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Role of ADAM23 in Normal Human Keratinocytes

A thesis submitted in partial satisfaction

of the requirements of the degree Master of Science

in Oral Biology

by

Soo Jung Yeon

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ABSTRACT OF THESIS

ADAM23 plays functional role in HPV-associated cancer development

By

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"High risk" human papillomaviruses (HPV) are known to cause or are closely associated with the development of head and neck cancers including oral squamous cell carcinomas (OSCCs). Integration of HPV DNA into host genome and subsequent expression of viral oncogenes, such as E6 and E7, are critical initial events for the HPV-associated cancer development; however, HPV infection alone is not sufficient to convert normal cells to malignant phenotypes, and other additional genetic and/or epigenetic events in the HPV-infected cells are required. Previously, we utilized the high-throughput RNAi Decode Library system on human oral keratinocyte-16B (HOK-16B), an immortalized but non-tumorigenic keratinocyte harboring HPV-16 genome, and identified ADAM23 as a putative tumor suppressor that functionally convert HOK-16B cells to tumorigenic. Here, we aimed to determine effects of ADAM23 in

proliferation potential (e.g., population doublings) in the absence of HPV backgrounds in normal human oral and epidermal keratinocytes (NHOKs and NHEKs) and OKF6, hTERT-immortalized human oral keratinocytes. During the serially subcultured NHOKs, ADAM23 expression was significant increased during replicative senescence. Overexpression ADAM23 in NHOKs and NHEKs did not cause any changes in population doubling although they exhibited increased expression of p16. Knockdown of ADAM23 in NHEKs had no significant changes in their population doubling. Similarly, overexpression of ADAM23 OKF6 cells yielded no gross alterations in proliferation potential. When the Wnt signaling pathway is examined by treating LiCl or Wnt3a, overexpression of ADAM23 activated Wnt signaling pathway as demonstrated by increased expression of beta-catenin. Our data suggest that ADAM23 has limited effects on keratinocytes in the absence of HPV. The thesis of Soo Jung Yeon is approved.

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TABLE OF CONTENTS

PAGES

INTRODUCTION	1
MATERIALS AND METHODS	5
RESULTS	8
DISCUSSION	12
FIGURE LEGENDS AND FIGURES	18
REFERENCES	26

1. INTRODUCTION

Despite recent advances in treatments, cancer still carries a major burden of disease with its high morbidity and mortality rates around the globe. Similar to other types of cancers, Oral Squamous Cell Carcinoma (OSCC) results from a multistep process which accumulates several irreversible genetic and epigenetic changes that escape tumor suppressive mechanisms [1]. In addition to tobacco uses and alcohol consumption, increasing number of reports suggest that oncogenic viral infection can also be an etiological factor [2]. In particular, Human Papillomavirus (HPV), a widely known cancer causing virus, has an ability to infect the host and modify intracellular growth signaling pathway that ultimately leads to cervical and head and neck squamous cell carcinomas (HNSCC) including OSCC.

HPVs are grouped into the mucosal or cutaneous types [3]. The mucosal HPV types are further divided into low- or high-risk types. Among the high-risk subtypes, HPV 16, 18, 31, 33, and 35 are considered oncogenic that play a role in development of oropharyngeal head and neck cancer [4]. In particular, HPV 16 accounts for 90-95% of oropharyngeal cancer cases [5]. Incidences of HPV associated cervical cancer is undoubtedly declining as vaccines are readily available. Nevertheless, cases of HPV positive HNSCC including OSCCs are continuously increasing in spite of the decline in alcohol consumption. A unique etiological factor explaining this occurrence can be found in increased sexual behaviors such as oral sex in young male population which make them more susceptible as their exposure to HPV increases [6]. Interestingly, the prognosis for HPV positive cancer patients is better than that of HPV negative cancer patients but the reason for this better response to treatment remains unknown [4]. Even though HPV positive HNSCC may seem to only affect a specific population, the number of cases is expected to exceed the annual occurrences of cervical cancer by 2020 [6]. In fact, OSCC is the sixth most common cancer worldwide and more than 300,000 new cases are diagnosed each year

[2]. Therefore, further understanding of the underlying molecular alterations contributing to the development of HPV related OSCC is necessary for better interventions and therapeutics.

HPVs are small double-stranded DNA viruses and approximately consist 8,000 base pairs in the genome [7]. HPV contains coding sequences such as the early structural genes (E) encoding E1, E2, E3, E4, E5, E6 and E7 proteins and the late structural genes (L) encoding the major (L1) and minor (L2) capsid proteins [7]. HPV has a strong affinity for squamous epithelial cells, keratinocytes, and are mainly found in the anogenital tract, urethra, larynx, skin, or oral mucosa. The life cycle of HPV is closely linked with differentiation factors of host epithelial cell [8]. As keratinocytes differentiate, viral DNA synthesis and expression of viral genes occur [9]. Oncoproteins, E6 and E7, have developed strategies to block host cellular proliferation regulatory pathways [10]. E6 activity is characterized by its ability to induce degradation of the tumor suppressor protein p53 via the ubiquitin pathway [11]. P53 is a transcription factor that activates cell cycle arrest or apoptosis in response to cellular stress or DNA damages. However, E6 binds to E6-associated protein (E6AP) and this E6/E6AP complex can bind to p53 and ubiquitinates p53 [12]. Ubiquitination of p53 results in proteasome degradation which degrades p53 and bypasses protection mechanism to keep the integrity of the genome. However, HPV proliferation tends to accumulate chromosomal abnormalities, greatly increasing the probability of the infected cells to become malignant [13]. E6 can also target Notch signaling pathway which is responsible for differentiation of keratinocytes. Notch genes are known to be the key players in cell-fate determination and differentiation. It has been reported that there is an increase in Notch activity which leads to cell cycle exit and commitment to differentiation pathway in primary epithelial cells [14]. Since p53 targets Notch1 gene, HPV can downregulate Notch1

through E6 protein that degrades p53 [15]. Additionally, degradation of p53 by E6 is known to be associated with cellular immortalization [16].

Another oncoprotein E7 binds to tumor suppressor protein retinoblastoma (pRb) and deregulates G1/S and G2/M cell cycle. Under the normal cell cycle, cyclin-dependent kinase (CDK) phosphorylates pRb which in turn disrupts pRb/E2F complexes and releases E2F, a transcription factor responsible for genes necessary for cell cycle progression such as cyclin A and cyclin E [13]. E7 can also induce cellular proliferation by blocking activity of CDK inhibitors p21 and p27. Without the proper cell cycle regulation through pRb, viral genes can enter cell cycle countless times and continue to proliferate. And as HPV infected cells continuously divide without the proper growth control, they are more prone to accumulate chromosomal abnormalities that increase the possibility of developing malignancy.

Aforementioned E6 and E7 oncoproteins cooperate in order to escape any negative growth regulation of the host cell and effectively replicate the viral genome. In theory, every HPV infection should lead to carcinogenesis since all HPV harbors E6 and E7 in the genome. However, most individuals who contracts HPV infection are able to fight off the virus. Only a fraction of infected individuals will acquire cancer. This suggests the genomic integration of the oncoproteins itself is not capable of HPV-mediated carcinogenesis. For example, E6 or E7 protein alone cannot immortalize human keratinocytes [17]. Considering HPV can be detected in 10-13% of normal oral mucosa, only a fraction of infected individuals will undergo carcinogenesis by HPV [3]. This suggests that other additional genetic and/or epigenetic events in the HPV-infected cells are required. According to Zhong et.al, viral oncogenes that changed Notch1 expression promoted HPV positive tumor growth in mice [18]. There was also increase in the rate of HPV16 in tissues with severe chronic inflammation [19]. There are other reports that show oxidative damage was related to high-risk HPV carcinogenesis [20, 21]. These studies altogether confirms the necessity of other events to promote carcinogenesis. Epigenetic regulation is necessary for development and differentiation but it can also occur by random change or external environmental influences[22]. Epigenetic changes such as DNA methylation, histone modification, and microRNAs (miRNAs) can contribute to carcinogenesis by selectively modulating gene expressions [23, 24]. Epigenetic mechanism along with the molecular characterization of HPV-mediated cancer development in growth is an important step to develop personalized treatments.

Previously, we utilized the high-throughput RNAi Decode Library system to introduce additional changes into the usual cellular processes. MicroRNA-adapted shRNA (shRNAmir) were able to introduce stable loss-of-function phenotypes in human oral keratinocyte-16B (HOK16B), an immortalized but non-tumorigenic keratinocyte harboring HPV-16 genome. With the convenient barcode system that allows locating the alterations taken place in the transfected cell, we have identified a putative tumor suppressor, A Disintegrin And Metalloproteinase 23 (ADAM 23).

Our previous study (unpublished) demonstrates that knocking down ADAM23 from HOK-16B increases proliferative potential as well as tumorigenic potential in vitro and in vivo. However, it is unknown what role ADAM23 has in keratinocytes without HPV background such as Normal Human Oral Keratinocytes (NHOK) and Normal Human Epidermal Keratinocytes (NHEK). Therefore, this study investigates the effects of ADAM23 in proliferation potential and the phenotypic or molecular changes that possibly affect Wnt signaling pathway.

2. MATERIALS AND METHODS

2.1 Cells and cell culture

Primary normal human oral keratinocyte (NHOK) and normal human epithelial keratinocyte (NHEK) cultures were established from separated keratinizing oral epithelial tissue or foreskins respectively. The detailed method of primary culture establishment can be found elsewhere (Kang *et al*, 2000). These keratinocytes were serially subcultured in EpiLife supplemented with HKGS (Cascade Biologics, Portland, OR. USA). OKF6 cells were cultured in Keratinocyte Growth Medium (KGM) containing a low level (0.15mM) of Ca++ and supplementary growth factor bullet kit (Cambrex, East Rutherford, NJ).

2.2 Western Blotting

The following whole cell extracts were isolated using the lysis buffer (1% Triton X-100, 20mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mg/ml PMSF): 10-09 Normal Human Oral Keratinocytes (NHOK), 10-15 NHOK, SCC4 (HPV-), SCC9 (HPV-), SCC15 (HPV-), Tu139 (HPV-), Tu177 (HPV-) and FaDu (HPV-). HPV associated HOK-16B, BapT, CaSki, HeLa, SiHa's whole cell extracts were also isolated using the same lysis buffer. These extracts were run through 10% SDS-PAGE gel and transferred to protein membranes (Milipor, Billerica, MA). BenchMark Pre-stained Protein Ladder (Invitrogen) and MagicMark XP Western Protein Standard (Invitrogen) were mixed in 1:1 ratio and used as a protein ladder. The membranes were then blocked with 5% nonfat milk and incubated with the primary antibody beta-catenin (Santa Cruz) overnight and secondary Anti-Rabbit-Horse radish peroxide (HRP) antibody (Santa Cruz) for 1.5 hrs. After antibody incubation, the membranes were exposed to the chemiluminescence reagent (Denville) for detection of proteins.

2.3 Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen), and the quality of RNA was assessed using NanoDrop Spectrophotometer (Thermo Fisher Sicentific). cDNA was generated from 5 µg of total RNA extracted using SuperScript II (Invitrogen). Then 2.5 ul cDNA was amplified using SYBR Green I Master Mix (Roche Applied Sciences) with the LightCycler 480 II real-time PCR system with primers. The primer sequences were obtained from the Universal Probe Library database. The cDNA samples were loaded in triplicates in LightCycler 96 well plates (Roche). GAPDH was used as internal control for the reaction. Second derivatives Cq values of the genes and GAPDH were compared to assess the fold-differences of amplification following the manufacturer's instruction (Roche).

2.4 Knockdown and overexpression of ADAM23 using lentiviral and retroviral vectors

To rule out the effects of other constructs of shRNA present in the pool conferring to the phenotypical changes observed, independent constructs of ADAM23 shRNAs 1-3 (Thermo Scientific) were used on NHEK, NHOK, and OKF6 cells. Lentiviral shRNA expression plasmids pGIPZ and ADAM23SH were prepared by transfecting 293FT cells with packaging plasmid delta 8.2 and the envelope plasmid pVSVG. Retroviral vectors pLPCX and pLPCX-ADAM23 were prepared by transfecting GP2-293 universal packaging cells (Clonetech, Mountain View, CA) with pVSV-G envelope plasmid. 24 hours after the transfection, the viral supernatant was collected after ultracentrifugation. 2 x 10^5 cells of individual cells were plated two days before infection in EpiLife supplemented with HKGS (Cascade Biologics, Portland, OR, USA) or Keratinocyte Growth Medium (KGM) (Lonza). Upon reaching 70% confluency, cells were infected with 1ml of the concentrated virus with polybrene for 3-4 hours. Selection of cells with 1 ug/ml puromycin began 48 hours after the infection and was continued for one week. Then

successfully infected cells were grown in either KGM or Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% super calf serum (Germini Bio-products) and 5 μ g/ml Gentamycin aminoglycoside antibiotic (Invitrogen) for further selection and isolation of surviving colonies of OKF6 cells.

2.5 LiCl treatment

30mM LiCl was added to the control and ADAM23 overexpressed OKF6 cells. Cells were treated for 18 hours then RNAs were isolated with the aforementioned method.

2.6 Wnt3a treatment

200 ng/ml Wnt3a recombinant protein was added to OKF6 control and ADAM23 overexpressed cells. Cells were treated for 18 hours and their RNAs were isolated with the method mentioned above.

3. **RESULTS**

3. 1 Expression of ADAM during replicative senescence in NHOK

To investigate specific target gene that is involved in replicative senescence, we utilized Normal Human Oral Keratinocytes (NHOK). We looked at the mRNA expression levels in both young and senescent population of NHOK. ADAM 11 and ADAM 22 did not show significant increase in the mRNA expression in either young or senescent cells. However, ADAM23 showed increase in mRNA expression in the senescent NHOK population (Figure 1). This suggests that ADAM23 is associated with replicative senescence.

3.2 Effect of ADAM23 overexpression in NHOK

In order to investigate the effect of ADAM23 in NHOK, we overexpressed ADAM23 using retroviral vectors. Figure 2A shows cell morphology of the control and overexpressed NHOK cells. When ADAM23 is overexpressed, NHOK cells looked more flattened out and enlarged compared to the control. We then verified overexpression efficiency by measuring the mRNA level of ADAM23. We can see thatADAM23 has been successfully overexpressed (Figure 2B). To further validate the association of ADAM23 and senescence, we measured the population doublings over 60 days. Proliferative potential of ADAM23 overexpressed cells was less than the control as they grew at a slower rate. This strongly suggested ADAM23 could potentially play a role in senescence; therefore we decided to further investigate with different senescent markers such as p16, p21, and p53. P16 is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6. It showed increase in mRNA expression in ADAM23 overexpressed NHOK cells. P53 which is a tumor suppressor also showed increase in mRNA levels as well. P21, a factor that mediates cellular senescence, interestingly showed decrease in the mRNA expression in ADAM23 overexpressed cells (Figure 2D).

3.3 Effect of ADAM23 overexpression in NHEK did not increase proliferative potential.

In order to verify the involvement of ADAM23 in senescence, we then utilized Normal Human Epidermal Keratinocytes (NHEK) which has similar characteristics to NHOK. We checked for overexpression efficiency by measuring ADAM23 mRNA levels. From Figure 3A, we can see that ADAM23 was successfully overexpressed in NHEK. Following infection with ADAM23 retroviruses, we again measured population doublings to study the proliferative potential of NHEK cells. Interestingly, NHEK cells overexpressed with ADAM23 does not show difference in growth potential (Figure 3B). We then checked for the mRNA expression levels of the same senescent markers and found that expression of p16 increased when ADAM23 was overexpressed (Figure 3C). With this data, we then wanted to investigate the effect of knocking down ADAM23 in NHEK cells.

3.4 Effect of ADAM23 knockdown in NHEK.

We used independent shRNAs specific targeting three different regions of ADAM23 to attenuate the expression. Among three shRNA constructs, we saw that construct number three inhibited the ADAM23 expression the most (data not shown). By using lentiviral vectors with ADAM23shRNA, we successfully knocked down the expression of ADAM23 in NHEK (Figure 4A). We then measured population doublings which does not show difference in proliferative potential between the control and ADAM23 knocked down NHEK cells (Figure 4B). The lentiviral construct includes GFP which is helpful to determine the infection efficiency. We further confirmed that both the control and ADAM23 knockdown was done successfully by looking at the green fluroescence (Figure 4C).

3.5 Effect of ADAM23 in OKF6.

Since both NHOK and NHEK cell cultures are difficult to maintain due to the limitations such as obtaining the human samples and proliferative restrictions, OKF6 cells were used in lieu of normal human keratinocytes. OKF6 cells are Human Oral Keratinocytes (HOKs) that have human telomerase reverse transcriptase (hTERT) immortalized. This enables OKF6 to maintain its full proliferative potential. The same vectors were used to knockdown or overexpress ADAM23 in OKF6 cells. From population doublings curve, we can see that knocking down and overexpressing ADAM23 did not change proliferative potential of OKF6 (Figure 5A and 5B).

3.6 Effect of LiCl in OKF6 cells.

LiCl is known to activate the Wnt signaling pathway by inhibiting GSK3 which is a component of the Wnt pathway [25, 26]. By inhibiting a factor such as GSK3, we examined how ADAM23 overexpression affects Wnt signaling pathway. When 30mM of LiCl was added, overexpressed ADAM23 OKF6 cells showed decrease in ADAM 23 expression at the mRNA level (Figure 6A). Similarly, Wnt target genes such as Axin2 and LEF1 showed decrease in overexpressed ADAM23 cells with LiCl treatment (Figure 6B and 6C). Protein expression level of beta-catenin was evaluated by a western blot. When ADAM23 is overexpressed, beta-catenin expression was increased with LiCl treatment (Figure 6D).

3.7 Effect of Wnt3a recombinant protein in OKF6 cells.

After confirming the differential gene expression of beta-catenin, we decided to further investigate the effect of Wnt3a signaling pathway more directly by using more specific Wnt3a recombinant protein. Overexpression of ADAM23 was verified from qRT-PCR (Figure 7A). We also confirmed that Axin2 showed decrease in mRNA expression when ADAM23 was overexpressed and treated with 200ng of Wnt3a recombinant protein (Figure 7B). LEF1mRNA

expression shows a similar pattern (Figure 7C). When the beta-catenin protein expression level was measured by western blot, we saw a significant increase in ADAM23 overexpressed cells with Wnt3a recombinant protein treatment (Figure 7D).

3.8 Replicative potential does not change in OKF6 ADAM23 knocked down cells in DMEM.

We further investigated the effect of knocking down ADAM23 by using OKF6 cells. Both the control and ADAM23 knockdown cells were grown in DMEM. DMEM medium was used as a selective control. There was no difference in the growth (Figure 8).

4. Discussion

The aim of this study was to determine effects of ADAM23 in proliferation potential in the absence of HPV backgrounds in normal human oral and epidermal keratinocytes (NHOK and NHEK) as well as OKF6. From our previous study, we hypothesized ADAM23 as a putative tumor suppressor that may be associated with HPV-mediated carcinogenesis.

ADAM23, A Disintegrin And Metalloproteinase 23, is a member of transmembrane proteins [27]. It is involved in many biological functions such as fertilization, adhesion, migration, proteolysis, muscle development, and neurogenesis [28, 29]. Unlike some ADAM proteins, ADAM23does not have catalytic domain and is mainly involved in direct cell to cell signaling by binding through the disintegrin domain [27]. ADAM23 is highly expressed in brain and regulates neurite outgrowth [27, 30]. Several studies on knockout mice have provided information on functional importance of ADAM23. ADAM23 knockdown has been reported to have an association with epilepsy during development [27]. Other studies on ADAM23 knockout mice have demonstrated short life spans as well as developments of seizures, tremor, and ataxia [31]. Some suggest this change from adhesion to invasion allowed increased migration ability that may be involved in tumor progression and metastasis formation through an unknown mechanism. [29] In fact, ADAM23 knockdown has been reported to increase cancer metastasis [32]. The disintegrin domain of ADAM23 specifically interacts with $\alpha_{\rm v}\beta_3$ integrin and negatively regulates integrin activation. This modification of $\alpha_{v}\beta_{3}$ integrin is associated with different tumors and metastasis formation. It is also reported that ADAM23 expression is silenced in pancreatic, breast, brain, gastric, and colorectal cancer [33-35]. Specifically the promoter region of ADAM23 was frequently hypermethylated in the lung, colorectal, and gastric tumors [34, 36, 37]. In the heterogeneous population of cancer cells, ADAM23 negative cells within ADAM23

positive tumor promoted tumor growth and metastasis [37]. Subclonal cells of heterogenous cancer cells show that silenced ADAM23 expression increases tumorigenic and metastatic potential. ADAM22 and ADAM23 have been implicated as tumor suppressors but the exact mechanism is yet unknown [38]. Specific ADAMs have been shown to promote cancer initiation and progression [29]. However, the mechanism how ADAM23 promotes cancer progression is still unknown. Previously, our laboratory investigated the functional role of ADAM23 in HPV mediated carcinogenesis. But this is the first study investigating its role in normal human oral and epidermal keratinocytes (NHOK and NHEK) as well as OKF6.

To determine the effects of ADAM23 in proliferative potential in the absence of HPV background, we first examined expression of several ADAMs; ADAM11, ADAM22, and ADAM23. During the serially subcultured NHOKs, ADAM23 expression was significantly increased during replicative senescence. This suggests that ADAM23 may be correlated to replicative senescence in NHOK. Replicative senescence is a regulated process that occurs in normal human somatic cells that results in irreversible growth arrest [39]. But it is also an important mechanism to investigate in order to prevent the proliferative potential of cancer cells. Our laboratory previously discovered that replicative senescence occurs independent of shortening of telomere ends in NHOK [40]. NHOK is also known to undergo 22±3 population doublings (PDs) before becoming senescent [41]. Increased expression of ADAM23 in senescent NHOK population suggests that ADAM23 may be associated with the proliferative potential.

In order to investigate possible proliferative potential of ADAM23, we amplified ADAM23 expression in NHOK. From the population doubling curve, we observed slower growth rate in ADAM23 overexpressed cells. This strongly suggested ADAM23 could potentially play a role in senescence. When ADAM23 was overexpressed, NHOK cells showed

morphological changes that indicates senescence. Their shapes look more flattened and differentiated. Since the hallmark of cellular senescence is inability to continue through the cell cycle, we further conducted experiments with different senescent markers [42]. Relative mRNA expression levels of p16, p21, and p 53 showed that ADAM23 overexpression alone does not permit NHOK to escape from the growth arrest. The p16 protein belongs to INK4 family of CDK inhibitors and controls the cell cycle [43]. In order to progress through cell cycle, cells need to pass through the checkpoints. Among the four cell cycle checkpoints, p16 controls G1 phase to S phase through p16-Rb pathway [44]. P16 binds to CDK4/6 and inhibits phosphorylation of Rb [45]. As previously mentioned in the introduction, p16 can inhibit pRb from phosphorylation by CDK and ultimately frees E2F from pRb control and allows cell cycle to progress. Rb is a retinoblastoma protein that is often mutated in many cancers and is a well known tumor suppressor protein. It is well known that pRb stays hypophosphorylated in senescent cells which implies pRb downregulation during senescence [45]. According to Alcorta et. al, p16 expression is upregulated during senescence in human fibroblasts [45, 46]. This gradual increase in p16 expression level initially led us to speculate its role in cellular senescence in NHOK. We see the similar expression pattern when ADAM23 is overexpressed as there is increase in p16 mRNA expression. This indicates that overexpressing ADAM23 did not cause any significant changes to p16 expression. We further wanted to elucidate our speculation with other senescent markers.

P53 is a well known senescence regulator as it can activate genetic programs that stop cell proliferation either through the cell cycle arrest or the apoptosis [47, 48]. P53 can regulate cell growth by activating the transcription of growth inhibitory genes such as p21. P21 is an inhibitor of CDKs and proliferating cell nuclear antigen (PCNA) and mediates replicative

senescence [49]. P21 is related to p53 as activated p53 can induce p21 expression to prevent Rb from phosphorylation ultimately prevents progressing through the cell cycle [50]. Min et. al have previously reported that there was a progressive loss of p53 and p21 expressions as NHOK senescence [51]. In ADAM23 overexpressed NHOK cells, we found that p53 expression levels slightly increased and p21 levels decreased. It is unclear why p53showed increased expression when ADAM23 is overexpressed. The difference in p21 expression level was not significant to consolidate the involvement of ADAM23 in NHOK replicative senescence. Similarly, overexpression of ADAM23 in NHEK had no significant changes in their population doubling. We further investigated the effect of ADAM23 knockdown in NHEK populations. Surprisingly, decreasing ADAM23 expression did not change the proliferative potential in NHEK. Based on our previous study, we expected improved growth potential when ADAM23 was knocked down because it suggested being a putative tumor suppressor. However, the proliferative potential of NHOK and NHEK remained the same through unknown mechanism.

One of the challenges in our study was unknown function of ADAM23. ADAM23 has previously been suggested as a putative tumor suppressor as its expression decreases in many cancers cells. However, there is limited information as which mechanism ADAM23uses to regulate the proliferative potential of the cells. Through the microarray analysis, our laboratory was able to elucidate the upregulated and downregulated genes (unpublished). In particular, we found genes involved in Wnt signaling pathway upregulated. We investigated further to better understand the effect of ADAM23 overexpression in Wnt signaling pathway. Specifically, there are two different types of Wnt pathway; one is canonical pathway which is dependent on β catenin and the other is non-canonical pathway which takes place independent of β -catenin. Wnt/ β -catenin signaling pathway is involved in many biological processes such as embryonic

development, tissue regeneration, hematopoiesis, survival, cellular proliferation, and differentiation [52]. This pathway is also indispensable for tooth development and dental epithelial cell proliferation and differentiation [53]. Among the Wnt proteins, Wnt3a is known to activate the Wnt/ β -catenin signaling pathway [53]. β -catenin is involved in interceullar adhesion and forms stable structure of the cytoskeleton [54]. Wnt signaling pathway is also considerable since HNSCC and HNSCC-derived cell lines overexpress Wnt signaling pathway components [55, 56]. In OSCC, β -catenin is generally known to accumulate in the cytoplasm. Taken together, Wnt/ β -catenin signaling may play a role in OSCC. By examing its effect in OKF6 in lieu of NHOK and NHEK due to limitations of growing serial cultures and reaching senescence, we expected to study the relevance of ADAM23 overexpression with in Wnt signaling pathway.

We activated Wnt signaling pathway by adding 30mM of LiCl. As previously mentioned, LiCl activates the Wnt signaling pathway by inhibiting GSK3 [25]. Overexpression of ADAM23 activated Wnt signaling pathway as β -catenin protein expression increased. However, the downstream target genes of Wnt signaling, AXIN2 and LEF1, did not show increase at the mRNA level. This suggests the possibility of epigenetic regulation occurring at the posttranscriptional level. Previous studies have shown that there are mutations prevalently present in Axin and β -catenin among many types of cancers such as colon, gastric, and liver cancer [57]. Nevertheless, mutations in Axin and β -catenin are rare in OSCC which suggests that they may not be associated with β -catenin accretion in oral cancer [58, 59].

We further validated our results by using Wnt3a recombinant protein to activate Wnt signaling pathway. We found a similar pattern as LiCl treatment to the cells. There was decreased expression in Wnt downstream target genes at the mRNA level while there was an increase in the protein expression level. As we can see there are several lines of evidence which

implicate the β-catenin accumulation in terms of cancer, but the results presented here indicate that a different mechanism is responsible for determining replicative life span of human keratinocytes in subcultures. Consistent with previous findings, knocking down of ADAM23 from OKF6 did not improve proliferative potential of the cells. By using non-permissive growth medium, DMEM, we were able to observe the continuous proliferation as cells escaped senescence. A better understanding of the underlying molecular mechanism of activation of Wnt signaling will contribute to the development of therapeutic applications.

In summary, this study provides novel information about the role of ADAM23 in normal human keratinocytes in the absence of HPV backgrounds. We confirmed that overexpression of ADAM23 in NHOKs and NHEKs did not show any gross changes in proliferative potential as well as in OKF6 cells. When LiCl or Wnt3a treatments activated Wnt signaling pathway, overexpression of ADAM23 allowed increase in β -catenin expression. Our data suggest that ADAM23 has limited effects on keratinocytes in the absence of HPV. In the future study, we would need to investigate how ADAM23 specifically mediates growth control by determining interacting signaling pathways and direct downstream effectors of Wnt3 pathway. A more thorough study of Wnt3 pathway activation to oral carcinogenesis will be a prerequisite for the development of future treatment. Taken together with the role of ADAM23 in HPV-positive HNSCC, this study may have significant potential value for a diagnostic marker for screening individuals.

Figure Legends and Figures

Figure 1: Expression of ADAM during replicative senescence in NHOK



A) Relative mRNA level ADAMs in both young and senescent NHOK cells. ADAM23 shows the highest mRNA expression in senescent NHOK cells.



Figure 2: Effect of ADAM23 overexpression in NHOK

A) Cell morphology of the control and ADAM23 overexpressed NHOK cells. B) Relative mRNA expression level of ADAM23. C) Population doublings curve showing proliferative potential of the control and ADAM23 overexpressed NHOK cells. D)
 Relative mRNA expression level of senescent markers in the control and ADAM23 overexpressed NHOK cells.

Figure 3: Effect of ADAM23 overexpression in NHEK



A) Relative mRNA expression level of ADAM23. B) Population doublings curve showing proliferative potential of the control and ADAM23 overexpressed NHEK cells. C)
 Relative mRNA expression level of senescent markers in the control and ADAM23 overexpressed NHEK cells.

Figure 4: Effect of ADAM23 knockdown in NHEK



A) Knockdown efficiency of ADAM23 confirmed by mRNA expression levels. B) Cell proliferation curve depicting the rate of population doublings between NHEK transfected with control pGIPZ and knockdown vector ADAM23SH. The cells were maintained for >1 month. C) Phase contrast and green fluorescence microscopy of control and ADAM23SH shows transfection efficiency.



A) Cell proliferation curves depicting the rate of population doublings between OKF6 transfected with control pGIPZ and knockdown vector ADAM23SH. The cells were maintained for >1 month. B) Population doublings curve with the control vector pLPCX and ADAM23 overexpressed vector pLPCX-AD23 transfected to OKF6 cells.





A) ADAM23 mRNA expression levels checked for the control and overexpressed ADAM23 OKF6 cells. The cells were treated with or without 30mM LiCl for 18 hours. B) mRNA expression level of Axin2 involved in Wnt signaling pathway of the control and overexpressed ADAM23 OKF6 cells. C) LEF1 expression in the same sets of cells from A and B. D) Western blotting analysis evaluating protein levels β-catenin in OKF6 cells treated with or without 30mM LiCl. β-catenin expression significantly increased in ADAM23 overexpressed OKF6 cells (most right lane).



Figure 7: Effect of Wnt3a recombinant protein in OKF6 cells

A) ADAM23 mRNA expression levels checked for the control and overexpressed ADAM23 OKF6 cells. The cells were treated with or without 200ng/ml of Wnt3a recombinant protein for 18 hours. B) mRNA expression level of Axin2 involved in Wnt signaling pathway of the control and overexpressed ADAM23 OKF6 cells. C) LEF1 expression in the same sets of cells from A and B. D) Western blotting analysis evaluating protein levels β-catenin in OKF6 cells treated with or without 200ng/ml Wnt3a. β-catenin expression significantly increased in ADAM23 overexpressed OKF6 cells (most right lane).





A) Population doubling after ADAM23sh used to knock down ADAM23 expression in OKF6 cells. Same proliferation pattern was observed as previously.

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