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## MUC4 Regulates Cellular Senescence in Head and Neck Squamous Cell Carcinoma (HNSCC) through p16/Rb Pathway

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### Abstract

The limited effectiveness of therapy for patients with advanced stage Head and Neck Squamous Cell Carcinoma (HNSCC) or recurrent disease is a reflection of an incomplete understanding of the molecular basis of HNSCC pathogenesis. MUC4, a high molecular weight glycoprotein, is differentially overexpressed in many human cancers and implicated in cancer progression and resistance to several chemotherapies. However its clinical relevance and the molecular mechanisms through which it mediates HNSCC progression are not well understood. The present study revealed a significant up-regulation of MUC4 in 78% (68/87) of HNSCC tissues compared to 10% (1/10) in benign samples [p= 0.006, OR (95% C.I) = 10.74 (2.0 - 57.56)]. MUC4 knockdown (KD) in SCC1 and SCC10B HNSCC cell lines resulted in significant inhibition of growth *in vitro* and *in vivo*, increased senescence as indicated by an increase in the number of flat, enlarged and senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) positive cells. Decreased cellular proliferation was associated with G<sub>0</sub>/G<sub>1</sub> cell cycle arrest and decrease expression of cell cycle regulatory proteins like cyclin E, cyclin D1 and decrease in BrdU incorporation. Mechanistic

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Conflicts of Interest

There are no potential conflicts of interest involved with this work.

studies revealed upregulation of p16, pRb dephosphorylation and its interaction with HDAC1/2. This resulted in decreased histone acetylation (H3K9) at *Cyclin E* promoter leading to its downregulation. Orthotopic implantation of MUC4 KD SCC1 cells into the floor of the mouth of nude mice resulted in the formation of significantly small tumors ( $170 \pm 18.30$  mg) compared to bigger tumors ( $375 \pm 17.29$  mg) formed by control cells ( $p = 0.00007$ ). In conclusion, our findings showed that MUC4 overexpression plays a critical role by regulating proliferation and cellular senescence of HNSCC cells. Downregulation of MUC4 may be a promising therapeutic approach for treating HNSCC patients.

## Keywords

MUC4; Head and Neck squamous cell Carcinoma; Senescence; cell cycle arrest

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6<sup>th</sup> most common cancer with approximately 650,000 incidences and 350,000 deaths worldwide annually.<sup>1</sup> In the USA, 41,380 new cases and 7,850 HNSCC related deaths are expected in 2013.<sup>2</sup> Despite multiple treatment modalities including surgery, chemotherapy and /or radiotherapy, the 5 year survival rate has not improved beyond 40-50% in last three decades<sup>3</sup> and can be attributed to the incomplete understanding of the molecular basis of HNSCC pathogenesis. Therefore, unraveling the cellular pathways for improved understanding of HNSCC pathogenesis is urgently needed.

Mucins are a family of heavily O-glycosylated proteins protecting epithelial cell surfaces under normal physiological conditions. Several studies from our lab and others have identified mucins as potential tumor markers and attractive therapeutic targets.<sup>4, 5</sup> MUC4, a membrane-bound mucin is expressed in several normal tissues, but its expression is elevated in malignancies of ovaries, thyroid, pancreas and breast (reviewed in<sup>6</sup>). Studies from our lab have shown that ectopic overexpression of MUC4 induces neoplastic transformation of fibroblasts suggesting the oncogenic potential of MUC4.<sup>7</sup> MUC4 activates Src/Focal adhesion kinase (FAK) signaling by physical interaction and stabilization of HER2<sup>8, 9</sup> thereby promoting survival, invasion and metastasis.<sup>8, 9</sup> More recently, we have shown that MUC4 stabilizes N-Cadherin expression and promotes epithelial to mechenchymal transition (EMT) in pancreatic cancer cells.<sup>10</sup>

We studied its expression in human HNSCC tissues and investigated its functional role in HNSCC cell lines. We observed significant upregulation of MUC4 in HNSCC tissues compared to normal tissues. Knockdown (KD) of MUC4 in HNSCC cells reduced proliferation; led to cell cycle arrest and induced cellular senescence by modulating p16/Rb tumor suppressor pathway. Furthermore, MUC4 KD decreased pRb and HDAC1/2 mediated histone acetylation (H3K9 acetylation) at cyclin E promoter resulting in transcriptional silencing of *cyclin E*. The present study is the first to propose that MUC4 KD triggers a senescence response by regulating p16, cyclin D1 and cyclin E expression.

## Results

### MUC4 is over expressed in HNSCC tissues and cell lines

Immunohistochemical (IHC) analysis showed overexpression of MUC4 in 78% of the tumor samples compared to only 10% in adjacent normal tissues [ $p=0.006$ , OR (95% C.I.) = 10.74 (2.0 - 57.56)] (Figure 1a). No significant association of MUC4 expression was observed with differentiation status, nodal metastasis, tumor size, age, gender and cigarette smoking (Table 1). HNSCC tissues showed a median composite score of 4 (range 0-12) for MUC4 as compared to the benign tissues with a median composite score of 0 ( $p=0.01$ ), as shown in the box-plot analysis in Figure 1b. Receiver Operating Characteristic (ROC) curve analysis was used to evaluate the potential of MUC4 protein as a biomarker for differentiating HNSCC from normal oral tissues. The value of area-under-the-curve (AUC) calculated on the basis of total score obtained for immune-staining of MUC4 protein were 0.78, with respect to normal tissues (Supplementary Figure 1a). These results suggest that MUC4 is deregulated in HNSCC tumors and raises the possibility that overexpression of MUC4 may contribute to pathogenesis of HNSCC.

We analyzed the expression of MUC4 in a panel of HNSCC cell lines and observed expression of MUC4 in 88% (7/8) of the cell lines at both RNA and protein level (Figure 1c). SCC1, SCC10B, SCC11B, SCC38, SCC47 and SCC74A showed robust expression of MUC4, while SCC23 cells showed mild expression of MUC4 at both RNA and protein level. SCC104 cells were negative for MUC4 expression (Figure 1c). MUC4 expression in HNSCC cell lines was independent of their site or origin. Cell lines derived from oral cavity (SCC1, SCC47 and SCC74A) and larynx (SCC10B and SCC11B) expressed similar levels of MUC4. Further, cell lines derived from both primary (SCC1, SCC38, SCC47, SCC74) and recurrent metastatic tumors (SCC10B and SCC11B) showed similar MUC4 expression. We also did not observe any association between MUC4 expression and aggressiveness of the cells as very aggressive SCC1 and non-aggressive SCC10B cells<sup>11</sup> express equal amount of MUC4. In addition, highly aggressive SCC104 cells<sup>12</sup> did not express MUC4.

### MUC4 silencing decreased cell proliferation and induced G<sub>0</sub>/G<sub>1</sub> cell cycle arrest *in vitro*

To dissect its functional role in HNSCC pathogenesis, MUC4 was stably knocked down in SCC1 and SCC10B cells as previously described.<sup>10</sup> These two cell lines were selected, because SCC1 cells is derived from the primary tumor in oral cavity with wild type with no expression of p53 and while SCC10B is derived from recurrent metastatic tissue in larynx with mutated and over expressed p53 (Figure 3b). Validation of MUC4 KD revealed a significant down-regulation of MUC4 in shMUC4 SCC1 and SCC10B cells (Figure 1d). Further, Real time PCR and immunofluorescence analysis confirmed the MUC4 knockdown (Supplementary Figure 2a and b).

To analyze the effect of MUC4 KD on proliferation of HNSCC cells, growth kinetics studies were performed. A significant decrease in growth rates of MUC4 KD SCC1 and SCC10B cells was observed ( $p=0.004$  and  $p=0.015$  respectively) (Figure 2a) indicating the role of MUC4 in cell proliferation. The doubling time increased from 26 h to 36 h and 34 h to 42 h in SCC1 and SCC10B cells respectively following MUC4 silencing. Similarly, MUC4 KD

also notably reduced BrdU incorporation by 56% and 53% in MUC4 KD SCC1 and SCC10B cells respectively (Supplementary Figure 2c). Furthermore, MUC4 KD reduced clonogenic potential (inhibiting colony formation) in both SCC1 and SCC10B cells. The number of colonies was reduced from 327 to 151 (54 % decrease) and 233 to 125 (46% decrease) in SCC1 and SCC10B cells respectively (Figure 2b). The number of larger colonies was reduced from 301/327 (92%) and 201/233 (86%) to 97/151 (64%) and 27/125 (21.6%); in SCC1 and SCC10B MUC4 KD cells respectively.

To investigate the mechanism underlying the growth suppression of HNSCC cells following MUC4 KD, we performed cell cycle analysis of the MUC4 KD cells by FACS analysis. As shown in Figure 2c and d, MUC4 KD induced the G<sub>1</sub> arrest; the percentage of cells in G<sub>1</sub> phase increased from 70.9% to 81% (p=0.05) and 69.9% to 82% (p=0.02) and the percentage of cells in S phase decreased from 20% to 14.8% (p=0.04) and 17.89% to 13.54% (p=0.012) in SCC1 and SCC10B cells respectively. However, MUC4 KD did not induce apoptosis in SCC1 and SCC10B cells, as demonstrated by the lack of sub-G<sub>1</sub> peak and Annexin-V positive cells (Figure 2c and d) and (Supplementary Figure 3a and b) and absence of any change in expression of Bcl2 and caspase-9 expression (Supplementary Figure 3b).

#### **MUC4 knockdown reduces motility/invasive potential in HNSCC cells**

MUC4 KD has been shown to decrease cell motility and invasion in various cancer cells.<sup>8-10</sup> We analyzed the motility of MUC4 KD SCC1 and SCC10B cells by Transwell migration (Boyden chamber) and scratch assay and observed a significant decrease in motility (P < 0.001 and P < 0.002) of MUC4 KD cells compared to control cells (Supplementary Figure 4a and b) respectively. We also observed a concordant decrease in invasive capability of MUC4 KD cells compared to control cells (P < 0.005 and P < 0.004, respectively) (Supplementary Figure 4c).

FAK, a non-receptor protein-tyrosine kinase is involved in cellular motility and invasion. Src-mediated phosphorylation and activation of FAK is required for focal adhesion assembly and cell spreading. Therefore, we determined if the decreased motility and invasion following MUC4 KD is mediated through Src/FAK signaling. We observed decreased phosphorylated Src and FAK in both SCC1 and SCC10B MUC4 KD cells compared to control cells (Supplementary Figure 4d) signifying the importance of Src/FAK pathway in MUC4 mediated increased cellular motility and invasion.

#### **MUC4 silencing induces senescence in HNSCC cells**

Cell cycle arrest is the central feature of senescent cells *in vitro* and *in vivo*. Morphologically, MUC4-KD SCC1 and SCC10B cells were vacuolated, flattened and much larger in size as compared to control cells (Figure 3a), a characteristic phenotype of senescent cells.<sup>13</sup> To test whether MUC4 KD induced cell cycle arrest was accompanied by senescence induction; we examined the expression of SA-β-gal activity and SAHF, which are markers for cellular senescence, in MUC4-silenced and control cells. Our SA-β-gal staining showed 37±4% and 27±6% positive in MUC4 KD SCC1 and SCC10B cells compared to 3±2% and 4±1% in control cells respectively (Figure 3a). Furthermore, 4, 6-

diamidino-2-phenylindole (DAPI) stained nuclei in MUC4 KD cells showed distinct senescence-associated heterochromatin foci compared to control cells as observed by immunofluorescence microscopy (Figure 3a). Overall these results indicate that MUC4 KD leads to cell cycle arrest and senescence.

MUC4 modulates PI3K/Akt and ERK1/2 signaling pathways in several cancers.<sup>9, 10, 14</sup> and these pathways are important in regulating cellular senescence.<sup>15, 16</sup> Our results revealed decreased levels of pAkt at Thr-308/Ser-473 and pERK1/2 in MUC4 KD SCC1 and SCC10B cells compared to control cells (Figure 3b) suggesting that MUC4 depletion induces cellular senescence by inactivation of PI3K/Akt or ERK1/2 signaling pathways. No change in expression of total Akt and ERK1/2 was observed.

### **MUC4 KD induces senescence through p16/pRb pathway**

p16/pRb and p53/p21 pathways are master regulators of cell cycle progression and cell senescence<sup>17</sup>. The effect of MUC4 KD on delayed G<sub>1</sub>/S progression and induction of senescence prompted us to examine the expression of key molecules controlling G<sub>1</sub>/S transition. We observed significant upregulation of p16 protein and downregulation of cyclin E, cyclin D1, decreased phosphorylation of pRb in MUC4 KD cells compared to control cells (Figure 3b). However, no changes in the expression of p21 and p27 were observed (Figure 3b).

Upregulation of p16 in MUC4 KD cells suggested p16-mediated senescence in HNSCC cells. To directly establish the role of p16 in senescence, we silenced p16 expression by p16 specific siRNA in MUC4 KD cells. We observed a significant downregulation of p16 after 72h of transfection in MUC4 KD cells compared to control siRNA transfected cells (Figure 3c). We also observed a clear rescue of the morphological effects of MUC4 KD with a significant reduction in SA- $\beta$ -Gal positive cells among siRNA transfected MUC4 KD SCC1 (9 $\pm$ 3%) and SCC10B (8 $\pm$ 4%) as compared to scrambled oligos transfected MUC4 KD SCC1 (39 $\pm$ 4%) and SCC10B (32 $\pm$ 6%) cells (Figure 3d). Importantly, the level of cyclin E, which is known to be expressed during G<sub>1</sub>/S transition and peak in the S phase and was noted to be downregulated in MUC4 silenced cells, was fully restored in p16 and MUC4 double KD (Figure 3c) SCC1 and SCC10B cells. These results suggest that MUC4 KD induces a p16/Rb dependent cellular senescence to suppress the growth of HNSCC cells.

### **MUC4 KD induces chromatin remodeling to down regulate cyclin E expression**

SAHF formation during cellular senescence is accompanied by loss of heterochromatin markers like Lys9 tri-methyl (H3K9me3), H3K27me3 of histone H3 and exclusion of euchromatic markers histone H3K9 acetyl, H3K4me2 and H3K4me3 on promoters of various cell cycle genes.<sup>18</sup> Histone acetylation/deacetylation on promoter has been shown to regulate cyclin E expression.<sup>19</sup> MUC4 KD resulted in decreased acetylated H3K9 and H3K4me2 with concomitant increase in H3K27me3 compared to control cells (Figure 4a). However, no change in acetylated H2BK5 and H2AK5 were observed in MUC4 KD cells as compared to control cells. Furthermore, the ratio of H3K4me2 to H3K27me3 shifted more towards the inactive chromatin in MUC4 KD cells with respect to control cells

(Supplementary Figure 5a) suggesting that deregulation of cell cycle regulated genes particularly cyclin E may be due to heterochromatin formation.

Differential binding of Histone acetyl transferases (HATs) and (Histone deacetylases) HDACs regulate cyclin E promoter activity.<sup>20</sup> Hypophosphorylated Rb interacts with HDAC 1, 2, and 3 and recruits them to the promoters of E2F target genes leading to repression of their expression.<sup>18</sup> To investigate the role of HDAC1/2 and Rb in senescence, we immunoprecipitated Rb complexes from MUC4 KD SCC1, SCC10B and observed increased levels of HDAC1/2 bound to unphosphorylated Rb in MUC4 KD cells compared to control cells (Figure 4b) indicating the involvement of HDAC 1 and 2 mediated cyclin E downregulation. More pronounced interaction between Rb and HDAC2 was observed in MUC4 KD cells as compared between HDAC1 and Rb. To further confirm the involvement of HDACs, we treated MUC4 KD cells with 5 $\mu$ M Trichostatin A (TSA), an HDAC inhibitor for 48 h and observed the re-expression of cyclin E compared to untreated MUC4 KD cells (Figure 4c). But on the contrary, we observed increased SA- $\beta$ -gal stained cells (Figure 4c). Therefore, we investigated if MUC4 mediated cyclin E down regulation was due to loss of acetylated chromatin on its promoter by performing ChIP assay using anti-acetylated histone H3 antibody. The H3 occupancy at E2F consensus binding sites of cyclin E promoter was analyzed using specific primers spanning the nucleotides between 1098 and 1795 bp on cyclin E promoter.<sup>21</sup> Less acetylated histone H3 was observed on the cyclin E promoter in both SCC1 and SCC10B MUC4 KD cells as compared to the control cells (Figure 4d) confirming that cyclin E downregulation upon MUC4 KD is mediated by histone deacetylation and heterochromatin formation.

### MUC4 knockdown suppresses tumorigenicity *in vivo*

MUC4 KD HNSCC cells were less proliferative, migratory and invasive *in vitro*; we thus tested if our *in vitro* observations impacted tumorigenicity and metastasis *in vivo*. We orthotopically implanted ( $2.5 \times 10^5$  in 50 $\mu$ l of PBS) either SCC1 MUC4 KD or control cells/ animal into the floor of mouth of nude mice (7 mice per group). Because of the large tumors formed by controls cells, animals were euthanized after 15 days of implantation, whereas animals implanted with MUC4 KD cells formed relatively smaller tumors and were euthanized after 30 days post implantation. Tumors formed by control cells were significantly larger (median weight of  $375 \pm 17.29$  mg) than MUC4 KD cells of only ( $170 \pm 18.30$  mg) ( $p = 0.00007$ ) (Figure 5a) indicating that KD of MUC4 in HNSCC cancer cells decreased tumorigenicity. IHC analysis of the harvested tumors revealed decreased MUC4 expression compared to control animals, indicating that MUC4 KD was maintained *in vivo* (Figure 5b). Furthermore, reduced Ki-67 positive cells were observed in tumors from MUC4 KD implanted animals compared to control cells (Figure 5b). Similar to *in vitro* observations, we also observed increased p16 expression and decreased cyclin E expression in tumors from MUC4 KD cells implanted animals compared to control cells (Figure 5b). Further, the percentage of SA- $\beta$ -gal positive cells was higher (~70%) in tumors from MUC4 KD cells as compared to control cells (~15%) (Figure 5c), strongly indicating cellular senescence *in vivo* is driven by MUC4 KD. Overall, our results suggest that MUC4 KD significantly suppressed tumor size by inhibiting proliferation and inducing cellular senescence *in vivo*.



## Discussion

MUC4 has recently emerged as a useful diagnostic marker and potential target for therapeutic intervention in several malignancies due to its functional involvement in promoting cell proliferation, invasion, metastasis and inhibition of apoptosis.<sup>9, 14, 22-24</sup> Several studies have reported aberrant expression of mucins (MUC1, MUC2, MUC4 and MUC5AC), but no functional study has yet been reported in HNSCC.<sup>25-29</sup> Using 1G8 antibody, MUC4 over expression has been reported in HNSCC (oral cavity, oropharynx, larynx, and hypopharynx) and associated with a worse prognosis.<sup>27</sup> However, head-to-head comparison of 1G8 with Mab 8G7 which recognizes human MUC4 has conclusively demonstrated that 1G8 neither recognizes human MUC4, nor exhibits staining pattern similar to human MUC4 antibody.<sup>30, 31</sup> A recent study using MAb 8G7 reported MUC4 overexpression (58%) in OSCC and its association with higher T classification, positive nodal metastasis, advanced tumor stage, diffuse invasion of cancer cells and bad prognosis.<sup>29</sup> In accordance with this report, we also observed a significant upregulation of MUC4 (68%) in HNSCC tissues compared to benign samples (P= 0.006), but contrary we did not observe any significant association of MUC4 overexpression with any clinical or pathological characteristics of patients (Figure 1 and Table 1). The major difference between the two studies was that we analyzed tissues samples of HNSCC which includes oral cavity, oropharynx, larynx, and hypopharynx, whereas the previous study used tissues from oral cavity only. Therefore lack of comprehensive knowledge about mucins prompted us to analyze the clinical and functional significance of mucins particularly MUC4 in HNSCC.

Our functional analyses using gene silencing approach indicated that MUC4 is involved in the regulation of cell growth, motility and invasion of HNSCC cells. We have previously demonstrated that MUC4 upregulates N-Cadherin expression which in turn interacts and stabilizes FGFR1 thereby promoting epithelial to mesenchymal transition (EMT) and therefore aggressive metastatic behavior of pancreatic cancer cells.<sup>10</sup> Further, previous studies in breast, ovarian and pancreatic cancers have established that MUC4 interacts with and stabilizes human epidermal growth factor receptor 2 (HER2), also known as ErbB2 leading to Src, FAK, Akt and ERK activation implicated in cell survival, cell motility/ invasion and metastasis.<sup>8, 10, 14, 32, 33</sup> Similarly, in the present study, MUC4 KD in HNSCC cells resulted in decreased phosphorylation of Src, FAK, Akt and ERK and resulted in decreased proliferation, motility and invasion (Supplementary Figures 4a-d). Further we observed that MUC4 augments proliferation by modulating cell cycle regulatory proteins like p16, cyclin E and cyclin D1. MUC4 silencing suppressed growth *via* a unique mechanism involving G<sub>0</sub>/G<sub>1</sub> cell cycle arrest. Interestingly, MUC4 silencing in HNSCC cell lines resulted in cellular senescence as suggested by large and flat cell morphology, increased SA- $\beta$ -galactosidase stained cells and SAHF formation (Figure 3a) which are considered to be characteristics of senescent cells.<sup>34</sup> This is the first report demonstrating that MUC4 expression augments senescence in cancer cells.

Cellular senescence is a potent tumor suppressor mechanism preventing unregulated growth and malignant transformation. p53 and p16/Rb signal transduction cascades are master regulators for cell cycle and promotion of cellular senescence.<sup>35</sup> Often lost in a variety of malignancies, p16 acts as an allosteric inhibitor of cdk4/6 complex to prevent its interaction

with cyclin D1, inducing the cell cycle arrest and senescence by activating Rb pathway. Cdk4/6-cyclin D1 complex mediated phosphorylation and inactivation of Rb allows the transcription of E2F-dependent various cell cycle regulatory genes including cyclin E. MUC4 silencing induced cellular senescence in HNSCC cells in a p16 dependent manner as indicated by: (a) increased p16 expression in MUC4 KD cells (b) abrogation of MUC4 silencing-induced senescence phenotype following p16 knockdown (Figure 3c-d). Our studies further indicated that senescence induction in MUC4 KD cells involved pRb dephosphorylation and chromatin remodeling to regulate cell cycle regulating protein cyclin E (Figure 3b and Figure 4a-d). Both P53 and p16/Rb signaling pathways are almost universally disrupted in 60-70% of HNSCC patients either by mutation, gene disruption or by promoter hypermethylation.<sup>36, 37</sup> Even though the involvement of p16 in cellular senescence and its downregulation in HNSCC is well established, there is still lack of a comprehensive study of its role in HNSCC senescence. Overexpression of p16 and p53 induced growth arrest of HNSCC cells<sup>38</sup>, suggesting that p53 or p16 restoration would be enough to decrease cell proliferation and tumor growth. Intriguingly, MUC4 silencing-induced senescence seemed to occur in a p53 independent manner as MUC4 KD induced growth arrest and senescence in both SCC1 and SCC10B cells (Figure 3b). Furthermore, western blot analysis revealed no difference in expression level of p53 between MUC4 knockdown and control shRNA transfected cells (Figure 3b). Besides the p53 and p16/Rb pathway, PTEN is also involved in the decision making and maintenance of oncogene-driven senescence; however no change in PTEN expression in MUC4 KD cells suggested the involvement of only p16/Rb pathway in senescence induction on MUC4 KD (Figure 3b).

Increased proliferation is mostly driven by altered cell cycle progression.<sup>32</sup> In HNSCC, cyclin E overexpression<sup>39</sup> has been shown to promote G<sub>1</sub> to S phase transition and induce escape from Ras induced senescence in mouse embryonic fibroblasts.<sup>40</sup> Conversely, a decrease in cyclin E-Cdk2 activity results in inhibition of the G<sub>1</sub> to S phase transition and induction of senescence.<sup>19</sup> Silencing of MUC4 resulted in decreased proliferation and G<sub>0</sub>/G<sub>1</sub> cell cycle arrest, providing evidence that MUC4 is a modulator of the cell cycle (Figure 2a-d). The decreased proliferation and cell cycle arrest was associated with downregulation of cell cycle regulatory proteins such as cyclin E and cyclin D1 (Figure 3b). Previously we have shown G<sub>0</sub>/G<sub>1</sub> cell cycle arrest and decreased proliferation upon MUC4 KD in breast and pancreatic cancer cells.<sup>14, 41</sup> In contrast to oncogenic role of MUC4 in PC, ovarian and breast, our recent work has shown a tumor suppressor role of MUC4 in lung cancer with decreased proliferation and induction of G<sub>2</sub>/M cell cycle arrest upon MUC4 over expression<sup>42</sup> along with downregulation of cyclin A, cyclin D1 and upregulation of cyclin dependent kinase inhibitors p21 and p27, suggesting that MUC4 has the potential to regulate (i.e. either promote or suppress) proliferation by altering cell cycle regulatory proteins.<sup>42</sup> In accordance with all these observations, we also observed increased expression of p16 and decreased expression of pRb, cyclin D1 and cyclin E expression upon KD of MUC4 in HNSCC cells and it appears that cyclin E/D down-regulation appears to be a direct consequence of MUC4 depletion (Figure 3b).

Local perturbations of chromatin structure could alter the accessibility and/or function of transcriptional regulatory proteins that bind DNA sequences in the region where histone acetylation or de-acetylation occurs.<sup>8, 43</sup> previous studies have shown that p300



downregulation decreased cyclin E expression through decreased histone acetylation and increased methylation of cyclin E promoter at the E2F binding site<sup>19</sup> leading to chromatin rearrangement accompanied by senescence associated hetero-chromatin foci (SAHF).<sup>8, 18</sup> As seen by DAPI staining, we also observed numerous SAHF in MUC4 KD cells compared to control cells (Figure 3a) suggesting that heterochromatin modification may be involved in cyclin E downregulation. These SAHF were shown to be enriched with heterochromatin markers like Lys9 tri-methyl (K9me3), H3K27me3 and heterochromatin protein 1 (HP1) of histone H3, and excluded euchromatic markers, such as histone H3K9 acetyl and H3K4me3.<sup>18</sup> Acetylation and methylation of H3K9 are mutually exclusive and therefore, de-acetylation of H3K9 by HDAC, which leads to repression, also allows for H3K9 methylation by methyl-transferases like Suv39H.<sup>44</sup> Similarly, our results revealed that MUC4 KD in HNSCC cells resulted in decreased expression of acetylated H3K9 and increased expression of H3K27me3 compared to control cells (Figure 4a) with no global changes in expression of acetylated H2BK5 and H2AK5. Ratios of H3K4me3/H3K27me3 were also decreased in MUC4 KD cells than the control cells. Similar to this observation, an earlier study has also shown the differential gene expression upon either H3K4me3/H3K27me3 methylation.<sup>45</sup> Histone modification at *cyclin E* promoter and its associated downregulation was further confirmed by CHIP assay using H3K9 acetylated antibody. In accordance with the results obtained by Bandyopadhyay *et al*<sup>19</sup> in human melanocytes that decreased acetylated histone at the cyclin E promoter resulted in decreased cyclin E expression, we also observed cyclin E downregulation by MUC4 mediated reduced H3K9 acetylation (Figure 4d). Under-phosphorylated pRb can recruit histone de-acetylases (HDAC 1, 2, and 3) to E2F dependent promoters like cyclin E, de-acetylating nearby histones and repressing gene expression.<sup>46, 47</sup> So we investigated whether HDAC may be involved in deacetylating H3K9 at cyclin E promoter. Co-immunoprecipitation and immunoblot analysis showed increased association between Rb and HDAC1 and HDAC2 in MUC4 KD cells compared to control cells with no change in total HDACs (Figure 4b). HDAC inhibitor Trichostatin A (TSA) has been shown to abolish Rb repression on some E2F target promoters like cyclin E.<sup>19</sup> Although TSA treatment to the MUC4 KD cells induced cyclin E expression comparable to control cells (Figure. 4c), we observed increased SA- $\beta$ -gal staining cells (Figure 4c). A likely explanation could be the necessity of the cells to maintain a critical balance between acetylated and deacetylated chromatin domains to sustain proliferation.<sup>19</sup> While hyper-acetylation caused by loss of HDAC activity could activate anti-proliferative genes, loss of acetylation may shift the balance toward repressive heterochromatin, causing silencing of genes associated with cell cycle progression.

HPV is an important etiological factor for oropharyngeal cancers among head and neck cancers.<sup>48</sup> These tumors have distinct molecular profile and significantly better prognosis than the traditional HPV negative and tobacco and alcohol related cancers.<sup>49</sup> Recently exome sequencing, revealed several mutations in MUC4 as well as in other mucins including MUC6 and MUC16 in HPV positive tumors compared to HPV negative tumors.<sup>50, 51</sup> Although, functional impact of these mutations remain undetermined, it is possible that these mutations may affect mucin expression, stability and post-translational modifications. While it is of interest to determine MUC4 expression in the context of HPV infections particularly in oropharyngeal cancer, due to small sample size and unknown HPV

status such correlations could not be investigated in the present study. However, we plan to study this association in a large cohort of oropharyngeal patients in future.

Overall, we have shown that MUC4 KD inhibits cell proliferation both *in vitro* and *in vivo* through induction of senescence programming pathways (Figure 5d). Several studies have shown that loss of oncogene function induces cellular senescence.<sup>19, 52</sup> Knockdown of MUC4 is crucial for ensuring the irreversibility of the senescence arrest even in p53 mutant cells. Our findings not only offer new perspectives in the modulation of senescence by MUC4 but also provide strong evidence for MUC4 based therapeutic interventions in HNSCC patients.

## Materials and methods

### Chemicals and antibodies

Trichostatin A (TSA) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The protein assay kit was from Bio-Rad (Hercules, CA, USA). Annexin-V conjugated AlexaFluor488 Apoptosis Detection Kit from Molecular Probes, Inc., (Eugene, OR). p16 siRNA was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MUC4 monoclonal antibody (8G7) was developed in our laboratory.<sup>53</sup> Horseradish peroxidase conjugated anti-mouse and anti-rabbit IgG were procured from GE Healthcare Biosciences (Uppsala, Sweden) and FITC-conjugated anti-mouse IgG was obtained from Invitrogen (California, USA). All other antibodies used are listed in supplementary table 1.

### Cell culture and MUC4 knockdown

Head and Neck Cancer cell lines UMSCC1, UMSCC10B, UMSCC11B, UMSCC23, UMSCC38, UMSCC47, UMSCC74 and UMSCC104 were a kind gift from Dr. Thomas Carey (University of Michigan, USA) and maintained as described in previous reports.<sup>54</sup> Stable MUC4 KD HNSCC cells were generated as described previously.<sup>8</sup> Stable MUC4 shRNA or scramble transfected clones were selected, pooled and tested for MUC4 expression by real time PCR<sup>10</sup>, confocal microscopy<sup>33</sup> and by western blot analysis.<sup>54</sup> Co-immunoprecipitation (co-IP) assay was done as previously described.<sup>55</sup>

### Cell proliferation, colony formation and BrdU incorporation

For growth rate analysis,  $1 \times 10^3$  cells/well were grown in 1%  $\alpha$ MEM and viable cell numbers were determined every 24 h for 8 days using a Vi-Cell XR instrument (Beckman Coulter, Fullerton, CA). Cell proliferation was also determined by measuring the incorporation of BrdU using a BrdU *In-Situ* Detection Kit (BD Pharmingen™, USA). In brief, control and MUC4 KD ( $10 \times 10^3$ ) cells were incubated overnight with 10 $\mu$ M BrdU for 24 h at 37°C. The incorporated BrdU was detected with an HRP conjugated anti-BrdU antibody and using tetramethylbenzidine (TMB) as a substrate. After stopping the reaction with H<sub>2</sub>SO<sub>4</sub>, plate was read at 450 nm. Effect of MUC4 KD on cell cycle, *in vitro* colony formation, cell motility and invasion was done as described by us previously.<sup>42, 54</sup>

### Quantitative *in situ* SA-β-gal assay

Senescence associated-β-galactosidase (SA-β-gal) activity was measured according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). SA-β-Gal activity was detected using X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) staining at pH 6.0. Using light microscope several representative fields (n=10) were randomly selected for the quantification of the percentage of SA-β-gal positive cells. Similarly, frozen tumor tissues from mice were stained for SA-β-gal activity as above.

### Tumorigenicity assay

Control and MUC4 KD SCC1 cells of >97% viability ( $2.5 \times 10^5$  cells/50 μl) were orthotopically implanted at the base of tongue (seven animals/group).<sup>56</sup> Athymic nude mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD, USA.). The mice were treated in accordance with Institutional Animal Care and Use Committee (IACUC) of UNMC guidelines. Based on the size of tumor, mice bearing control cells were euthanized after 15 days of implantation whereas MUC4 KD implanted mice were euthanized after 30 days. Tumors were excised, weighed, measured and formalin fixed. A part of the tumor tissue was also snap-frozen for SA-β-gal analysis. Experiment was repeated twice.

### Immunohistochemistry

The study was approved by the institutional review board of University of Nebraska Medical Center, USA. Eighty seven primary HNSCC samples and 10 benign tissues were analyzed by immunohistochemical (IHC) analysis for MUC4 as described previously.<sup>14</sup> Tumors from orthotopically implanted animals were also analyzed for MUC4, p16, cyclin E and Ki-67 expression. Replacement of the primary antibody with isotype IgG was used as negative control. Slide were analyzed by two pathologists (SL and SJ) blinded to the study. Intensity of expression and percentage of cells stained were analyzed and overall staining was then represented by a composite score as done by us earlier.<sup>14</sup>

### Statistical analysis

The data was analyzed using the Medcalc software (version 9.6.4.0) for windows. The relationship between MUC4 protein expression and clinicopathological parameters was tested using Chi-Square and Fischer's test. Mean tumor weights were compared between groups using a two-tailed independent sample student t-test. The criterion for statistical significance was \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.005.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

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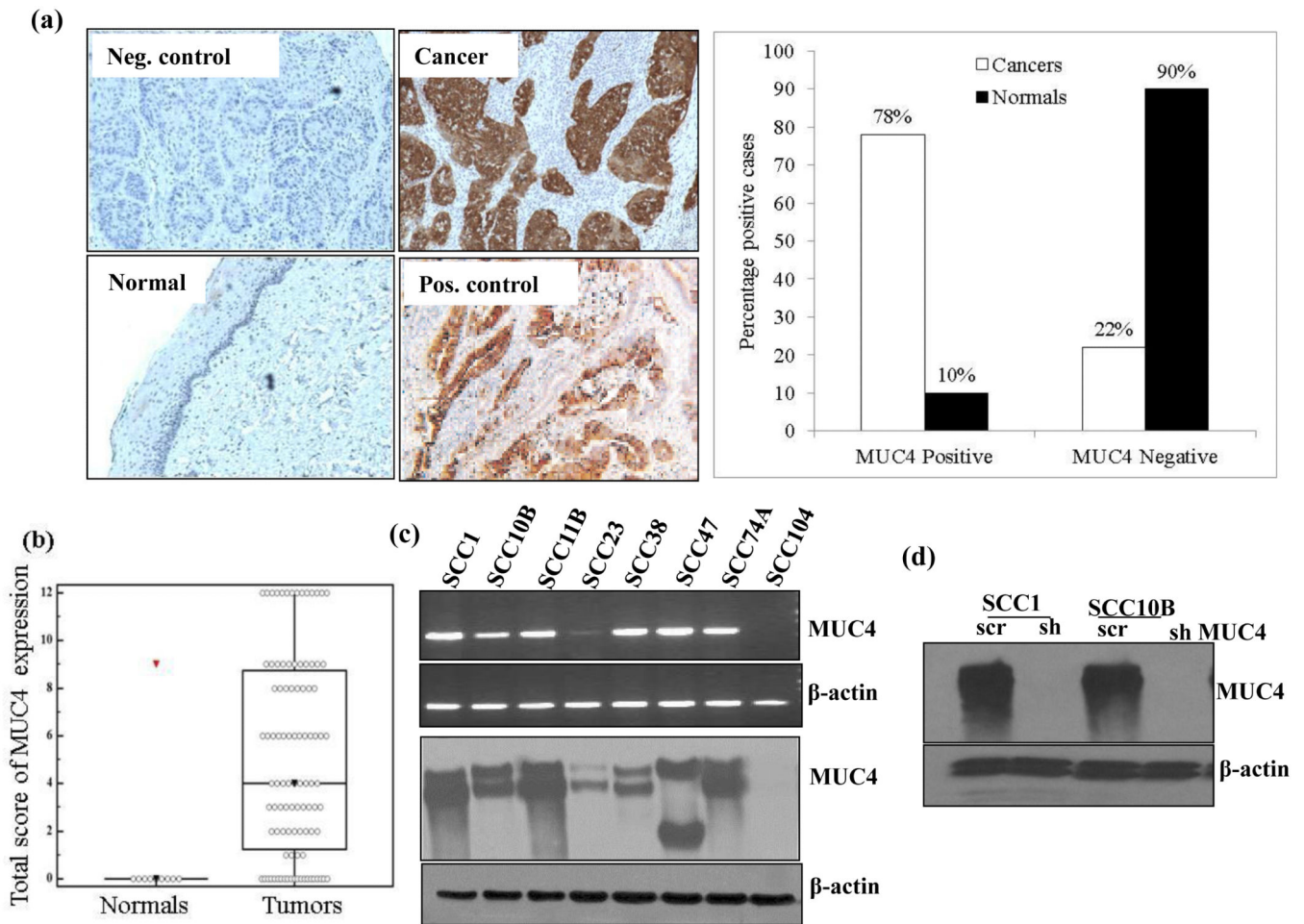
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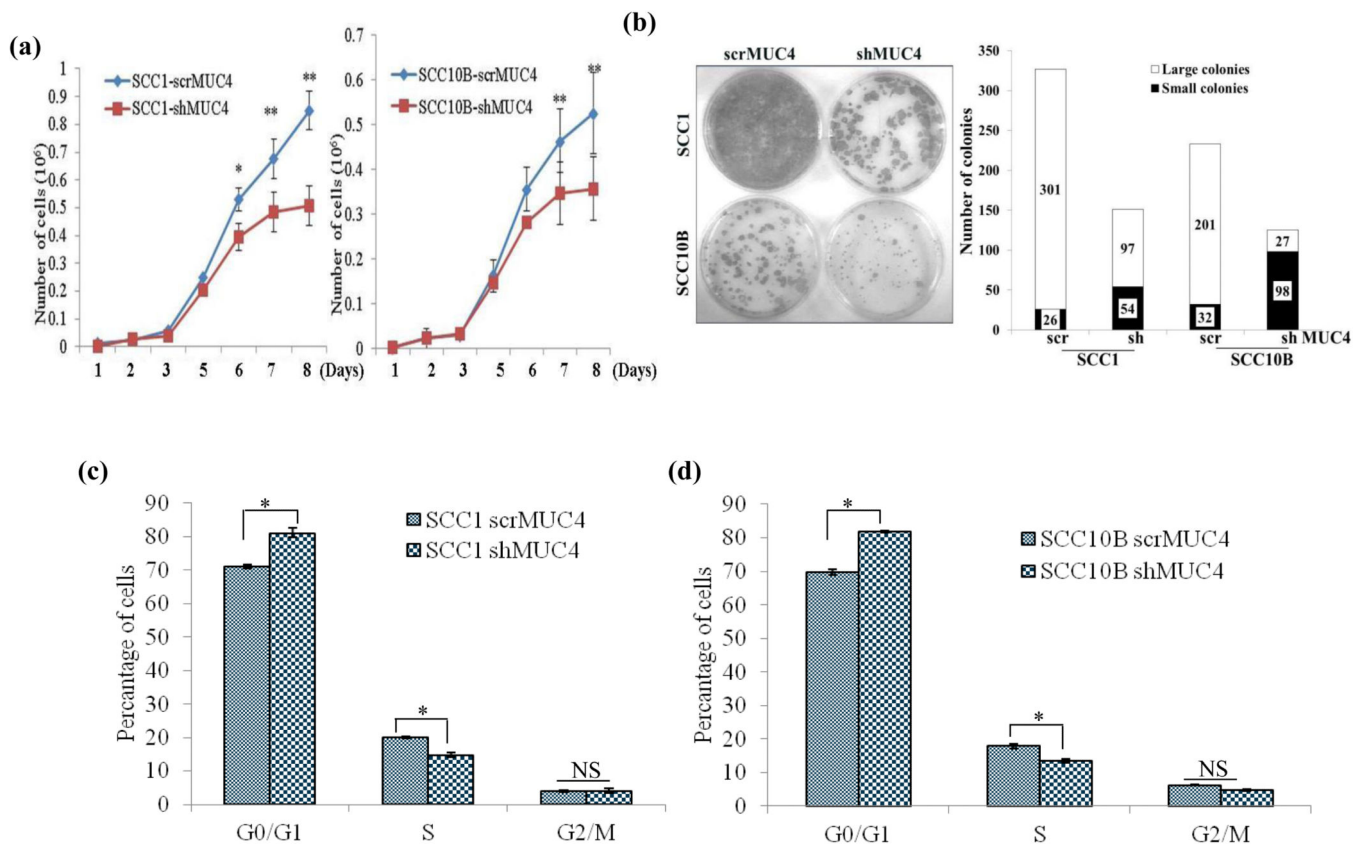




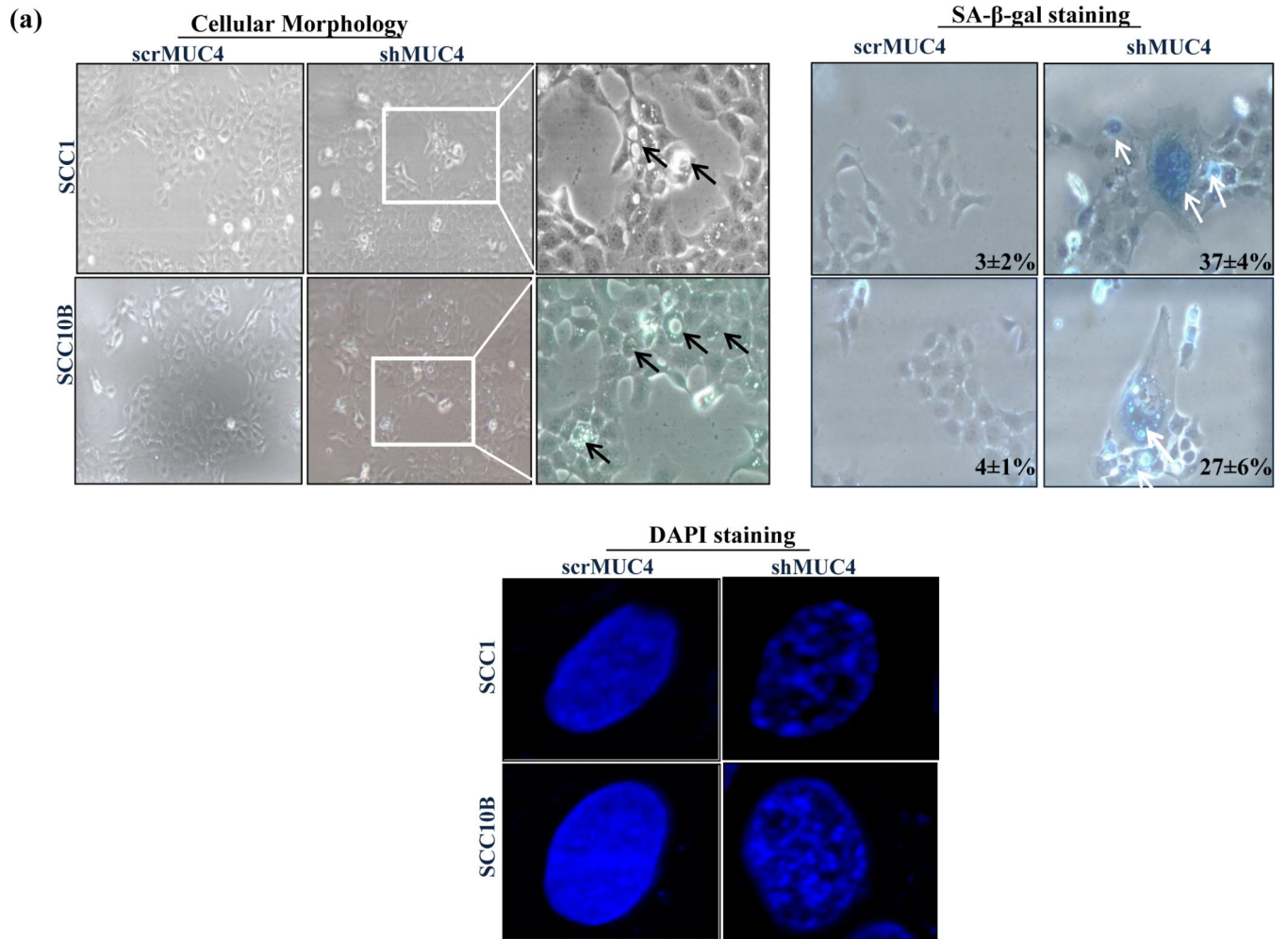
**Figure 1.**

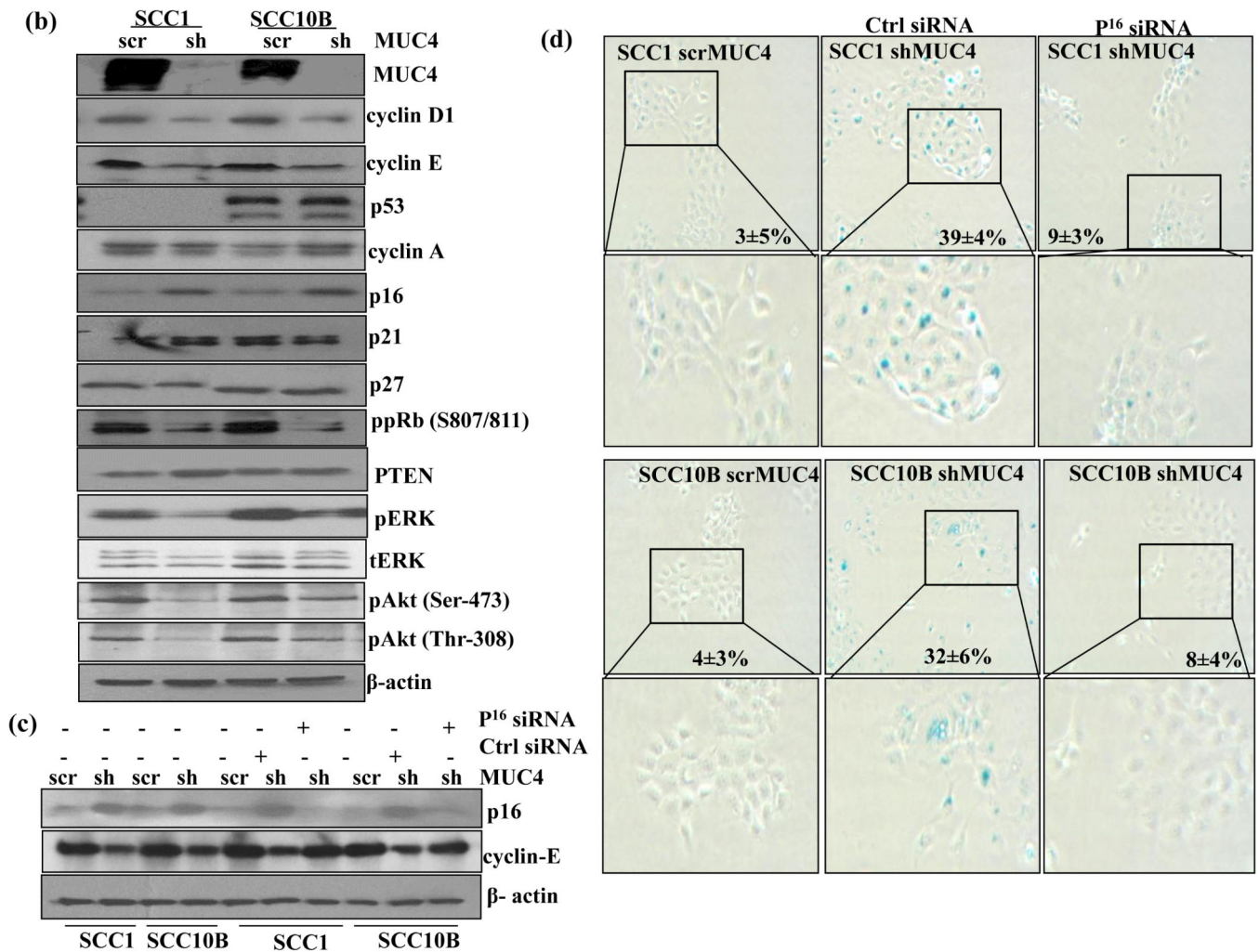
Over expression of MUC4 in HNSCC tissues and HNSCC cell lines. (a)

Immunohistochemical analysis of MUC4 paraffin embedded sections of normal tissues (n = 10) and HNSCC tissue (n = 87) using anti-MUC4 Mab 8G7. Anti-KLH Mab K2G6 was used as negative control. Pancreatic cancer tissue sample was used as positive control for MUC4 expression. Magnification  $\times 100$ . (b) Box plots showing distribution of composite scores based on IHC of MUC4 protein. Increased expression of MUC4 was observed in HNSCC with a median score of 4 (range 0–12), as compared to the histologically normal oral tissues with a median score of 0. (c) MUC4 expression in a panel of HNSCC cell lines was determined at protein level by immunoblotting while mRNA was analyzed by RT-PCR.  $\beta$ -actin was used as loading control for both western blot and RT-PCR analysis. (d) Silencing of MUC4 expression in SCC1 and SCC10B cells. Cells were infected with lentiviruses carrying shRNA against MUC4 in pLKO.1 hairpin vector or with scrambled sequence containing vector and used as control. Transfected cells were selected using Puromycin and positive clones were checked for MUC4 expression using 2% agarose SDS gel.  $\beta$ -actin was used as a loading control.



**Figure 2.** MUC4 knockdown decreases cell proliferation and induces cell cycle arrest in HNSCC cells. **(a)** Growth kinetics analysis showing that MUC4 KD in HNSCC SCC1 and SCC10B cells has lower proliferative activity in comparison with control cells. **(b)** SCC1 and SCC10B MUC4 KD and control cells ( $1 \times 10^3$ ) were seeded in triplicate in 10% DMEM in a 12-well plate, and allowed them to grow in 10% DMEM for 2 weeks. Colonies formed were washed with PBS, fixed in methanol and then stained with 0.1% crystal violet in PBS. The number of colonies (per well) were counted with the automatic colony counting tool of the Quantity One Imaging software. The graphs represent the mean ( $\pm$ SE) number of colonies. **(c-d)** SCC1 and SCC10B KD and control cells were synchronized with double thymidine block. After synchronization, cells were stained with propidium iodide (PI) and analyzed by fluorescence-activated cell sorting to evaluate the number of cells in different stages of cell cycles.

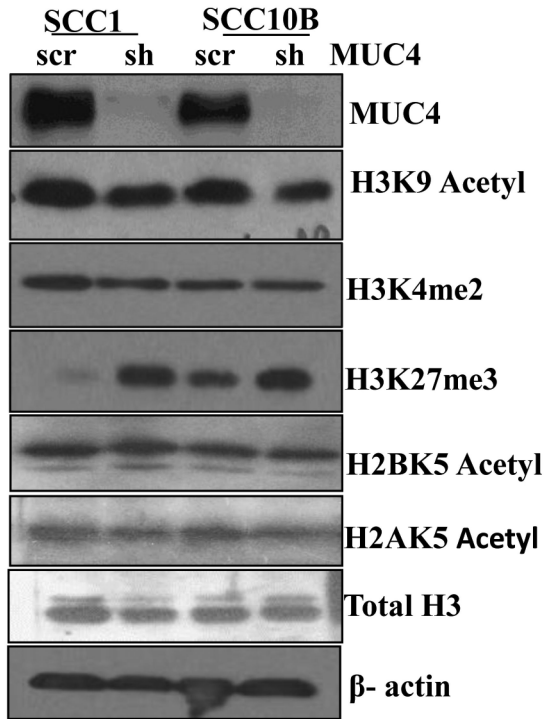


**Figure 3.**

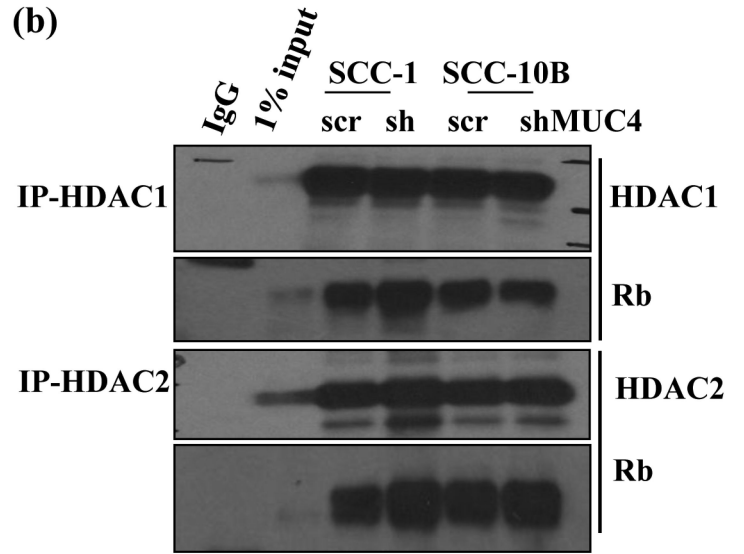
MUC4 knockdown induces cell senescence by modulating cell cycle regulators proteins in HNSCC cells. (a) HNSCC cells were subjected to cellular morphological observation, SA- $\beta$ -gal and DAPI staining and observed under microscopy. MUC4 KD caused SCC1 and SCC10B cells are large, flattened and vacuolated, characteristics of senescent cell. SA- $\beta$ -gal positive cells for senescence and DAPI for SAHF formation cells were observed under microscope and quantified. (b) Protein lysates from control and MUC4 KD HNSCC cells were analyzed for p16, p21, p27, p53, cyclin D1, cyclin E, cyclin A PTEN, phosphorylated and total Akt, ERK<sub>1/2</sub> proteins. Western blot analysis showed upregulation of p16 and downregulation of cyclin D1, cyclin E, pAkt and pERK<sub>1/2</sub> with no change in expression of total Akt and ERK<sub>1/2</sub>.  $\beta$ -actin was used as a loading control. (c-d) p16 depletion partially rescues senescent phenotype and induces cyclin E expression. MUC4 KD SCC1 and SCC10B cells were transfected with p16 siRNA or with control siRNA. After 72 hrs, proteins lysates were analyzed for p16, cyclin E expression and stained for SA- $\beta$ -gal positive cells.  $\beta$ -actin was used as a loading control for western blot analysis.

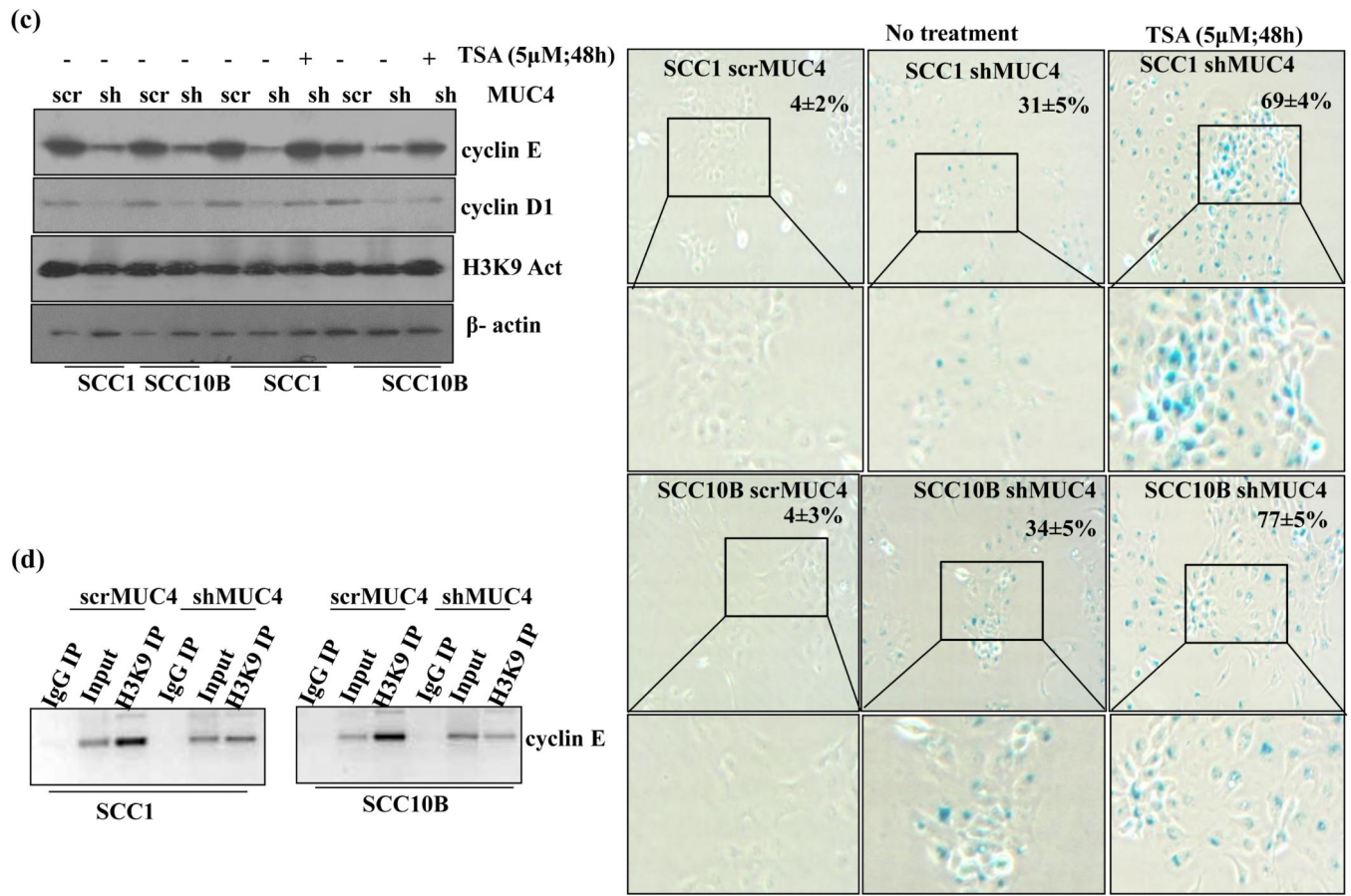


(a)



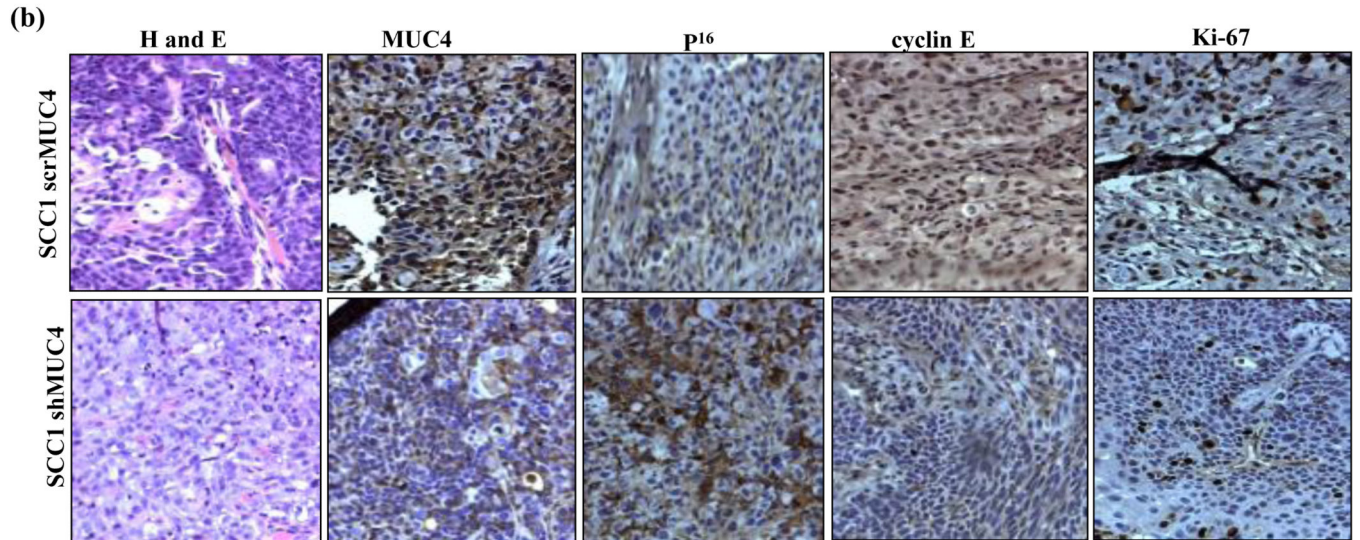
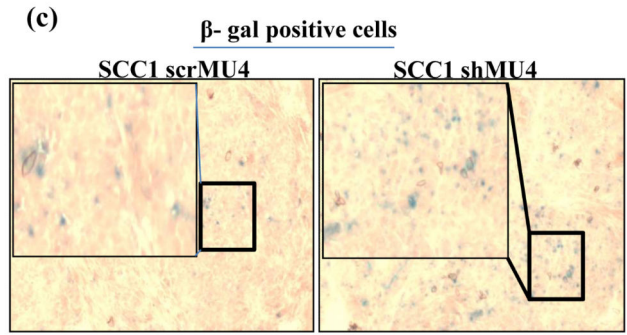
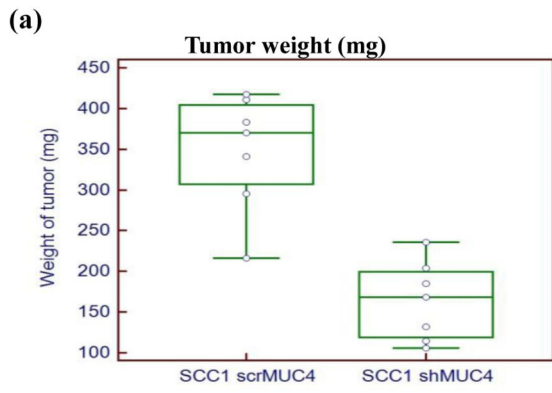
(b)

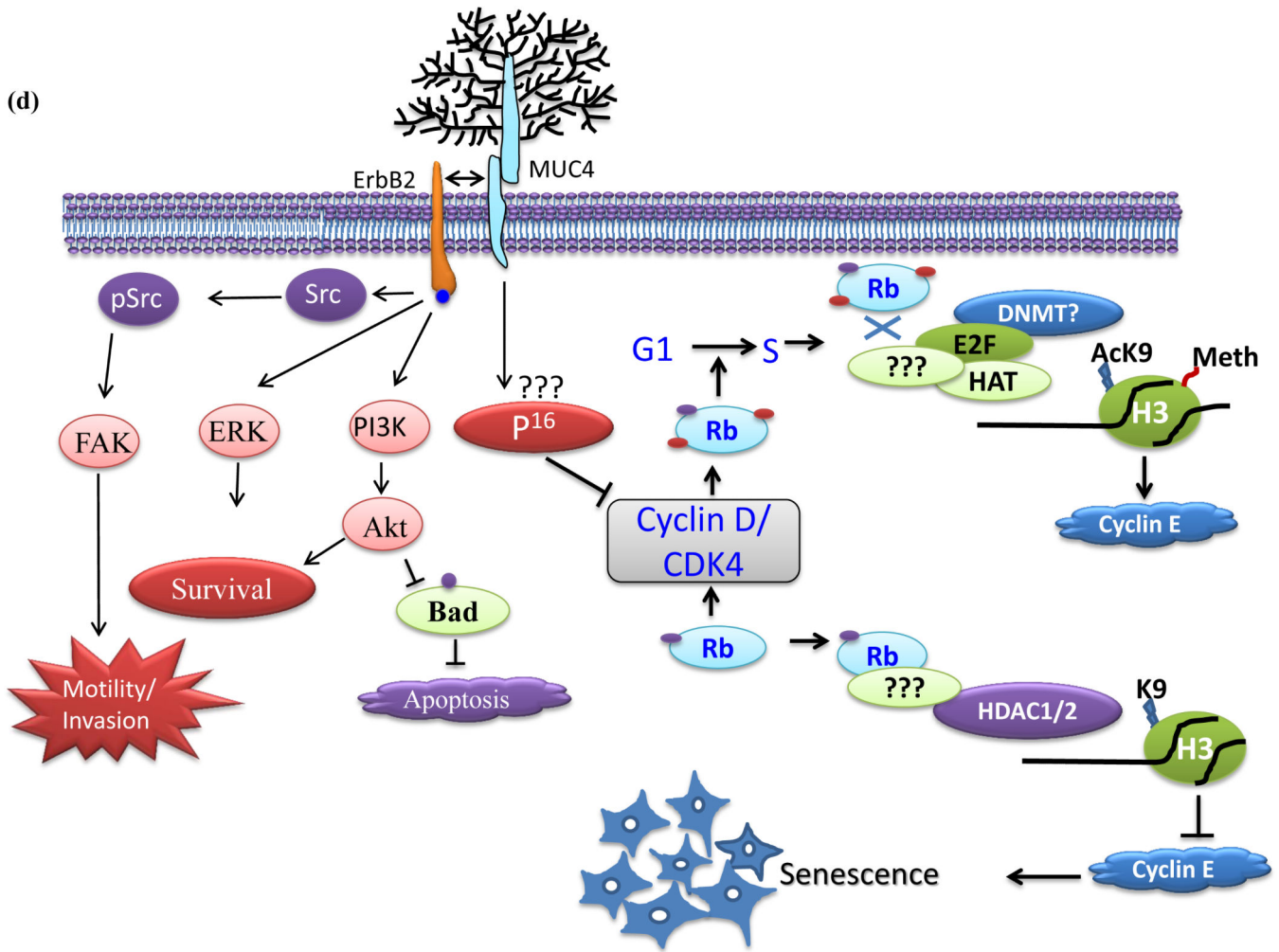


**Figure 4.**

MUC4 knockdown induces histone modification to modulate cyclin E expression. **(a)** Western blot analysis of lysates from control and MUC4 KD SCC1 and SCC10B cells using specific antibodies recognizing acetylated and methylated histones. **(b)** Enhanced interaction between pRb and HDAC1/2 in MUC4 KD cells. Association of pRb and HDAC1/2 was studied in the lysates of control and MUC4 KD SCC1 and SCC10B cells by immunoprecipitation and western blotting as described in Materials and Methods. HDAC1 and 2 were immunoprecipitated using specific antibodies and the bound levels of pRb co-precipitated with HDAC1/2 were determined by western blot analysis. **(c)** Trichostatin A (TSA) treatment induces cyclin E expression and increases SA- $\beta$ -gal stained cells. MUC4 KD SCC1 and SCC10B cells were treated with TSA (5 $\mu$ M) and 48 h and protein lysates were analyzed by western blot analysis and probed with cyclin E, cyclin D1 and acetylated H3K9 specific antibodies.  $\beta$ -actin was used as a loading control. Similarly, TSA treated cells were analyzed for SA- $\beta$ -gal staining and senescent cells were observed and counted. **(d)** Decreased H3K9 acetylation at cyclin E promoter. Cross-linked, sheared chromatin was prepared from control and MUC4 KD SCC1 and SCC10B cells and subjected to immunoprecipitation with the H3K9 acetylated antibody. The immunoprecipitated complexes were subjected to PCR analysis using primer pairs spanning the human cyclin E promoter. Isotypic IgG Ab was used as negative control, whereas chromatin obtained before immunoprecipitation was used as internal control.







**Figure 5.**

MUC4 knockdown inhibits HNSCC cell tumorigenicity. (a) Box-plot showing the distribution of tumor weight isolated from mice injected with MUC4 KD cells versus control cells. Error bars represent SE for  $n = 7$  mice. (b) Immunohistochemistry of MUC4, P16, cyclin E and Ki-67 in tumor sections obtained from mice injected with MUC4 KD HNSCC cells (bottom) and control cells (top) along with corresponding H&E sections. (c) Showing SA- $\beta$ -gal stained cells in tumor sections obtained from mice injected with MUC4 KD SCC1 cells (right) and control cells (left). (d) Proposed model for MUC4-mediated cellular senescence. MUC4 KD induces p16 expression which in turn decreased phosphorylated pRb. Under-phosphorylated pRb recruits HDAC1/2 to gene promoters like cyclin E, induces histone de acetylation and heterochromatin formation. Decreased cell cycle proliferating genes like cyclin E as a result of heterochromatin formation leads to cell cycle arrest and finally cell senescence. MUC4 *via* physical interaction and subsequent stabilization of HER2/ErbB2 leads to activation of Src/FAK, PI3K/Akt and ERK signaling pathways for enhanced motility, viability and increased cell proliferation.

**Table 1**

Correlation of MUC4 expression with clinicopathological characteristics of patients

Patient characteristics	MUC4 Negative (n=19) N (%)	MUC4 Positive (n=68) N (%)	p-value
<b>Age (median 59.5, range 42-95)</b>			
<58 years	8 (42%)	31 (46%)	0.75
≥ 58 years	11 (58%)	36 (54%)	
<b>Race</b>			
African American	7 (37%)	20 (29%)	0.83
White	11 (58%)	44 (65%)	
Unknown	1 (5%)	4 (6%)	
<b>Sex</b>			
Male	12 (92%)	49 (73%)	0.40
Female	7 (8%)	18 (27%)	
<b>Tobacco</b>			
Never	5 (28%)	12 (18%)	0.23
Current	12 (67%)	37 (56%)	
Former	1 (6%)	17 (26%)	
<b>Site Group 1</b>			
Oral cavity	6 (32%)	13 (19%)	0.25
Oropharyngeal	13 (68%)	55 (81%)	
<b>Site Group 2</b>			
Oral cavity	8 (42%)	13 (19%)	N/A
Base of tongue	1 (5%)	18 (26%)	
Tonsil	5 (26%)	28 (41%)	
Pharynx	5 (26%)	9 (13%)	
<b>Grade</b>			
Well differentiated	1 (6%)	6 (9%)	0.56
Moderately differentiated	9 (50%)	38 (59%)	
Poorly differentiated	8 (44%)	20 (31%)	
<b>T-stage</b>			
T1/T2	7 (41%)	32 (51%)	0.48
T3/T4	10 (59%)	31 (49%)	
<b>N-stage</b>			
NO	5 (29%)	17 (27%)	0.84
N1-N3	12 (71%)	46 (73%)	

N/A - analysis not conducted because of unstable OR estimates due to small sample size.