



ROLE OF BPAG1e IN NEOPLASTIC PROGRESSION OF ORAL SQUAMOUS CELL CARCINOMA DERIVED CELLS.

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ABSTRACT

Aberrant expression of Keratin8/18 has been consistently shown in squamous cell carcinomas. Further, knockdown of K8 in oral squamous cell carcinoma (OSCC) derived cell line resulted in alterations in levels of $\alpha 6\beta 4$ integrin proteins, $\alpha 6\beta 4$ integrin mediated signalling, reduction in cell motility, tumorigenic potential and Fascin expression. However, how Keratin8/18 pair modulates $\beta 4$ integrin downstream signalling events is still obscure. BPAG1e and Plectin anchor the keratin network to the cell surface via $\beta 4$ integrin. In order to understand the role of BPAG1e in K8/18 mediated regulation of $\beta 4$ integrin signalling, it was downregulated in an OSCC derived AW13516 cell line. BPAG1e knockdown resulted in alterations in $\alpha 6\beta 4$ integrin mediated signalling, reduction in cell migration, cell invasion, tumorigenic potential and altered actin organization. In summary, BPAG1e promotes cell migration and neoplastic progression by modulating $\alpha 6\beta 4$ integrin mediated signalling in OSCC cells and may prove useful in prognostication of human oral cancer.

KEYWORDS: *BPAG1e, $\alpha 6\beta 4$ integrin, keratin8/18, oral squamous cell carcinoma, neoplastic progression*



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INTRODUCTION

Hemidesmosomes (HDs) are anchoring junctions which connect basal epidermal cells to the extracellular matrix. In complex epithelia like skin, type 1 HDs are present which comprise of the transmembrane proteins like $\alpha 6\beta 4$ integrin, BP180 and CD151 that form the outer plaque of HDs. The inner plaque of HDs is formed by cytoplasmic linker proteins, BPAG1e and Plectin.¹⁻² These linker proteins connect the intermediate filament proteins to the transmembrane proteins, which in turn interact with the extracellular matrix protein laminin 5. BP230 protein, which is encoded by the DST gene, has several isoforms namely BPAG1n, BPAG1e, BPAG1a, BPAG1b, based on alternative splicing of the gene. BPAG1e is a major isoform expressed in the epidermis.³ Ablation of BPAG1e expression in keratinocytes resulted in the formation of blisters. Moreover, BPAG1e knockout animals displayed impaired wound healing *in vivo*.⁴ This study indirectly implicates the role of BPAG1e in keratinocyte migration. Hamill *et al* have shown that BPAG1e regulates keratinocyte migration by acting as a scaffold for $\beta 4$ integrin mediated modulation of Rac1 and cofilin activities.⁵ Conversely, Michael *et al* have reported reduced adhesion but increased spreading and migration in human keratinocytes carrying homozygous nonsense mutations in BPAG1e encoding gene.⁶ In another study, it has been reported that upregulation of BPAG1e and $\alpha 6\beta 4$ integrin expression is found in invasive squamous cell carcinomas. The polarized localization of BPAG1e was lost in highly invasive tumours.⁷ Contrary to this; Lo *et al* have reported downregulated levels of hemidesmosomal components, including BPAG1e in nasopharyngeal carcinoma as compared to non-malignant nasopharyngeal epithelia.⁸ These observations together suggest that the role of BPAG1e in cancer development could be context dependent. Keratins (K) are the largest subgroup of intermediate filament proteins expressed in a tissue specific and differentiation dependent manner. They are obligate noncovalent heteropolymers that include at least one type I and one type II keratin which pair together during filament formation. K8 and K18 are expressed in all simple epithelial cells.⁹ Normally, K8 and K18 expression is not observed in stratified adult epithelial tissues. However, these proteins are often aberrantly expressed in squamous cell carcinomas (SCC), where their expression is correlated with invasion and poor prognosis.¹⁰⁻¹² Previous work from our laboratory has shown that K8 overexpression led to neoplastic transformation and increased invasive and metastatic potential in a cell line derived from the buccal mucosa of normal human fetus.¹³ Subsequent work from our laboratory revealed that knockdown of K8 in OSCC derived cell line (AW13516) resulted in alterations in the levels of $\alpha 6\beta 4$ integrin proteins, $\alpha 6\beta 4$ integrin mediated signalling, reduction in cell motility, downregulation of cell motility associated protein fascin and alterations in reorganization of the actin cytoskeleton.¹⁴ However, the mechanism by which K8/18 pair modulates $\beta 4$ integrin

signalling and its downstream events is still obscure. As linker proteins like BPAG1e and Plectin anchor keratin proteins to the cell surface via $\beta 4$ integrin, we hypothesized that these linker proteins may have a role in keratin mediated regulation of $\beta 4$ integrin signalling and thereby regulation of cell motility and neoplastic progression of oral squamous cell carcinoma OSCC. In the present communication, we show that the knockdown of BPAG1e in OSCC derived cell line resulted in significant reduction in cell motility, cell invasion, *in vivo* and *in vitro* tumorigenicity, and alterations in actin organization. Moreover, $\alpha 6\beta 4$ integrin mediated signalling was found to be affected upon BPAG1e knockdown in OSCC derived cells.

MATERIALS AND METHODS

Cell line, Plasmids and Transduction

The cell line AW13516, derived from a tongue SCC was cultured in IMDM supplemented with 10% fetal bovine serum and antibiotics under a 5% CO₂ atmosphere and grown at 37°C.¹⁵ The pLKO1.puro plasmid was purchased from Addgene (Plasmid #10878).¹⁶ The pLKO1.puro plasmid containing shRNA sequence against BPAG1e (AGCAAGCTCTGTATTACTC) was gifted by Dr. Jonathan Jones, Washington State University, USA. Briefly, AW13516 cells were infected with lentivirus encoding a BPAG1e specific shRNA. The stable clones were selected in a medium containing 0.5 μ g/ml puromycin. The knockdown of BPAG1e was confirmed using RT-PCR and Western blotting.

Antibodies

The following antibodies were used: BPAG1e (goat polyclonal, Santacruz Biotechnology, sc-13776), β actin (mouse monoclonal, Sigma, A5316), $\beta 4$ integrin (rabbit polyclonal, Santacruz Biotechnology, sc-9090), Keratin 8 (mouse monoclonal, Sigma, C5301), Plectin (mouse monoclonal, Santacruz Biotechnology, sc-33649), Fascin (mouse monoclonal, Thermo Scientific, MA1-20912), Akt (rabbit polyclonal, Cell signalling technology, 9272), Erk1/2 (rabbit polyclonal, Cell signalling technology, 9102), Phospho Akt (S473) (rabbit polyclonal, Cell signalling technology, 9272), Phospho Erk1/2 (T185+T202) (rabbit polyclonal, Abcam, ab4819), $\beta 4$ Integrin 3E1 (mouse monoclonal, EMD Millipore, MAB1964), Phalloidin - fluorescein isothiocyanate labeled (Sigma, P5282), Anti mouse HRP (Sigma, A4416), Anti rabbit HRP (Sigma, A0545), Anti mouse Alexa Fluor 488 (Life technologies, A11001).

RNA isolation and reverse transcriptase (RT) PCR

RNA was isolated by TRI reagent and RT-PCR was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's protocol. The primers used to amplify BPAG1e and GAPDH (as internal control) are shown in Table 1. RT-PCR conditions were as follows: denaturation at 94°C for 30 s; annealing at 58°C for 30 s; and extension at 72°C for 60 s. Number of cycles: 27 and 30 for GAPDH and BPAG1e respectively.

Table 1
RT-PCR primer sequences

Primer name	Primer Sequence (5'-3')
GAPDH (F)	GAAGGTGAAGGTCGGAGTC
GAPDH (R)	GAAGATGGTGATGGGATTTC
BPAG1e (F)	TACTGCCCTGGTCACTCTCAT
BPAG1e (R)	CACTGTTGGCTTCTGACGCT

F: Forward, R: Reverse

Western blotting

Whole cell lysates were prepared in SDS lysis buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol) with protease inhibitor cocktail. Western blotting was performed as previously described.¹³

Immunoprecipitation assay

The cell lysates were made by pooling both NP-40 (1%) and Empigen (2%) fractions. Immunoprecipitation was carried out as described previously.¹⁷

Wound healing assay for migration

The cells were grown in 6 well plates to 95% confluency. Media was replaced with fresh IMDM containing 0.1% serum for 24 h. After incubation, the medium was discarded and wounds were scratched using a sterile 2 μ l pipette tip. The cells were fed with fresh IMDM with 0.1% serum medium and observed under an Axiovert 200 M Inverted Carl Zeiss microscope fitted with a stage maintained at 37°C and 5% CO₂. The images were taken every 20 minutes for 20 h using an AxioCam MRm camera. The rate of migration was calculated using ImageJ software.

Matrigel invasion assay

40 μ l Matrigel (1mg/ml) was applied to 8 μ m pore size polycarbonate membrane filters and the bottom chamber was filled with 0.6 ml of complete IMDM. 2 x 10⁵ cells were seeded in the upper chamber in serum-free medium, and then incubated at 37°C. At the 15th h, 4 μ g/ml Calcein AM was added to the lower chamber and incubated for 1 h at 37°C. The cells on the upper surface were carefully removed with a cotton swab. Fluorescence of the invaded cells was read at wavelengths of 488/535nm (Ex/Em) on a bottom reading fluorescent plate reader at the 16th h.

Soft agar colony forming assay

The soft agar colony forming assay was performed as described previously.¹⁴ Opaque and dense colonies were imaged and counted microscopically.

In vivo Tumorigenicity assay

All protocols for animal studies were reviewed and approved by the Institutional Animal Ethics Committee constituted under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Sanction No. 04/2015). The cells were suspended in plain medium without serum and 6 x 10⁶ cells were injected subcutaneously into the dorsal flank of 6–8 weeks old NOD-SCID mice. 6 mice were injected per clone and were observed for tumor formation over a period of time. Tumor volume was determined using a digital vernier

caliper and calculated by the modified ellipsoidal formula, [Tumor volume = 1/2(length x width²).

Cell proliferation assay

1000 cells were seeded in triplicate in a 96 well plate. 20 μ l 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to each well. The plate was incubated at 37°C in a CO₂ incubator for 4 h, then 100 μ l of acidified SDS (10% SDS in 0.01 N HCl) was added to each well, and the plate was incubated overnight at 37°C. The next day, optical density (OD) was measured at 540 nm against a reference wavelength of 690 nm. Proliferation was studied every 24 h upto a period of 4 days.

Stimulation of β 4 integrin signalling

About 60 -70% confluent cells were serum starved for 18 h and then treated with 5 μ g/ml of 3E1 antibody (β 4 integrin activating antibody).¹⁸ 1 μ g/ml mouse IgG treated cells were used as control. After 30 minutes of treatment, proteins were extracted and used for western blot analysis.

Immunofluorescence

Immunofluorescence staining was performed as previously described.¹³ Confocal images were obtained with a LSM720 Carl Zeiss Confocal system.

Actin organization

The wounds were scratched in confluent cells. The cells surrounding the scratch were allowed to migrate into the wounded region for 8 h. Subsequently, cells were fixed using 4% Paraformaldehyde. Further, cells were subjected to Phalloidin staining for 1 hr and visualized under confocal microscope.

Statistical Analysis

All the statistical analyses were performed using GraphPad Prism software (version 6.01). Two groups of data were compared by performing unpaired t-test. p<0.05 was considered statistically significant.

RESULTS

Knockdown of BPAG1e in OSCC derived cells using shRNA technology

To understand the role of BPAG1e in OSCC derived cells, BPAG1e was downregulated in AW13516 cells using shRNA technology. Downregulation of BPAG1e was confirmed at mRNA and protein level (Fig 1a, 1b). BPAG1e knockdown clones C4 and C12 displayed 77% and 81% decrease in BPAG1e protein respectively, when compared to vector control clone BVC (Fig 1b).

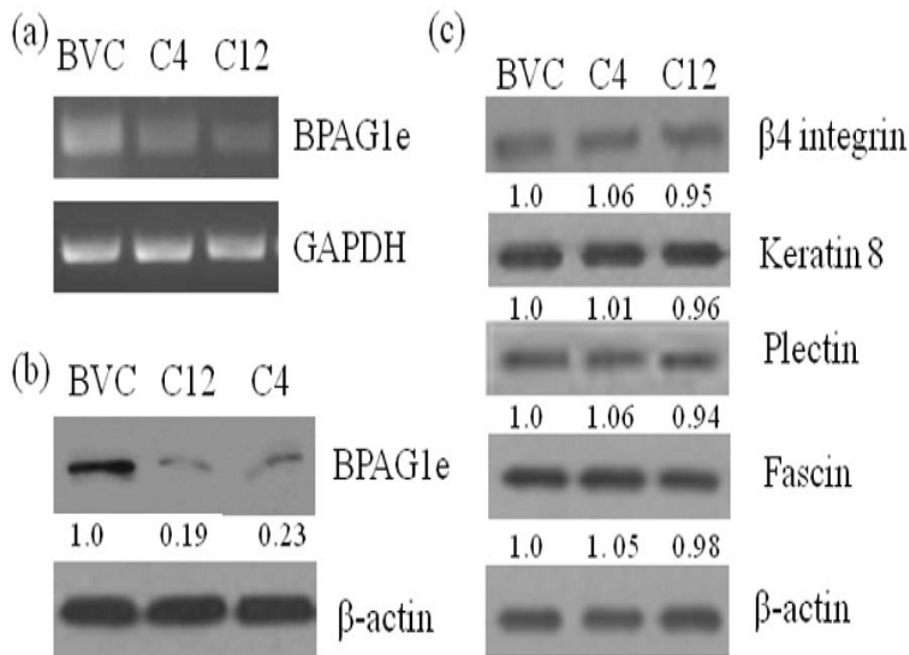


Figure 1

BPAG1e knockdown and its effect on other associated proteins in AW13516 cells

(a) RT-PCR analysis of BPAG1e knockdown clones (C4, C12) and its vector control clone (BVC); (b) Western blot analysis of BPAG1e knockdown clones (C4, C12) and its vector control clone (BVC) (c) Western blot analysis of BPAG1e associated proteins in the BPAG1e knockdown (C4, C12) and vector control clones (BVC). The numbers below the blot indicate relative intensity of the bands using densitometric analysis.

Phenotypic assays for cell transformation for BPAG1e knockdown cells

In our previous study, we have demonstrated that loss of K8 in AW13516 cells leads to reduction in cell migration, cell invasion, *in vitro* and *in vivo* tumorigenic potential and alterations in actin organization.¹⁴ Therefore, to understand whether knockdown of BPAG1e leads to similar phenotypic changes as seen in response to K8 knockdown, we first carried out phenotypic assays using BPAG1e knockdown cells.

Scratch wound healing motility assay and actin organization

We saw significant reduction in the rate of cell migration in BPAG1e knockdown clones C4 (29%) and C12 (32%) as compared to the vector control clone BVC ($p < 0.001$) (Fig 2a, 2b). It is well documented that changes in cell migration are many times associated with alterations in actin organization.¹⁹⁻²⁰ Therefore, phalloidin staining was carried out to check alterations in actin organization in response to BPAG1e knockdown (Fig 3a). The membrane protrusions in BVC were found to be uniformly present whereas in case of loss of BPAG1e, C12 clone showed non-uniformly organized membrane protrusions. Moreover, C12 clone showed reduction in the length of membrane protrusions (28.11 μ m) as compared to BVC (45.15 μ m) ($p < 0.001$) (Fig 3b).

Boyden chamber invasion assay

Boyden chamber invasion assay demonstrated that invasion was reduced significantly in BPAG1e knockdown clones, C4 (33%) and C12 (38%) as compared to vector control clone BVC ($p = 0.001$) (Fig 2c).

Soft agar colony forming assay

Both BPAG1e knockdown clones C4 and C12 showed a significant reduction in the number of colonies by 20% ($p = 0.002$) (Fig 2e). Moreover, C4 and C12 displayed a decrease in the colony size by 64% and 61% respectively as compared with BVC ($p < 0.001$) (Fig 2d, 2f).

In vivo Tumorigenicity assay

At the end of 8 weeks, the mean tumour volume of the mice bearing C12 cells was reduced by 32% as compared with the mean tumour volume formed by the BVC cells ($p < 0.001$) (Fig 2g, 2h).

Cell proliferation assay

No significant difference was observed between proliferation of BPAG1e knockdown clones C4, C12 and Vector control clone BVC ($p = 0.1$) (Fig 2i).

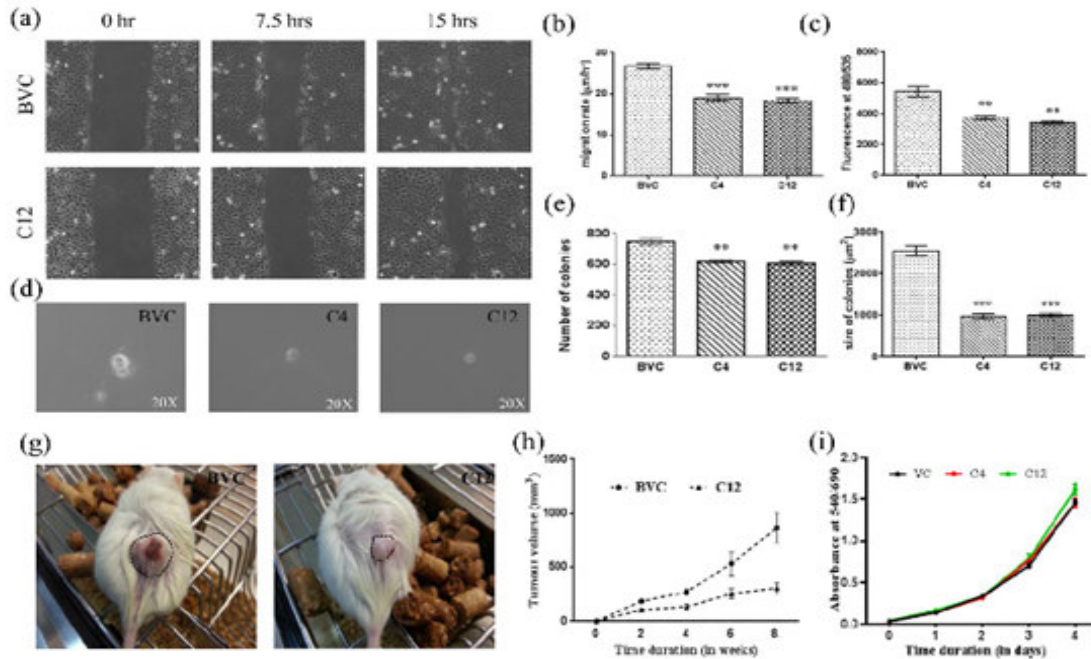


Figure 2
Phenotypic assays for cell transformation for BPAG1e knockdown cells

(a) Representative time lapse microscopy images showing wound healing for vector control clone (BVC) and BPAG1e knockdown clone (C12); (b) The graph shows rate of migration for vector control clone (BVC) and BPAG1e knockdown clones (C4, C12) ($p < 0.001$); (c) The graph shows fluorescence of invaded vector control clone (BVC) and BPAG1e knockdown clones (C4, C12) which is read at wavelengths of 488/535nm (Ex/Em) ($p = 0.001$); (d) Representative images of colonies formed by vector control clone (BVC) and BPAG1e knockdown clones (C4, C12) in soft agar; (e) Graphical representation of the number of colonies formed by vector control clone (BVC) and BPAG1e knockdown clones (C4, C12) ($p = 0.002$); (f) Graphical representation of the size of colonies formed by vector control clone (BVC) and BPAG1e knockdown clones (C4, C12) ($p < 0.001$); (g) Representative images of NOD-SCID mice bearing tumors formed by vector control cells (BVC) and BPAG1e knockdown cells (C12) after 8 weeks of injection. The tumors are indicated by dotted circles; (h) Tumor volume was plotted against time. It represents mean \pm SEM for 6 animals injected for each clone ($p < 0.001$); (i) Graphical representation of MTT assay for vector control clone (BVC) and BPAG1e knockdown clones (C4, C12) ($p = 0.1$)

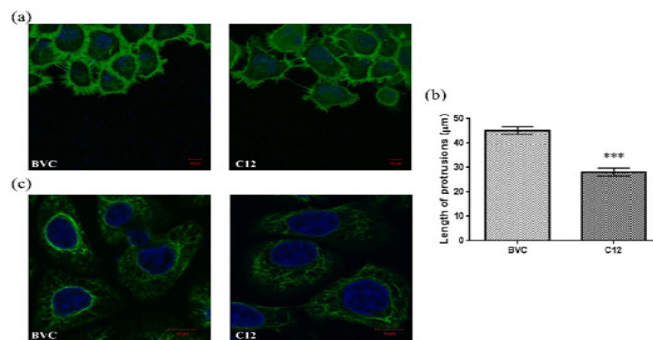


Figure 3
Effect of BPAG1e knockdown on cytoskeletal elements

(a) Immunofluorescence analysis of actin organization at the wound edge using phalloidin-FITC staining in vector control clone (BVC) and BPAG1e knockdown clone (C12). The scale bar represents 10 μ m; (b) The graph shows length of membrane protrusions in vector control clone (BVC) and BPAG1e knockdown clone (C12). For quantification, data represents mean \pm SEM of 40 cells from BVC and BC12 clones ($p < 0.001$); (c) Immunofluorescence analysis showing K8 filament organization in vector control clone (BVC) and BPAG1e knockdown clone (C12). The scale bar represents 10 μ m.

BPAG1e interacts with K8 in OSCC derived cells

BPAG1e is expressed in the basal layer of squamous cell epithelia whereas K8 is normally expressed in simple epithelia.²¹⁻²² In case of OSCC, there is an aberrant expression of K8/18.^{11, 23} In OSCC, whether BPAG1e interacts with K8 is not known. Co-

immunoprecipitation (Co-IP) of K8 was observed when antibody for BPAG1e was used for IP (Fig 4). Similarly, pull down fraction of K8 IP showed presence of BPAG1e (Fig 4). These results indicated that K8 interacts with BPAG1e in OSCC cells.

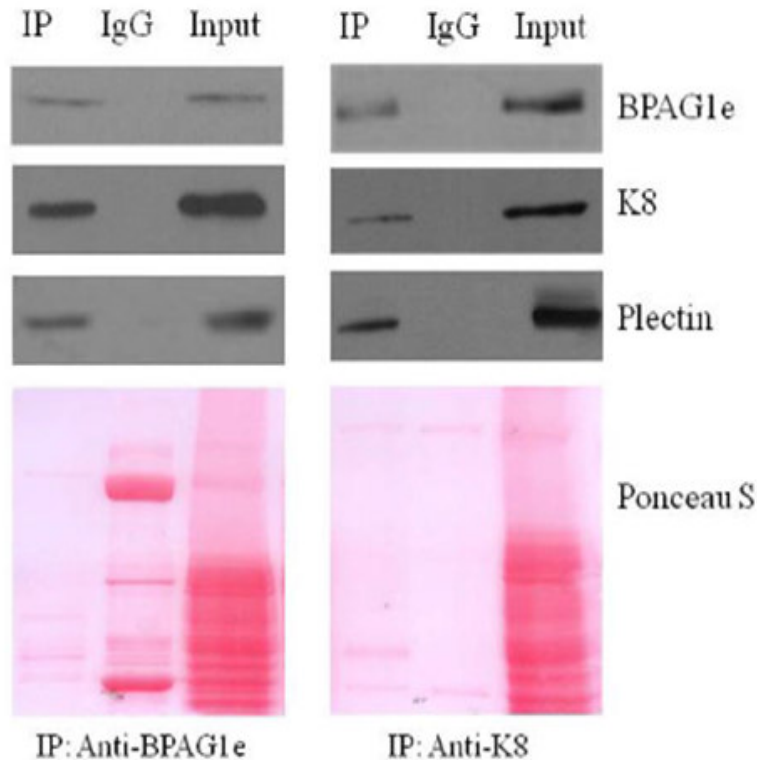


Figure 4
BPAG1e interacts with K8 in AW13516 cells

Endogenous pull down experiments with either anti-BPAG1e or anti-K8 antibody performed using cell lysate from AW13516 cells. Anti-goat and Anti-mouse IgG antibody was used as secondary antibody control for BPAG1e IP and K8 IP respectively. The immunoprecipitated complex was examined for the presence of BPAG1e, Keratin 8 and Plectin. Input represented 10% of cell lysate used in the experiment. This experiment was performed in triplicate.

Loss of BPAG1e does not alter expression of associated proteins

BPAG1e and Plectin are known linker proteins between $\beta 4$ integrin and keratin filaments.²⁴ In earlier studies, we have shown that $\beta 4$ integrin and actin bundling protein Fascin were downregulated in response to K8 knockdown in AW13516 cells.¹⁴ Surprisingly, upon knockdown of BPAG1e, there were no changes observed in expression of $\beta 4$ integrin, Fascin and K8 (Fig 1c). Moreover, we could not see any changes in K8 filament organization in BPAG1e knockdown cells as compared to vector control cells (Fig 3c).

Knockdown of BPAG1e results in reduced activation of $\beta 4$ integrin mediated signalling

In a previous study, we have shown that K8 knockdown in AW13516 cells resulted in reduction in the activation of $\beta 4$ integrin signalling upon ligation of the 3E1 antibody.¹⁴ In order to verify the effect of BPAG1e depletion on $\beta 4$ integrin signalling, activation of its downstream effectors in response to ligation of $\beta 4$ integrin by the activating antibody 3E1 was studied. The levels of p-Akt and p-Erk1/2 were found to be reduced in BPAG1e knockdown clone BC12 as compared to vector control clone BVC upon 3E1 ligation (Fig 5). These results confirmed that depletion of BPAG1e led to downregulation of $\beta 4$ integrin signalling in these cells.

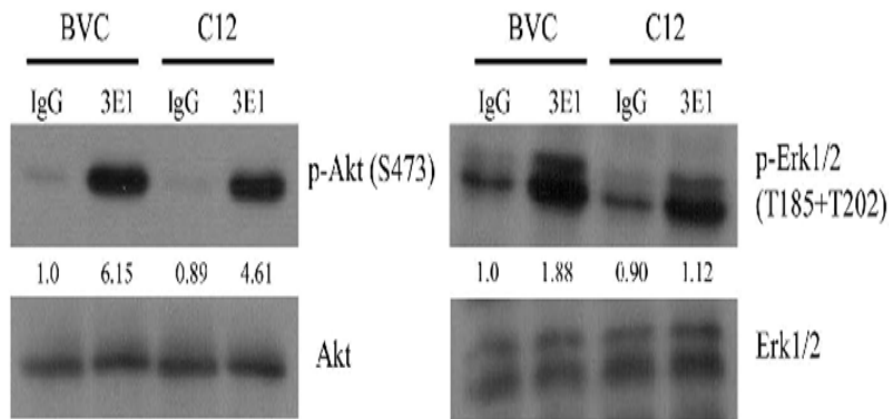


Figure 5
BPAG1e loss affects β 4 integrin mediated signalling

BPAG1e knockdown and vector control cells were stimulated with 5 μ g/ml of 3E1 antibody or mouse IgG for 30 minutes after 18 hours of serum starvation. Subsequently protein extracts were analysed by western blotting using antibodies against phosphorylated forms of Akt and Erk1/2. Total Akt, Erk1/2 were used as loading controls.

DISCUSSION

Recent studies have shown the role of hemidesmosomal linker proteins (BPAG1e and Plectin) in various cellular processes other than their known anchoring function. Further, these reports suggest that the role of BPAG1e may be context dependent.⁵⁻⁶ The literature regarding BPAG1e expression in human cancers is very scanty.^{8, 25} Moreover, very little information is available regarding molecular mechanisms underlying BPAG1e associated phenotype in cancer. Previously, we have reported that K8 knockdown in AW13516 cells resulted in reduced cell motility, cell invasion, altered actin organization and decreased tumorigenic potential.¹⁴ In the current study, we show that cell motility, cell invasion as well as tumorigenic potential were reduced upon downregulation of BPAG1e in OSCC derived AW13516 cells. Moreover, actin organization was found to be altered upon BPAG1e loss. Reduction in cell motility is often associated with alterations in actin organization.¹⁹⁻²⁰ Indeed, BPAG1e knockdown cells displayed sparsely arranged filopodia. A number of proteins are involved in the regulation of cell motility out of which Rho GTPases are one of the most important proteins that play a crucial role in the reorganization of the actin cytoskeleton.²⁶⁻²⁷ One of the Rho GTPases family member, Cdc42, regulates cellular motility by controlling filopodia formation.²⁸ Thus, in case of BPAG1e loss in AW13516 cells, reduced cell motility can possibly be attributed to reduced Cdc42 activity. Many studies have reported that altered cell motility is accompanied by altered cell invasion.²⁹⁻³⁰ The cellular invasion was found to be reduced in BPAG1e knockdown cells. It is well documented that increased activity of Matrix Metalloproteinases (MMPs), MMP2 and MMP9 in particular, correlates with invasive potential of OSCC.³¹⁻³² Therefore, it would be interesting to check activity of MMPs in BPAG1e downregulated cells. Additionally, loss of BPAG1e in AW13516 cells resulted in reduced

tumorigenic potential, both *in vitro* (anchorage independent growth) and *in vivo* (tumour formation in immunocompromised mice) indicating that BPAG1e plays a role in the process of cell transformation. To our knowledge, this is the first study reporting a role of BPAG1e in cancer cell migration, invasion and cell transformation. The shRNA mediated knockdown of BPAG1e did not alter expression of the other linker protein Plectin, demonstrating the specificity of BPAG1e shRNA (Fig 1c). This also underlines the fact that the phenotypic changes observed in AW13516 cells are solely due to the loss of BPAG1e. K8/18 pair is normally expressed in simple epithelia where it interacts with integrins via Plectin, as BPAG1e is not expressed in simple epithelia.³³ In case of OSCC, K8/18 pair is aberrantly expressed.^{11, 23} Therefore, it was important to investigate whether K8/18 filaments interact with BPAG1e in AW13516 cells. Our immunoprecipitation experiments suggested that K8 interacts with BPAG1e. Next, we studied the effect of β 4 integrin activating antibody in BPAG1e knockdown cells. In our previous study, we have shown that there are alterations in levels of α 6 β 4 integrin proteins, α 6 β 4 integrin mediated signalling and actin bundling protein Fascin upon knockdown of K8 in AW13516 cells.¹⁴ Surprisingly, in the present study we did not see changes in β 4 integrin and Fascin protein levels in response to BPAG1e knockdown. But we could notice alterations in α 6 β 4 integrin mediated signalling in BPAG1e knockdown cells in response to treatment with 3E1 antibody. Activation of α 6 β 4 integrin mediated signalling with the 3E1 antibody resulted in reduced activation of downstream effector molecules of β 4 integrin signalling, such as Akt and Erk1/2 in BPAG1e depleted cells. Although we did not see any change in β 4 integrin levels, it is possible that β 4 integrin phosphorylation levels were upon BPAG1e loss.³⁴ Therefore, it would be interesting to check phosphorylation levels of β 4 integrin in BPAG1e knockdown cells. Previously, it has been reported that loss of BPAG1e does not affect keratin filament organization.⁵ In this study, we could show that K8 filament organization is unaltered upon BPAG1e

knockdown, indicating that in the absence of one linker protein, the other linker protein is sufficient to anchor keratin proteins.

CONCLUSION

Taken together, these results suggest that BPAG1e promotes cell migration and neoplastic progression by modulating $\beta 4$ integrin mediated signalling in OSCC cells. Thus, it would be interesting to check the levels of BPAG1e expression in oral tumour tissues as compared to normal tissues. In future, BPAG1e may prove to be a useful candidate in prognostication of Human OSCC.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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