipid rafts, further perturbing EGFR or other oncogenic signaling networks [76].

Preliminary studies show that when FASN was first characterized as a potential targetable enzyme in cancer, cerulenin was the inhibitor of choice [41]. Cerulenin is a naturally occurring antifungal antibiotic but not a specific FASN inhibitor [129]. Although cerulenin inhibits the β-ketoacyl-acyl-carrier-protein (ACP) synthase activity of FASN, which is responsible for priming acyl-ACP with malonyl-ACP, it also negatively affects the synthase activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), a key mediator of steroid biosynthesis [130, 131]. Moreover, cerulenin stimulates fatty acid oxidation by activating carnitine palmitoyltransferase I (CPT1), which causes weight loss and raised concerns about the clinical translational relevance [132]. C75, a synthetic analog of cerulenin, was later developed with the expectation that this would be more specific to

FASN and not as toxic *in vivo*. Unfortunately, despite potent FASN inhibition and noticeable toxicity in cancer cells, C75 treatment also resulted in anorexia and weight loss in mouse models [55]. Due to the strong reliance of cancer cells on FASN function, the gap in research into novel, specific, and non-toxic inhibitors became more glaring.

To this end, newly developed FASN inhibitory agents are emerging with reported anti-neoplastic effects and the advantage of not inducing weight loss and anorexia in mouse models. In particular, TVB-2640 was recently developed by 3-V Biosciences (Menlo Park, CA) and has progressed to Phase II clinical trials for breast cancer treatment [75]. Although TVB-2640 has been described as a potent FASN inhibitor with anti-tumor properties, the exact mechanism underlying these effects has not been disclosed [102]. TVB-3166, which was also developed by 3-V Biosciences, is commercially available and inhibits the keto-reductase activity of FASN, which is responsible for reducing a ketone group in malonyl-ACP to a hydroxyl group, and does not exhibit any significant weight loss in mouse models [76]. Although FASN is normally expressed in liver and adipose tissue, its inhibition is not expected to negatively affect these tissues, as *de novo* lipogenesis is only a minor contributor to serum lipid homeostasis [42].

The IC_{50} of TVB-3166 is approximately 100 nM, and it has been reported that TVB-3166 inhibits both the PI3K-AKT-mTOR pathway as well as the β-catenin pathway. It also induces apoptosis in prostate and lung cancer cell lines and xenografts [76]. Despite the newly established relationship between FASN and EGFR, the effect of TVB-3166 on EGFR has yet to be identified. Citing the strong evidence supporting pro-oncogenic activity of EGFR in oral carcinogenesis, this study was conducted to examine the role of TVB-3166 in EGFR-expressing OSCC cells [14, 15, 18-20]. We hypothesized that FASN

inhibition with TVB-3166 inhibits EGFR activation, and affects *in vitro* OSCC cell growth and migration

Our data show that TVB-3166 induced anti-neoplastic effects by significantly reducing cell viability in OSCC cells but not in normal oral keratinocytes derived from human gingiva (NOK) providing promising evidence that the drug may not have side effects on normal oral tissues. TVB-3166 also downregulated EGFR activation, as observed by reduced phosphorylation of Y1173 in OSCC cells with basal EGFR activity. Interestingly, spontaneous and EGF-induced OSCC cell migration was also markedly decreased by TVB-3166 treatment. Lastly, we investigated the effect of TVB-3166 on a panel of established oncogenic proteins, and determined that TVB-3166 altered protein expression of several cancer-associated proteins. Overall, this work offers new evidence on the possibility of using TVB-3166 in oral oncology, specifically in OSCC tumors with basal EGFR activation. Moreover, this study presents a comprehensive list of other potential proteins affected by TVB-3166, which warrants further investigation in order to gain a better understanding of the underlying basic mechanisms of this class of FASN inhibitors in oral carcinogenesis.

Materials and Methods

Cell Culture and reagents

Human-derived cell lines Cal-27, HN4, HN6, HN13 were kindly provided by Dr. J. Silvio Gutkind (University of California, San Diego), and were grown and maintained in high glucose Dulbecco's modified Eagle's media (DMEM, Millipore Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Millipore Sigma), and 1%

antibiotic/antimycotic (Millipore Sigma). Normal oral keratinocytes (NOK) derived from human gingiva (ATCC PCS-200-014) served as a non-malignant keratinocyte cell line control, and were grown, maintained, and treated in Dermal Cell Basal Medium (ATCC PCS-200-030) supplemented with Keratinocyte Growth Kit (ATCC PCS-200-040). Of note, NOK-SI used in previous studies are spontaneously immortalized, whereas NOK in this study are primary cells. Cells were cultured at 37°C and 5% CO_2.

In specific experiments, Cal-27, HN4, HN13 and HN6 cells were serum starved in low-glucose serum-free DMEM (Gibco, Grand Island, NY) overnight. Then, cells were cultured in low-glucose DMEM with 1% FBS, along with TVB-3166 (Millipore Sigma), 100 ng/mL human recombinant EGF (#AF-100-15, Peprotech, Rocky Hill, NJ), or vehicle control (DMSO) for the indicated time points.

Western blotting

Western blotting was performed as previously described in **Chapter 2 (page 29)**. To determine PARP cleavage, floating cells were collected in the culture medium, and adherent cells were scraped in ice cold PBS. Following cell collection, cells were spun down in 4°C, rinsed with ice cold PBS, and subsequently lysed with the aforementioned lysis buffer.

The following Cell Signaling primary antibodies were used: rabbit monoclonal against FASN (#3180, 1:1000), rabbit monoclonal against phospho-EGFR Y1173 (#4407, 1:500), and PARP (#9542, 1:1000). Rabbit polyclonal against total EGFR (#sc-03, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal against β-Actin (#A5441, 1:20000, Millipore Sigma), and mouse monoclonal against maspin (#sc-166260, 1:500,

Santa Cruz Biotechnology, Santa Cruz, CA) were also used as primary antibodies. Peroxidase labeled goat anti-rabbit IgG (#5220-0458, 1:10000) and goat anti-mouse IgG (#5450-0011, 1:20000) were purchased from Seracare (Milford, MA) and used as secondary antibodies.

MTS cell viability assay

Cell viability was determined as described in **Chapter 2 (page 31)**. Cells were plated at a density of 5,000 cells per well in 96-well plates. Following seeding, cells were serum starved overnight, then treated with the indicated doses of TVB-3166 in low-glucose DMEM supplemented with 1% FBS for 48 hours.

Cell counting

Cal-27, HN4, HN6 and HN13 cells were plated at a density of 5,000 cells per well in a 96-well plate, serum starved overnight, and treated with the indicated reagents for up to 72 hours in 1% FBS supplemented low-glucose DMEM. NOK were continuously treated in supplemented Dermal Cell Basal Medium as described above. Images and subsequent analysis was carried out as described in **Chapter 3 (page 59).** Changes in cell number over time was determined by (TimeX/Time0). *Of note, cell counting as control experiments for cell migration assays were carried out in serum-free low-glucose DMEM.

Wound closure migration assay

Cells were plated to confluency in 24-well plates, serum starved overnight, and pre-treated with TVB-3166 for 6 hours. Following pre-treatment, the cell monolayer was scratched with a 200 µL pipet tip, and washed with PBS. Cells were then continuously treated in serum-free low-glucose DMEM with TVB-3166 for the indicated time points.

Images were acquired at time of scratching and at the final time point with the Biotek Cytation 5 imaging system. Cell migration was calculated as percentage of gap closure using the formula ([Initial Area-Final Area]/Initial Area) x100. Area of the acellular gap was measured with ImageJ software (National Institutes of Health, Bethesda, MD). **EGF-induced migration experiments were carried out with EGF and TVB-3166 treatments occurring overnight along with serum starvation prior to scratching. Treatments were continued following scratching as well.

*Of note, in migration assays and corresponding control experiments for migration assays, cells were continuously cultured and treated in serum-free conditions.

Human XL oncology array ™

Proteome profiler Human XL Oncology Array ™ kit (#ARY026, R&D Systems, Minneapolis, MN) was used according to the manufacturer's protocol. Briefly, Cal-27 cells were lysed with Lysis Buffer 17 (#895943, R&D Systems, Minneapolis, MN) and incubated at 4°C overnight with membranes containing capture antibodies, followed by incubation with a detection antibody cocktail, streptavidin-HRP, and "chemi" reagent mix for signal detection. Changes in protein expression levels were quantified by signal densitometry using Image J software (National Institutes of Health, Bethesda, MD). Densitometry of duplicate signals were averaged, and fold change was calculated by [average vehicle densitometry/average TVB-3166 densitometry].

Statistics

Experiments were carried out in triplicates. Similar statistical analyses were performed as described in **Chapter 2** (**page 31**)**.**

Results

TVB-3166 reduces cell viability and cell number in OSCC cells, but not normal oral keratinocytes.

We first set out to determine a relevant dose for TVB-3166 in OSCC cells. It has been previously reported that TVB-3166 has an IC_{50} of approximately 100 nM [4]. Through the use of an MTS cell viability assay, HN4, Cal-27, HN13 and HN6 cells were treated with 0, 1, 10, 100 and 1000 nM TVB-3166 (Figure 4.1A). HN6 and Cal-27 both exhibited a 50% reduction in cell viability over 48 hours with 100 nM TVB-3166. Interestingly, HN4 and HN13 cells did not have significant responses to TVB-3166 over a 48-hour period. To confirm that TVB-3166 reduces OSCC cell number *in vitro*, we treated cells with 100 nM TVB-3166 and tracked cell number changes at 0, 24 and 48 hours for Cal-27 and HN6 cells, and up to 72 hours in HN4 and HN13 cells (Figure 4.1B). Again, we observed that total cell number significantly decreased in HN6 and Cal-27 cells at 48 hours. We also found that by extending the time of cell number counting to 72 hours, HN4 and HN13 cell numbers were markedly reduced following treatment with TVB-3166.

FASN inhibition has been previously indicated to not affect normal, non-malignant cells, as typically their FASN expression is low and not necessary for cell function. Therefore, we tracked cell number changes in normal oral keratinocytes derived from the human gingiva (NOK) over 72 hours with up to 10 µM TVB-3166. We found that there were no significant effects on cell growth, indicating that this drug may not be detrimental to normal cells (Figure 4.1C).

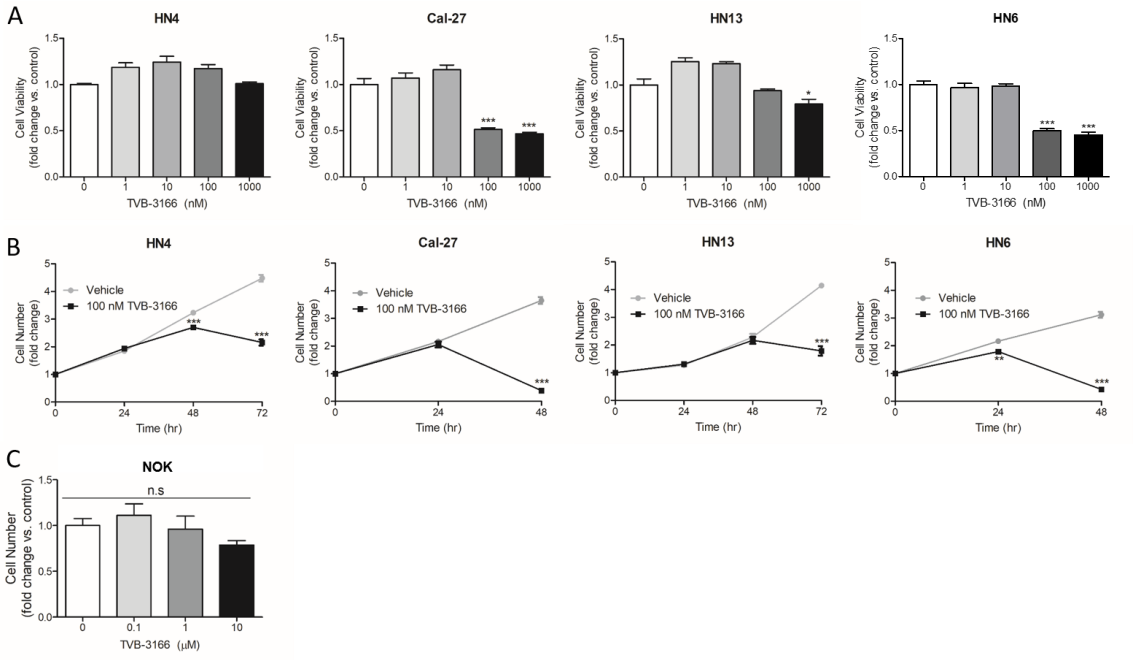

Figure 4.1. TVB-3166 reduces cell viability and cell number in OSCC cells, but not primary gingival keratinocytes.

(A) HN4, Cal-27, HN13 and HN6 cells were plated, serum starved overnight, and treated with indicated doses of TVB-3166 in low-glucose DMEM (1% FBS) for 48 hours. MTS reagent was added and subsequent absorbance reading was recorded at 490 nm. Data represent mean ± S.E.M, with *$p<0.05$ and ***$p<0.001$. (B) HN4, Cal-27, HN13 and HN6 cells were plated, serum starved overnight, and treated with vehicle (DMSO) or TVB-3166 (100 nM) in low-glucose DMEM (1% FBS) for up to 48 hours (Cal-27, HN6) or 72 hours (HN4, HN13). Cell number for individual wells was tracked through cell counting analysis. Data represent mean ± S.E.M, with **$p<0.01$ or ***$p<0.001$ when comparing vehicle vs TVB-3166 at individual time points with unpaired two-tailed Students *t* test. (C) Normal oral keratinocytes (NOK) were plated and treated with indicated TVB-3166 doses over 72 hours in complete Dermal Cell Basal Medium (with supplements). Cell number for individual wells was tracked through cell counting analysis. Data represent mean ± S.E.M, with $p>0.05$, indicating data is not significant (n.s).

TVB-3166 induces apoptosis in OSCC cells

Considering the reduction in OSCC cell number observed in Figure 4.1, we next determined whether this response was due to apoptotic, programmed cell death. HN4 and HN13 exhibited PARP cleavage, a well-established indicator of apoptosis, after 72 hours of treatment with TVB-3166, which coincided with the reduction in cell number in Figure 4.1 (Figure 4.2A and 4.2C). HN6 and Cal-27 cells exhibited PARP cleavage at 48 hours following TVB-3166 treatment, also coinciding with their reduction in cell number (Figure 4.2B and 4.2D). Taking into account that PARP cleavage is a well-established indicator of apoptotic cell death, these data suggest that TVB-3166-induced reduction in OSCC cell number occurred through apoptosis.

TVB-3166 reduces OSCC cell migration

FASN has recently been implicated in cancer cell migration, although to our knowledge the effects of TVB-3166 on cancer cell migration is unknown [134-136]. Therefore, we decided to examine whether TVB-3166 could reduce OSCC cell migration *in vitro.* Both HN6 and HN4 are migratory *in vitro*, whereas Cal-27 and HN13 are not (data not shown). Using serum-free conditions, we determined that both HN6 (Figure 4.3A) and HN4 (Figure 4.3B) had statistically significant reductions in cell migration when treated with TVB-3166, suggesting that FASN plays a significant role in OSCC cell migration. Both HN6 and HN4 cell number was unaffected over these time periods; therefore, the observed gap closure was indeed due to a cell migratory response and not cell proliferation (Figure 4.3C).

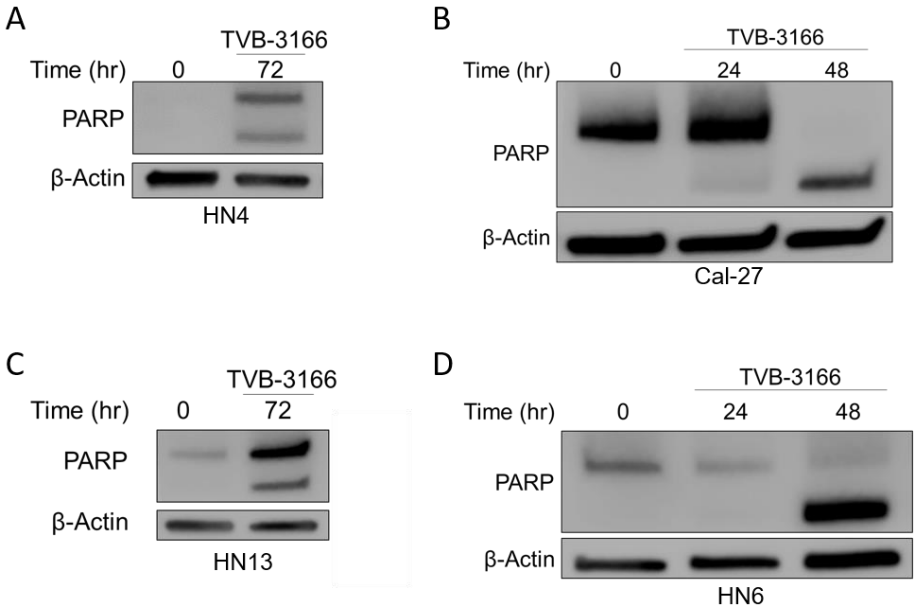

Figure 4.2. TVB-3166 induces PARP cleavage in OSCC cells.

(A) HN4, (B) Cal-27, (C) HN13, and (D) HN6 cells were plated, serum starved overnight, and treated with vehicle control (DMSO) or TVB-3166 (100 nM) for the indicated time points in low-glucose DMEM (1% FBS). All cells (floating and seeded) were collected at the end-point, with Time 0 indicating vehicle control treatments. Cells were lysed and subjected to western blotting for PARP and β-Actin (loading control).

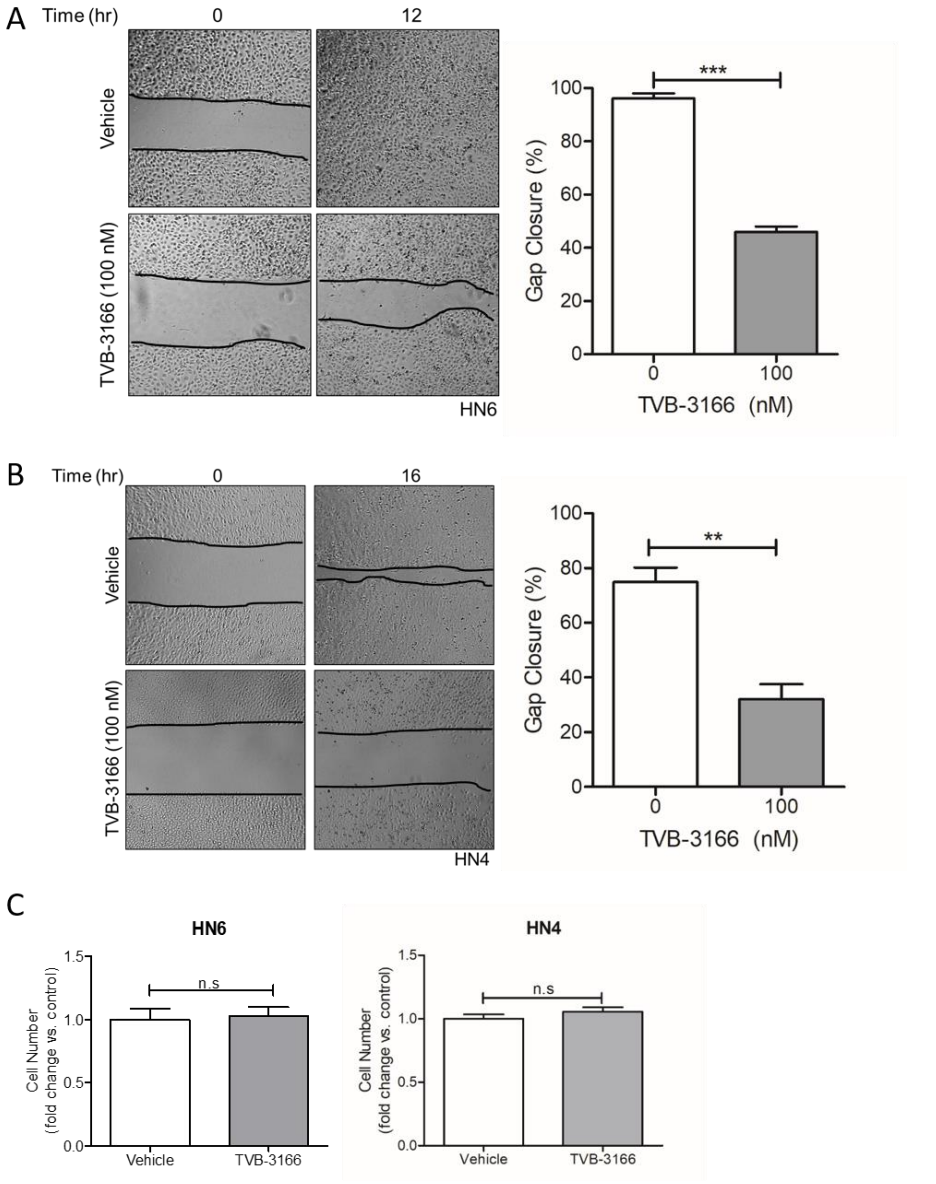

Figure 4.3. TVB-3166 reduces OSCC cell migration.

(A) HN6 and (B) HN4 cells were plated to confluency, serum starved overnight, and then pre-treated for 6 hours with the indicated TVB-3166 doses in serum-free low-glucose DMEM. Following scratching of the cell monolayer to create a gap, cells were continuously treated with TVB-3166 or vehicle (DMSO) in serum-free conditions. Migrating cells were imaged at 0 and (A) 12 or (B) 16 hours. Quantification of percentage acellular gap closure is shown. Data represent mean ± S.E.M. **$p<0.01$ and ***$p<0.001$. (C) In a separate parallel experiment, cell number was tracked through cell counting analysis at time 0 and either 12 (HN6) or 16 (HN4). Data represent mean ± S.E.M, with $p>0.05$ indicating data is not statistically significant (n.s).

OSCC cells exhibit varying FASN expression and EGFR activity

TVB-3166 is known to affect the PI3K pathway, which is downstream of EGFR signaling [76]. A recent study indicated that EGFR activity appears to be affected by palmitoylation, which is the addition of palmitate, the product of FASN, to a cytoplasmic cysteine on EGFR, causing ligand-independent dimerization and activation [54]. This led us to hypothesize that EGFR activity in OSCC cells may be affected by FASN inhibition with TVB-3166, and further could provide insight into the differential sensitivity of the four OSCC cell lines used in this study to TVB-3166. Since the FASN protein expression level of these cells remains unknown, we decided to investigate whether FASN expression contributed to the varied sensitivity to the drug.

Through western blotting, we determined that indeed all four OSCC cell lines had varied FASN expression levels, with HN6 and Cal-27 (the most sensitive to TVB-3166) having the highest expression (Figure 4.4A). In addition, HN6 and Cal-27 exhibited high basal EGFR activity, as reflected by high levels of Y1173 phosphorylation (note: HN6 has significantly higher pEGFR expression than Cal-27, as the pEGFR for HN6 are short exposures (S.E) and the pEGFR for NOK, HN4, Cal-27 and HN13 are long exposures (L.E)). NOK have no observable FASN or phosphorylated EGFR, whereas HN4 and HN13 have minimal expression of these proteins, which may indicate the difference in sensitivity observed in prior experiments to TVB-3166.

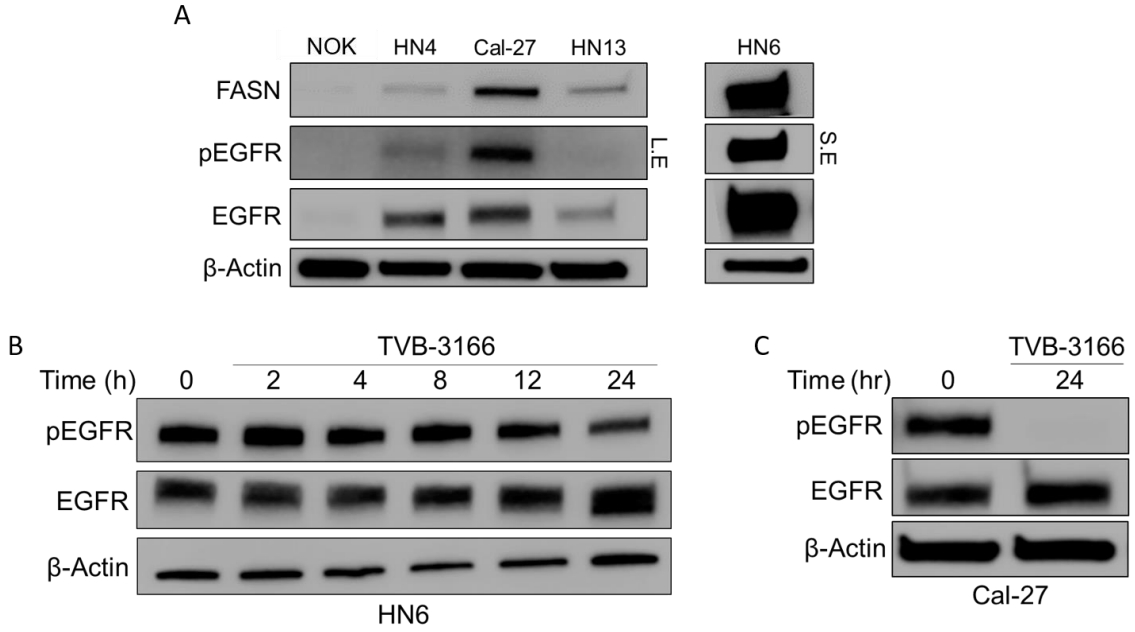

Figure 4.4. TVB-3166 inhibits EGFR signaling in OSCC cells with basal EGFR activity.

(A) NOK were seeded and cultured in complete Dermal Cell Basal Medium (with supplements) for 24 hours. HN4, Cal-27, HN13 and HN6 cells were seeded, serum starved overnight, and subsequently cultured in low-glucose DMEM (1% FBS) for 24 hours, followed by cell lysis and western blotting for FASN, pEGFR (Y1173), EGFR, and β-Actin (loading control). *Note: HN6 pEGFR images are from a short exposure (S.E) time, and NOK, HN4, Cal-27 and HN13 long exposure (L.E) time. (B) HN6 cells were seeded, serum starved overnight, then cultured in low-glucose DMEM (1% FBS) with TVB-3166 (100 nM) for the indicated time points. Cells were lysed and subjected to western blotting for pEGFR (Y1173), EGFR, and β-Actin (loading control). (C) Cal-27 cells were seeded, serum starved overnight, then cultured in low-glucose DMEM (1% FBS) with TVB-3166 (100 nM) for 24 hours. Cells were lysed and subjected to western blotting for pEGFR (Y1173), EGFR, and β-Actin (loading control). *Note, exposure between Figure 4B and 4C are not comparable.

TVB-3166 inhibits EGFR signaling in OSCC cells with basal EGFR activity

Since HN6 and Cal-27 cells were the most sensitive to TVB-3166, and they both exhibited basal EGFR phosphorylation, we treated HN6 cells with TVB-3166 at various time points. Inhibition of EGFR phosphorylation was noticeable at 12-24 hours (Figure 4.4B). As shown in Figure 4.4C, TVB-3166 treatment also inhibited EGFR phosphorylation in Cal 27 at 24 hours, indicating that TVB-3166 is capable of reducing EGFR signaling in two OSCC cell lines with high basal EGFR activity.

TVB-3166 reduces EGFR-dependent OSCC migration

As previously mentioned, HN13 cells do not spontaneously migrate *in vitro*, and also do not exhibit basal EGFR activity (Figure 4.4A). Therefore, in order to determine whether EGFR signaling does indeed play a role in TVB-3166 sensitivity, we stimulated HN13 cell migration with 100 ng/mL recombinant human epidermal growth factor (EGF) (Figure 4.5A). Over an 8-hour period post-scratch, EGF significantly induced HN13 cell migration, which was reduced with TVB-3166 treatment by approximately 50%. These data imply that EGFR signaling-dependent HN13 cell migration is affected by FASN function as treatment with TVB-3166 markedly decreased the migratory response to EGF.

The relationship between FASN inhibition and EGFR activation was confirmed by western blotting, where EGF stimulation increased EGFR Y1173 phosphorylation (Figure 4.5B). Further, EGFR activation was reduced by TVB-3166 treatment. Overall, this proves that EGF induced EGFR signaling and subsequent cell migration relies partly on FASN.

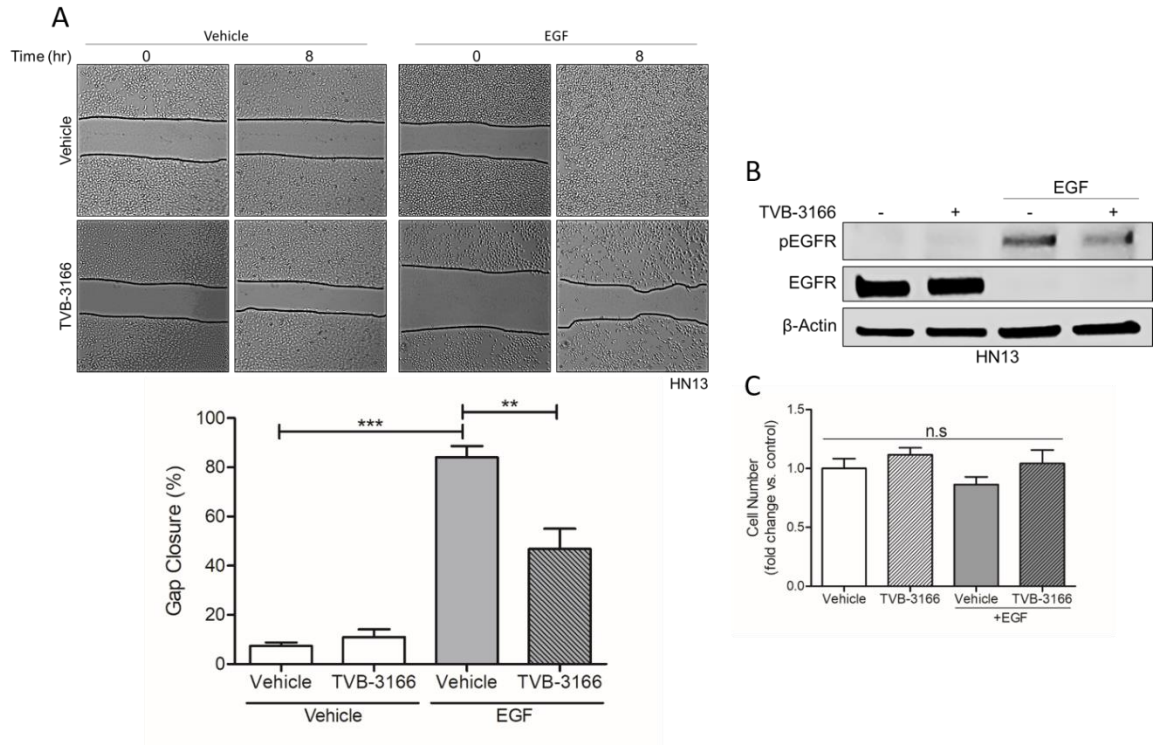

Figure 4.5. TVB-3166 inhibits EGF-induced HN13 cell migration and EGFR activation.

(A) HN13 cells were plated to confluency, and serum-starved overnight along with TVB-3166 (100 nM) and/or EGF (100 ng/mL). Following this starvation and pre-treatment, the cell monolayer was scratched to form a wound, and cells were continuously treated with TVB-3166 and/or EGF for 8 hours, in serum-free low-glucose DMEM. Migrating cells were imaged at 0 and 8 hours, and the percentage of acellular gap closure was quantified. Data represent mean ± S.E.M. $^{**}p<0.01$ and $^{***}p<0.001$. (B) In parallel, whole cell lysates were collected and subjected to western blotting for p-EGFR (Y1173), total EGFR and β-Actin (loading control), and (C) cell number was tracked through counting analysis in a separate experiment at both 0 and 8 hours. Changes in cell number were calculated. Data represent mean ± S.E.M, with $p>0.05$ indicating results were not significant (n.s).

Of note, EGF and TVB-3166 treatment did not affect cell number in HN13 cells over 8 hours, proving that the migratory effects were not due to changes in cell number (Figure 4.5C). EGF treatment induced EGFR phosphorylation, and reduced total EGFR protein levels. This could be due to the recycling of non-activated EGFR upon ligand-stimulation, and likely is independent of the effects of TVB-3166 and more directly related to EGF treatment, as TVB-3166 treatment alone does not affect total EGFR.

TVB-3166 affects multiple oncogenic signaling proteins

Considering that FASN inhibition with TVB-3166 affects EGFR activation, we hypothesized that FASN inhibition may be affecting other receptor tyrosine kinases, as well as other oncogenic signaling pathways and proteins. Therefore, we used a commercially available antibody array to simultaneously detect differences in the relative expression of 84 human cancer-related proteins following treatment of Cal-27 with vehicle control or TVB-3166. Overall, TVB-3166 treatment was found to reduce expression of 15 proteins, and up-regulated expression of 3 proteins (Table 4.1).

Discussion

Although FASN inhibition has recently been implicated in reducing OSCC growth and metastasis, there has yet to be an inhibitor to reach the clinic, mainly due to off-target toxicity resulting in severe anorexia and weight loss in mice [55, 132]. Recently, TVB-3166 was synthesized, and it exhibited none of the toxic side effects observed with other FASN inhibitors, which led us to investigate whether this drug could be useful for OSCC treatment, considering that 5-year survival is still poor, especially in later-stage, aggressive carcinomas [6-8].

Fold Change	Protein	Vehicle	TVB-3166
27.78	Dkk-1		
23.11	Axl		
12.77	CCL20		
9.74	GM-CSF		
5.64	Hif1α		
3.52	Fox01		
2.90	Cathepsin S		
2.29	Survivin		
1.94	p53		
1.83	Cathepsin B		
1.66	uPA		
1.60	Galectin-3		
1.52	Enolase 2		
1.39	EGFR		
1.28	EpCAM		
0.59	Maspin		
0.43	Tenascin C		
0.41	Osteonectin		
0.98	Loading Control		
1.03	Loading Control		
1.00	Loading Control		

Table 4.1. TVB-3166 affects cancer associated protein expression.

Cal-27 cells were seeded, serum starved overnight, and treated with vehicle (DMSO) or TVB-3166 (100 nM) for 24 hours in low-glucose DMEM (1% FBS). Cells were lysed and incubated with Proteome Profiler Human XL Oncology Array ™ membranes. Green indicates decreases in protein expression with TVB-3166 treatment, and red indicates an increase in protein expression.

FASN inhibition has recently been shown to affect EGFR signaling, and considering that EGFR signaling is a common oncogenic driver in OSCC, we wanted to investigate the inhibitory role that TVB-3166 could have on EGFR activation [14, 15, 18-20]. Here, we show for the first time that TVB-3166 reduces OSCC cell number by inducing apoptosis, while not affecting the growth of normal oral keratinocytes, with doses corresponding to a previously established IC_{50} of 100 nM [76].

TVB-3166 reduced OSCC migration *in vitro*, providing evidence that TVB-3166 may affect tumor cell growth as well as metastasis, as *in vitro* migration suggest a potential metastatic ability of cancer cells. Treatment with TVB-3166 also reduced EGFR activation in OSCC cells that exhibited basal EGFR activation, as determined by phosphorylation of Y1173. Of note, HN4 cells did not display much basal EGFR activity as evidenced by very low Y1173 phosphorylation. However, these cells still spontaneously migrated, and TVB-3166 treatment was able to reduce this response, indicating that the anti-migratory effects of TVB-3166 in HN4 cells may be EGFR-independent. Future studies are needed to elucidate which other pathways are being affected by TVB-3166 to reduce the migratory capability of OSCC cells that are not highly dependent on EGFR activation. On the other hand, HN6 cells reveal very high basal Y1173 phosphorylation, which was markedly inhibited by TVB-3166 treatment over 12-24 hours, which coincided with the inhibitory action of TVB-3166 on HN6 cell migration. These data strongly suggest that EGFR inhibition may play a major role in the reduced HN6 cell migration triggered by TVB-3166.

Further, when HN13 cell migration and EGFR phosphorylation were stimulated with EGF, both of these observed effects were reduced by TVB-3166, further implicating

the role of FASN on EGFR activity, whether it be basally active or stimulated with a ligand. This presents an interesting hypothesis that the effects observed here may indeed be due to membrane remodeling as opposed to palmitoylation, as palmitoylation of EGFR has been described to be ligand independent. This sheds light on the fact that further investigation is required to more comprehensively define the effect FASN has on EGFR in OSCC in response to TVB-3166, specifically in regards to EGFR palmitoylation or membrane remodeling [50, 54, 76].

EGFR is the most well-known oncogenic signaling pathway that is aberrant in OSCC, therefore it is promising that FASN inhibition by TVB-3166 de-regulates this pathway, especially considering EGFR targeted therapies are less efficacious in the clinic than expected [78]. FASN inhibition has been hypothesized to alter other membrane-associated oncogenic signaling networks, although it is unclear how dependent OSCC is on these pathways. For example, targeting KRAS has been suggested as a potential target for FASN inhibition, as it is a membrane-associated oncogenic signaling protein, however KRAS mutations typically do not play much of a role in OSCC development and progression [137]. Her-2, an EGFR family member, is also commonly targeted by FASN inhibition, specifically in breast cancer where Her-2 drives many tumors; however, Her-2 does not seem to be a logical target in OSCC as there are not significantly observed increases in Her-2 levels [138].

Although FASN inhibitors are hypothesized to alter membrane remodeling by deforming the membrane, affecting transmembrane receptor recycling or altering the fluidity of membranes, the potential effect of disruption of palmitoylation is another novel hypothesis [50]. Recently, the “palmitoylome”, or list of proteins that may be

palmitoylated by post-translational modifications, has been described to contain approximately 1,800 genes which may encode proteins that can be palmitoylated [139]. Inhibiting FASN would reduce the amount of palmitate available to be covalently attached to cysteines on proteins targeting them for localization to the membrane. Considering the efficacy of TVB-3166 in this study, future work can elucidate proteins within the "palmitoylome" that are particularly associated with oral carcinogenesis, and determine if these may play a role in OSCC sensitivity to TVB-3166 or similar FASN inhibitors.

Taking into account the possibility that TVB-3166 could be altering the expression of proteins other than EGFR, or downstream of EGFR, we used a protein array that contains capture antibodies for 84 cancer-related proteins. We used Cal-27 cells, as these were sensitive to TVB-3166 induced apoptosis, and also exhibited a complete inhibition of EGFR compared to the approximately 50% reduction in phosphorylated EGFR in HN6, which would allow us to more clearly elucidate potential proteins downstream of EGFR inhibition. We observed an increase in expression of 3 proteins, and a noticeable reduction of expression of 15 proteins (Table 4.1). There are several noteworthy proteins affected by TVB-3166 in this study, which can be studied in the future in other OSCC cell lines or in mouse models of oral carcinogenesis, and investigated in their relationship regarding FASN and EGFR.

AXL is a membrane-bound receptor tyrosine kinase, which has recently been associated with increased cell invasion and migration, with overexpression correlating to poor prognosis in OSCC [140]. To our knowledge, this is the first evidence that FASN inhibition reduces Axl protein expression, and presents a logical future direction of study, considering the impact of this receptor in OSCC, and the lack of current studies regarding

its role in OSCC biology and therapeutics. TVB-3166 also reduced the protein expression of the protease Cathepsin B, which is overexpressed in OSCC, correlates with lymph node metastasis and tumor grade, and is secreted in response to EGFR activation to promote cancer cell migration through extracellular matrix (ECM) remodeling [141-143]. Interestingly, inhibition of Cathepsin B may actually inhibit apoptosis in OSCC, indicating a need for further characterization of the role of this protein in OSCC, specifically in response to TVB-3166 [144]. Another member of the Cathepsin protease family, Cathepsin S, was downregulated in response to TVB-3166. To our knowledge, this study is the first to determine that FASN inhibition can regulate Cathepsin S protein expression, and as opposed to Cathepsin B, inhibition of Cathepsin S can help induce apoptosis in OSCC, indicating a potential future direction of study elucidating the roles of Cathepsins in TVB-3166 induced apoptosis in OSCC [145]. Similarly to Cathepsin B, Cathepsin S promotes ECM cleavage, therefore playing a role in cancer migration [146]. Interestingly, Cathepsin S may play a role in EGFR degradation, suggesting a potential relationship between FASN inhibition and EGFR activity [147].

TVB-3166 also reduced expression of urokinase-type plasminogen activator (uPA), a serine protease that is known to bind and activate urokinase-type plasminogen activator receptor (uPAR), which is expressed in a tumor-specific manner in OSCC, associated with tumor invasion, poor prognosis, and negatively associated with overall survival [148- 150]. uPA mediates cancer cell invasion downstream of EGFR, and is also associated with inducing cell survival in glioblastoma's with EGFR tyrosine kinase inhibitor (TKI) resistance [151-154]. However, to the best of our knowledge, FASN has yet to be shown to regulate uPA or uPAR. TVB-3166 reduced expression of galactin-3, part of the lectin

family, which is a transmembrane protein which modulates the cell membrane, and affects EGFR activation and cancer cell survival, invasion and metastasis [155, 156]. Galectin-3 is associated with tumor size and grade in OSCC, although the relationship between EGFR, FASN and galectin-3 has not been elucidated in OSCC [157, 158]. Epithelial cell adhesion molecule (EpCAM) was also reduced by TVB-3166, and is involved in proliferation and migration of cancer. EpCAM is overexpressed in OSCC and correlates with tumor size, grade and poor survival, although it does not seem to have a direct relationship with EGFR or FASN, instead regulating adenosine monophosphate activated protein kinase (AMPK) signaling [159, 160].

TVB-3166 reduced survivin protein expression, and although there is no known relationship with FASN, survivin has increased expression in OSCC, correlates with increased cancer stage and metastasis, and inhibits apoptosis by inhibiting caspase 3 [161-163]. Survivin protein expression correlates with EGFR expression, and although the relationship between the two has yet to be elucidated fully in OSCC, survivin has been hypothesized to be a target in cancers resistant to EGFR TKI [164-166]. CCL20 (C-C motif chemokine ligand 20), a chemokine which is associated with motility in OSCC, and has also been implicated in tumor promotion through attraction of immunosuppressive cells, affecting evasion of the immune system by tumor cells, had reduced expression in response to TVB-3166 [167, 168]. CCL20 increases cancer cell proliferation and migration through trans-activation of EGFR, although there are currently no known associations with FASN in response to CCL20 activation of EGFR [169]. This is another potential future direction investigating the effects of FASN inhibition on EGFR activation. FOXO1 (forkhead box protein O1) is a transcription factor, which is commonly considered

a tumor suppressor that is known to regulate FASN expression [170-173], although the role of FASN on FOX01 has yet to be investigated. Considering TVB-3166 reduced FOX01 protein expression, and FOX01 is considered a tumor suppressor, there is a need to further characterize its role in OSCC. Osteonectin affects degradation of the ECM and tissue remodeling, and is expressed in OSCC [174, 175]. Interestingly, TVB-3166 increased osteonectin protein expression, again demonstrating a need to further characterize what effect this could potentially have in OSCC.

TVB-3166 reduced Dkk-1 (Dickkopf-1) protein expression, which is typically upregulated in OSCC and negatively regulates the Wnt pathway. However, the role of Dkk1 has been reported to be both pro and anti-migratory, indicating a need for further study in OSCC, specifically in relation to FASN, as there is currently no evidence that this protein is regulated by FASN [176, 177]. TVB-3166 also reduced expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), which has an unknown role in OSCC and FASN inhibition. GM-CSF has been associated with EGFR activation, as well as angiogenesis and immune suppression in cancer [178, 179]. Interestingly, tenascin C expression was increased by TVB-3166, which affects cancer cell invasion through ECM reorganization [180, 181]. However, tenascin c exhibits “egf-like repeats” which can activate EGFR, meaning that this up-regulation could be in response to EGFR inhibition, where the cell is trying to re-activate EGFR with another ligand [182].

P53, a widely established tumor suppressor, was reduced by TVB-3166 [183]. However, p53 has been shown to regulate FASN expression, and non-functioning p53 has increased cancer cell sensitivity to FASN inhibitors [184, 185]. P53 overexpression has been correlated with EGFR overexpression in hepatocellular carcinoma, and in OSCC both

p53 and EGFR co-expression were associated with an increased overall response and survival to chemoradiation [186-188]. Our findings indicate that future experimentation is warranted into the effects of p53 inhibition by TVB-3166 in OSCC, and whether or not p53 functions solely as a tumor suppressor in our OSCC cell lines, especially considering that these OSCC cells harbor p53 mutations [15]. TVB-3166 reduces hypoxia-inducible factor 1 alpha (Hif-1α) expression. Although this is the first study showing TVB-3166 reduces Hif-1α, FASN inhibition has indeed been linked to Hif-1α inhibition, affecting cancer angiogenesis, invasion and growth [189, 190]. Hif-1α is overexpressed in OSCC, regulated by EGFR, and is also a well-known regulator of FASN protein expression [191-193].

Maspin (mammary serine protease inhibitor), a tumor suppressor encoded by the SERPINB5 gene, was increased in response to TVB-3166, and has been recently implicated to be upregulated by EGFR knockdown in head and neck cancer, which encompasses OSCC [133]. The exact mechanisms by which maspin affects cancer cell proliferation and metastasis, however, is less known, although maspin has previously been shown to inhibit proliferation, migration and invasion of cancer cells [194-196]. These protein arrays can also be repeated with the other cell lines in the future to determine common proteins inhibited by TVB-3166, which may help elucidate the mechanism of action in these cells.

Since TVB-3166 has been shown to not exhibit side effects *in vivo*, future studies elucidating the effects of TVB-3166 on OSCC *in vivo* will help confirm the findings from this study in regards to tumor growth and metastasis, as well as confirm that it is non-toxic in mice. Taking this into account, along with the fact that a similar FASN inhibitor, TVB-

2640, is currently in clinical trials, this presents excitement for two separate FASN inhibitors to potentially be considered for *in vivo* and clinical trials for OSCC. Further, elucidation of potential pathways targeted by this drug in OSCC are important to define potential proteins which may contribute to drug sensitivity in patients, which will help create prognostic indicators that may help determine patient populations who will best respond to treatment with this novel drug.

In summary, this study presents novel findings that the FASN inhibitor TVB-3166 induces apoptosis and reduces migration in OSCC, and exciting evidence that this drug should be further pursued as a potential clinical treatment, due to its lack of toxicity in normal oral keratinocytes. We present evidence that the sensitivity to this drug may be due to the inhibitory effect of TVB-3166 on EGFR activity, which has yet to be shown in other studies examining pathways affected by this drug. Lastly, we provide evidence that TVB-3166 regulates the expression of multiple cancer-associated proteins, which suggests future studies aimed at identifying specific networks that could impact TVB-3166 sensitivity in oral cancer.

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Chapter Five

Conclusions

The goal of this dissertation work was to investigate potential risk factors associated with oral dysplasia progression to fully established OSCC, as well as novel therapies for OSCC. This insight can improve the overall survival in OSCC patients by prevention of disease development through monitoring or avoidance of risk factors, as well as providing novel drugs which may have an improved therapeutic response in OSCC patients. We first investigated two incredibly topical risk factors for cancer development (nicotine and high glucose), and their role in inducing malignant-like behavior in oral dysplastic keratinocytes. The major pitfall of these two individual studies, however, is that they are exhibiting changes in dysplasias *in vitro*. Future experiments examining these effects *in vivo* are required to confirm, and shed further light on, the reported results. Further, we describe the effects of FASN-dependent EGFR activation on the observed increases in cell growth and migration, however elucidation of both upstream and downstream signaling mediators will provide more insight in the future.

Along with the identification of potential risk factors affecting OSCC development, the field requires development of novel therapeutics for OSCC, and this work explored the FASN inhibitor TVB-3166. This dissertation work established the anti-neoplastic effects of TVB-3166, and provides evidence it inhibits active EGFR signaling in OSCC. Taking into account that EGFR signaling commonly drives OSCC malignancy, this finding offers excitement that this novel inhibitor can improve clinical outcomes. Although this study is limited to *in vitro* work, it provides sufficient pre-clinical data to warrant future *in vivo*

work, which will procure the efficacy of TVB-3166 in relevant animal models. **The major contributions of this work** (Chapters 2, 3, and 4), are summarized below:

Nicotine Induces Oral Dysplastic Keratinocyte Migration via Fatty Acid Synthase dependent Epidermal Growth Factor Receptor Activation

Despite the rising popularity of electronic cigarettes and other nicotine vaporizing devices, the effects of these products on oral tissues is still understudied. Nicotine induces proliferation and migration in OSCC, however the effect of nicotine, deemed relatively safe, has yet to be examined in normal or oral dysplastic cells [59-64]. This *in vitro* study presents the first evidence, to the best of our knowledge, that nicotine promotes migration in oral dysplastic keratinocytes, associating nicotine with a more malignant phenotype in non-malignant oral dysplastic keratinocytes. These data are clinically relevant to current and former smokers who may have developed oral premalignant lesions and decide to use nicotine containing devices for smoking cessation or in addition to tobacco smoking.

FASN plays a role in the malignancy of various cancers, including OSCC, and this study presents novel evidence that nicotine increases FASN protein expression in oral dysplastic keratinocytes [84, 102]. FASN synthesizes palmitate, a free fatty acid, which is either added post-translationally to proteins for cell membrane targeting or interconverted to phospholipids and other lipid species, affecting cell membrane biosynthesis and fluidity. Further, FASN can activate EGFR signaling in cancer, and this study presents novel evidence that nicotine is capable of activating EGFR signaling in a manner dependent on FASN expression in oral dysplastic keratinocytes [53, 54, 85]. This nicotine induced FASN/EGFR signaling axis drives oral dysplastic keratinocyte migration, which is a

hallmark of cancer, suggesting that nicotine induced FASN/EGFR signaling may affect the malignancy of oral dysplasias.

Although we show that FASN expression affects EGFR activation in oral dysplastic keratinocytes, it is unclear whether this effect is due to palmitoylation of EGFR, which has been shown to activate EGFR in a ligand-independent manner, or if there is another pathway responsible for this activation, such as alterations in cell membrane dynamics, effects on EGFR ligands, or other unestablished pathways [54]. Studies investigating specific palmitoyltransferases, the enzymes necessary for catalyzing the transfer of palmitate to cysteines on proteins, in response to nicotine on EGFR activity may provide insight into this question. An acyl-biotin exchange (ABE) assay can also determine the effects of nicotine on EGFR palmitoylation through detection by immunoblotting. Experiments focusing on nicotine stimulation of FASN and EGFR localization to the cell membrane, specifically in relation to lipid rafts or other membrane structures, can provide insight into what exactly is occurring in this model. Lastly, addition of palmitate to cell culture independent of nicotine or FASN can help elucidate whether palmitate alone is responsible for the observed effects, or whether FASN is affecting EGFR in a manner that is not reliant on palmitate.

The effect of FASN on EGFR is pertinent since the majority of OSCC's rely on EGFR signaling, providing evidence that nicotine may promote the malignant progression of oral dysplastic keratinocytes to OSCC through stimulation of this pathway [78, 86]. The characterization of nicotine-induced FASN in this study is noteworthy as well, since FASN is typically not considered a cancer-causing protein, and this study provides novel insight

that FASN is capable of activating EGFR-dependent cell migration, and therefore may play a more important role in the early stages of oral carcinogenesis than initially thought [102].

This study was unable to elucidate the upstream signaling mediators in response to nicotine, however it is EGFR/PI3K/mTOR independent, which is the pathway most commonly associated with FASN regulation in cancer. Consequently, future work is warranted in order to determine specifically how nicotine is regulating FASN expression. FASN expression is known to be regulated by a de-ubiquitinating enzyme USP2a, and is also transcriptionally regulated by SREBP1c and ChREBP [44, 105, 127]. These present as a logical starting point for future investigation into upstream signaling mechanisms for nicotine induced FASN. Nicotine typically signals through nicotinic acetylcholine receptors (nAChR's), which are now known to be expressed on non-neuronal cells, and investigation into these receptors either through chemical inhibition or genetic silencing can also provide insight into the upstream signaling responsible for nicotine induced FASN and migration [99]. Potential mechanisms for the observed response to nicotine are outlined in Figure 5.1.

In summary, nicotine is capable of activating cellular migration of oral dysplastic keratinocytes, a traditional hallmark of cancer. Nicotine increases FASN protein expression, which activates EGFR, a known pro-oncogenic signaling pathway that greatly contributes to OSCC development and progression. These data suggest that further study of nicotine vaporizing products in association with the oral cavity are warranted, as these devices which are publically considered safe may drive pro-oncogenic signaling.

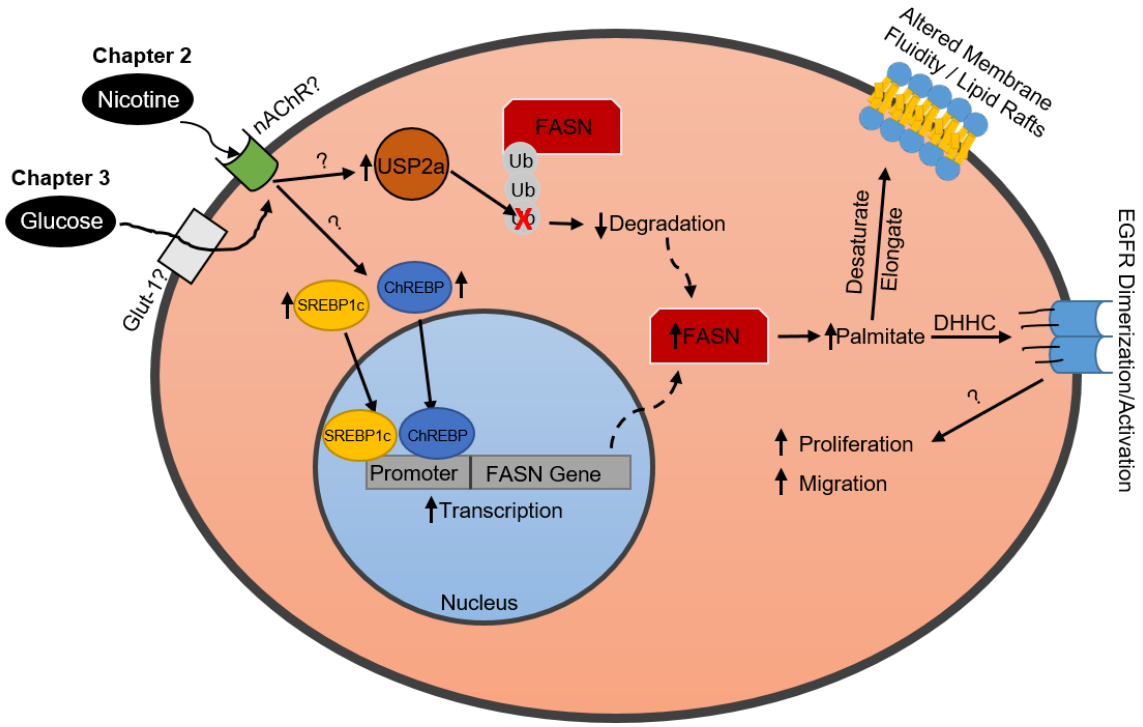

Figure 5.1. Proposed mechanisms for nicotine and high glucose induced FASN and EGFR in oral dysplastic keratinocytes.

Nicotine may bind to nicotinic acetylcholine receptors (nAChR) in oral dysplastic keratinocytes whereas glucose is likely transported by glucose transporter 1 (Glut-1). This study proposes that fatty acid synthase (FASN) protein expression is increased either by de-ubiquitination of FASN by ubiquitin specific protease 2a (USP2a), or increases in transcription by either sterol regulatory element binding protein 1c (SREBP1c) or carbohydrate-responsive element binding protein (ChREBP). Palmitate increases may increase palmitoylation and ligand-independent activation of epidermal growth factor receptor (EGFR) through palmitoyltransferases (DHHC), or through alterations of the cell membrane fluidity or lipid raft localization, resulting in observed increases in proliferation and/or migration of dysplastic oral keratinocytes.

High Glucose Increases Oral Dysplastic Keratinocyte Growth and Migration via Fatty Acid Synthase dependent Epidermal Growth Factor Receptor Activation

This study set out to define the effects of high glucose on oral dysplastic keratinocytes *in vitro*, since hyperglycemia has been recently described as a risk factor for OSCC development, and smoking carcinogenic tobacco is now linked to the onset of type II diabetes [68-70, 74, 111]. We present the first evidence that high glucose drives oral dysplastic keratinocyte growth and migration, which are two well-established hallmarks of cancer. Hyperglycemia has been considered a risk factor for OSCC development through longitudinal studies, and we present the first pre-clinical *in vitro* evidence confirming this relationship.

High glucose stimulates FASN expression in breast cancer and the liver, though the influence of high glucose on FASN has not been investigated in oral dysplastic cells or OSCC [71, 72]. In **Chapter 2**, we described that FASN stimulated by nicotine was capable of driving oral dysplastic keratinocyte migration, which led us to investigate whether the high glucose induced changes in oral dysplastic keratinocyte growth and migration was also due to this pathway [117]. This study determined that high glucose does increase FASN protein expression in oral dysplastic keratinocytes, with the high glucose induced FASN directly contributing to increases in both growth and migration. One of the main goals of this dissertation work was to elucidate the role of FASN on EGFR signaling in oral dysplasias, and this study specifically set out to determine whether this increase in FASN expression by high glucose was capable of activating EGFR, as observed through nicotine stimulation in **Chapter 2.** This hypothesis was established by previous studies in

cancer that indicate FASN affects membrane remodeling and palmitoylation of EGFR, both of which could affect EGFR activation [53, 54, 125].

We report that high glucose activated EGFR signaling in a FASN-dependent manner in oral dysplastic keratinocytes. This EGFR activation in response to high glucose also affected the observed increases in growth and migration. Although we presented that high glucose induced FASN expression is affecting EGFR activity, we did not investigate specifically how FASN is inducing EGFR activation. We hypothesize that palmitoylation of EGFR is inducing ligand-independent activation, however future experiments are needed to test this hypothesis, such as the aforementioned ABE assay to determine if high glucose increases EGFR palmitoylation. Also, inhibition of palmitoyltransferases, the enzymes which catalyze the attachment of palmitate to proteins, could elucidate whether this observed EGFR activation is due to palmitoylation, whereas imaging studies could elucidate changes in membrane dynamics in response to FASN induction and inhibition. Lastly, addition of palmitate to cell culture independent of high glucose could determine if palmitate addition alone is capable of activating EGFR.

Unexpectedly, in spontaneously immortalized normal oral keratinocytes (NOK-SI), high glucose increased both FASN and total EGFR, although it did not affect EGFR activation. EGFR amplification is common in OSCC development, and potentially longer exposure of normal cells to high glucose could sustain FASN and/or EGFR protein expression increases which may have chronic downstream effects [29, 30]. Future experiments should address this observed effect to determine if high glucose also produces oncogenic effects in normal cells, not only dysplastic cells.

Another question raised by this study relates to the signaling responsible for the upregulation of FASN protein expression. Glucose transporter 1 (Glut1) is the likely glucose transporter for glucose uptake by the oral dysplastic keratinocytes, as it is the most heavily implicated and widely distributed glucose transporter in cancer [126]. Future experiments will determine whether the observed FASN/EGFR signaling, growth and migration, are Glut1 dependent through use of genetic silencing of the Glut1 gene. Future experiments will also determine whether FASN is upregulated transcriptionally by ChREBP and SREBP1c, through de-ubiquitination by USP2a, or through other regulatory networks [105, 127]. Elucidation of regulatory networks contributing to FASN/EGFR signaling in oral dysplastic keratinocytes that contribute to increased cellular growth and migration will help determine targetable pathways which relate to cancer development and progression in OSCC. The proposed mechanisms for FASN regulation in response to high glucose, and potential upstream and downstream pathways, are outlined in Figure 5.1.

The next step for clinical translation will be an examination of the effects of hyperglycemia and FASN/EGFR signaling in mouse models. Hyperglycemic mouse models are common, such as the Akita mouse, the nonobese diabetic mouse, and the ob/ob mouse [124]. OSCC can be triggered through the introduction of 4-Nitroquinoline 1-oxide (4-NQO) to the drinking water of these mice, which will determine whether hyperglycemic mice are more likely to develop lesions on their tongues with 4-NQO treatment. Analysis of FASN and EGFR expression in these lesions could determine whether their expression is altered in hyperglycemic mice. Lastly, cetuximab and TVB-3166 can be used in conjunction with this study to determine whether EGFR or FASN, respectively, affect hyperglycemic induction of OSCC in response to 4-NQO.

As a whole, this study presents evidence that high glucose drives FASN/EGFR dependent oral dysplastic keratinocyte growth and migration. We establish that FASN is a potential pro-oncogenic enzyme by activating EGFR, and taken together this indicates that oral premalignant lesions should be monitored along with blood glucose levels.

FASN Inhibition with TVB-3166 Induces Apoptosis, and Reduces Migration and EGFR Signaling in OSCC Cells

In **Chapter 4,** the focus shifted to the effect of FASN inhibition on EGFR signaling in OSCC cells which already exhibit high FASN and/or EGFR expression, independent of either nicotine or high glucose. There is currently a gap in clinical practicality of FASN inhibitors, as previous FASN inhibitors caused severe anorexia and weight loss in mice [55, 132]. This study targets that gap through testing of the non-toxic, potent FASN inhibitor TVB-3166 in OSCC, which has yet to be done. Determination of the efficacy of this drug in OSCC is important, as 5-year survival in late stage, aggressive carcinomas is still incredibly poor, and the clinic lacks successful targeted therapies for OSCC treatment [6-8]. Further, we wanted to determine whether the FASN/EGFR relationship established in **Chapter 2** and **Chapter 3** also occurred in OSCC which exhibit basal overexpression of these proteins, especially since EGFR is a common oncogenic driver in OSCC [14, 15, 18-20].

This study presents novel evidence that TVB-3166 induces apoptosis in OSCC, and reduces migration *in vitro*, two important functions for anti-cancer therapy. We also display that this drug is non-toxic to normal oral keratinocytes derived from the human gingiva, which institutes excitement going forward that this drug will have limited side effects in non-cancerous oral tissues. This study describes for the first time that TVB-3166

reduces EGFR activation, and also reduces EGF-induced EGFR activation and cancer cell migration.

It is clear, however, that TVB-3166 may not only elicit these anti-cancer effects through EGFR inhibition, considering HN4 cells did not exhibit EGFR activity but still had induced apoptosis and reduced migration. This indicates that, although TVB-3166 clearly inhibits EGFR activity, this is likely not the only pathway inhibited. In conjunction with the limitations of **Chapter 2** and **Chapter 3**, this study also does not determine specifically how FASN inhibition reduces EGFR activation, and future experimentation should be carried out to determine the exact relationship between FASN inhibition and EGFR inhibition by TVB-3166. Since the majority of OSCC rely on aberrant EGFR signaling, these findings provide excitement that the FASN inhibitor TVB-3166 may be a novel drug for use in OSCC, and should be further investigated with preclinical and clinical work [78]. Potential mechanisms for FASN inhibition by TVB-3166 on EGFR and other signaling networks are outlined in Figure 5.2.

A protein array was used to determine a comprehensive list of proteins that were either increased or decreased in response to TVB-3166 treatment. Further experimentation is required to determine the proteins affected in other OSCC cell lines, as in this study only one cell line was studied. Cross-referencing these lists of proteins affected by TVB-3166 in the future could determine common pathways that are regulated. Experiments deciphering the effects of these networks on apoptosis, migration, and invasion will help shed light on the mechanism of action of this drug. Future elucidation of pathways affected by this drug is important, as it will define proteins which may contribute to drug sensitivity

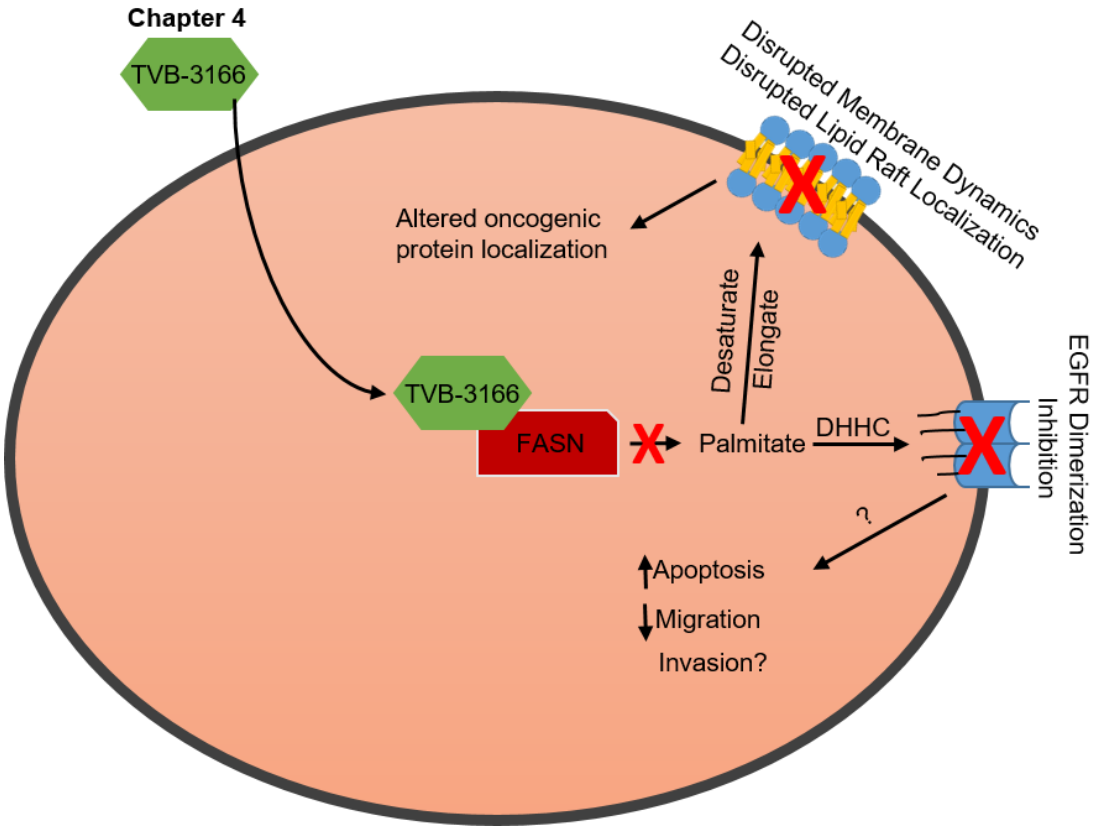

Figure 5.2. Proposed mechanism for TVB-3166 inhibition of EGFR and membrane dynamic alteration in OSCC.

TVB-3166 inhibition of FASN activity in OSCC will reduce palmitate accumulation in cells. This reduction is proposed to reduce the substrate for DHHC (palmitoyltransferases), which will reduce palmitoylation of EGFR, in turn reducing palmitoylation-dependent EGFR activation. This alters unknown downstream pathways, affecting the observed apoptosis and migration decreases. Reduction of palmitate also reduces substrate which can be desaturated and elongated into fatty acids for phospholipid incorporation into the membrane, which can also alter oncogenic protein localization and activity at the membrane.

in patients, helping create prognostic indicators and patient populations which will best respond to treatment.

TVB-3166 was previously established to not exhibit side effects *in vivo*, therefore future studies elucidating the effects of TVB-3166 on OSCC mouse models will help confirm the findings from this study in regards to tumor growth and metastasis, as well as confirm that it is non-toxic in mice. The findings from this study, combined with the fact that a similar FASN inhibitor, TVB-2640, is currently in clinical trials, grants excitement that FASN inhibitors have potential for further pre-clinical and clinical study in OSCC.

Taken together, this study shows that TVB-3166 causes apoptosis and a reduction in OSCC migration *in vitro* for the first time, and is also the first to illustrate that TVB-3166 inhibits EGFR signaling, and de-regulates various cancer-associated proteins, which present as exciting future pathways of study in relationship to the mechanism of action of TVB-3166 in OSCC. Thus, TVB-3166 should be studied further in the future to determine efficacy *in vivo* as a potential novel targeted therapy in OSCC.

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