

# Alcohol Treatment of Oral Streptococcus Spp. Increased the Entry of Human Papillomavirus Type 16 into Non-Malignant and Oral Squamous Cell Carcinoma Cells

**Keywords:** Oral Bacteria; Keratinocyte; Alcohol; Acetaldehyde; Human Papilloma Virus; Non-malignant; Immortalized; Malignant Transformation

## Abstract

**Background:** Oropharyngeal carcinoma is often associated with human papilloma virus subtype 16 (HPV) infections; however events that lead to HPV entry and carcinoma are poorly understood. We simulated a clinical situation in the laboratory by examining human epithelial and oral keratinocyte (HOK) responses to oral commensal bacteria (*Streptococcus* spp.) and metabolism of ethyl alcohol (ETOH). These studies led us to conclude that oral bacteria and ethyl alcohol through keratinocyte membrane signals act as co-factors for HPV 16 entry.

**Methods:** We tested ETOH exposure responses of *Streptococcus* spp., ETOH-sensitive, acetaldehyde (AA) producers: *S. mutans* (LT11), *S. salivarius* strain, 109-2 and *S. gordonii* (V2016; wild type). In comparison to ETOH-resistant, non-producers of AA: *S. salivarius* strain, 101-7 or *S. gordonii* (adh ABE mutant).

$5 \times 10^5$  per cfu/mL of bacteria were exposed to ETOH (5%,v/v) for 1h and then co-incubated for 3h with epithelial targets: immortalized: 293T; HPV 16 (HOK/HPV 16B cells), or telomerase: hTERT HOK. *Streptococci* spp. attachment to epithelial cells and subsequent effects were enumerated with immune cytochemistry; Western immunoblotting, and immunoprecipitation techniques. These methods delineated expressions of syndecans-1, 4; phospho-L-tyrosine epidermal growth factor receptor (EGFR), and furin convertase (FC) with HPV 16 pseudo virus (PsV) entry into 293T, hTERT HOKs and oral squamous cell carcinoma (SCC-25). To verify these relationships we used inhibitors of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), and FC.

**Results:** *Streptococcus* spp. metabolism of ETOH released AA to trigger HOK expressions of syndecans 1 and 4 and heparin sulfate binding proteins were degraded by heparanase. Following these events enhanced phosphorylated EGFR expression and HPV 16 entry through FC activity were recorded.

**Conclusions:** Some oral *Streptococcus* spp. response to ETOH mediates epithelial cell susceptibility to HPV 16 entry. This occurs through membrane associated expression changes of proteoglycans and EGFR. Moreover, oral cancer cell differentiation and growth may be dependent upon these associations.

## Abbreviations

ETOH: Ethyl Alcohol; ADH: Alcohol Dehydrogenase; ADLH: Aldehyde Dehydrogenase; AA: Acetaldehyde; HSBP: Heparan Sulfate Binding Protein; BAC: Blood Alcohol Concentration; OSCC:



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Oral Squamous Cell Carcinoma; Htert: Human Telomerase; HOK: Human Oral Keratinocyte; FC: Furinconvertase; HPV: Human Papilloma Virus; Psv: HPV 16 Pseudo Virus; 4Mpyr: Methylpyrazole Hydrochloride; CMK: Chloromethylketone; TDS: Tetraethylthiuram Disulfide (Disulfiram)

## Introduction

Human Papilloma Virus (HPV) family contains many types (120) and 16 different genera. Some are linked to enhanced incidence of HPV-related oropharyngeal carcinoma (OPC) [1]. Furthermore, 80% of OPCs and a low percentage of oral squamous cell carcinoma (OSCC) are often HPV subtype 16 seropositive [2,3]. In parallel younger aged Caucasian males present more often with HPV 16 related OPC compared to patients that present with OSCC.

The study of HPV 16 infection has led to seemingly contradictory results and puzzling survival outcomes. It is known that HPV 16 specificity (e.g., about 38%) is dependent upon sites but also method of detection [4]. This has led to unanswered questions about squamous cell carcinomas aggressive growth without HPV 16 infection. In contrast to squamous cell carcinomas with HPV 16 infection that exhibit reduced growth and better survival outcome at identical sites [5-7]. Furthermore, HPV 16 related OPC often shows less HPV 16 infection at other sites in the same patient [8]. For example, lateral border of the tongue, a common site for OSCC is often HPV 16 negative while a carcinoma at the base of the tongue is more likely to be HPV 16 infected [9]. If HPV selective mucotropism and the factors that contribute to reinfection were better understood, the chance for improved treatment of HPV related oral diseases such as focal epithelial hyperplasia (Heck's disease) or OPC is expected to be increased.

We hypothesize that exposure to environmental DNA damaging agents which contribute to oral carcinogenesis; for example, metabolism of ETOH with synthesis of acetaldehyde, also influence HPV entry into oral sites. However, study of HPV 16 should not occur with-

out acknowledging the complex microbial biofilm which is expected to influence HPV 16 entry biology. Moreover, ETOH metabolism is distributed throughout the oropharynx and cavity as a physiology product of both host oral epithelium and microbe [10].

*Streptococcus spp.* is an oral commensal genus and common within the oral microbiome. This genus is also often identified with inflammatory oral diseases (e.g., caries, pharyngitis, gingivitis and periodontitis) [11,12]. An epithelial derived molecular inflammatory cascade is also often identified with the presence of gram positive microbes from this genus and presence of *Streptococcus spp.* bacterial antigen activates Toll-like receptors and growth receptors on oral keratinocytes. Our additional hypothesis is *Streptococcus spp.* presence consequently triggers EGFR activity and HPV 16 entry [13,14]. *Streptococcus spp.* express: AdhA, AdhB or AdhE genotypes for metabolism of ETOH with release of acetaldehyde in a protective process similar to *Listeria* [15,16]. AA in turn contributes to a mutator gene environment that can be characterized indirectly through enhanced expression of markers such as, Annexin A2, a heterotetramer protein. This protein is also a marker for carcinoma induction and functions as a cofactor for HPV 16 entry through EGFR signaling [18,20].

In addition, humans metabolize ETOH through expression of polymorphic variant genotypes of ADH and ALDH (e.g. fast *ADH1B\**, *ADH1C\*1*, and *ALDH2\*2* genotypes that result in accumulation of AA in comparison to slow *ADH1B\*1/1*, *ADH1C\*1/2* genotypes and almost all Caucasians carry an additional *ALDH2\*1/1* genotype) [17,31].

Among human populations AA is a product of both tobacco (e.g., smoke and E-cigarette) [21] and alcohol metabolism which increases risk for oral disorders such as premalignant and malignant carcinoma [21-27]. AA facilitates these problems by entering epithelium through membrane to induce DNA damage, bulky adduct formation; N<sup>2</sup>-acetaldehyde deoxyguanine. Furthermore, AA modifies covalent binding of proteins (e.g., microtubules, actin polymerization); suppresses or modifies enzyme activities (e.g., convertases; such as furin convertase) and binds with GSH<sub>red</sub> to produce a disruption of cellular oxidative state, with lipid per oxidation and formation of hydroxyl radicals [28,29].

Our understanding of the interaction between common oral commensal microbes, epithelium, and viral mucotropism of HPV-16 is very limited. However, it is recognized that selective anatomic sites for HPV 16 infection, a sign of mucotropism occurs in concert with sexually transmission among young Caucasian males [2,30]. This group also demonstrates a unique alcohol behavior that is designated as binge drinking but it is identified with low blood alcohol concentration (BAC) and accompanied by periodic spikes of AA synthesis [31-33]. We suggest that this behavior pattern aids in opportunistic *Streptococcus spp.* proliferation and HPV 16 interactions leading to enhanced risk for OPC in contrast to a lower incidence of HPV 16 related OPC among a comparable female population [31,35,36].

Other ETOH related variables include alcohol per volume or type of beverage (e.g., beer, 12 or 16 oz. 5.0%; wine 2.5 or 5.0 oz.; 12.0%). Moreover, specificities for ETOH metabolism involve conversion of ETOH beverage % to BAC per unit time based on body water content; basal metabolic index (e.g., type of fat content); weight, height, age,

sex, diet, and carbonation etc. In this study a comparative low cellular exposure is used: 5%, v/v (0.0009 ppm) of ETOH to mimic exposure experience and to reflect rapid dilution of an initial concentration such as,  $\geq 20\%$ , v/v; for a constant exposure rate of human oral keratinocytes (HOK) among alcohol users [37].

In addition to AA linked DNA damages to HOK, microbe metabolism of ETOH releases other toxic agents (4-hydroxynonenal (HNE) that contributes to DNA damage and these induce a variety of human diseases (e.g., periodontal diseases; liver and kidney) [37].

This study shows a novel participatory effect from oral commensal *Streptococcus spp.* treated with ETOH. These bacteria attach to HOK cell surface, influence membrane signals linked to phosphorylated EGFR and facilitate HPV 16 entry into HOK. This association was verified by selective inhibitors (e.g., ADH, ALDH), FC and phosphorylated EGFR expression changes.

## Material and Methods

### Chemicals

Acetaldehyde (100  $\mu$ M stock, working concentration: 1.0 and 1.1  $\mu$ M) (Sigma-Aldrich). Ethyl Alcohol (200 proof) was diluted using phosphate physiologic saline buffer (1X) to (5%,v/v) 50 mg/ mL; 10.75  $\mu$ M) (Sigma-Aldrich, St. Louis,MO). Heparanase, endoglycosidase; recombinant human active form, was used at a concentration of 100 ng/mL (R&D Systems, Inc. Minneapolis, Mn).

dec-Arg-Val-Lys-Arg chloromethyl ketone (CMK) ketone (CMK) (Bachem, ENZO, Life Sciences. Farmingdale, NY; stock: 100  $\mu$ M, working concentration 1.0 microMolar ( $\mu$ M) is an irreversible cell permeable competitive inhibitor of the proprotein convertase of proteases which include furin.

4-Methylpyrazole hydrochloride (4Mpyr) (1.0 mM (mL) stock; working concentration: 0.05-0.025 mM (Sigma-Aldrich) is an inhibitor of alcohol dehydrogenase.

Tetraethylthiuram disulfide (TDS) (Disulfiram; 0.5% stock; working concentrations: 0.25%-0.12%-0.012%) is an inhibitor of acetaldehyde dehydrogenase and proteosome inhibitor.

Epidermal growth factor (EGF); Sigma Chemical, St. Louis MO is used at a working concentration of 4  $\mu$ g/mL

**Cells:** HOK/HPV 16B, and human telomerase immortalized (hTERT) human oral keratinocytes (HOK) and HPV 16 immortalized HOK (HOK/HPV 16B cells) are used [38]. Growth medium is Dulbecco's Modified Eagle's Medium (DMEM) +/- Enhanced GLU (GIBCO, Life Technologies, Grand Island, NY) with the dipeptide L-alanyl-L-glutamine (GIBCO, Life technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis) and 10,000 units/mL of streptomycin and penicillin in co-incubation assays without bacteria; for human embryo kidney cells, 293TT [39].

### Viability of Keratinocytes Exposed to ETOH or AA

Keratinocytes viability was assessed as previously described [10]. Keratinocytes were directly exposed to ethyl alcohol (ETOH) (5%,v/v; 50 mg/mL, 10.75  $\mu$ M), and AA (AA: 4.4-5.0 mg/ml or 1.0 to 1.1  $\mu$ M) to test viability (AA spectrometric assay test from Megazyme, UK) [40].

## Bacteria

*Streptococcus mutans* (LT11) is a highly transformable variant of UA159 [41]. *S. salivarius* strains (109-2 producer of AA; 101-7 non-producer of AA), and *S. gordonii* (V288, isogenic strain for alcohol dehydrogenase (ADH wild type, or ADH/ null ABE genes) were all obtained from Dr. Tao (University of Illinois, College of Dentistry) [41].

## Bacteria Attachment Assay

After exposure to ETOH (50 mg/mL/10-11 $\mu$ M at 1h) 50 $\times$ 10<sup>3</sup> *S. mutans*, *S. salivarius*, and *S. gordonii*, were plated on to tissue chambers (Nunc Tissue Chambers) or 6 well tissue culture plates (Fisher Scientific) containing 40-60% confluent keratinocyte populations. Bacteria were gently washed at least 3 times in medium identical to growth medium, but with no antibiotics or serum. Microbe populations were added as a co-culture and incubated for 3h at 37°C, 5% CO<sub>2</sub> to permit attachment. After this period of time incubation chamber is gently washed at least 3 times to remove non-adherent bacteria. Cultures can be fixed if required with 1% para formaldehyde and used for immune cytochemistry.

## Heparin Sulfate or Heparanase Treatments

Bacteria culture is added to HOK population that are pre-treated with heparin sulfate (100 units) and/or heparanase (100 ng/ml) for 1-2 hours.

## Expression and Purification of Recombinant S100 Proteins

Recombinant S100A8 and S100A9 protein were produced and purified based on standard methods as previously described [38]. Briefly, both proteins were cloned in a pGEX-2T GST vector (Amersham, Piscataway, NJ). The proteins were expressed in Top-10 F<sup>+</sup> E-coli as GST fusion proteins. The GST tag was cleaved during the purification process. Protein concentration was assessed through a Bradford protein assay (Pierce, Rockford, IL). Antibody for Western immunoblotting (0.1 mg/ml) and immunoprecipitation (5 mg/ml) with polyclonal S100 alpha antibody PA1-932, Pierce Antibodies, Thermo Scientific; to detect complexes (1:400).

## Immunocytochemistry and Immunodetection of Fusion Protein GST-S100 Complex Binding, Phosphorylated EGFR, and Furin

Monoclonal antibody for syndecan-1, DL-101 (mouse monoclonal) and syndecan-4 H-140 (rabbit, polyclonal) (Santa Cruz, CA); anti-phospho-L-tyrosine epidermal growth factor antibody (rabbit polyclonal, Sigma Chemical, Saint Louis, MO (1:400 (0.4 microgram/mL.) and anti-furin antibody (AbCam rabbit polyclonal 28547 reacts with amino-terminal end) were used at 1:200 for cell immunocytochemistry and Western immunoblot assay.

Dilutions used for immune detection were between 1:50 to 1:400 and detection used was chemi-luminescence (ECL, Amersham, Piscataway, NJ). Detection for immune cytochemistry was accomplished using diaminebenzidine (DAB) and horseradish peroxidase (HRP).

## Production of Human Papilloma Virus Pseudo Virus (Psv)

Human Embryonic Kidney 293TT cells are an adenovirus trans-

formed cell line with a stably integrated SV<sub>40</sub> genome with high levels of large T antigen. PsV production and an Opt prep purification method using overnight incubation of crude cell lysate at 37°C (Buck CB Curr Protocol.2007) which is available from <http://home.ccr.cancer.gov/lco/PseudovirusProduction.htm>. A map of HPV 16 PsV packaging plasmid (p16SheLL for HPV and pCDNA-GFP for GFP gene) and driven by the cytomegalovirus (CMV) promoter is also found at this website. Our system relies upon co-propagation of L1/L2 expression plasmid together with a reporter plasmid (green fluorescent protein (GFP)) to generate high titer of mature PsV stocks for visualization.

## Pseudo virus (PsV) Infection

Pseudo viral like particles (PsV) were plated into wells in a 6 well plate coated with collagen (Type IV) containing 293TT or hTERT HOK cells at a 50-60% confluence (5X10<sup>5</sup> cells). Addition of PsV (50 microliter.) occurred after inhibitors and/or chemicals were added during a 24h-72h incubation and washing with medium. Entry was monitored in 293TT cells for at least 3-7 days for 293TT cells and hTERT HOK using GFP expression as a consequence of plasmid replication. Before addition of PsV for experimental studies we conducted a titration assay (200-100-50-25-10-5 microliter.) for each cell line to determine maximum expression of GFP through a week. In addition, HPV 16 PsV are not oncogenic viable virions because they lack transformation potential and cannot be transferred between individuals.

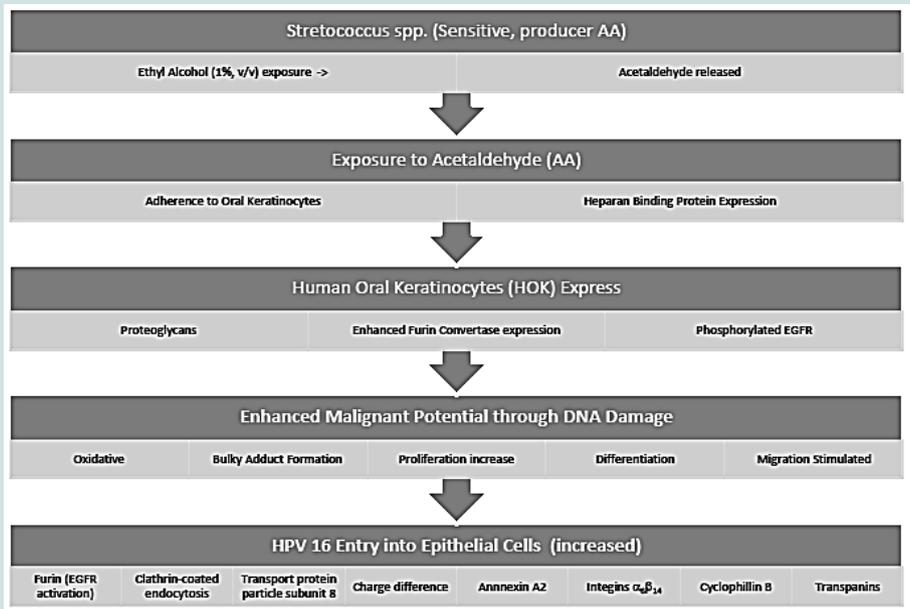
## Statistical Analysis

A paired dependent two tailed Student's t test with a confidence limit of p $\leq$ 0.01 determined level of significance between two comparative groups. Results presented in the figures are a product of triplicate or quadruplicate mean counts from chambers or wells and this resulted in fractions of whole numbers which were rounded to the nearest whole number to reflect the practical situation.

## Results and Discussion

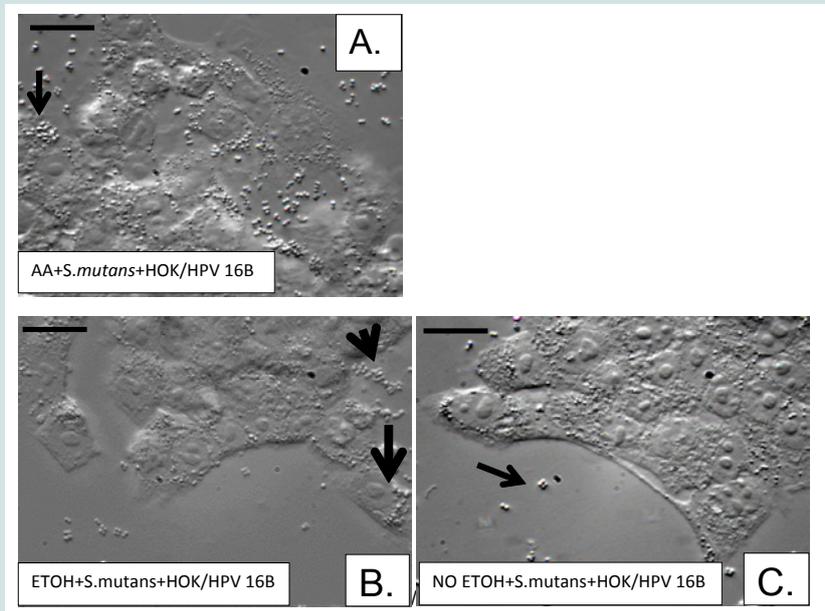
### Experimental Design to Evaluate Oral Commensal *Streptococcus spp.* Exposure to ETOH

Previously reported, oral *Streptococci spp.* could be selected based on association with squamous cell carcinomas, ETOH metabolism and release of DNA damaging agents, AA and malondialdehyde [10,41,42]. These latter DNA damaging agents assist with formation of bulky DNA adducts, and inappropriate keratinocyte proliferation, repair, and migration [10,40]. Clinically HPV infection is associated with epithelial proliferation (e.g., focal epithelial hyperplasia), but little is known about entry events that precede cell growth or linkage to EGFR. There are presently documented eight cofactors for HPV 16 entry into epithelial cells that contribute to selective mucotropism. These include: (1) clathrin coated vesicles, (2) endocytosis, (3) transport protein particle subunit 8, (4) a negative charge molecular display on entry membrane, (5) annexin A2 expression, (6) integrins $\alpha_6\beta_1$  expression, (7) cyclophilin B activity and (8) growth factor receptors activation, such as EGFR, and tetraspanins [18-20,43-45]. In addition entry event studies for HPV 16 in HOK are sadly lacking. In this study we show *Streptococcus spp.* metabolism of ETOH increased adhesion to the surface of HOKs that enhance expression of proteoglycans in



**Figure 1:** Experimental Design to Evaluate Oral Commensal *Streptococcus spp.* Exposure to ETOH: Release of AA Enhances HPV 16 Entry to Contribute to Malignant Transformation of HOK.

Our experimental design explores the relationship between ETOH treatment of *Streptococcus spp.* and a change in physiology among these microbes. This change involves attachment to a variety of epithelial cell targets but it is dependent upon strain specific ETOH metabolism. Attachment complements HOK changes in expression of cell surface heparan sulfate binding proteins (HSBP; syndecans 1,4) and phosphorylation of epidermal growth factor receptor (EGFR). Furthermore, this study explores the possibility for enhancement of HPV 16 entry as a consequence of fore-mentioned HOK protein expressions and enhanced proprotein convertase/serine endo peptidase furin (FC) expression. Taken together this study demonstrates novel relationships between oral commensal bacteria and HOK and HPV 16 entry.



**Figure 2:** *S. mutans* Attachment to HOK/HPV 16B Following Exposure to ETOH or AA.

Panels:

A. Group 1: *S. mutans* treatment by ETOH (5%, v/v; 50 mg/ml or 10.75 μM) for 1h results in AA synthesis (4.4 μg/ml or 1.0 μM). In panel A: AA (1.0 μM) was added to a population of *S. mutans* and incubated for 1h. Then *S. mutans* was washed free of exogenous AA and incubated for 3h with HOK/HPV 16B cells. Results identify colonies of *S. mutans* attached to HOK/HPV 16B cells (Arrow). Bar scale = 10 μM.

B. Group 2: *S. mutans* were treated with ETOH (10.75 μM) for 1h and washed free of exogenous ETOH and incubated with HOK/HPV 16B cells for 3h. Identification of colonies of *S. mutans* attached (Arrow). Bar scale= 10 μM.

C. Group 3: No ETOH treatment only *S. mutans* and HOK/HPV 16B cells. Identification of small colonies of *S. mutans*, however they are not attached to HOK/HPV 16B cells (Arrow).

These photomicrographs display a requirement for ETOH metabolism and/or presence of AA for attachment by *S. mutans* to HOK/HPV 16B cells. Bar scale= 10 μM

conjunction with activity of phosphorylated-EGFR, FC activity and HPV 16 entry in HOK (Figure 1).

### ***S. mutans* Attachment to HOK/ HPV 16B Following Exposure to ETOH or AA**

In response to ETOH treatment (50 mg/mL for 1h), *S. mutans* expresses histone-like protein A, a heparan sulfate binding protein (HlpA) and this expression increases our expectation for attachment to oral epithelial cells using HSBP affinity [46-48].

For example, HSBP proteoglycans contribute to attachment by microbes to various protein surfaces on normal HOKs, during malignant transformation, or on carcinoma cells [47-50]. *In vitro* or tissue section studies have also shown this relationship [51,52]. Here we show ETOH treatment and presence of AA (10 to 1.0  $\mu$ M 1h) from *Streptococci spp.* such as, *S. mutans* and *S. salivarius* (109-2) enhanced adherence [10] to foster increased syndecan-4 expression after 3h co-incubations with HOK/HPV 16B cells (Figure 2).

### **HOK/HPV 16B Expression of Syndecan-4 Following Incubation with AA-Producer *S. mutans* or *S. salivarius* and Heparanase Treatment**

Immunocytochemistry detected a significant (see p values below) enhanced cytosolic, peri-nuclear and nuclear expression in HOK/HPV 16B cells for syndecan-4 expression after incubations (3h) with *S. mutans* + ETOH (52.9% $\pm$  6.5%;  $p \leq 0.0007$ ) or *S. salivarius*+ ETOH (109-2) (82.4% $\pm$  16.2%;  $p \leq 0.0001$ ) (Figure 3A).

However, without ETOH co-incubation of HOK/HPV 16B cells with *S. mutans* (41.6% $\pm$  17.1%;  $p \leq 0.0153$ ) or *S. salivarius* (44.6 $\pm$  17.5;  $p \leq 0.0101$ ) produced syndecan-4 expressions that were only borderline significant (Figure 3A, B). Respectively, syndecan-4 expressions  $p < 0.2605$  and  $p < 0.2256$  are not significant following only ETOH treatment (50 mg/mL, 10.75  $\mu$ M) with no *Streptococcus spp.* present and no treatment (Figure 3D). We also compared (images not shown) of only ETOH (10.75  $\mu$ M) to only AA treatments (1.1  $\mu$ M) for 3h incubation with HOK/HPV 16B cells. Syndecan-4 expression increased with time but it was not significant ( $p \leq 0.0566$ ). Furthermore, heparanase pre-treatment of HOK/HPV 16B cells before co-incubation with ETOH treated *S. mutans* ( $p \leq 0.2529$ ) or *S. salivarius* ( $p \leq 0.0304$ ) showed a decrease in syndecan-4 expressions (respectively: 23.3% $\pm$  18.0% or 31.5% $\pm$  5.0%) that were not significant (Figure 3.C: a. and b.)

Direct exposure of HOK/HPV 16B cells to ETOH or AA treated *S. mutans* or *S. salivarius* (AA producer) significantly enhanced syndecan-4 expression but this effect is reduced with heparanase pre-treatment. These findings suggest a relationship between *Streptococcus spp.* attachment, metabolism of ETOH and a requirement for HOK synthesis of HSBPs, such as syndecan. In the next assay we explored specificity for syndecan-1 which is also linked to selective cell membrane protein exemplified by calprotectin (S100) [49,54-56].

### **Syndecan-1 in Fusion Protein Complex with GST-Calprotectin (S100A8/A9)**

Syndecan-1 is a proteoglycan reported to characterize malignant cells [49-53]. Syndecan-1 will bind with a glutathione-S-transferase GST tag-calprotectin complex linked to calprotectin. This is interesting because calprotectin is an inflammatory cell membrane marker observed with oropharynx and oral carcinoma [54,55].

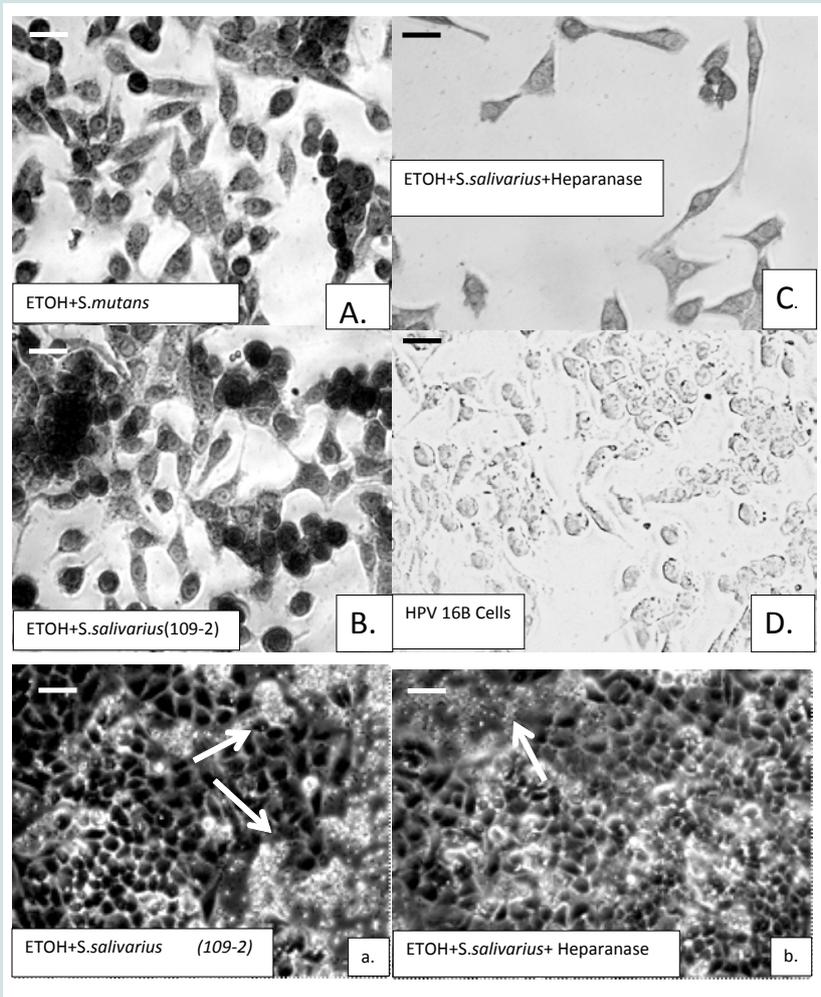
To obtain specificity we used a pull down immune blot approach to identify a fusion complex of GST, calprotectin (S100A8/A9 proteins) and syndecan-1. Furthermore this method provided an opportunity to assess linkage of syndecan-1 to S100A8 or S100A9 in the identical complex. Syndecan-1 expression was a product of exposure for 1h of ETOH (10nM) with *S. salivarius* (109-2, AA producer) and incubation (3h) with HOK/HPV 16B cells (Figure 4).

**ETOH<sub>ST</sub> assay:** A protein extract was obtained from HOK/HPV 16B cells after co-incubation with *S. salivarius* (109-2) for 3h and ETOH treatment for 1h. Described in the legend we note a selective expression of related calprotectin proteins in complex with syndecan-1. This result indicates a linkage to migratory related protein (MRP14 (kd) and calgranulin B; (A9) but not (MRP-8kd) and calgranulin A (A8) in association with syndecan-1 following ETOH treatment of *Streptococcus spp* (Figure 4).

**ETOH<sub>LT</sub> assay:** An extension of incubation time from 1h to 3h of ETOH (50mg/mL.) of HOK/ HPV 16B cells in co-incubation with *S. salivarius* (109-2) was used to assess increased concentrations of AA from metabolism of ETOH by *S. salivarius* syndecan (add)-1 expression to the GST-tagged calprotectin complex. Although an enhanced syndecan-1 complex with the GST-calprotectin is noted (densitometric unit/ETOH<sub>ST</sub> %=71) an examination of densities of bands for A8 (densitometric units/ETOH<sub>ST</sub> %= 3.1) and A9 (densitometric unit/ ETOH<sub>ST</sub> %= 29.5) showed a relative lack of syndecan-1 with S100 (S100A8) with a corresponding reduction in of S100A9 in comparison to ETOH<sub>ST</sub> associated syndecan-1 expression.

**ETOH<sub>ST</sub>+HS assay:** Noted above we observed a loss of syndecan-4 expression with pretreatment of heparanase (Figure 3). We suggest an enhanced expression of syndecan-1 would be observable with a pre-treatment of HOK/HOK 16B cells with HS. Heparan sulfate pre-treatment of HOK/HPV 16 B cells was performed before co-incubation with ETOH<sub>ST</sub> treated *S. salivarius* (109-2). The GST-calprotectin complexed with syndecan-1 was enhanced in comparison to ETOH<sub>ST</sub> treatment syndecan-1 expression response (densitometric units/ETOH<sub>ST</sub> %=64.7) but the isolated S100A8 (densitometric unit/ ETOH<sub>ST</sub> %=2.8) and (S100A9) (densitometric unit/ETOH<sub>ST</sub> %= 63.0) related protein complexes showed a similar pattern as previously stated above. However, with HS pre-treatment calprotectin S100A9 showed an enhanced syndecan-1 complex expression; as shown by density band analysis.

ETOH treatment time of *S. salivarius* and HS pre-treatment of HOK/HPV 16 mediated syndecan-1 complex expression and complex formation with specific enhancement of syndecan-1 to (A9) MRP14 and calgranulin B. Significance to this finding is suggested by these facts for syndecan expression. Syndecan-1,4 activities require NADPH oxidase enzyme activity and heavy metal (zinc) deposition through S100A8/A9 complex formation which may influence metalloproteinase degradation of extracellular proteins, an important function for transformed cell migration [56]. S100A8/A9 is also reported to be a mediator in growth regulation of keratinocytes and epithelial inducible inflammatory activity which can be initiated through Toll-like receptors that recognize *Streptococci spp* [56]. Another S100; A10 sub unit is found with Annexin A2 and as noted



**Figure 3:** HOK/HPV 16B Cell Expression of Syndecan-4 Following Incubation with AA-Producer *S. mutans* or *S. salivarius* and Heparanase Treatment.

A. The photomicrograph shows following ETOH (5%, v/v; 10 to 50 mg/mL or 10.75  $\mu$ M) treatment of *S. mutans* ( $5 \times 10^3$  cfu/mL) for 1h results in a release of AA (1.0  $\mu$ M). After washing of bacteria and then incubation with HOK/HPV 16B cells (3h incubation) we stained using horseradish peroxidase/DAB detection. We observed cytosolic and perinuclear (black staining) expression of syndecan-4 in HOK/HPV 16B cells. In some cells there is noted an increase in intensity compared to others which suggests bacteria contact and or cell cycle phase specificity prior to syndecan-4 expression. Bar scale = 5  $\mu$ M

B. Another photomicrograph shows enhanced syndecan-4 expression in HOK/HPV 16B cells following ETOH (10  $\mu$ M) treatment and incubation with *S. salivarius* (109-2, AA: 1.16  $\mu$ M) ( $5 \times 10^3$  cfu/mL). We noted a cytosolic and perinuclear expression and intensity of syndecan-4 expression as stated above. Bar scale = 5  $\mu$ M

C. Syndecan-4 is a proteoglycan and heparan binding protein. To demonstrate a relationship between ETOH treatment of *S. salivarius* (109-2, AA: 1.16  $\mu$ M) ( $5 \times 10^3$  cfu/mL) and effect on HOK/HPV 16B cell membrane proteoglycan, syndecan-4 we pretreated cells with the endoglycosidase, heparanase (100 ng/ml). Prior treatment with heparanase resulted in a reduction in Syndecan-4 expression in the cytosol and perinuclear areas compared to HOK/HPV 16 cells not treated with heparanase but incubated with *S. salivarius* and capable of release of AA.

D. Control population of HOK/HPV 16B cells with no co-incubation or treatment does not express highly syndecan-4. Bar scale = 5  $\mu$ M

Attachment Effect on *S. salivarius* from heparanase pre-treatment of HOK/HPV 16B cells

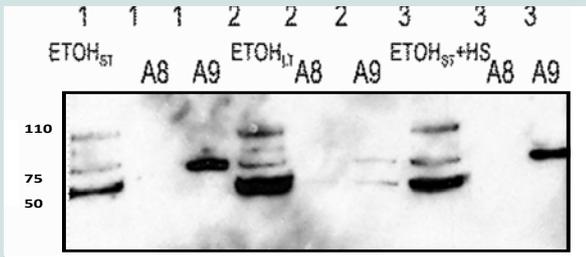
a. *S. salivarius* (109-2, AA: 1.16  $\mu$ M) ( $5 \times 10^3$  cfu/mL) treatment with ETOH (50mg/mL/10  $\mu$ M) 1h and washed then incubated with HOK/HPV 16B cells for 3h. Photomicrograph shows *S. salivarius* attached to HOK/HPV 16B cells (74% $\pm$ 15.0%). (see arrows)

b. *S. salivarius* (109-2, AA: 11.6  $\mu$ M) ( $5 \times 10^3$  cfu/mL) treatment with ETOH (50 mg/mL/10  $\mu$ M) and washed then incubated with HOK/HPV 16B cells (3h), which were pre-treated with heparanase (100 ng/mL). A reduction in attachment of *S. salivarius* to HOK/HPV 16B with heparanase treatment was observed (37% $\pm$ 10.5%) ( $p < .0005$  significant) (see arrows).

These assays show attachment of oral *Streptococcus spp.* treated with ETOH (5%,v/v) resulted in significant increase in attachment to HOK/HPV 16B cells. A corresponding expression of syndecan-4, a HSBP was observed which is susceptible to heparanase activity to effect *Streptococcus spp.* attachment to HOK.

above a HPV 16 entry factor in response to ETOH exposure. Annexin A2 expression leads to activation of EGFR and HPV 16 entry [57]. AA is also noted to induce apoptosis and proteoglycan/syndecan-calprotectin interaction [56]. Stated above syndecan 1,4 expressions are recognized as factors for enhanced migratory activity of transformed and malignant HOK. Syndecan also binds to collagens

(I, III, and V), fibronectin, tenascin and other extracellular matrix non-collagenous proteins that facilitate migration [58]. Moreover, syndecan-1 can degrade HSBPs in a heparanase like manner to increase proliferation and metastatic potential of keratinocytes [50,51,56]. Lastly, syndecan-4 stimulates tumor cell proliferation with a decrease in binding of fibronectin by epithelial cells to accelerate



**Figure 4:** Syndecan-1 In Fusion Protein Complex with GST-Calprotectin (S100A8/A9): A testing of Membrane Proteins Following Attachment of ETOH Treated *S. salivarius*.

**ETOH<sub>ST</sub> Assay:**

a. HOK/HPV 16B cells were exposed to ETOH treated *S. salivarius* (109-2) (1h) as stated in figure 3. Antibodies binding to syndecan-1 resulted in immunoprecipitation of syndecan-1 and a fusion protein complex GST-S100A8/A9 (110, 75, 50 kd).

b. Syndecan-1 expression was not demonstrated to be expressed in this GST-S100A8 complex.

c. Syndecan -1 expression is identified with a GST-S100A9 complex (75 kd). ETOH treated *S. salivarius*(109-2) which releases AA will bind to membrane associated calprotectin (S100 complex) with specificity to the S100A9 region.

**ETOH<sub>LT</sub> Assay:**

a. ETOH treatment time of *S. salivarius* was extended (3h) and identically incubated with HOK/HPV 16B cells as described above. This incubation is associated with increased proliferation of *S. salivarius* (109-2) and AA release (30-40 μM; 13.2-17.8 to 1.32 μg/mL). Antibodies bind to syndecan-1 and fusion protein complex GST-S100A8/A9 (110, 80, 75, 50 kd).

b. Syndecan-1 is not identified with a GST-S100A8 complex.

c. Comparatively, syndecan -1 is identified with a GST-S100A9 complex (50-75kd) but reduced in expression level.

**ETOH<sub>ST</sub>+HS:**

a. ETOH treatment of *S. salivarius* identical to the first group with incubation with HOK/HPV 16B cells These HOK cells were pretreated with heparan sulfate (100 units) to assess possible association with syndecan-1 expression and attachment by *S. salivarius* after 3h. Antibody identified expression of syndecan-1 bound to a fusion protein complex GST-S100A8/A9 (110, 75, 50 kd).

b. Syndecan-1 is not identified with a GST-S100A8 complex.

c. Syndecan -1 is identified with a GST-S100A9 complex (75 kd). This assay showed (see text for densitometric % comparison) increased release of AA alters syndecan-1 complex with calprotectin domain calgranulin B (S100A9 complex) but not S100 A8; calgranulin A. An increase in the total complex with syndecan-1 was noted at 50 kd for extended time for ETOH treatment of *S. salivarius* (109-2) or HS pre-treatment of HOK/HPV 16B cells. A8= MRP-8 kd; calgranulin A; A9= MRP-14 kd; calgranulin B.

migration [58-60]. Results from this assay support prior published results that exposure of HOK to *Streptococcus spp.* facilitate migration [10].

**Set of Comparative Assays to Examine Associations of *S. salivarius* and *S. gordonii* following treatment with ETOH: Phosphorylated EGFR and HPV 16 (PsV) Entry**

We state above that ETOH exposure of *Streptococcus spp.* effects complex associations of calprotectin S100 proteins and surface proteins such as syndecans in HPV 16 immortalized HOK cells. Subsequently we sought to determine whether there was an association with HPV 16 entry events. Furthermore, there were two reports by Surviladze et al. (2012, 2013) that described EGFR expression coincident with HPV 16 entry [61,62]. This is important because activation/phosphorylation of EGFR participates in epithelial differentiation through the junctional pathway designated PI3K/Akt/PTEN/ mTor [63-65]. In this assay we

examined phosphorylation of EGFR as a mediator of HPV 16 entry into hTERT HOK cells or SCC-25, OSCC.

We used comparative immunocytochemistry and a Western immunoblot to assess phosphorylated EGFR expressions and a fluorescent (GFP) assay to evaluate HPV 16 entry.

A comparison between Panel A (Control hTERT HOK) and Panel B (hTERT HOK (3h) + ETOH<sub>ST</sub> (50 mg/mL.) and 1h incubation with *S. gordonii* (strain 4) showed a significant difference (p<0.0007) in phospho-tyrosine- EGFR expressions: Panel A (a): 23.3+/-9.5% vs. Panel B (a). *S. gordonii* (strain 4, ETOH treated): 52.0+/-11.0%. In contrast, Panel A. (a) vs. Panel B. (b) *S. gordonii* (no adh) strain (b) that cannot metabolize ETOH to synthesize AA; was not significantly different (p<0.1914) in expression of phosphorylated EGFR:31.4+/-10.5% in hTERT HOK cells.

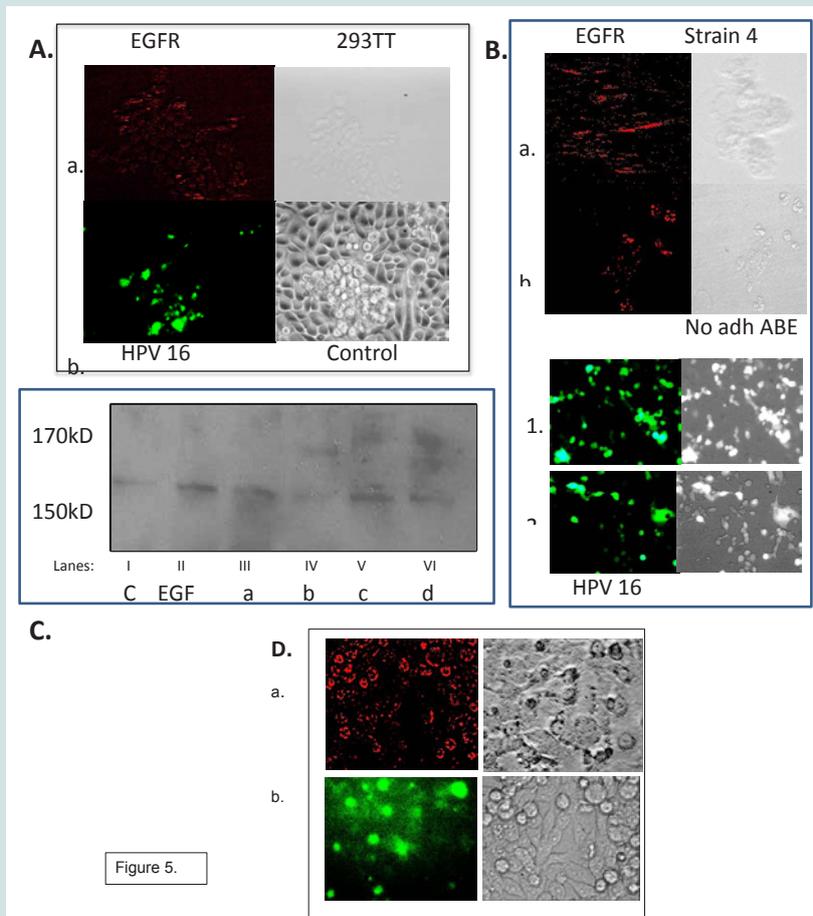
A second comparison between Panel A (Control hTERT HOK) and Panel B (hTERT HOK (3h) + ETOH<sub>ST</sub> (50 mg/mL.) and 1h incubation with *S. gordonii* is presented to demonstrate a corresponding effect on HPV 16 entry. This comparison produced a significant difference (p<0.0001) in HPV 16 PsV entry. Panel A. (a): 27.1+/-5.4% vs. Panel B *S. gordonii* (strain 4, ETOH treated) (1); 77.5+/-10.1%

In contrast, *S. gordonii* (no adh) strain (2.) does not significantly enhance (p<0.0117) HPV 16 PsV entry in hTERT HOK cells (40.0+/-9.2%).

**Panel C:** A Western immunoblot confirmed phosphorylated EGFR expression changes noted above. Identical amounts of proteins added to each lane documented by densitometric unit % a comparative difference between untreated control (lane I) EGFR expression and the positive control, EGF (lane II) (Figure 5).

Another comparative exposure and phosphorylated EGFR (150kd) band between ETOH<sub>ST</sub> treated *S. salivarius* (109-2) in lane III and band expression for phosphorylated EGFR obtained from EGF administration to hTERT HOK cells is noted. However, *S. salivarius* (107-1) which can-not metabolize ETOH to AA has nearly one-half the level of phosphorylated EGFR; although a second band (170 kD) did appear.

AA is a genotoxic stress inducer that not only is a product of ETOH metabolism but also alters fatty acid metabolism and effects cell physiology through: 1) increases in acetylation and suppression of deacetylation (e.g., HDAC SIRT proteins); 2) mediation of transcription factor function; such as, 3) sterol regulatory binding protein (e.g., 1c); 4) alteration in expression of heat shock proteins, and 5) enhancement of oxido-reductase activity (e.g., long chain acyl coenzyme A dehydrogenase) [66]. Here we record in a second *Streptococcus spp.* comparison; dependence on AA release and increase in expression of phosphorylated EGFR (150 and 170 kD) in hTERT HOK following co-incubation with *S. gordonii* (strain 4, ETOH treated) (lane V). However, unexpectedly we observe only a slight reduction for phosphorylated EGFR expression from *S. gordonii* (no adh.) (lane VI). Therefore a difference between *S. salivarius* and *S. gordonii* in phosphorylated EGFR expression is indicated by but in phosphorylation of EGFR is also dependent on species and strain.



**Figure 5:** A Set of Comparative Assays to Examine Associations of *S. salivarius* and *S. gordonii* Following Treatment with ETOH: Expression of Phosphorylated EGFR phospho- L-tyrosine and HPV 16 Entry

Results described in previous figures used HOK/HPV 16 cells and indicated a possible link between ETOH treated *Streptococcus spp.* and a role for HPV 16. To shed light on this association the following assays were performed.

**Panel A:**

Comparative immunocytochemistry photomicrographs (dark and light fields) in epithelial cells, hTERT HOK cells.

a. EGFR expression (red, 660 nm) in hTERT HOK is on cell surface (32.1+/-8.8%)

b. HPV entry is detected after replication of plasmid containing Green Fluorescent Protein expression (550 nm) (19.1+/-10.2%).

**Panel B:**

Comparative immunocytochemistry photomicrographs (dark and light fields) of hTERT HOK cells that show phosphorylated EGFR expression (red) and HPV-16 (PsV) (Green) entry between non-treated and identical hTERT HOK populations following exposure to ETOH ( 50 mg/mL) treated *S. gordonii* (1h) (strain 4) or *S. gordonii* ( no adh (ABE)

(Panel A vs. Panel B) Results described in text and shown here: phosphorylated EGFR (a.) levels significantly elevated in hTERT HOK cells following ETOH treatment of *S. gordonii* strain 4 (p<0.0007).

(Panel A vs. Panel B) Results described in text and shown here a comparison of 293TT cells with HPV 16 entry levels significantly elevated in hTERT HOK cells following ETOH treatment of *S. gordonii* strain 4 (p<0.0001).

**Panel C:**

Expressions of phosphorylated EGFR expression using Western immunoblot approach.

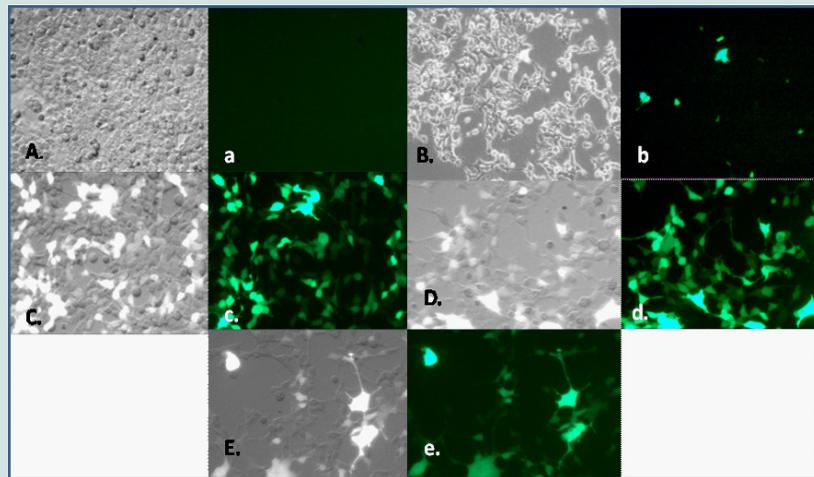
Control=C (lane I); EGF(50 ng/ml) (lane II); *S. salivarius* (109-2, AA producer)(lane III); *S. salivarius* (101-7, non AA producer)(lane IV); *S. gordonii* ((strain 4)(lane V) and *S. gordonii* (non adh) (lane VI)

Densitometry analysis at 150kd recorded EGF treatment of hTERT HOK cells elicited a 10.2% increase in expression compared to cell control (Lane I): 146.4 density units; EGF (Lane II) 161.4 density units. Exposure to ETOH treatment (50mg/mL) *S. salivarius* (AA producer) (Lane III) elicited 17.4% increase in expression: 171.9 density units). Exposure to ETOH treatment *S. salivarius* (non AA producer) (Lane IV) elicited 9.5% increase in expression: 160.0 density units. Exposure to ETOH treatment (50 mg/mL) *S. gordonii* (strain 4,AA producer) (Lane V) elicited an 18.5% increase in expression: 172.4 density units. In contrast, exposure to ETOH treated *S. gordonii* (no adh, non AA producer) elicited 8.9% increase in expression: 159 density units. However, a 170 kd band is also detected for Lanes IV, V, and VI and is consistent with phospho-L-tyrosine heterodimerization band detection.

**Panel D:**

a. SCC-25, oral squamous cell carcinoma cells were exposed to *S. gordonii* (strain 4) following ETOH treatment (50 mg/mL) as previously described. Phosphorylated EGFR immunocytochemistry expression compared to non-cancer epithelial cells (hTERT HOK, Panel A, a) showed a significant increase in expression (p=0.001).

b. HPV 16 PsV entry assay also demonstrated a significant increase (p=0.0004) in entry among SCC-25 cells exposed to ETOH treated *S. gordonii* (strain 4) (44.4+/-6.0%) in comparison to control hTERT HOK (non *S. gordonii* exposed), non-cancer epithelial cells (Panel A, b) (19.1+/-10.2%). However, a comparison of *S. gordonii* (strain 4 treated with ETOH) of Panel B, 1 (63.6+/-7.3%) to Panel D, (b) treated cells showed a significant (p=0.0006) higher level of HPV 16 entry in the non-cancer hTERT cells.



**Figure 6:** Quantitative Analysis of HPV 16 PsV Entry into 293TT Cells Following Exposure to *S.gordonii*, and/or CMK, a Furin Inhibitor. (A., a) Epithelial target, 293TT cells were treated with the furin convertase inhibitor, chloromethyl ketone (CMK: 1.0  $\mu$ M). We observed as shown in the dark field in comparison to light field, a significant reduction ( $p < 0.0001$ ) in HPV 16 entry (2.1 $\pm$ 3.8%) compared to non CMK treated 293 TT cells after 72h incubation with HPV 16 PsV particles (E.,e.) (57.5 $\pm$ 7.2%). (B.b.) 293TT cells were pre-treated with CMK and then co-incubated (3h) with *S. gordonii* (strain 4) + ETOH (50 mg/mL.) (1h). We observe a slight increase in HPV 16 PsV entry (7.6 $\pm$ 5.5%) compared to results shown in A.,a. but significantly reduced from control (no CMK, *S. gordonii* or ETOH (E.,e.) ( $p < 0.0001$ )). (C.,c.) 293 TT cells were not pre-treated with CMK but treated in an identical manner described above for B., b. We observe HPV 16 entry (64.1 $\pm$ 3.7%) is responsive to FC activity as discussed in text. (D.,d) Using only *S. gordonii* in co-incubation with 293TT with no ETOH or CMK treatment conditions noted above we observe a significant increase (45.2 $\pm$ 2.9%) ( $p < 0.0001$ ) in HPV 16 entry compared to 293TT cells that underwent CMK treatment (D.,d vs. A.a). (see text)

The antibody used recognizes hetero dimerized forms of phosphorylated EGFR and this is expected to account for the banding. In addition, we suggest additional factors such as Annexin A2 or Toll-like receptor activation triggered by *Streptococcus spp.* while in close contact with hTERT HOK membrane influences phosphorylation of EGFR [13,14,57].

The *S. gordonii* (no adh) strain is interesting because it is capable of producing acetic acid/acetate and this synthesis can produce an imbalance in acid-base levels and foster cell stress leading to small increases in EGFR tyrosine phosphorylation which is detected with Western immunoblotting [66]. Another possibility for this observed pattern is expression of heparan-EGF like proteins from *S. salivarius* that bind and enhance expression of phosphorylated EGFR that would not be observed with immune cytochemistry [60].

**Panel D:** An immune cytochemistry result showed a significant increase ( $p = 0.001$ ) in phosphorylated EGFR in SCC-25 cells derived from the tongue (a) in comparison to a similar exposure to *S. gordonii* (strain 4 (Panel B, a) of hTERT HOK, non-cancer cells or non exposed hTERT HOKs (Panel A, a). This finding is interesting because epidemiology studies indicate a relatively high level of growth among cancers from the tongue which parallels phosphorylated EGFR levels. Moreover, we show an 2.3 times higher level of HPV 16 entry among SCC-25 compared to no *S. gordonii* exposed hTERT HOK. However, *S. gordonii* (strain 4) ETOH treatment produced a significant higher level for HPV 16 entry in the hTERT HOK cells compared to the SCC-25 cells. Although these finding still support evidence provided by Reddout, et.al, in 2007 that showed SCC-25 cells and HPV 16 transfection responses enhanced proliferation under *in vitro* conditions [67] (Figure 5).

Following ETOH exposure of selected strains of *S. gordonii* or *S. salivarius* activation of phosphorylated EGFR and HPV 16 entry were observed in hTERT HOKs. Enhanced expression of phosphorylated EGFR also suggests a suppression of EGFR should reduce HPV 16 entry and this is achievable through EGFR tyrosine kinase inhibitors (results not shown here). Moreover, other growth receptor expressions in association with EGFR expression are linked: vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin growth factors (IGFs) and transforming growth factor (TGF- $\beta$ ) [68,69]. In addition, EGFR pathway activation can shed EGFR (e.g., sheddases) to assist attachment of microbes to epithelial cells [70]. It is further noteworthy, EGFR has a cysteine -rich sequence which is FC like and suggests an assist with HPV 16 entry [71,72]. We further speculate *Streptococcus spp.* proliferation following low level (e.g., 5%, v/v, 50 mg/mL.) ETOH treatment will increase expression of Annexin A2, and EGFR to also enhance HPV 16 entry [13,14,18-32].

**Quantitative Analysis of HPV 16 PsV Entry into 293TT Cells Following Exposure to *S. gordonii* and/or CMK** In the previous assays we showed a linkage between exposures to oral commensal bacteria such as *S. gordonii* (strain 4) as they metabolize ETOH and phosphorylated EGFR to enhance HPV 16 entry. In this assay we identify the serine endopeptidase and convertase, furin (FC) as a critical factor for HPV 16 entry. FC is not only a recognized proprotein membrane “master “switch that governs HPV entry into epithelial cells but is also a marker of metastatic malignant behavior [35,71-73].

FC specificity in conjunction with HPV 16 entry is determined using CMK. CMK treatments reduced HPV 16 entry in these groups: *S. gordonii* (strain 4) co-incubations or in control 293TT cells (A.a

vs. B.b). But this difference was small and non-significant ( $p < 0.3921$ ) for HPV 16 entry. In contrast, ETOH treated *S. gordonii* without CMK, showed a significant increase in HPV 16 entry (A,a vs. C,c) ( $p < 0.003$ ) or in comparison we recorded significance ( $p < 0.0001$ ) for another group for no ETOH treatment with only *S. gordonii* (D,d) and untreated 293TT cell control E, e. (Figure 6).

To better understand this relationship between ETOH exposure and HPV entry we performed another assay with pre-treatment using inhibitors for ADH (4-Mpyr) and ALDH (TDS) in 293TT human epithelial cells.

ETOH metabolism in the 293TT cells was assessed following co-cultivation of *S. gordonii* (strain 4, AA producer). We recorded a significant increased effect on HPV 16 entry which was modified with treatment of TDS, an inhibitor of ALDH. TDS causes an accumulation of AA (3.83  $\mu$ M) with a blockage of AA degradation to acetate/acetic acid. A significant increase ( $p \leq 0.006$ ) in HPV 16 entry in 293TT cells following co-incubation with ETOH treated *S. gordonii* was recorded (55.0% $\pm$  8.0%) compared to no TDS treatment or ETOH treatment of *S. gordonii* before co-incubation (18.0% $\pm$  6.0%). However, TDS treatment, and AA accumulation effect on HPV 16 entry could be abrogated when the epithelial cells were initially treated with 4-Mpyr to block ADH activity (1-2% reduction in baseline control levels) which we suggest resides on *S. gordonii* membrane. An assay demonstrated untreated *S. gordonii* to have an ADH  $OD_{340} = 0.370$  level in comparison to ETOH (50 mg/mL) treatment of *S. gordonii*  $ADH_{340} = 2.182$ .

**Conclusion:** An ADH block suppresses ETOH metabolism and AA synthesis which can be fostered by ADH activity of *S. gordonii* with ETOH exposure which also releases AA and produces enhancement of HPV 16 entry. Attachment by *S. gordonii* to HOK is facilitated through surface proteoglycan such as syndecans. This process also activates phosphorylation of EGFR and FC activity leading to HPV 16 entry. However, in clinical settings, other pathways such as retinol dehydrogenase are likely candidates for amplification of this process [76]. We suggest markers expressed in these assays form a set of detectors for HPV 16 infection in conjunction with *Streptococcus spp.* after metabolism of ETOH.

In association with HPV 16 entry FC cycles from the cell membrane where it is under the influence of EGFR and forms complexes with viral derived substrates on cytosol organelles; such as the golgi. At the golgi FC acts as a convertase and cleaves virions glycoproteins [35,72,75]. In one case; RNA virus; lenti-virus (HIV) glycoprotein (gp160) undergoes FC enzymatic degradation to gp120 and gp44 [75]. Host immune response is also responsive to presence of *Streptococcus spp.* causes the release of immune regulatory factors such as, APRIL (B-cell stimulating factor) as part of an innate immunity through Toll-like receptors [13,14,78].

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