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Nicotine induces oral dysplastic keratinocyte migration via Fatty Acid Synthase-dependent Epidermal Growth Factor Receptor activation

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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.

Keywords

FASN; EGFR; nicotine; oral premalignant lesions; oral cancer; cell migration

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1. 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 [1]. In the United States, 51,540 newly diagnosed OSCC tumors and 10,030 deaths are expected to occur in 2018 [2]. Although treatments have unquestionably improved in recent years, the 5-year OSCC survival rate remains at around 50% [1]. 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 [3].

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 [3]. 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% [4]. Thus, defining determinants of neoplastic progression is critical to the field of oral carcinogenesis.

The use of electronic nicotine delivery systems (ENDS), including a variety of vaping devices such as electronic cigarettes (e-cigs), is increasing at an alarming rate around the world [5]. Although nicotine is the main trigger for addiction to tobacco and tobacco-derived products, it is not considered a carcinogenic chemical *per se* [6]. 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 [5]. 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; yet there is little evidence available on the role of nicotine in oral precancer [7–12]. 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 [13, 14]. Considering the potentially large population of individuals with occult, undiagnosed oral premalignant lesions who are using nicotine-containing e-cigs, 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 [15]. FASN is responsible for the final step of *de novo* lipogenesis, and is typically not expressed in non-hepatic tissues [16]. 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 [16, 17]. 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 [18]. Furthermore, FASN appears to be very relevant to cancer since it has been linked to Epidermal Growth Factor Receptor (EGFR) activation [19–21]. In OSCC, EGFR overexpression and its aberrant pro-oncogenic signaling are strongly associated with tumor progression, advanced clinical stages and worse survival rate outcomes [3, 22]. In fact, nicotine exposure causes EGFR-dependent growth and migratory signals in cancer cells [23–26]. 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.

2. MATERIALS AND METHODS

2.1. 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% CO₂.

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.

2.2. FASN RNA interference

Short interfering RNA (siRNA) experiments were performed as previously described [27]. Briefly, DOK and Leuk-1 cells were plated at a density of 15,000 cells/cm². 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.

2.3. 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°C with indicated antibodies as previously described [28]. 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.

2.4. Wound closure migration assay

Cells were plated onto six-well plates in complete media at a density of 100,000 cells/cm² 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 μ L 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 $([\text{Initial Area} - \text{Final Area}] / \text{Initial Area}) \times 100$.

2.5. 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.

2.6. 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 μ M nicotine. After 24 hours, 10 μ l 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.

2.7. 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 $\pm$ 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.

3. RESULTS

3.1. 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 [29], exposure to nicotine led to a statistically significant acellular gap closure at 8 and 24 hours when compared to unexposed controls (Figure 1A and 1C). As shown in Figure 1B and 1D, DOK cells, which were originally isolated from a dysplastic tongue leukoplakia in a 57-year-old man who was a heavy smoker [30], also responded to nicotine by increasing the closure of the acellular gap area. 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 [3]. Of note, nicotine did not affect normal oral keratinocyte migration (Figures S1A and S1B).

3.2. 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 [15], we next conducted experiments to elucidate whether this effect occurs in non-hepatic cells such

as Leuk-1 and DOK cells. Interestingly, we found that nicotine is capable of increasing FASN protein expression at time points that coincided with the stimulation of cellular migration (Figure 2A and 2B). 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 S1C).

3.3. 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 μ M), 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 2C and 2D) and DOK (Figure 2E and 2F), 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.

3.4. 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 [31–34]. As depicted in Figure 3A and 3B, 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.

3.5. Nicotine activates EGFR in a FASN-dependent manner

Recent studies suggest that FASN may affect EGFR signaling in lung and prostate cancer cells [20]. 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 3, nicotine did stimulate EGFR phosphorylation (Y1173) after a 24-hour exposure in both Leuk-1 and DOK cells (Figure 4A and 4B). Nicotine exposure, however, did not lead to EGFR activation in normal oral keratinocytes (Figure S1C). 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 4C and 4D). 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 4E and 4F). Of note, in DOK cells, FASN knockdown also resulted in a significant decrease in total EGFR levels (Figure 4F), whereas this response was less evident in Leuk-1 cells (Figure 4E).

3.6. 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 5).

3.7. 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 6A). Further, when compared to parental cells and pPUR-transfected cells, FASN overexpression led to a marked increase in DOK cell migration (Figure 6B and 6C). Interestingly, we observed a significant reduction in cell migration when FASN-overexpressing DOK cells were pre-treated with AG1478 (Figure 6D and 6E). 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.

4. 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 [7–12]. 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 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 μ M nicotine [35–37]. In this study, the lowest dose observed to have migratory and protein expression effects was 10 μ M (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 μ M range [38]. 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, however, so further 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 [35–37].

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 [39]. Interestingly, FASN has been recently shown to affect OSCC proliferation and migration [18]. 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 [19–21]. 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.* [20]. However, another study indicates that palmitoylation inhibits EGFR signaling [40]. Interestingly, when FASN was knocked down with siRNA in DOK cells (Figure 4F), 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 S1A). 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 [3, 41]. FASN has been considered more of a cancer-associated protein as opposed to a cancer-causing protein [39]; 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 S2). 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 3 and 4. This, along with the rapid (<2 hours) increase in FASN protein, indicates that nicotine may potentially be affecting protein translation or degradation. In OSCC, FASN expression correlates with USP2a, a de-ubiquitinating enzyme, however this is a correlation based on immunohistochemical, and not functional, analysis [42]. In prostate cancer, USP2a stabilizes FASN expression by de-ubiquitinating FASN, therefore blocking it from proteasomal degradation [43]. 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.

4.1. Conclusions

The present study provides the first evidence that in oral dysplastic keratinocytes nicotine markedly increases cell migration by activating EGFR signaling through a FASN-dependent

mechanism. These findings raise concerns about the role of nicotine on the cellular behavior of premalignant cells, and whether nicotine acts as a promoter of malignant progression by activating, through a *de novo* lipogenic biosynthetic pathway, a strong pro-oncogenic signaling network in precancerous lesions developing in the aerodigestive tract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 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 μ M 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 $\pm$ S.E.M. ** $p < 0.01$, when compared to untreated control.

Figure 2. 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 μ M). The cell monolayers were next scratched with a pipet tip. Following scratching, cells were left untreated or treated with 10 μ M nicotine for 24 hours. Quantification of percentage acellular gap closure is shown for Leuk-1 (D) and DOK (F) cells. Data represent mean $\pm$ S.E.M. * p <0.05, ** p <0.01, and *** p <0.001.

Figure 3. 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 μ g/mL cetuximab (CTX), 10 μ M AG1478 (AG), 10 μ M 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 4. 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 μ M 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).

Figure 5. 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 μ M 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 $\pm$ S.E.M. ** p <0.01 and *** p <0.001.

Figure 6. 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 μ M 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 $\pm$ S.E.M. ** $p < 0.01$ and *** $p < 0.001$.