Oral Microbiome Induced Tumor Stemness Pathway in Oral Cancer

Oral Microbiome Induced Tumor Stemness Pathway in Oral Cancer

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ABSTRACT

Background: The mechanisms of Oral squamous cell carcinoma (OSCC) development and progression are largely unknown. Various studies have identified self-renewing population of cancer cells, the cancer stem cells (CSCs) among the heterogeneous cell population of OSCC. These CSCs maintain stemness (self-renewal and undifferentiated state) by activating molecular pathways of stemness. Our lab's previous work has characterized the hypoxia inducible factor 2 alpha (HIF-2α) tumor stemness pathway in lymphoma, as well as OSCC. The purpose of the study was to develop an in vitro assay platform that can identify oral bacteria having potential ability to modulate the tumor stemness pathways. We hypothesized that the oral microbiome of patients affected with OSCC activates the HIF-2α tumor stemness pathway. **Innovation:** The completion of the study may help to identify oral bacteria that may mediate tumor stemness pathway, and therefore facilitate in early diagnosis, as well as therapeutic monitoring of OSCC treatment. **Materials and Methods:** The SCC-25 cell line that contain ABCG2+ CSCs were treated with lipopolysaccharide (LPS) to define the toll-like receptor (TLR) mediated activation of tumor stemness switch. The assay for tumor stemness switch included the qPCR study of genes involved in the HIF-2α tumor stemness pathway, invasion/migration in a Boyden Chamber assay, self-sufficiency (the ability of the ABCG2+ cells to maintain stemness in the serum-free media), and niche-modulation (ability of the conditioned media of the ABCG2+ CSCs to reprogram cell components of tumor microenvironment such as mesenchymal stem cells and or immune cells). Next, the assays for the tumor stemness switch was repeated in SCC-25 cells treated with the processed saliva of OSCC patients. Finally, we attempted to recover live bacteria from the SCC-25 cells that exhibited tumor stemness switch following saliva treatment. Moreover, the identified bacteria was then tested for its direct ability to induce tumor stemness switch in SCC-25 cells. **Results:** SCC-25 cells tolerated the treatment of 5/22 saliva samples obtained from 22 OSCC patients. Out of these 5 saliva samples, 2 (patient #21 and 22) showed significant induction of genes involved in the tumor stemness switch. From the patient #21 saliva sample, we identified Fusobacterium nucleatum that internalized into ABCG2+ CSCs and modulated tumor stemness switch. **Conclusion:** The in vitro assay of tumor stemness switch using SCC-25 cell line has shown to be effective in identifying oral bacteria relevant in OSCC progression. Therefore, there is a great relevance of this assay to be used as a platform to study the role of oral microbiome induced tumor stemness switch, a cellular and molecular mechanism having clinical significance in OSCC diagnosis and management.

FIGURES AND TABLES

CHAPTER 1: SPECIFIC AIMS

Oral squamous cell carcinoma (OSCC) is the most common oral cavity malignancy. It has been associated with many risk factors such as smoking and alcohol consumption, and many molecular abnormalities have been linked to OSCC such as mutations in *p53* and mutations in NOTCH1 and Wnt signaling pathways (1, 2). Moreover, stemness (self-renewal and de-differentiated state) may also play key role in OSCC. Cancer stem cells (CSCs) have been identified in OSCC, and they express stemness factors Nanog, Sox2, and Oct4 (3-5). Epithelial-mesenchymal transition (EMT) may also induce stemness to OSCCs. In the OSCC microenvironment, stemness may induce an immunosuppressive microenvironment, and may thus drive tumor growth, drug resistant and relapse. In this context, our laboratory has identified molecular mechanism of stemness in human embryonic stem cells, as well as solid tumors including oral cancer. Importantly, our lab identified hypoxia inducible factor 2 alpha (HIF-2α) stemness pathway in embryonic stem cells (ESC) (6), and a MYC-HIF-2α tumor stemness pathway in lymphoma (7) and OSCC (8). Furthermore, our lab showed that bacteria may modulate the stemness pathway in bone marrow mesenchymal stem cells (9). However, it is not yet clear if oral bacteria may modulate the tumor stemness pathway to drive OSCC growth.

The goal of this project is to develop an experimental plan to identify oral bacteria that modulate tumor stemness, and to study the role of the oral microbiome in oral cancer stemness. The oral microbiome is defined as the collection of microorganisms that are present within the oral cavity in several habitats, including the teeth, gingiva, gingival sulcus, tongue and generally all oral mucosa (10). The oral microbiome is comprised of over 600 species, and some species are prevalent more at specific habitats within the oral cavity than others (11). The potential effect of the oral microbiome on the initiation and/or progression oral cancer is not yet fully understood. A recent study from our lab and collaborators have shown that transfer of oral microbiomes from

tumor bearing or healthy mice into germfree recipients significantly increased the numbers and sizes of tumors compared to those in mice that remained germfree but that were exposed to 4-NQ (12). Additionally, Ha et al have shown that prolonged and repetitive infection with P. gingivalis promotes epithelial-mesenchymal transition (EMT)-like changes and stemness in OSCC cells (13). Furthemore, we found that bacteria may modulate stemness by regulating the TLR-based signaling pathway (14). The study of the oral microbiome can help in screening for cancer and also in early diagnosis. It has been speculated that bacteria can be a driving factor in oral carcinogenesis, or play a role in the progression of cancer, showing increased population of certain bacteria in the setting of oral cancer (15). These preliminary studies provide rational to develop an experimental plan to identify oral bacteria that modulate tumor stemness pathway in OSCC.

Based on the above work, we hypothesize that the specific bacteria of oral microbiome may modulate OSCC stemness. To study this hypothesis, we have proposed the following Aims:

Aim 1: Identify an appropriate OSCC cell line to study the role of oral microbiome in oral cancer stemness.

Hypothesis: OSCC cell line that exhibit tumor stemness phenotype following treatment with the bacterial product lipopolysaccharide (LPS) is suitable to study the role of oral microbiome in oral cancer stemness.

To test the hypothesis, we will use an OSCC representative cell line, the SCC-25. We have previously shown that this cell line contains ABCG2+ CSCs that mildly express the gene involved in the MYC/HIF-2α stemness pathway (16). We first propose to characterize the tumor stemness phenotype in this cell line by using the TLR receptor agonist LPS, as performed previously in our

lab (14). Next, we propose to treat the cell line with the processed saliva obtained from OSCC patients either under remission or relapse after chemotherapy. The saliva treated SCC-25 cells will be subjected to study gene/protein expression of HIF-2α tumor stemness pathway. The role of TLR 2 or 4 pathway in the oral microbiome induced tumor stemness will be evaluated by treating the saliva treated cells with the neutralizing antibodies against TLR 2 and 4.

Aim 2: Identify oral bacteria that induces stemness switch in oral cancer.

Hypothesis: Specific bacteria present in the saliva of oral cancer patients activates tumor stemness switch in the SCC-25 cell line.

To test the hypothesis, we will first repeat the experiments in Aim 1 by using antibiotic treated saliva; antibiotic will be chosen based on the microbial culture and sensitivity test performed on saliva that induces tumor stemness switch to SCC-25 cells (Aim 1 results). Then, the cell extracts (CSCs and non-CSCs of saliva treated SCC-25 cells) of both the experimental groups (antibiotic treated versus non-treated saliva) will be subjected to microbial culture to identify bacterial presence. It is expected that anti-biotic treated saliva will fail to exhibit tumor stemness switch in SCC-25 cells confirming the role of live bacteria required to induce the tumor stemness pathway. It is also expected that the live bacteria will be detected in the CSC of the experimental group, where the processed saliva was not treated with antibiotics. Once a specific bacteria is identified in the CSC population, its role in tumor stemness switch will be confirmed by infecting SCC-25 cells with the identified bacteria, and then repeating the experiments described in Aim 1.

CHAPTER 2: INTRODUCTION

Oral Squamous cell carcinoma (OSCC)

OSCC is the eighth leading malignancy worldwide (17, 18). South Asia has the second highest incidence of OSCC, probably due to associated poor oral hygiene, tobacco and betel nut chewing as well as smoking (19). Treatment modalities involve surgery, radiation, chemotherapy, and immunotherapy including checkpoint inhibitors. However, despite progress in treatment, the mortality rate of oral cavity cancer remains very high and estimated to be about 50% within 5 years (17).

One of the most difficult clinical challenges of oral cancer is drug resistance and recurrence with more aggressive, invasive and metastatic behavior (20). A study of 275 patients with OSCC have estimated the recurrence rate to be approximately 32.7%, and this was strongly associated with T stage, differentiation degree, Pn stage, and flap repair (21). Other studies relate the relapse to reasons such as field cancerization phenomenon i.e. oral mucosa adjacent to the tumor showing microscopic changes of premalignant nature suggesting that the whole field may be susceptible to recurrence (22). Another contributing factor to drug resistance is the tumor microenvironment which is comprised of immune cells, stromal cells as well as mesenchymal stem cells. The microenvironment in oral cancer is often hypoxic, which also contribute to drug resistance as well as metastasis. Furthermore, OSCC may contain inherently drug resistant, Cancer stem cells (CSC). In fact, a decade of work now indicates that CSCs plays a key role in cancer progression, invasion, distant metastasis and recurrence (23). CSCs are more resistant to chemotherapy than the cells that do not express CSC markers (24). CSCs in OSCC has been identified and shown to express CD44, ALDH1, and side population markers. We found that OSCC contain ABCG2+ CSCs exhibiting drug resistance (25), and that these CSCs may modulate tumor microenvironment by reprograming

mesenchymal stem cells (MSCs) (25). Thus, field cancerization, immunosuppressive microenvironment and CSCs contributes to drug resistance, aggressiveness and metastasis of OSCC. Specially, molecular pathways that confer CSC or stemness property to cancer cells may play an important role OSCC progression. Importantly, *p53* tumor suppressor gene plays key role in stemness, and it is either mutated and or lose function in the majority of oral cancer (26).

The major challenge for the management of oral cancer is that the exact mechanisms of carcinogenesis and progression are still unclear, which hamper our ability of early diagnosis and treatment. Whereas, 5-year survival for local stage I and II disease in the oral cavity is 82%, the late stage IV with metastasis is only 26%, which highlights the importance of early diagnosis (27). Therefore, novel insights into the pathogenesis of oral cancer including the mechanism of carcinogenesis and progression may greatly contribute to early diagnosis and cure of oral cancer. The pathogenesis of oral cancer is not yet clear, but many risk factors have been identified, including smoking, alcohol, HPV, immunosuppression, and areca nut chewing that contribute to initiation as well as progression of oral cancer (19). These factors may contribute to pathogenesis by modulating p53 tumor suppressor genes, and inducing oxidative stress, DNA damage, mutation and a chronic inflammatory response (26). In fact, Human papillomavirus (HPV) has been strongly associated with the pathogenesis of oropharyngeal cancer (28, 29). Interestingly, this virus may also be involved in the activation of tumor stemness program (30).

However, potential influence of the resident oral bacteria on the pathogenesis and prognosis of OSCC is not yet fully understood. The oral microbiome is defined as the collection of microorganisms present within the oral cavity in several habitats, including the teeth, gingiva,

gingival sulcus, tongue and generally all oral mucosa (10). The oral microbiome is comprised of over 600 bacterial species (11). Previous studies indicate these oral bacteria may contribute to the pathogenesis of distant organs such as heart and lung, as well as metabolic diseases (12). Changes in oral microbial flora and periodontal disease may contribute to cardiovascular disease, diabetes mellitus, as well as respiratory disease (31). These studies have increased awareness of the association of the oral microbiome with systemic diseases.

Microorganisms and carcinogenesis

Viruses have been found to be associated with the carcinogenesis of many cancer types. Human papilloma virus (HPV) has been correlated with many oropharyngeal, nasopharyngeal, cervical, anal, and penile squamous cell carcinoma (32). HPV associated carcinogenesis is associated with the upregulation of the E6 and E7 oncoproteins that induces oncogenesis by altering p53 (33). Another virus is the Human T cell lymphoma virus that causes T cell lymphomas by inducing Tax protein that effects the NFkB and PI3K-Akt signaling pathways (34). The mechanism of viral induced cancer differs from the bacterial induced cancer where the latter is derived from progressive changes caused by chronic inflammation (35).

Previous studies have highlighted the important role that some bacteria play in carcinogenesis of cancers. For instance, Helicobacter Pylori infection has been linked with the development of stomach cancer. H.pylori causes progressive changes in the gastric mucosa through chronic gastritis, atrophic gastritis and eventually intestinal metaplasia, all of which are established risk factors for gastric cancer (35). Many other bacterial and viral induced cancer have been reported

in the literature. Bacteria and viruses have distinct mechanisms by which they induce carcinogenesis, and previous studies have increased interest in this field.

Oral microbiome and carcinogenesis

It has been speculated that bacteria can be a driving factor in oral carcinogenesis, or play a role in the progression of cancer, showing increased population of certain bacteria in the setting of oral cancer (15). Other studies have indicated that some bacteria may correlate strongly with OSCC, such as P. gingivalis, F. nucleatum, P. intermedia and other species (36). Candida, a fungal organism present within the oral microbiome, was also identified in oral lesions (36), such as oral leukoplakia (37). Other studies have reported reduction in the abundance of the majority of oral microbiome in oral cavity and oropharyngeal cancer subjects when compared to healthy patients (15, 38, 39).

Even though the correlation of oral microbiome with OSCC was shown in these studies and many others, the molecular mechanism that bacteria employ to drive oncogenesis or progression is not yet fully understood. Some of the proposed mechanisms of bacteria-induced oncogenesis include chronic inflammation (10, 35), direct manipulation of host cell biology or altering of tissue stem-cell homeostasis (10). Bacteria-secreted endotoxins (40), such as N-nitroso compounds and acetaldehyde may induce oxidative DNA damage (36). However, it is not yet explained whether cancer can be caused by a single pathogen or require the coordinated action of multiple bacteria (40).

Ha N et al showed that prolonged and repetitive infection with P. gingivalis promotes epithelial-mesenchymal transition (EMT)-like changes and stemness in OSCC cells (13). Additionally, a recent study have shown that transfer of microbiomes from tumor bearing or healthy mice into germfree recipients significantly increased the numbers and sizes of tumors compared to those in mice that remained germfree but that were exposed to 4-NQO (12). These studies indicate that bacteria may modulate cancer stem cells (CSCs). This is interesting, and need to be explored, as it may reveal a new avenue of research on microbiota/cancer interaction.

Stemness, enhanced stemness and carcinogenesis

Stem cells are a state of undifferentiation with the capability of self-renewal and proliferation into stem cell and non-stem cell progeny. Stemness is the functional attribute of stem cells, providing it with the ability to remain in an undifferentiated state (41, 42). In Embryonic stem cells (ESCs), stemness is maintained by transcription factors Nanog, Sox2, and Oct4. Stemness in differentiated cells can be induced by these transcription factors in combination with MYC, and KLF4. HIF-2α, another transcription factor that modulates Nanog, and Oct4 in ESCs, can also be considered as a key transcription factor for embryonic stemness (6). In hematopoietic stem cells (HSCs), stemness is regulated by developmental pathways such as Wnt and Notch pathways, as well as VEGF/HIF-1α autocrine pathways (43). These pathways regulate the integrated gene expression networks that maintain the stemness state, which may be visualized as an attractor state in the Waddington's epigenetic landscape metaphor (44) (Figure 1).

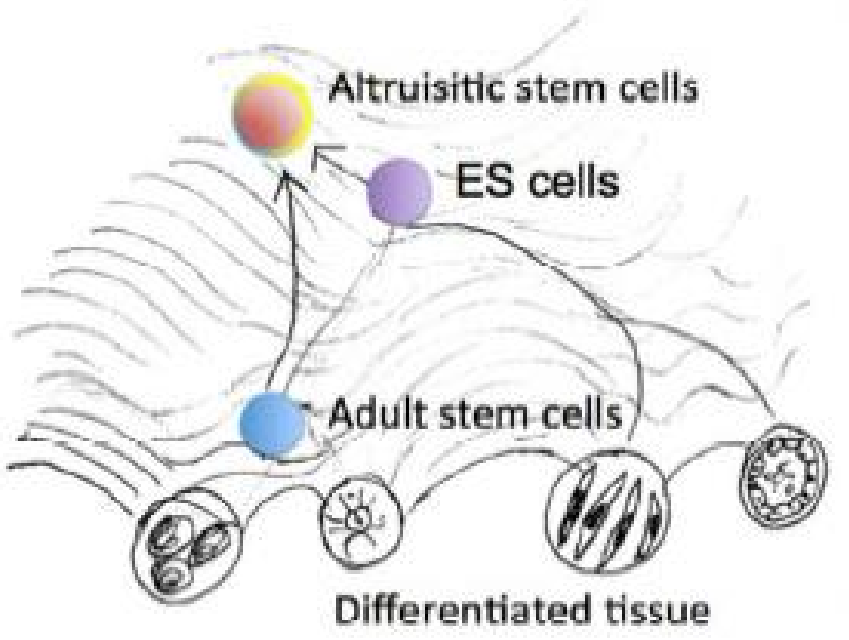


Figure 1. Schematic representation to depicting stemness as valleys in Waddington's epigenetic landscape metaphor.
The valleys are the stable cell state attractor in the dynamical system governed by genetic interaction networks, whereas trajectories (shown with arrows) are pathways that attractors follow to reach a stable state. Thus, the trajectories may represent stemness pathway that can lead a cell state from one attractor to another attractor state, or lower degree of stemness to higher degree of stemness state. ESCs undergo reprogramming to the enhanced stemness state (6) that corresponds to the altruistic stem cell (45) phenotype (46). In a similar manner, adult stem cells may also reprogram to the ASC phenotype. ASCs are transient, and therefore, could be considered as the intermediate cell state (46), as proposed by Waddington (47). The image has been adapted from Talukdar J et al (25).

This "epigenetic landscape" metaphor has been a popular metaphor to explain cell identity and lineage specification during tissue development. Conrad Waddington first described this landscape, where a ball rolling down on an inclined surface of valleys and ridges. In this "epigenetic landscape" metaphor, branching valleys represent cell state trajectories that ends in a stable cellular states, such as ESC, adult stem cell, or differentiated cell states, whereas ridges represents barrier between those trajectories and corresponding stable cell states (Figure 1). If we consider the stemness of a cell state as a dynamical system governed by genetic interaction

networks, then, molecular pathways that regulate genetic networks can be visualized as the "switch" to move a cell state from a lower to a higher valley, or lower to higher degree of stemness. For example, epithelial cells may undergo EMT to enhance stemness, and acquire a new stable cell state of mesenchymal phenotype. In the EMT process, transcription factors snail, and twist plays key roles, and these transcription factors regulate integrated gene expression networks that maintain the mesenchymal stem cell state, and therefore, the putative transcriptional network of these transcription factors serve as a stemness pathway that reprogram epithelial cells to mesenchymal stem cell state (41). Thus, in the epigenetic landscape, the trajectories may represent stemness pathway that can lead a cell state from one attractor to another attractor state, or lower degree of stemness to higher degree of stemness state. The reprograming of an epithelial cell state to an ESC state through the process of induced pluripotency is an example of moving from a lower to higher valley in the inclined "epigenetic landscape".

Our lab has identified an unstable stemness state, the enhanced stemness state having significance in the carcinogenesis process of stem cells. Briefly, when the human ESCs were exposed to extreme hypoxia/oxidative stress, while most of the cells activated p53, and underwent differentiation, in a few of the ABCG2+/SSEA3+ cells., p53 activation was transiently suppressed by the HIF-2α stemness pathway, which permitted the genetically unstable cells to propagate, and these cells acquired a higher degree of stemness state, i.e. they become niche-independent (6). Thus, the "enhanced stemness" state is a p53 deficient stemness state being characterized in ESCs. Nanog, Sox2 and Oct4 are highly expressed in this enhanced stemness state, as the p53 mediated inhibition of these proteins are absent. The high expression of these factors may provide self-sufficiency state by yet unknown mechanisms (6). Interestingly, ESCs exhibiting enhanced

stemness exhibited altruistic behavior, as they secreted cytoprotective factors including glutathione that conferred protection to less-fit ESCs, and in the process losing self-fitness (sacrificed self-fitness to enhance group fitness) (6). In the epigenetic landscape metaphor, the altruistic stem cell (45) state is on the top of a ridge (Figure 1), as the cell state is unstable as it exhibits enhanced stemness phenotype.

Based on Waddington's epigenetic landscape model, and the stem cell niche (the specific site of a tissue, where stem cell resides to maintain quiescent state), the stemness can be broadly divided into three types: niche-dependent, niche-independent and niche-modulatory (Figure 2) (44). The niche-modulatory stemness corresponds to the enhanced stemness state, where the stem cells acquire extreme self-sufficiency and capable of modulating the niche by cytoprotective activity, as demonstrated in ESCs (6, 46).

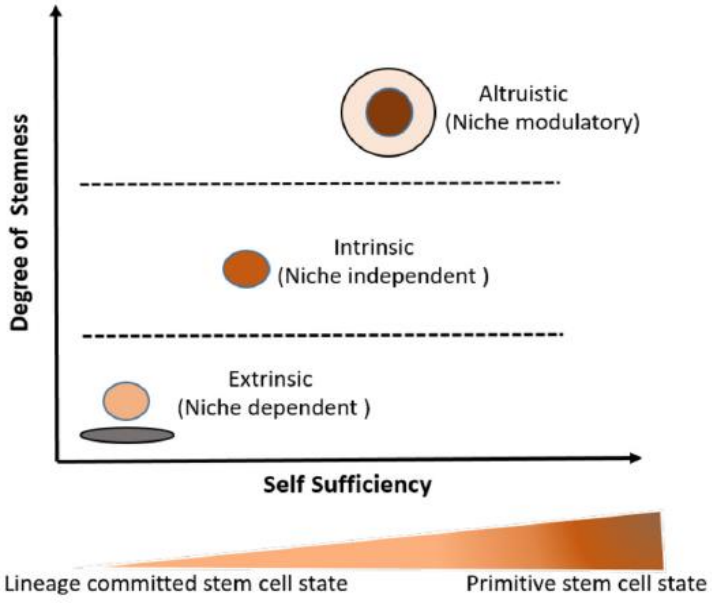


Figure 2. Schematic of developmental/hierarchical ontogeny of stem cells.
Three broad types of stemness (self-renewal and undifferentiated state) as a relational attribute to stem cell niche and developmental ontogeny of the stem cell undifferentiated state. The idea of the degree of stemness incorporates both stem cell niche and developmental/hierarchical ontogeny of a given stem cell state. Higher the degree of intrinsic stemness, higher is the level of

self-sufficiency and the hierarchical position of the stem cell state in the developmental ontogeny of stem cells. In this model, primitive stem cell state such as naïve ESCs exhibit higher degree of intrinsic stemness and are more self-sufficient than lineage committed stem cells such as CD34+ HSCs (46). Adapted from Pal B and Das B, 2017. (44)

Our ongoing research indicates that the enhanced stemness state may serve as a pre-malignant state in oral cancer carcinogenesis. The oral carcinogen 4-NQO treated mice showed the presence of CD271+/ABCG2+ oral mucosa cells exhibiting enhanced stemness phenotype, and a rare fraction of these cells (1 in $1x10^7$ cells) showed malignant conversion (25). Notably, the enhanced stemness to tumor stemness switch of the 4-NQO treated oral mucosa cells involved the mutant p53 conformation (48) suggesting the clinical relevance of this model, as OSCC exhibit mutant p53 conformation.

Comprehensive genomic and functional analysis has revealed several critical genes and pathways critical in Oral cancer, including *p53*, *CDKN2A*, *CASP8*, *FAT1*, *NOTCH1*, *HRAS*, *PIK3CA*, *MLL2*, and *FBXW7* (1, 26, 49). Even though multiple genes contribute to the cellular transformation process, the importance of *p53* stands out, especially in oral cancer where our knowledge of key driver oncogenes is still limited. Clinically, *p53* mutations correlates with poor prognosis and worse patient survival. The Cancer Genome Atlas (TCGA) findings reveal explicit *p53* mutations at different exon locations, majorly limited to the DNA-binding domain and representing the hotspot mutations for oral cancer. Mutations in *p53* either hinders its binding directly to the p53-responsive element in DNA (e.g., p53R273H, p53R280K), or alters the protein conformation to disrupt its functionality (26). However, in both cases, not only is the transcriptional function of WT *p53* is lost but the mutant form gains either dominant negative activity by hetero-oligomerization with the WT *p53* or a gain of function property that contributes to malignancy.

Additionally, changes in epigenetic levels in p14/ARF promoters, methylation of the p53 promoter or persistent expression of Mdm2 and Mdmx may also contribute to loss of function of wtP53 (50). *P53* mutation is one of the most common mutations in head and neck cancers (Figure 3). In this context, the *p53* deficient enhanced stemness state may have clinical relevance as a pre-malignant phenotype in oral cancer.

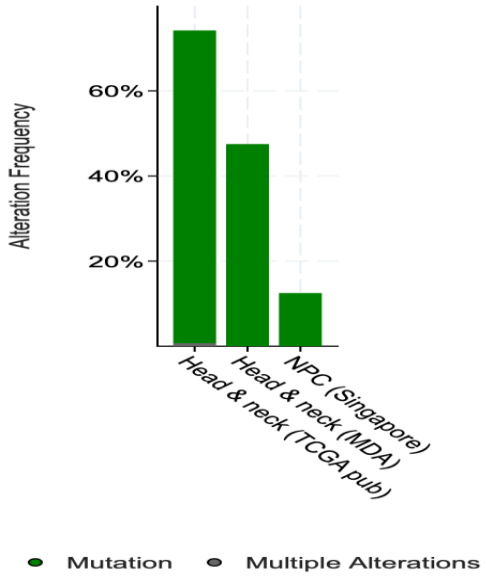


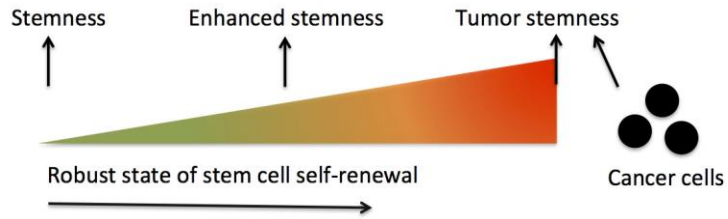
Figure 3. Frequency of *p53* mutation in head and neck cancer.
Histogram representing the frequency in p53 gene alterations across Head & Neck cancer and Nasopharyngeal carcinoma patient samples obtained from different publicly available datasets.

Induction of enhanced stemness reprogramming in cancer stem cells: the tumor stemness switch

Cancer stem cells (CSCs) have been identified in many cancer types. CSCs reside in the specific niches of tumor microenvironment, and may be niche-dependent and or niche-independent. Studies of oral CSCs, the ALDH+ and CD44+ cells suggest that these cells are niche dependent, as these cells require growth factors to maintain their stemness (51-53). We speculated that cancer

cells might hijack the enhanced stemness pathways for tumor progression and metastasis (54). Enhanced stemness state in ESCs is characterized by the activation of the HIF-2α stemness pathway, where Nanog transcriptionally regulates HIF-2α. Indeed, many studies have shown that ESC related gene signatures are upregulated in highly aggressive cancer. The activation of this ESC stemness pathway in CSCs may lead to rapid tumor progression as often seen in blast crisis of leukemia. This process of the activation of enhanced stemness state in CSCs can be termed as the tumor stemness switch (6) (Figure 4). Our lab recently confirmed the activation of the enhanced stemness pathway in lymphoma stem cells (7), thus confirming the occurrence of tumor stemness switch. We noted that the tumor stemness switch in lymphoma is characterized by the regulation of the HIF-2α stemness pathway by an oncogene such as MYC. Consistent with our findings, several studies have shown that *MYC*, *HIF-2α*, *Nanog*, *SOX-2* and *OCT-4* are the stemness associated genes (3-5) and their enhanced expression in OSCC, particularly Nanog, has been reported (55).

A.



B.

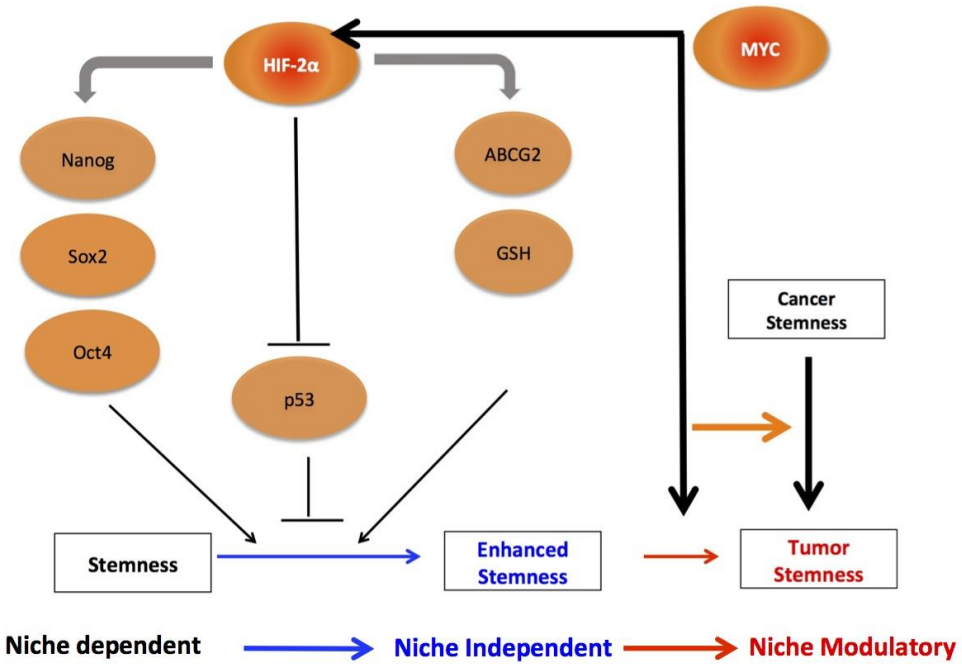


Figure 4. Schematic diagram of the HIF-2α stemness switch pathway and its role in tumor stemness switch.

A) The de-differentiation of stemness state to higher degree of stemness: the enhanced stemness state (6), and tumor stemness (7). HIF-2α stemness pathway is activated during the enhanced stemness, and tumor stemness switch (6, 7). The schematic is taken from the Das B: Altruistic stem cells and cancer stem cells, Cancer Stem Cells, 2014 (46). B) HIF-2α mediated stemness pathway. High levels of HIF-2α leads to enhanced transcriptional activity of Nanog, Sox2 and Oct4, and the inhibition of p53 that leads to the reprogramming of stemness to a highly cytoprotective and altruistic, the Enhanced Stemness state (6). MYC hijacks this pathway by regulating HIF-2α and Nanog transcriptional activity. In lymphoma, the pathway reprograms cancer stemness to tumor stemness state (7). In oral cancer stem cells, the tumor stemness state exhibit niche modulatory activity i.e. ability to reprogram mesenchymal stem cells to secrete factors that can modulate the tumor microenvironment to a highly pro-tumorigenic and immunosuppressive state (25).

Emerging role of bacteria in modulating tumor stemness switch in CSCs

Since the discovery that M.tuberculosis invade and modulate mesenchymal stem cell stemness (9), there has been a growing recognition of the potential implications of bacteria/stem cell interaction in infectious diseases and cancer. Bacteria may modulate stemness. Indeed, we found that M.tuberculosis modulate the HIF-1α signaling pathway, and enhances the stemness of CD271+ BM-MSCs (9).

The tumor stemness may also be associated with immunosuppression as CSCs exhibiting tumor stemness switch modulate the tumor microenvironment to be highly immunosuppressive by secreting anti-inflamatory cytokines (56). Considering that immunotherapy is playing an increasing role, it is important to study if bacteria induced tumor stemness switch involves immunosuppression.

Significance

1) Test the contribution of oral microbiome in modulating the stemness potential of tumor niche and induce tumorigenesis.
2) Determine how CSCs modulate F. nucleatum to transform the non-CSCs to highly immunosuppressive CSCs.
3) Develop in vitro assays to demonstrate the host/pathogen interactions between ABCG2+ oral CSCs and F. nucleatum and target them for therapeutic outcomes.

Innovation

1) Use the in vitro ABCG2+ oral CSCs and F. nucleatum host/pathogen interaction model to identify specific modulators of MYC-HIF-2α stemness pathway that mediates the EMT-phenotype, CSC production and enrichment, and oncogenic immunosuppressive niche modulatory phenotypes.
2) Probe sustained F. nucleatum infection and new CSC-related enhanced stemness mechanisms as key contributors to F. nucleatum-associated Oral SCC.

CHAPTER 2: SPECIFIC AIM 1: IDENTIFY AN APPROPRIATE OSCC CELL LINE TO STUDY THE ROLE OF ORAL MICROBIOME IN ORAL CANCER STEMNESS.

Background and rationale

The oral microbiome is defined as the collection of microorganisms that are present within the oral cavity in several habitats, including the teeth, gingiva, gingival sulcus, tongue and generally all oral mucosa (10) as well as in saliva (57). Existing research indicates the presence of over 600 species, and some species that forms the microbiome at specific habitats within the oral cavity than others (11). The potential effect of the oral microbiome on the initiation and/or progression oral cancer is not yet fully understood. However, it has been speculated that oral bacteria may be a contributing factor in oral carcinogenesis and/or progression. Bacterial population are found to be increased in the setting of oral cancer (15). Further studies identified specific bacterial species that correlate strongly with OSCC, such as Porphyromonas gingivalis, Fusobacterium nucleatum, and Prevotella intermedia. Other correlated microorganisms include Leptotrichia, Campylobacter, and Rothia species (36). Oral leukoplakia lesions, which are considered an oral potentially malignant lesion, may be enriched with specific bacterial species that include Fusobacterium, Leptotrichia, Campylobacter, and Rothia species, with Fusobacterium being the most consistent finding (36). A recent study reported significant reduction in the abundance of Firmicutes (Streptococcus) and Actinobacteria (Rothia), and an increase in abundance of Fusobacteria (Fusobacterium) in OSCC tumors in 13 patients compared to healthy samples (15). Another study showed increase in abundance of Oribacterium in oral cancer and oral pre-cancer patients (39), while a recent study showed that the abundance of Fusobacteria increased significantly in correlation with the progression of oral cancer from the healthy controls at 2.98%, to OSCC stage 1 at 4.35%, and finally OSCC stage 4 at 7.92% (58).

Although such correlation of specific bacterial species with OSCC has been identified, the molecular mechanism that bacteria employ to drive oncogenesis or progression is not yet understood. The proposed mechanisms of bacteria induced oncogenesis include chronic inflammation (10, 35), direct manipulation of host cell biology and altering of tissue stem-cell homeostasis (10). Another proposed mechanism is the inflammation-induced DNA damage that occurs in the epithelial cells caused by microorganism-secreted endotoxins (40), such as N-nitroso compounds and acetaldehyde may induce oxidative DNA damage (36).

Oral bacteria may also directly induce tumor stemness switch of oral cancer. A positive correlation is reported between F. nucleatum level and expression of Oct-4, Nanog and SOX2 in colorectal cancer patients (59). These Oct4, Nanog, Sox2 are three core transcription factors in embryonic stem-cell-ness (6). Interestingly, upregulation of these transcription factors may induce stemness in differentiated cells, and also in non-stem cancer cells (60). Bacteria may also induce stemness by regulating the mechanisms of epithelial-mesenchymal transition (EMT). A study has shown that prolonged and repetitive infection with P. gingivalis promotes EMT-like changes and stemness in OSCC cells (13). A recent study has shown that transfer of microbiomes from tumor bearing or healthy mice into germfree recipients significantly increased the numbers and sizes of tumors compared to those in mice that remained germfree but that were exposed to 4-NQO (12). It is possible that this increase in tumor size and number was due to the activation of the tumor stemness pathway.

One of the key bacterial products is called lipopolysaccharide (LPS), which is an endotoxin that is secreted from the outer membrane of Gram negative bacteria (61). LPS is known to induce immune

response, and many studies have shown that some bacteria, such as P. multocida are dependent on their LPS for infection (62). Another important factor that bacteria may play a role in is the activation of toll-like receptors (TLR). TLR is activated via an inflammatory pathway, which in turn activates NF-κB that promotes many proinflammatory pathways that affect both acute and chronic inflammation (63, 64). TLR plays an important role in inflammation induced tumor stemness (64) (Figure 5). These are possible mechanisms by which the oral microbiome may interact with the host and/or cancer cells.

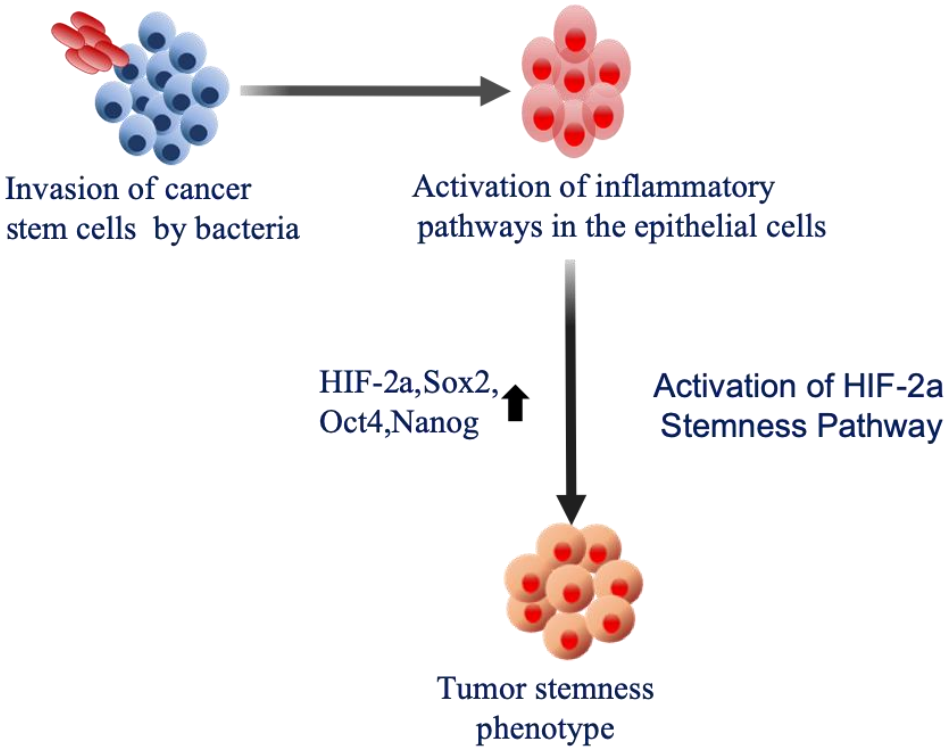


Figure 5. Proposed bacterial induced tumor stemness switch.
Invasion of oral cancer stem cells by microbiomes activates inflammatory pathways that then activates the HIF-2α stemness pathway, leading to CSC reprograming to tumor stemness phenotype characterized by invasive, and niche modulatory activities.

The concept of tumor stemness has been linked to tumor progression in different tumor models. Specific stemness pathways are being exploited by cancer cells that rewires host cellular machinery and promote stemness. Activation of these stemness pathways are upregulated in response to multiple microenvironmental cues including hypoxia. Previous reports from our lab indicate that ABCG2+ lymphoma stem cells activate MYC/HIF-2α stemness pathway to promote the self-renewal of cancer stem cells (7). Also, previously our lab showed that the ABCG2+ cells are localized at the hypoxic zone of sarcomas, neuroblastomas and small-cell lung carcinoma and exhibited increased Oct-4 expression (65). Additionally, we developed an in vitro oral cancer model based on SCC-25 human oral cancer cell line. Our results emphasize that hypoxic microenvironment induced activation of MYC/HIF-2α stemness pathway to upregulate stemness and cytoprotection in specific subpopulation of oral cancer stem cells (66). We enriched these population using specific biomarker ABCG2/Epcam, which are frequently used as cancer stem cell markers (67). Our lab has also identified the tumor stemness phenotype components in the SCC-25 cell line. This phenotype is characterized by activation of MYC/HIF-2α pathway, maintenance of cellular hierarchy of ABCG2+ and ABCG2- cell populations, exhibiting a niche modulatory activity that reprogram mesenchymal stem cells (MSCs) to be protumorigenic, and exhibiting invasive capability (7). Thus, the SCC-25 cell line is appropriate to test our hypothesis that saliva may induce the tumor stemness switch.

Hypothesis

OSCC cell line that exhibit tumor stemness phenotype following treatment with the bacterial product lipopolysaccharide (LPS) is suitable to study the role of oral microbiome in oral cancer stemness.

Materials and methods

SCC-25 cell line culture:

The SCC-25 cell line obtained from ATCC (CRL-1628) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 Mm L-glutamine, 15 Mm HEPES and 0.5 Mm sodium pyruvate and supplemented with 400 ng/ml hydrocortisone and 10% fetal bovine serum (FBS). The SCC-25 cells were counted via the trypan blue exclusion method and the cells (~1×10^5) were seeded in 6-well tissue culture plates and incubated overnight at 37°C in the 5% CO_2 incubator and then the cells were subjected to various assays.

Bacterial lipopolysaccharide (LPS) treatment:

After washing twice with phosphate-buffered saline (PBS; Life Technologies, USA), the cells in each well were treated with 1,000 ng/ml of LPS (Sigma, USA) as described previously (61). After 3 days, the cells are washed with PBS and a fresh DMEM medium is used, and cells are grown for 1 week.

Patient selection:

The study was approved by the ethical committee at KaviKrishna Laboratory, GBP, Indian Institute of Technology, Guwhati. Oral cancer patients (n=22, table 1) and four healthy individuals were enrolled in the study. Following written informed consent, saliva was collected from these individuals. The clinical cases with head & neck cancer were selected based on history, and diagnosis performed at Guwahati Medical College. Previously treated subjects of oral squamous cell cancer were recruited based on pathological data.

Patient	Sex/Age	SITE OF CANCER	HISTOPATHOLOGY REPORT
1	50/F	Right cheek	Well Differentiated Squamous Cell Carcinoma
2	75/F	Oral cavity and Right cheek	Well Differentiated Squamous Cell Carcinoma
3	69/M	Mouth	Well Differentiated Squamous Cell Carcinoma
4	63/M	Oral cavity	Well Differentiated Squamous Cell Carcinoma
5	60/F	Tongue	Well Differentiated Squamous Cell Carcinoma
6	48/M	Buccal mucosa	Well Differentiated Squamous Cell Carcinoma
7	60/F	Oral cavity	Well Differentiated Squamous Cell Carcinoma
8	53/F	Oral cavity	Well Differentiated Squamous Cell Carcinoma
9	68/M	Oral cavity	Verrucous Carcinoma
10	46/M	Oral cavity	Moderately Differentiated Squamous Cell Carcinoma
11	40/F	Oral cavity	Well Differentiated Squamous Cell Carcinoma
12	80/M	Right cheek	Well Differentiated Squamous Cell Carcinoma
13	45/F	Gums	Well Differentiated Squamous Cell Carcinoma
14	68/F	Mouth	Well Differentiated Squamous Cell Carcinoma
15	55/M	Mouth	Well Differentiated Squamous Cell Carcinoma
16	68/M	Tongue	Well Differentiated Squamous Cell Carcinoma
17	35/F	Gums	Well Differentiated Squamous Cell Carcinoma
18	50/M	Buccal mucosa	Well Differentiated Squamous Cell Carcinoma
19	70/M	Buccal mucosa	Well Differentiated Squamous Cell Carcinoma
20	42/F	Buccal mucosa	Well Differentiated Squamous Cell Carcinoma
21	60/M	Buccal mucosa	Moderately Differentiated Squamous Cell Carcinoma
22	78/M	Base of tongue	Poorly Differentiated Squamous Cell Carcinoma

Table 1. Patients clinical data.

Saliva collection and processing:

Saliva collection was done by the spitting method as described previously (68). Briefly, in the early morning, patients accumulate their saliva from the floor of the mouth and the patient spits it out into the collection tube every 60 seconds. 1 ml of saliva is collected and dissolved in DMEM media in a 15 ml tube, and transported on ice to the lab, and immediately centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet is then resuspended in 1 ml DMEM, and flask is treated with 100 µl of the processed saliva. After 3 days, each flask is treated with amikacin and metronidazole for 24 hours to kill the remaining extracellular bacteria, and the cells were grown for 1 week.

Immunomagnetic sorting of ABCG2+ cells:

The ABCG2 cells were sorted by immunomagnetic sorting method that we recently applied to purify CD271+ cells by using phycoerythrin (PE) conjugation method (69). The ABCG2 antibody (#ab 3380, Abcam) was first PE conjugated by SiteClick antibody labeling kit as previously described (69). We achieved a 94% purity by this method. We also used a biotin conjugated method, where the biotin conjugated ABCG2 (#ab95692, Abcam) were subjected to biotin positive selection (#18559, Stem Cell Technologies, BC). We obtained enrichment similar to PE conjugation.

Real-time PCR:

Total RNA was isolated from the cell lines 1 week after LPS or saliva treatment using Trizol (ThermoFisher Scientific, # 15596018), and cDNA was synthesized using reverse transcriptase M-MLV (Promega, Madison, WI). The qPCR was performed in a 7900 system (Applied Biosystems, Foster City, CA) as described previously (43) using 2ng of starting cDNA in 50 cycles at 94°C for

60 seconds. RNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control and RNA was quantified using the ΔΔCt method using the SDS software, version 2.2.1 (Applied Biosystems, Foster City, USA). The following TaqMan gene expression primers were used: HIF -2α (Hs01026149_m1), Nanog (Hs02387400_g1), Oct 4 (Hs03005111_g1), ABCG2 (Hs00184979_m1), Sox2 (Hs00602736_s1), MYC (Hs00153408_m1), p21 (Hs00355782_m1), and GAPDH (Hs00266705_g1).

Clonogenic assay:

The assay was performed as previously described (65). Briefly, a total of 1x10^3 freshly sorted ABCG2+ and ABCG2- cells were seeded in methylcellulose medium (Methocult M3134, Stem Cell Technologies, BC or the Methylcellulose powder, # AC182312500, Fisher Scientific, was reconstituted in RPMI media with 10% FBS at KaviKrishna Lab, Guwahati, India). The cells were seeded in 6 well plate, incubated at 37°C and 5% CO_2, and colonies were counted after two weeks.

Western blot:

Western blot was performed as previously described (43). Briefly, protein extracts were obtained using the cell lysis buffer (Cell Signaling Technology, Beverly, MA) mixed with protease inhibitor cocktail (Mini Pill, Roche, Nutley, NJ). 50 μg of the clear protein lysate was resolved on 4% to 12% SDS-PAGE gels and transferred to 0.45-μm nitrocellulose membranes (Bio-Rad, CA, USA). The blocked membranes were incubated overnight with the following antibodies: HIF-2α (Novus Biologicals, Littleton, CO), MYC, Nanog, Oct-4, SOX-2 and β-actin (Cell Signaling technology). Immunoblotting was detected by enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Densitometry study was done using the Alpha

View software (Alpha InfoTech Alpha Imager 3400 Light Cabinet Camera Transilluminator Imaging UV, CA, USA). Following antibodies were used: Hm-MYC, Hm-HIF-2α, Hm-Nanog, Hm-Oct-4, and Hm-SOX-2 (Cell Signaling technology). Immunoblotting was detected by enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Densitometry study was done using the AlphaView software (Alpha Innotech Alpha Imager 3400 Light Cabinet Camera Transilluminator Imaging UV, CA, USA).

ELISA:

The protein levels of HIF-2α, MYC, Nanog, Oct-4 and SOX-2 were measured by In Cell ELISA using a horseradish peroxidase (HRP)-conjugated detection reagent (In-Cell ELISA Colorimetric detection kit, Thermo Fisher, #62200) and/or standard ELISA kits as described previously (65). HIF-2α -MBS-702348 was purchased from MyBiosource (San Diego, CA).

Matrigel invasion assay:

A Boyden chamber invasion assay was performed as previously described (65). Briefly, 8-μm pore size polyvinyl membrane-based chambers (Corning Life Sciences, Lowell, MA, http://www.corning.com/lifesciences) were coated with 100 μl of ice-cold Matrigel (7.5 mg/ml; BD Biosciences, San Diego, http://www.bdbiosciences.com) and incubated at 37°C for 4 hours. Appropriate numbers of cells (following trypsin neutralization) were added to the upper chamber, and the lower chamber was filled with appropriate media as desired. The chamber was incubated at 37°C for 8–24 hours, and invading cells were counted as described after crystal violet staining (70). When required, the invading and non-invading cells were isolated after brief trypsinization and expanded using the appropriate culture medium.

Statistical analysis:

The statistical calculations were performed with GraphPad Prism 4.0 (Hearne Scientific Software) using Student t test and One-Way ANOVA with Dunnett post-hoc test.

Results

Assessment of tumor stemness switch in the LPS treated SCC-25 cell line:

We have first assessed whether SCC-25 cell line is appropriate to study bacterial induced tumor stemness switch. For this purpose, we have developed an in-vitro assay using SCC-25 cell line. This cell line contains the highly tumorigenic ABCG2+ CSCs that have been shown to mildly express the MYC/HIF-2α stemness pathway (25). Using this assay, SCC-25 cell line was treated with LPS for three days as described previously (60) (Figure 6A). The LPS treated cells were then evaluated for the expression of genes involved in the HIF-2α stemness pathway. We found that 7-days of LPS treatment significantly increased expression of *MYC*, *HIF-2α*, *Nanog* and *Sox2* genes by 2-3-fold by RT-PCR (figure 6B). These results suggest that LPS treatment for 7 days upregulate the expression of genes involved in the HIF-2α stemness pathway.

Next, we performed flow cytometry and immunomagnetic sorting for ABCG2+ cells, and found that the ABCG2+ cell population was expanded (figure 6C). Next, we wanted to confirm that the expansion of the ABCG2+ cell population is clonogenic. We performed the clonogenic assay and found that the LPS treatment led to increase in clonogenic activity of these cells and maintained stemness, while the LPS negative group did not exhibit such increase (Figure 6D, E). In contrast, the effect of LPS treatment on the ABCG2- population had no effect on clonogenic activity (Figure

6F). This suggests that the LPS treatment specifically induce stemness and expand the ABCG2+ cell population while maintaining the hierarchical cell populations of ABCG2+ and ABCG2- cells. To further confirm the selective effect of LPS treatment, we compared the gene expression between the ABCG2+ and ABCG2- populations. We found that there is a significant increase in expression of genes involved in the HIF-2α stemness pathway of the ABCG2+ population (Figure 6G).

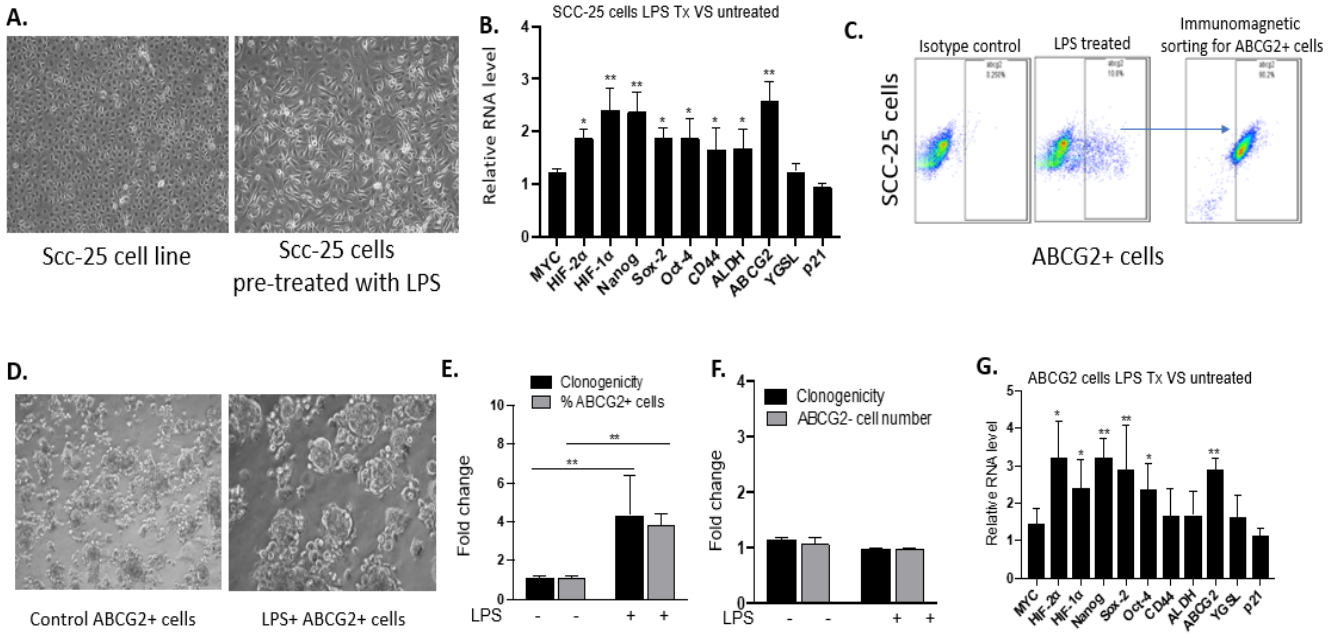


Figure 6. LPS induced tumor stemness switch in SCC-25 cell line.
*A) Representative phase contrast image of SCC-25 cells with and without LPS treatment. B) qPCR analysis shows upregulation of stemness associated genes involved in the HIF-2α stemness pathway in SCC-25 cells post LPS treatment. C) Representative flow cytometry shows further enrichment of ABCG2+ cells post LPS treatment. D-F) LPS treatment enhanced Clonogenic potential and ABCG2+ cell number. Additionally, the invasive and niche modulatory capacity of ABCG2+ vs ABCG2- cells was enhanced. G) qPCR analysis of immunomagnetically sorted ABCG2+ cells show increased expression of genes involved in the HIF-2α stemness pathway. N = 3 independent experiments; error bar represent mean ± SEM. *p<0.05, **p<0.001, ANOVA.*

Next, we were interested in whether the ABCG2+ population exhibit invasive potential, an important component of tumor stemness phenotype. To test this, we performed Matrigel migration assay. We found that the ABCG2+ cells exhibited increased number of invasive cells (Figure 7A, B). This suggests an increased invasive capability of ABCG2+ cell population.

Another important component of tumor stemness phenotype is the niche modulatory activity. To assess this, we collected the conditioned media of the ABCG2+ and ABCG2- cells and treated mesenchymal stem cells (MSCs) with this conditioned media. We used MSCs because they are known to be part of the tumor microenvironment, and are known to secrete growth factors important for tumor growth, such as stromal derived factor 1 alpha (SDF-1α), vascular endothelial growth factor (VEGF), glutathione, and interleukin 2 (IL-2) (25, 65). Indeed, we have found that the MSCs treated with the conditioned media of ABCG2+ cell population exhibited increased secretions of SDF-1α, VEGF, glutathione, and IL-2 (Figure 7C). This result suggests that LPS treated ABCG2+ cell population exhibited niche modulatory activity.

The increase in the ABCG2+ cell number was transient and only lasted for approximately two weeks after LPS treatment (Figure 7D).

Thus, LPS treatment led to the enrichment of highly clonogenic ABCG2+ cell population. The population lasted for two weeks and exhibited niche modulatory activity. Importantly, the ABCG2+ cell population exhibited increase expression of genes involved in the HIF-2α stemness pathway.

To evaluate the effect of LPS treatment on the expression of TLR 2 and 4, we performed qPCR to detect the expression level of these genes. We have found that LPS treatment led to increased expression of *TLR2* and *TLR4*. (Figure 7E).

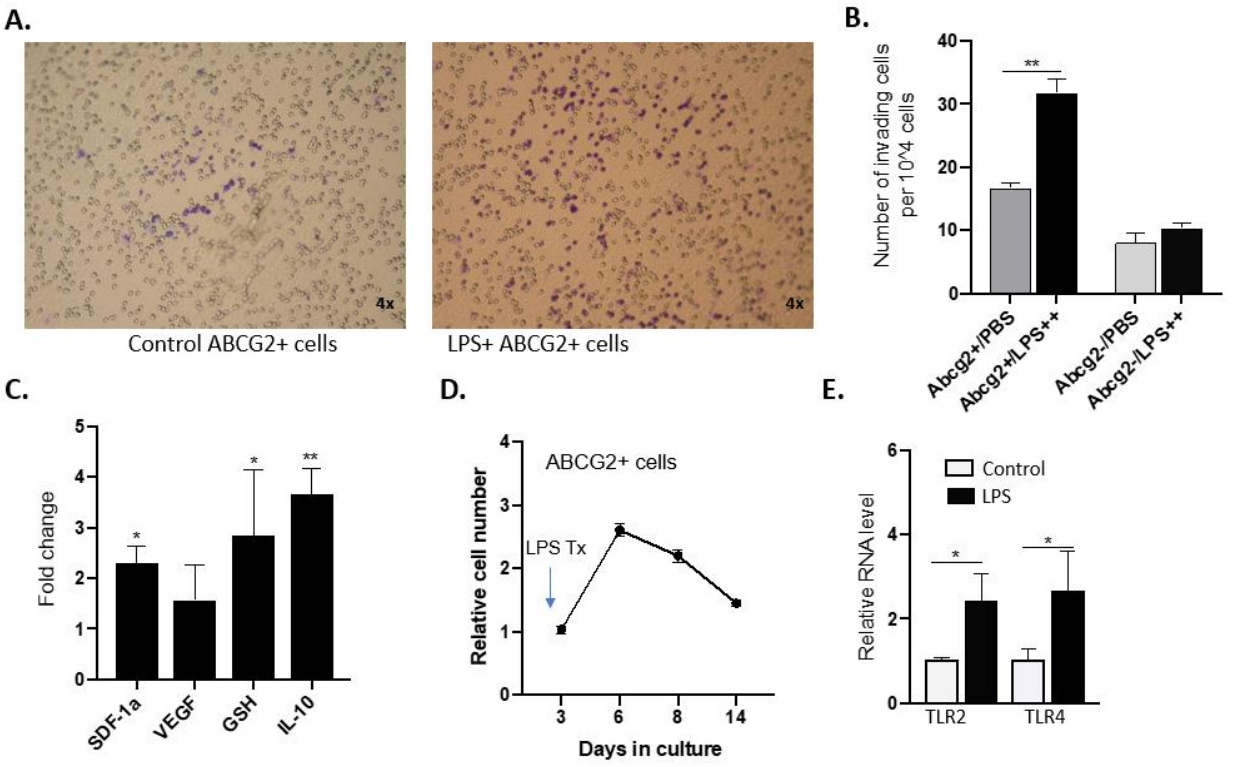


Figure 7. LPS treated ABCG2+ cells exhibit tumor stemness switch.
*A) Representative microscopic images of the Matrigel invasion assay show increased number of cells passing through the pores post LPS treatment. B) Comparison between the relative number of invading cells in LPS treatment group and PBS treatment group. Note the increased number is limited to the ABCG2+ cell population. C) Conditioned media of ABCG2+ cells exhibit niche modulatory activity. D) ABCG2+ cell number increased transiently and lasted for approximately two weeks. E) Day 7 after LPS treatment. qPCR analysis indicates that the LPS treatment led to increased expression of TLR2/4 genes. N = 3 independent experiments; error bar represent mean ± SEM. *p<0.05, **p<0.001, ANOVA.*

Assessment of tumor stemness switch in the saliva treated SCC-25 cell line:

After validating the SCC-25 cell line to study TLR mediated tumor stemness switch, we then evaluated the potential of human saliva of oral cancer subjects to induce tumor stemness in this cell line. We reasoned that bacteria present in the saliva may induce tumor stemness switch, and

therefore, wanted to treat the SCC-25 cells with processed saliva containing live bacteria. We, therefore, obtained saliva samples from 22 subjects with oral cancer (Table 1), processed in DMEM culture media, and added to SCC-25 cells. Then, 3-days later, as the cell culture get overwhelmed with bacterial growth, the extracellular bacteria were cleared using a protocol that our lab previously described for infecting stem cells with M.tuberculosis (9). The protocol involved treating the cells with amikacin and metronidazole for 24 hours, and then growing the cell culture for 1-2 weeks, and then performing qPCR gene expression to evaluate the stemness of the infected cells (9) (Figure 8A). We found that despite antibiotic treatment, the SCC-25 cells grown with the saliva samples 1-17 remained heavily contaminated with bacterial growth, and therefore, the cell culture was discarded. Only the patients 18-22 saliva treated SCC-25 cells continued to grow with limited contamination, and therefore, saliva of these patients were utilized to study saliva induced tumor stemness switch. Among these 18-22 patients, the two relapsed cases, 21 & 22, exhibited significant increase in *MYC*, *HIF-2α*, *Nanog*, *SOX-2* and *Oct-4* compared to cell cultures treated with saliva from patients on remission (Figure 8B). This suggests that the oral microbiome in the saliva from patients affected with OSCC may be involved in the activation of the tumor stemness pathway.

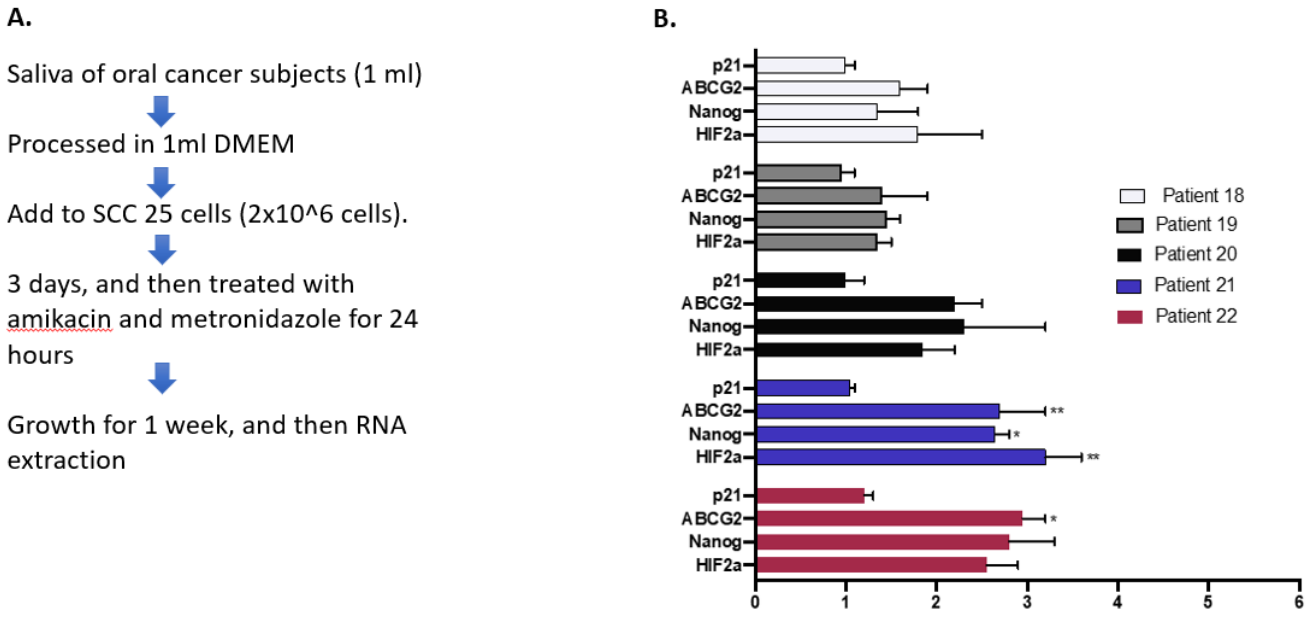


Figure 8. Saliva of oral cancer subjects modulates the genes related to HIF-2α stemness pathway.
*A) Schematic representation of the experimental design. Saliva from oral cancer subjects is processed in DMEM and added to SCC-25 cells (2x10^6 cells). Three days post treatment, cells in culture were treated with amikacin and metronidazole for 24 hours. Cells were then maintained for 7 days and then assessed for RNA. B) Oral cancer subject's saliva (table 1) treatment in SCC-25 cells upregulated the genes involved in the HIF-2α stemness pathway. PBS treated cells were used as control in the experiment to compare gene expression. N = 3 independent experiments; error bar represent mean ± SEM. *p<0.05, **p<0.001, ANOVA.*

We selected the saliva from patient 21, who is on relapse, to investigate the effects on tumor stemness switch. First, we performed the clonogenic assay. We found that saliva treatment led to increased expansion of ABCG2+ cell population (Figure 9A). Next, we performed qPCR to evaluate the expression of stemness genes. We have found that the saliva treatment led to increased expression of *MYC*, *HIF-2α*, *Nanog*, *Oct-4* and *Sox-2* in the ABCG2+ cell population, while the ABCG2- population did not show significant increase (figure 9B). To confirm our findings, we next performed western blot to evaluate the transcriptional activity of the stemness genes. We have found increased levels of *HIF-2α* in tumor cells treated with saliva compared to the control group (Figure 9C). These results suggest that the saliva from relapsed patients has the capability to induce

expansion of ABCG2+ cells with increased expression of genes associated with the tumor stemness switch.

Next, we wanted to investigate two of the tumor stemness phenotype components, the migration capability and the niche modulatory activity. To test the former, we performed the Matrigel migration assay. We found that the ABCG2+ cells of the patient 21 saliva treated group exhibited significantly higher invasive potential than control group (Figure 9D). To test the latter, we collected the conditioned media (CM) of the ABCG2+ cell population, and treated BM-MSCs with this media. We found that the MSCs treated with the CM of saliva treated ABCG2+ cells exhibited significantly increased secretions of SDF-1α, VEGF, glutathione, and IL-2 compared to control group (Figure 9E). Importantly, the increase in the ABCG2+ population was transient and only lasted for approximately two weeks after saliva treatment (Figure 9F). These results suggest that the saliva treatment led to induction of the tumor stemness phenotype in the ABCG2+ cell population of SCC-25 cells.

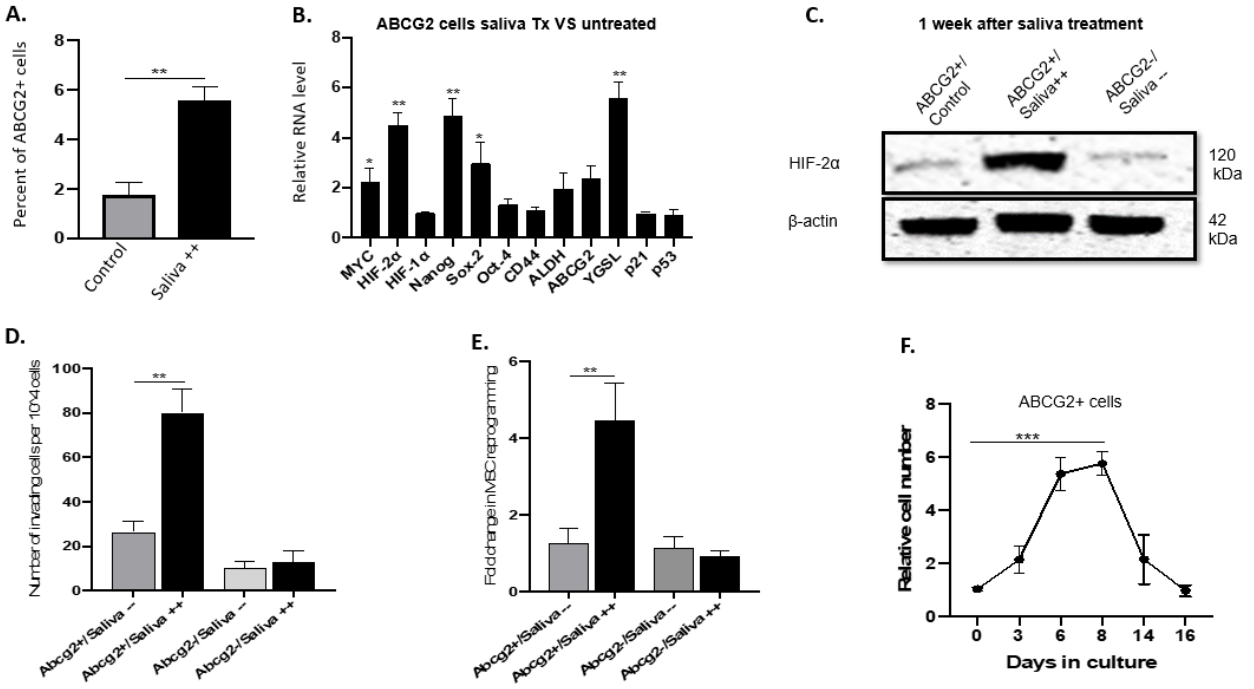


Figure 9. Patient 21 saliva induced tumor stemness switch in SCC-25 cell line.
*A) Saliva treatment expanded the ABCG2+ population. B) Saliva treatment enhanced the stemness of ABCG2+ cells, with significantly increased expression of genes involved in the HIF-2α stemness pathway. C) Western blot analysis indicates increased protein levels of HIF-2α in ABCG2+ cells of saliva treated group. D) Saliva treatment enhanced invasive potential of ABCG2+ vs ABCG2- cells. E) Saliva treatment enhanced the niche modulatory capacity of ABCG2+ vs ABCG2- cells. F) ABCG2+ cell number increased transiently and lasted for approximately two weeks. N = 3 independent experiments; error bar represent mean ± SEM. *p<0.05, **p<0.001, ***p<0.0001, ANOVA.*

To evaluate if the saliva induced tumor stemness is TLR4 pathway dependent, we performed qPCR analysis of *TLR2* and *TLR4* gene expression. We found that the saliva treated group exhibited significant increase in TLR4 expression (Figure 10A). We then evaluated the number and percentage of ABCG2+ cells with and without the addition of anti-TLR2 and TLR4 antibodies (Invivogen) (neutralizing antibodies against TLR2 and TLR4). We found that the addition of anti-TLR4 resulted in diminished number and percentage of ABCG2+ cells, while the addition of anti-TLR2 had no significant effect on that cell population (Figure 10 B&C). We also performed qPCR analysis of *HIF-2α* and *p21* gene expression with and without the addition of anti-TLR4. We have

found that the addition of anti TLR4 resulted in no increase in the expression of *HIF-2α*, or the decrease in *p21* (figure 10D). These results suggest that the saliva induced stemness is TLR4 pathway dependent.

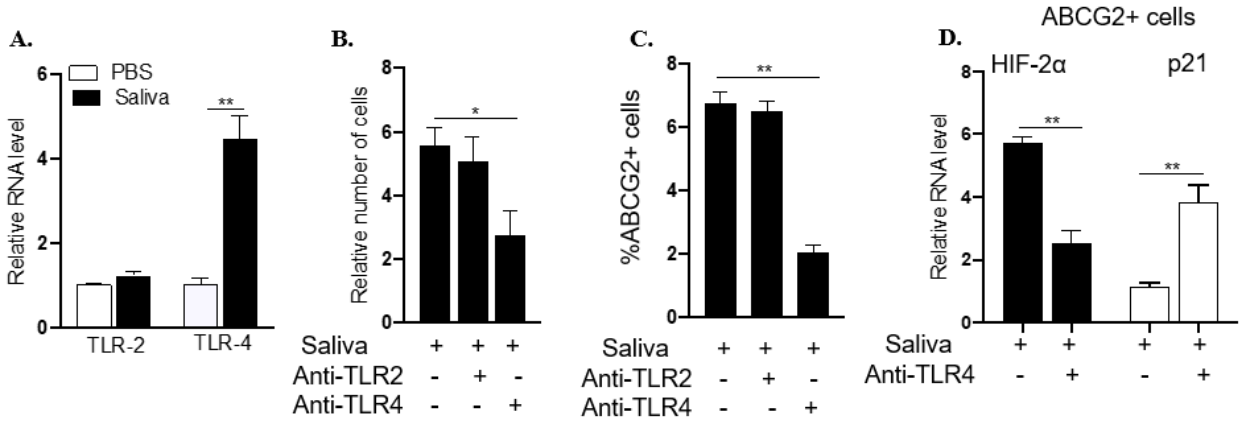


Figure 10. Patient 21 saliva induced tumor stemness is TLR4 pathway dependent.
*A) Day 7 after saliva treatment. qPCR analysis indicates that the saliva treatment increased TLR4 expression. B) Relative number of ABCG2+cells following treatment with anti-TLR2/4 antibodies (Invivogen) (neutralizing antibody against TLR2/4). C) Percentage of ABCG2+ cells following treatment with anti-TLR2/4 antibodies D) qPCR analysis of HIF-2α and p21 gene expression in saliva treated group with and without anti TLR4 indicating that the saliva induced stemness is TLR4 pathway dependent. N = 3 independent experiments; error bar represent mean ± SEM. *p<0.05, **p<0.001, ANOVA.*

Discussion

The oral microbiome has been associated with oral cancer in many studies (15, 36), however, to this date, there has been no confirmation of an exact mechanism by which the oral microbiome may induce and/or aid in the progression of oral cancer. The human saliva contains many of the species that reside within the oral microbiome (57). Since bacteria has been shown previously to modulate the stemness pathway in bone marrow mesenchymal stem cells (9), we sought to evaluate the effect of the saliva from subjects affected with OSCC on the SCC-25 cell line culture, particularly in regards to the activation of the tumor stemness pathway.

The tumor stemness pathway is a newly recognized pathway that was discovered by Das et al in 2008 (65). It has been shown that the hypoxic microenvironment in tumors induce a p53 deficient state that activates the HIF-2α stemness pathway (65). To the best of our knowledge, this was pathway was never explored in oral cancer. The interest in the contribution of the oral microbiome is the initiation and progression of cancer has been increasing in the last decade, and many studies have shown an association between certain bacteria and oral cancer. By studying the effect of saliva on the stemness of the SCC-25 cell culture, we hoped to shed light on a possible mechanism by which the oral microbiome may contribute in the development and/or progression of oral cancer.

Our results have shown that the LPS treatment and saliva from patients with OSCC increased the stemness of ABCG2+ cell population in the SCC-25 cell line culture. Cell lines treated with LPS exhibited similar results but to a lesser extent, suggesting that bacterial LPS products may also be

involved in the activation of this pathway. We have also shown that the effect of LPS and saliva treatment was selective for the ABCG2+ cell population, showing expansion and expression of the stemness genes, without affecting the ABCG2- population, maintaining the hierarchy. This expansion also exhibited other tumor stemness phenotype components, including niche modulatory activity and invasive capabilities. The expansion was also transient and only lasted for approximately 2 weeks. Our results agree with Ha N et al in that they have also found increased in stemness of OSCC cells when exposed to repetitive and prolonged infection with P. gingivalis (13). A recent study by Stashenko P et al has shown that transfer of microbiomes from tumor bearing or healthy mice into germfree recipients significantly increased the numbers and sizes of tumors compared to those in mice that remained germfree but that were exposed to 4-NQO (12). This suggests that the cancer cells modulate the microbiome to increase expression of the stemness genes. Our results have also shown that the bacterial LPS may activate the tumor stemness pathway, however, the effect is less pronounced when compared to saliva treated groups.

CHAPTER 3: SPECIFIC AIM 2: IDENTIFY ORAL BACTERIA THAT INDUCES TUMOR STEMNESS SWITCH IN ORAL CANCER

Background and rationale

Recent studies have shown that oral microbiome and dysbiosis may be associated with the stemness pathway, NOTCH1 and Wnt/beta catenin pathways (12). Another study have shown that prolonged and repetitive infection with Porphyromonas gingivalis promotes epithelial-mesenchymal transition (EMT)-like changes and stemness in OSCC cells with increased expression of CD44 and CD133 (13). F. nucleatum has been shown to promote colorectal cancer by modulating E-cadherin/beta catenin, induce EMT-like changes by upregulating miR-21 (71), as well as activation of toll-like receptor 4 (TLR-4) (72). NF-κB, an important transcription factor in inflammation and cancer, has been shown to be activated by TLRs and bacterial LPS (73).

F. nucleatum is a Gram-negative anaerobic bacillus that is present within the oral microbiome as well as the gastrointestinal flora, and is known to cause opportunistic infections. In the oral cavity, it has been shown to play an important role in biofilm organization by expressing many adhesins such as RadD that is associated with the adhesion to Streptococcus mutans (74, 75). Studies have suggested that F. nucleatum may play a role in carcinogensis, particularly colorectal cancer (75). In the colorectal environment, the induction of proinflammatory cytokines, as well as the expression of surface proteins of F. nucleatum has been associated with the development of colorectal cancer (76). The two most known surface proteins are called fibroblast activation protein 2 (Fap2), which is a galactose-sensitive hemagglutinin/adhesive protein, that is known to be part of the invasive ability of F. nucleatum (76, 77), and Fusobacterium adhesion A (FadA), that is thought to be an important factor in cell-cell attachment of F. nucleatum (72, 76). Since F.

nucleatum is present within the oral cavity, we postulate that it may also be associated with the initiation and/or progression of OSCC.

There are two proposed models for bacteria to influence cancer cells. External influence, where bacterial infection leads to secretions of cytokines and toxins within the extracellular matrix (78), and internal influence, where the bacteria internalizes into the host cells, leading to the secretion of cytokines intracellularly (13, 78). Ha et al also showed that internalized P. gingivalis within OSCC cells led to increased secretion of IL-8 and VEGF as well as increased stemness and expression of CD44 of OSCC cells (13). Our lab previously has shown that bacteria is able to internalize in MSCs and modulate stemness of the host cells from there (9). Thus, bacteria can internally modulate stemness. Hence, we may speculate that the F. nucleatum in the oral cavity might be internalized in the oral cancer cells and thereby activate the tumor stemness switch.

Hypothesis

Specific bacteria present in the saliva of oral cancer patients activates the tumor stemness switch in the SCC-25 cell line.

Materials and methods

SCC-25 cell line culture:

The SCC-25 cell line obtained from ATCC (CRL-1628) was cultured in the appropriate culture media as described in Aim 1.

Ciprofloxacin treatment of the saliva treated and LPS treated SCC-25 cell line:

10 µg/ml Ciprofloxacin was added to the processed saliva and LPS for 24 hours to make them sterile, then after adding this saliva and LPS to the SCC-25 cells, we continued to add 1µg/ml of ciprofloxacin and metronidazole for 7 days. This was done because the cell lines contained F. nucleatum and they were sensitive to metronidazole and ciprofloxacin. Ciprofloxacin has been shown to be effective for intracellular and extracellular bacteria (79). Among the patients 18-22, the 21 and 22 contained F. nucleatum as well as Staphylococcus, and streptococcus infection, and these bacteria were sensitive to ciprofloxacin as well as per the bacterial culture and sensitivity reports.

PCR:

The PCR was run for 32 cycles on a Peltier thermal cycler (PTC-200 DNA engineTM, MJ Research Inc., Watertown, MA, U.S.A.) as described previously (80). Briefly, each cycle consisting of denaturation at 94^{o}C for 1 min, primer annealing at 68^{o}C for 30 sec, and extension at 72^{o}C for 1 min. The final cycle has an additional 10 min extension at 72^{o}C. A 2 ml aliquot of the reaction mixture was analyzed by 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, (pH 8.0)) at 100 V for 30 min. The amplification products

obtained by the F. nucleatum were stained with ethidium bromide and visualized by UV transillumination. The following primers were used: conserved forward primer, 5'-CGG GAG GCA GCA GTG GGG AAT-3'; and F.nucleatum reverse primer, 5'-TTG CTT GGG CGC TGA GGT TC.

F. nucleatum culture and infection of SCC-25 cell line:

The F. nucleatum obtained from ATCC (#25586) was cultured anaerobically for 24 hours in brain heart infusion broth supplemented with yeast extract (5 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml) as described previously (81). The culture is then washed with PBS and the SCC-25 cell line was infected with F. nucleatum at a multiplicity of infection of 1:50, quantified using optical density (OD_{600}), and incubated at 37°C with 5% CO_2 incubator for 12 hours.

Immunomagnetic sorting of ABCG2+ cells:

The F. nucleatum treated SCC-25 cell line culture was subjected to immunomagnetic sorting for ABCG2+ cells as described in Aim 1.

Real time PCR:

Total RNA was extracted, and qPCR was performed as described in Aim 1. The following TaqMan gene expression primers used were HIF -2α (Hs01026149_m1), Nanog (Hs02387400_g1), Oct 4 (Hs03005111_g1), ABCG2 (Hs00184979_m1), Sox2 (Hs00602736_s1), MYC (Hs00153408_m1), p21 (Hs00355782_m1), and GAPDH (Hs00266705_g1). The following primers were used to detect specificity for 16S Rrna of *F. nucleatum*: 5′-CCGCGGTAATACGTATGTCACG-3′ and 5′-TCCGCTTACCTCTCCAGTACTC-3′.

Clonogenic assay:

The clonogenic assay was performed as previously described in Aim 1.

Western blot:

Western blot was performed as previously described in Aim 1. The following antibodies were used: Hm-MYC, Hm-HIF-2α, Hm-Nanog, Hm-Oct-4, and Hm-SOX-2 (Cell Signaling technology). Immunoblotting was detected by enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Densitometry study was done using the AlphaView software (Alpha Innotech Alpha Imager 3400 Light Cabinet Camera Transilluminator Imaging UV, CA, USA).

ELISA:

The protein levels of HIF-2α, MYC, Nanog, Oct-4 and SOX-2 were measured as described in Aim 1.

Matrigel invasion assay:

The Matrigel invasion assay was performed as previously described in Aim 1.

Statistical analysis:

The statistical calculations were performed with GraphPad Prism 4.0 (Hearne Scientific Software) using Student t test and One-Way ANOVA with Dunnett post-hoc test.

Results

SCC-25 cell line treated with ciprofloxacin-treated saliva exhibit reduction of tumor stemness switch:

To investigate whether the live bacteria in the saliva is needed for the activation of the tumor stemness pathway, we treated some of the saliva samples from patients on relapse with the broad-spectrum antibiotic ciprofloxacin to kill the live bacteria. We also treated some of the LPS group with the same antibiotic. We have found that, in contrast to our previous findings, the culture treated with saliva that had ciprofloxacin added to it exhibited no increase in the expression of MYC, HIF-2α, Sox-2 or Nanog. However, the LPS group treated with antibiotic still exhibited increased expression of the stemness genes (Figure 11). These results suggest that for saliva, live bacteria are needed to induce stemness. However, for the bacterial products LPS, the antibiotics had no effect.

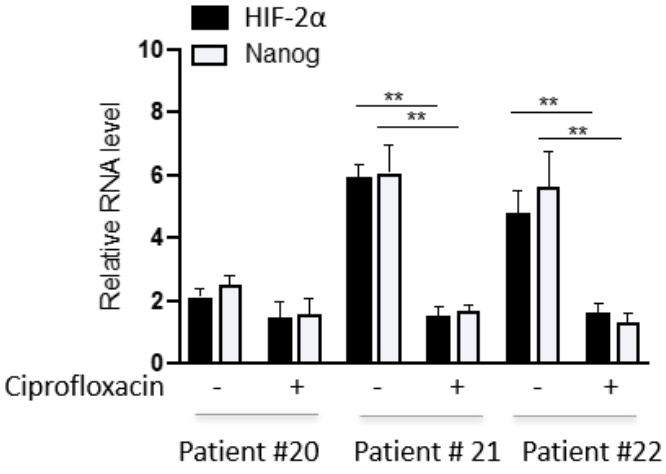


Figure 11. Ciprofloxacin treated saliva from patient 20, 21 and 22 effect on SCC-25 cells. *qPCR analysis of ABCG2+ cells immunomagnetically sorted from SCC-25 cells pre-treated with saliva from Patient 20, 21 and 22, with and without ciprofloxacin for 7 days and evaluated for expression of HIF-2α and Nanog. Data was normalized to GAPDH to obtain the ΔΔCT value as previously described. N = 3 independent experiments; error bar represent mean ± SEM. **p<0.001, ANOVA.*

ABCG2+ cells exhibit the presence of F. Nucleatum

To investigate whether specific bacteria may be involved in the tumor stemness pathway, we subjected the cell extracts from samples treated with the saliva from patients affected with relapsed disease. The microbial culture from the saliva of relapsed subjects showed that the ABCG2+ populations exhibited the presence of F. nucleatum within the cells. (Figure 12A-B). This suggests that F. nucleatum internalizes within the OSCC cells and may modulate the tumor stemness pathway.

A.

Patient	Culture organism (ABCG2+ cells)	Culture organism (ABCG2- cells)
Patient 21	F. Nucleatum	Negative
Patient 21 + antibiotics	Negative	Negative

B.

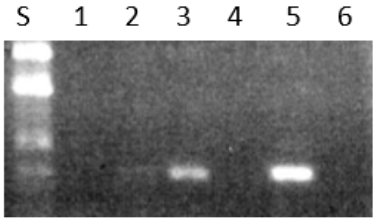


Figure 12. SCC-25 cells derived ABCG2+ cells treated with Patient 21 saliva is Fusobacterium nucleatum positive.
A) Microbiology culture indicated the presence of F. nucleatum within the ABCG2+ cells. B) Specificity test of the PCR performed with primers for F.Nucleatum . Agarose gel electrophoresis of the amplification products obtained by the F.Nucleatum was performed. Each (4 ng) of the bacterial genomic DNA was used as the PCR template. Lanes: S, 100 base pair DNA ladder (Bioneer Corp.); 1, sterilized deionized water (negative control); 2. Control SCC-25 cells. 3. ABCG2+ cells of Patient #4 saliva. 4. ABCG2- cells of Patient #4 saliva. 5., F. nucleatum ATCC 25586; 6. Sterilized deionized water (negative control). The filled arrowhead indicates the PCR products of F. nucleatum (495 bp).

F. nucleatum infected SCC-25 cell line exhibited tumor stemness switch:

To evaluate whether the F. nucleatum activates the tumor stemness pathway in cancer cells (7), we infected the SCC-25 cell line with F. nucleatum (Figure 13A). We have found that SCC-25 cell

culture infected with F. nucleatum exhibited significantly increased expansion of ABCG2+ cell population, with increased clonogeniety (Figure 13B, C).

Next, we performed qPCR to evaluate the gene expression of stemness genes. We found that the ABCG2+ cells exhibited increased gene expression of *MYC*, *HIF-2α*, *Nanog*, *SOX-2* and *Oct-4* (Figure 13D). This suggests that the F. nucleatum may be involved in the activation of the tumor stemness pathway.

To confirm our findings, we next performed western blot to evaluate the protein levels of MYC, HIF-2α and Nanog .We have found increased levels of *MYC*, *HIF-2α* and *Nanog* of the F.nucleatum treated group compared to the control group (Figure 13E).

Next, we wanted to investigate whether the F. nucleatum induced tumor stemness is TLR4 pathway dependent, similar to the saliva treated group in Aim 1. To test this, we performed qPCR to evaluate the gene expression of *HIF-2α* and *p21* with and without anti TLR4 (neutralizing antibody against TLR4). We found that the addition of anti TLR4 results in no increase in *HIF-2α* expression, and no decrease in *p21* expression (figure 13F). This result suggests that the F. nucleatum induced stemness is TLR4 pathway dependent.

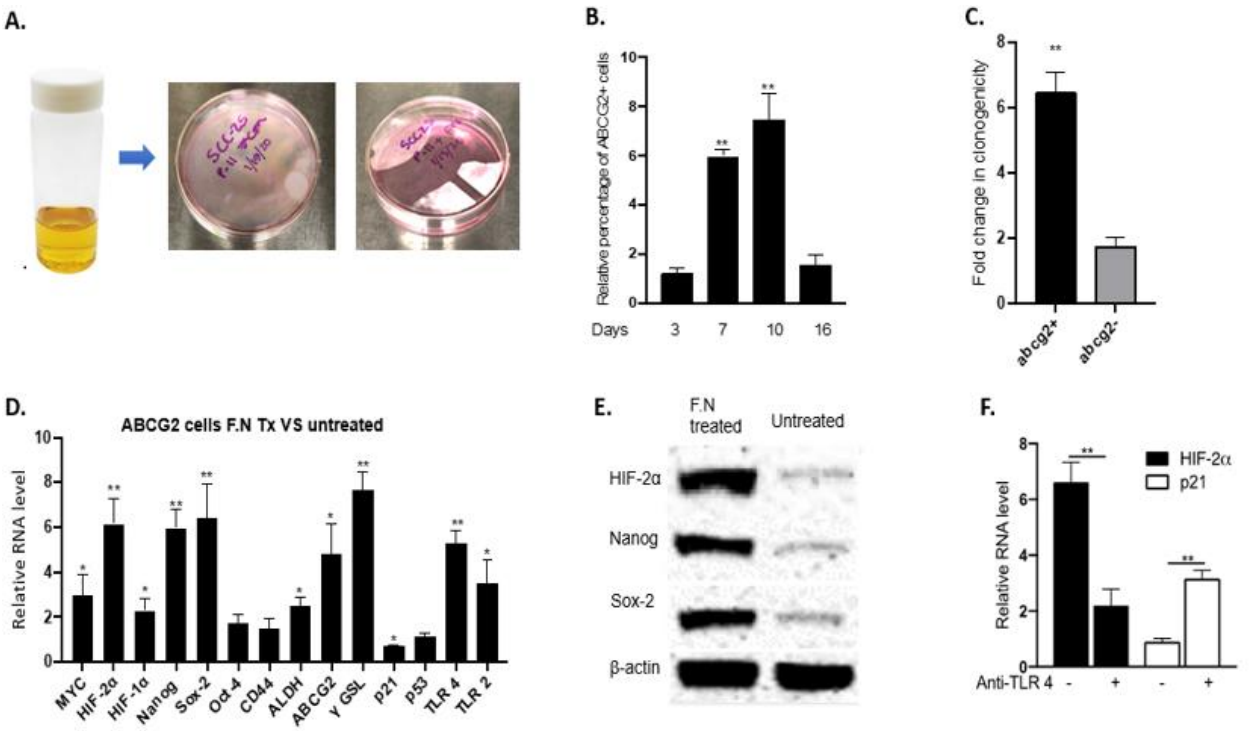


Figure 13. F-nucleatum induces tumor stemness switch in ABCG2+ cells.
*A) F.Nucleatum (ATCC:25586) grown in brain-heart infusion broth for 24 hours were added to SCC-25 culture in MOI 20. After 3 days, cells were treated with antibiotics amikacin and Metronidazole for 24 hours, and then incubated for two weeks. B, C) F. nucleatum infection led to expansion of ABCG2+ cell population vs ABCG- cells. D) qPCR analysis indicates increased expression of genes involved in the HIF-2α stemness pathway. E) Western blot analysis indicates increased protein levels of HIF-2α, Nanog and Sox-2 in the F. nucleatum treated group. F) qPCR analysis of HIF-2α and p21 gene expression in F. nucleatum treated group with and without anti TLR4 (neutralizing antibody against TLR4) indicating that the F. nucleatum induced stemness is TLR4 pathway dependent. N = 3 independent experiments; error bar represent mean ± SEM. *p<0.05, **p<0.001, ANOVA.*

Discussion

Fusobacterium nucleatum is a Gram-negative anaerobic bacillus that is present within the oral microbiome as well as the gastrointestinal flora. The bacteria has been explored within the oral cavity, and is thought to be a normal commensal within the oral microbiome, with functions primarily related to the organization of the bacterial biofilm (74, 75). In Aim 1, both LPS and

saliva from subjects affected with OSCC induced tumor stemness switch in ABCG2+ cells of SCC-25 cell line, and here in Aim 2, we have shown that live bacteria are needed for the activation of the stemness pathway, as using the broad-spectrum antibiotics on saliva resulted in no effect of the tumor stemness pathway. While these studies may suggest that the activation of the stemness pathway is not specific to certain bacteria, in our results, the microbial culture have shown the presence of F. nucleatum within the SCC-25 cells. This has led us to believe that F. nucleatum may play an important role in the tumor stemness switch. Indeed, in Aim 2, we have found direct evidence that F. nucleatum infected SCC-25 cells exhibit increased expression of tumor stemness genes.

Our results from the SCC-25 cells infected with F. nucleatum were similar to the cell lines treated with LPS or saliva from patients affected with OSCC. The infected cell lines exhibited increased expression of tumor stemness genes of ABCG2+ cell population, including MYC, HIF-2α, Nanog, Oct-4 and Sox-2. The cells also showed selective expansion of ABCG2+ population with maintenance of the hierarchy of ABCG2+ and ABCG2- populations. The ABCG2+ cells exhibited tumor stemness phenotype components, such as niche modulatory activity and invasive potential. The expansion of ABCG2+ population was transient and lasted for approximately 2 weeks. These results suggest that F. nucleatum induces tumor stemness switch in ABCG2+ cells of the SCC-25 cell line, similar to the LPS and saliva treated cell lines discussed in Aim 1.

Previous studies have found enrichment of the bacteria in oral malignant and premalignant lesions (15, 36). However, these studies did not report direct causal relationship between F. nucleatum and oral cancer progression. Our results suggest a direct involvement of F. nucleatum in the progression of cancer in SCC-25 cell line by promoting tumor stemness switch. This in vitro model

may be useful for future studies to further characterize this causal relationship by culturing pure F. nucleatum with primary oral cancer cells. Importantly, this in vitro model may be used to study the expression of surface protein on F. nucleatum, particularly FadA and Fap2, which have been shown to be associated with the pathogenicity of F. nucleatum, and are important for the host-pathogen interaction (72, 76, 77).

The molecular mechanism of F. nucleatum induced tumor stemness is switch is not fully understood. One potential mechanism is the TLR-mediated NF-Kb activation, which has been studied in F. nucleatum-associated colorectal cancer (75). NF-Kb is a transcription factor that mediates many of the proinflammatory cytokines that are associated with cancer (73). Another possible mechanism is the F. nucleatum modulation of E-cadherin/beta catenin pathway, and the induction of EMT-like changes by upregulating miR-21, which have also been studied in colorectal cancer (71). It is possible that F. nucleatum present within the oral microbiome may exert these same mechanisms in the initiation and/or progression of oral cancer, and are good targets for future studies.

F. nucleatum may also potentially affect the initiation and/or progression of OSCC by mechanisms other than the tumor stemness switch that we found in our results. Such unexplored mechanisms include angiogenesis, immune suppression and modulation of immune checkpoints, and increased mutation load and ROS. Future studies using our in vitro model may help shed light in these potential mechanisms.

A recent review has stated that while some bacteria may be associated with OSCC, such as F. nucleatum and P. gingivalis. There is, however, considerable variation in the bacterial composition between subjects affected with OSCC, indicating that no specific bacteria is implicated, but rather the microbiome within the tumor microenvironment contributes through sustaining chronic inflammation (82). In Aim 1, our results agree with Al-Hebshi et al in that there was considerable variation between the saliva of different patients. However, in Aim 2, we have identified a causal relationship between F. nucleatum and SCC-25 cells by the induction of tumor stemness switch, and this is suggestive that F. nucleatum plays an important role in the activation of this pathway. We also may speculate that F. nucleatum could play a cooperative role with other bacteria or viruses within the oral cavity in the initiation and/or progression of cancer.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In this project, we aimed to identify an appropriate OSCC cell line to study the oral microbiome induced tumor stemness, and to identify oral bacteria that induces the tumor stemness pathway. We were able to characterize SCC-25 cell line as an OSCC representative cell line to be appropriate for this purpose, as we have found that using the TLR agonist, LPS, activated the HIF-2α tumor stemness pathway. And by using the saliva from patients affected with OSCC, particularly those who are on relapse, the cell line also showed increased expression of genes involved in the tumor stemness pathway. Thus, the saliva from patients affected with OSCC may induce the tumor stemness switch in CSCs of SCC-25 cell line. We then showed that live bacteria is needed for this pathway, as applying the broad spectrum antibiotic ciprofloxacin lead to diminished expression of the genes involved in the tumor stemness pathway. We also identified the presence of F. nucleatum internalized within ABCG2+ CSCs post saliva treatment. We then showed evidence that F. nucleatum infection of SCC-25 cells lead to the activation of the HIF-2α tumor stemness pathway in a TLR4 dependent fashion. The in vitro assay of tumor stemness switch using SCC-25 cell line has shown to be effective in identifying oral bacteria relevant in OSCC progression. Therefore, there is a great relevance of this assay to be used as a platform to study the role of oral microbiome induced tumor stemness switch, a cellular and molecular mechanism having clinical significance in OSCC diagnosis and management.

Future directions

Our in vitro model now raises question about the relevance of our results in vivo. Unlike in vitro, the in vivo bacterial/host pathogen interaction may be complex, and bacteria may or may not internalize into the ABCG2+ cells. A potential next step would be to isolate F. nucleatum from the ABCG2+ CSCs of patient's tumors to confirm our findings. The sequencing of the bacterial population intracellular to ABCG2+ CSCs will also help to gain insight about the host/pathogen interaction.

Our work can be considered as a pilot study, and next steps may involve studying the tumor stemness pathway in a mouse model where we can quantify the effects on the developing tumors. The in vivo experiments may provide insight about the applicability of our findings in a clinical setting. We have identified F. nucleatum to be involved in the tumor stemness pathway. Thus, the effect of eliminating this bacterium needs to be investigated in clinical and in vivo model.

We would also like to find out if the F. nucleatum internalization inside the ABCG2+ CSCs is a part of CSC defense mechanism against the pathogen, so that the pathogen spread/replication can be contained. Previously, we proposed stem cell altruism or Vedic altruism is a component of innate altruistic defense mechanism of cells against oxidative stress (56), and we demonstrated this defense in human ESCs exposed to extreme oxidative stress (6). Briefly, when exposed to oxidative stress, a few ESCs reprogram to ASC phenotype of transient p53 deficiency, and secretion of antioxidants, and these cells then defended neighboring ESCs from the oxidative stress. Thus, we speculate that CSCs may also activate the ASC based defense against F. nucleatum

invasion. Here, the question arises: is the tumor stemness switch a defense mechanism of CSCs against bacterial invasion? If so, this aspect of CSCs may have clinical applicability. Is this the only bacterium involved in the tumor stemness phenotype? Does ASC defense modulate the bacterial phenotype to make the tumor more oncogenic? Thus, in the future, we would like to design experiments to find out if the ABCG2+ CSCs exhibited tumor stemness switch as a part of the ASC defense mechanism against the bacteria present in the saliva. This in vitro assay model of CSCs and F. nucleatum host/pathogen interaction may provide insight about a putative ASC based CSC defense mechanism against our immune system.

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