Loss of keratins 8 and 18 leads to alterations in α6β4-integrin-mediated signalling and decreased neoplastic progression in an oral-tumour-derived cell line

Hunain Alam, Samrat T. Kundu, Sorab N. Dalal and Milind M. Vaidya*

Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai 410 210, India

*Author for correspondence (mvaidya@actrec.gov.in)

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Summary

Keratins 8 and 18 (K8 and K18) are predominantly expressed in simple epithelial tissues and perform both mechanical and regulatory functions. Aberrant expression of K8 and K18 is associated with neoplastic progression and invasion in squamous cell carcinomas (SCCs). To understand the molecular basis by which K8 promotes neoplastic progression in oral SCC (OSCC), K8 expression was inhibited in AW13516 cells. The K8-knockdown clones showed a significant reduction in tumorigenic potential, which was accompanied by a reduction in cell motility, cell invasion, decreased fascin levels, alterations in the organization of the actin cytoskeleton and changes in cell shape. Furthermore, K8 knockdown led to a decrease in α6β4 integrin levels and α6β4-integrin-dependent signalling events, which have been reported to play an important role in neoplastic progression in epithelial tissues. Therefore, modulation of α6β4 integrin signalling might be one of the mechanisms by which K8 and K18 promote malignant transformation and/or progression in OSCCs.

Key words: Keratin 8, Keratin 18, β4 integrin, FAK, Cell motility, Squamous cell carcinoma (SCC)

Introduction

Keratins are cytoplasmic intermediate filament proteins preferentially expressed in epithelial tissues (Moll et al., 2008; Moll et al., 1982). They are subdivided into the type I acidic (K9–K28) and type II basic (K1–K8 and K71–K74) keratins (Schweizer et al., 2006). They are obligatory heteropolymers and are coexpressed in various epithelia in specific pairs consisting of one type I and one type II keratin (Coulombe and Omary, 2002; Fuchs and Weber, 1994). Epithelial tissues express different pairs of keratins depending upon the epithelial cell type and stage of differentiation (e.g. all stratified squamous epithelia express K5 and K14, whereas K8 and K18 are seen in all simple epithelia) (Coulombe and Omary, 2002). Keratin filaments are connected with cell adhesion complexes, such as hemidesmosomes and desmosomes, at the cell membrane (Green and Jones, 1996) and provide mechanical support to tissue architecture (Fuchs and Cleveland, 1998). At hemidesmosomes, keratin networks are associated with β4 integrin through plectin and dystonin (also known as BP230) (Borradori and Sonnenberg, 1996; Yang et al., 1996). In addition to their scaffolding function, keratins form complex signalling platforms and interact with various kinases, adaptors, receptors and apoptotic proteins, thus regulating and/or modulating associated signalling pathways (Paramio and Jorcano, 2002).

K8 and K18 are expressed in all simple epithelial cells and execute various regulatory functions (Coulombe and Omary, 2002; Owens and Lane, 2003), which include modulation of protein localization, targeting, trafficking and synthesis (Coulombe and Omary, 2002; Omary et al., 2009; Owens and Lane, 2003). K8 and K18 expression is not observed in stratified adult epithelial tissues; however, these proteins are often aberrantly expressed in squamous cell carcinoma (SCC), where their expression is correlated with invasion and poor prognosis (Fillies et al., 2006; Moll et al., 2008; Schaafsma et al., 1993; Vaidya et al., 1996). Our previous data has shown that K8 overexpression leads to neoplastic transformation and increased invasive and metastatic potential in the FBM cell line derived from the buccal mucosa of normal human foetus (Raul et al., 2004). Transgenic mice expressing human K8 in the epidermis have a dramatic increase in the progression of papillomas towards malignancy (Casanova et al., 2004). Overexpression of K8 and K18 in human melanoma cells leads to increased migratory and invasive activity in vitro (Hendrix et al., 1992). Mouse fibroblasts expressing complete K8 and K18 filaments showed a higher migratory and invasive ability (Chu et al., 1993). All these results suggest that aberrant expression of K8 and K18 contributes to neoplastic progression in various tissues, including stratified epithelial cells. However, the mechanism of K8- and K18-mediated regulation of neoplastic transformation and progression in SCC is not well understood.

Keratin filaments are associated with β4 integrin through plectin and dystonin at hemidesmosomes at the cell membrane in basal keratinocytes (Borradori and Sonnenberg, 1996). Interestingly, expression of β4 integrin is known to increase substantially during malignant progression in squamous carcinomas of multiple tissues, including head and neck (e.g. oral SCC; OSCC) (Giancotti, 2007). β4 integrin signalling promotes tumour cell proliferation, migration, invasion and metastasis by different mechanisms, for example, by activating FAK and downstream effectors, amplifying receptor tyrosine kinase (RTK) signalling and modulating extracellular matrix (ECM) components in different tissues (Giancotti, 1996; Guo and Giancotti,

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2004; Guo et al., 2006; Mercurio et al., 2001). It has been demonstrated that keratinocytes expressing altered keratin filaments showed reduced activation of Akt (PKB) and ERK1/2 in response to β4 integrin ligation by the activating antibody 3E1 (Kippenberger et al., 2010). In another recent report, modulation of integrin- and FAK-mediated cell migration by K8 and K18 through protein kinase C (PKC) δ in hepatocytes has been shown (Bordeleau et al., 2010). However, the role of K8 and K18 in association with β4 integrin signalling in neoplastic progression of SCC has not been documented yet.

In the present study, we demonstrate that a knockdown of K8 in the OSCC-derived cell line AW13516 leads to a substantial reduction in tumorigenicity, cell motility, cell invasion, fascin and α6β4 integrin expression and α6β4-integrin-associated signalling. Thus, our results indicate that K8 and K18 promote malignant transformation and play an important role in invasion and metastasis, possibly by modulating β4 integrin signalling.

Results

Generation of stable K8-knockdown OSCC cells

To generate effective short hairpin RNA (shRNA) constructs for knockdown of K8, four different sequences were used to generate pTU6-PURO-based shRNA constructs (Kundu et al., 2008). The constructs were validated by testing their ability to inhibit the expression of an exogenously expressed GFP–K8 construct in HEK-293 cells. shRNAK8.2 and shRNAK8.3 showed substantial knockdown of K8–GFP expression (supplementary material Fig. S1). The effective shRNA construct, shRNAK8.2 or the empty vector control were transfected into the OSCC cell line AW13516, to generate stable K8 knockdown and vector control clones. K8 expression (both protein and mRNA) (Fig. 1A,B) and filament formation (Fig. 1C) in K8.2-shRNA-transfected stable clones (shRNAK8.2-C1, -C2, -C4 and -C6) were significantly (P<0.0005) downregulated compared with that in cells transfected with the vector control (pTU6-Cont.). K8-knockdown clones showed a substantial reduction in protein level (Fig. 1A) and an ~60% decrease in filament formation (Fig. 1C) with its normal partner K18, as previously reported (Baribault et al., 1993). There was no change in mRNA levels of K18 (Fig. 1B) indicating that the reduction in K18 protein was due to the decreased stability of the protein.

Effect of K8 knockdown on the levels of other keratins and vimentin

To check the effect of K8 loss on the levels of other keratins, a keratin profile of the K8-knockdown and vector control cells was

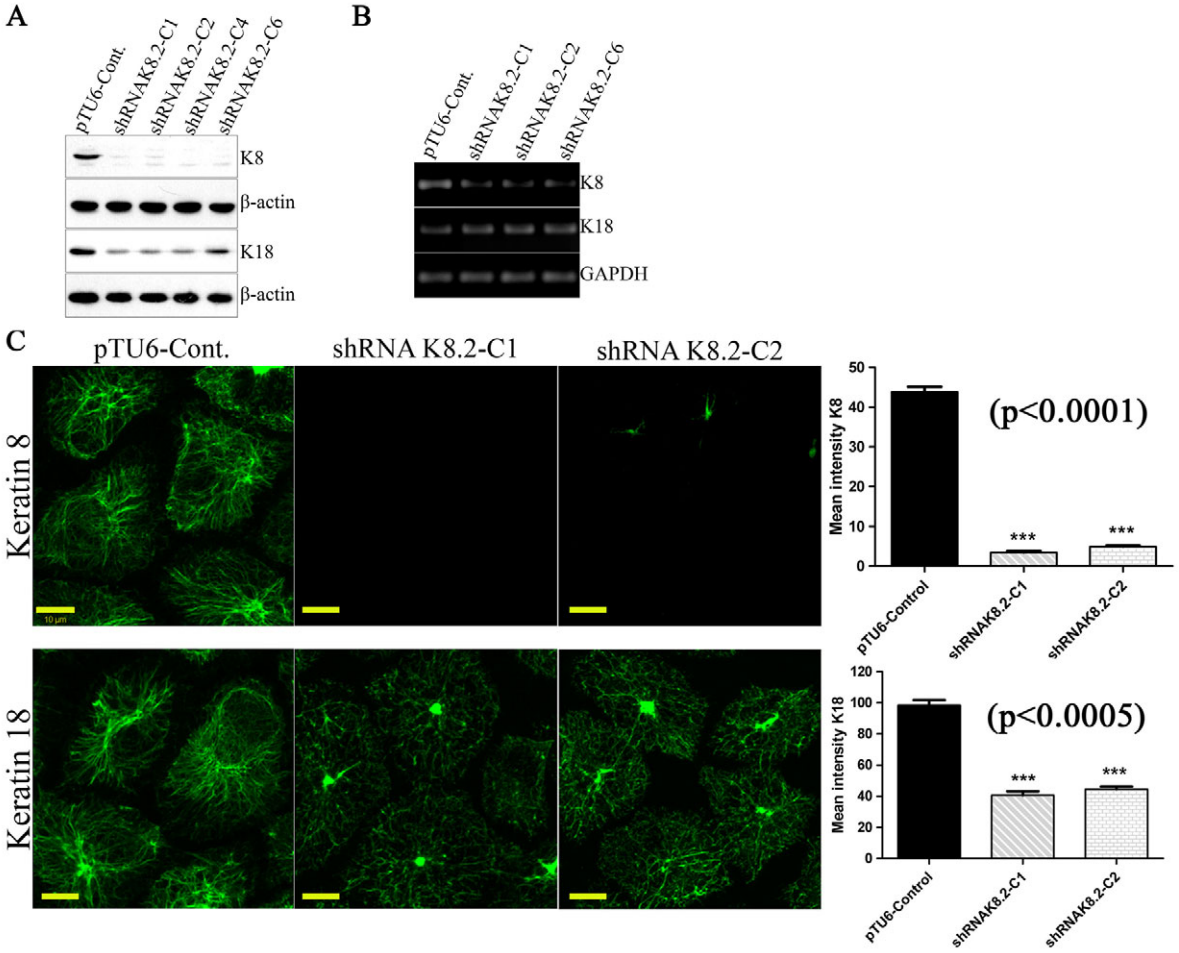

Fig. 1. Generation of K8-knockdown clones from AW13516 cells. (**A**) Western blot analysis of stable K8-knockdown clones (shRNAK8.2-C1, shRNAK8.2-C2, shRNAK8.2-C4 and shRNAK8.2-C6) and a vector control clone (pTU6-Cont.) derived from AW13516 cells with antibodies to K8 and K18. β-actin was used as a loading control. (**B**) RT-PCR analysis of the genes encoding K8 and K18 in stable K8-knockdown clones and a vector control clone. *GAPDH* was used as internal control. (**C**) Confocal analysis of K8 and K18 levels and filament networks in K8-knockdown and vector control clones. Scale bars: 10 µm. The mean fluorescence intensity (+s.d., n=3) of K8 and K18 is shown on the right-hand side. ***P<0.0001 for K8 and P<0.0005 for K18.

analysed by Coomassie-stained SDS-PAGE after high-salt extraction. The results showed that there was no change in the levels of keratins other than K8 and K18 (Fig. 2C). Furthermore, the levels and filament organization of K5 and K14 were determined by western blotting and confocal microscopy, respectively. There was no substantial difference in protein expression (Fig. 2A) and filament formation (Fig. 2B) of K5 or K14 in the K8-knockdown stable clones. In addition, both mRNA and protein levels of vimentin were checked in K8-knockdown clones using RT-PCR and western blot analysis respectively. We did not find any considerable change in the levels of vimentin expression in K8-depleted cells (Fig. 2D).

K8 knockdown leads to a reduction in the tumorigenic potential of AW13516 cells both in vitro and in vivo

In order to assess the effects of K8 downregulation on tumorigenic potential of AW13516 cells, a soft agar assay was performed with the K8-knockdown (shRNAK8.2-C1 and shRNAK8.2-C2) and vector control (pTU6-Cont.) clones. K8-knockdown clones showed a substantial reduction in size (by ~50%) and number of colonies formed in soft agar compared with vector control clones (Fig. 2E). Furthermore, the in vivo tumorigenicity of K8-knockdown and vector control clones was assessed by subcutaneous injection in nude mice. Prior to injection, the cell viability and cell proliferation was assessed and no substantial differences in survival and proliferation were found between the K8-knockdown and the vector control clones (data not shown). The mean tumour volume of the mice bearing K8-knockdown tumours was reduced ~50% compared with the average volume of tumours formed by the vector control cells (Fig. 2F).

K8 knockdown leads to a reduction in the levels of the cell-motility-associated protein, fascin

To identify signalling pathways that are altered upon K8 loss, protein extracts from vector control (pTU6-Cont.) and K8-knockdown clones

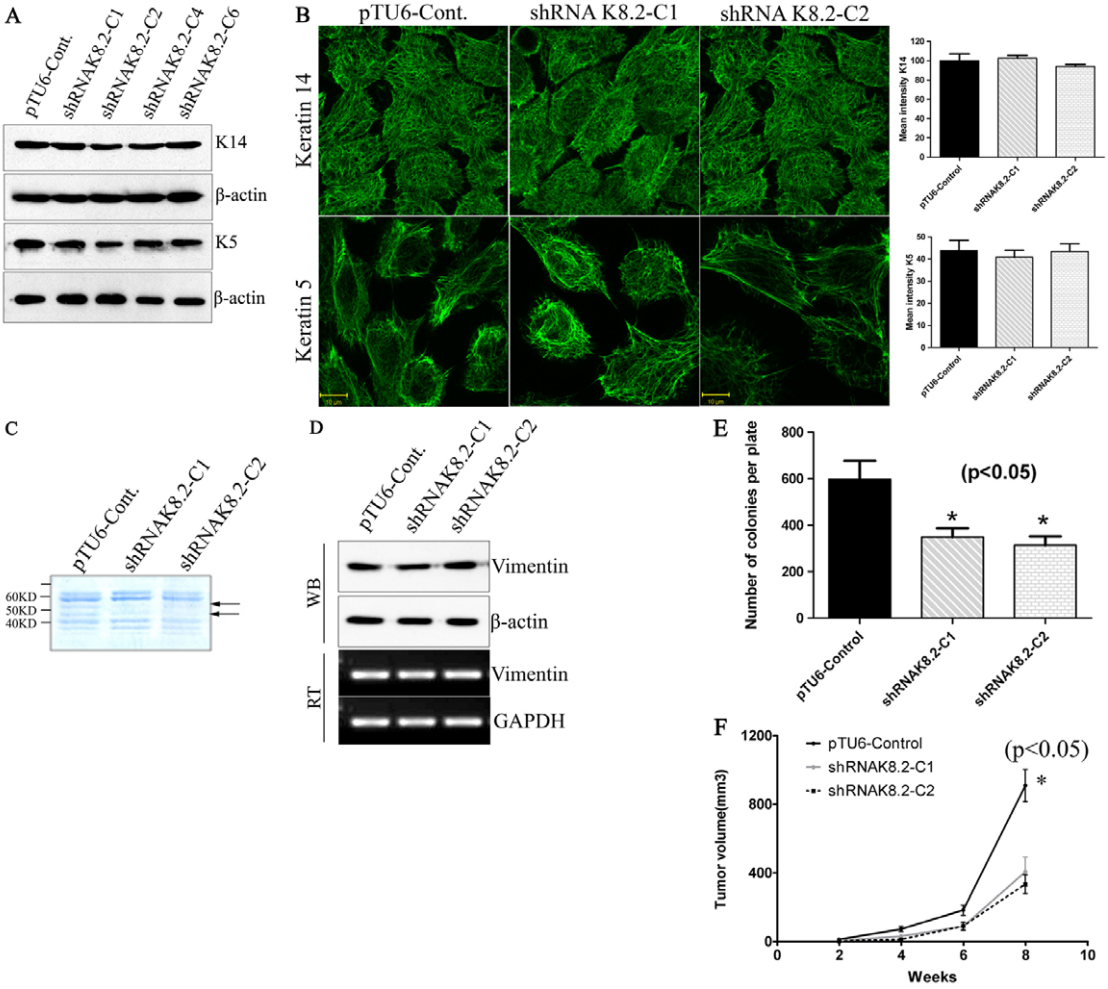

Fig. 2. Effect of K8 knockdown on other keratins, vimentin and tumorigenicity. (**A**) Western blot analysis of K5 and K14 in vector control (pTU6-Cont.) and K8 knockdown clones (shRNAK8.2-C1, shRNAK8.2-C2, shRNAK8.2-C4 and shRNAK8.2-C6). β-actin was used as a loading control. (**B**) Confocal analysis of K5 and K14 levels and filament networks in vector control and K8-knockdown clones. Scale bars: 10 μm. The mean fluorescence intensity (+s.d.) of K5 and K14 was calculated per cell by measuring fluorescence intensity of 20 cells of each experiment (using LSM10 software) and is shown in the graph on the right-hand side. (**C**) The keratin profile of K8 knockdown and vector control cells after high-salt extraction. The arrows indicate K8 (molecular mass ~53 kDa) and K18 (molecular mass ~48 kDa) bands on Commassie-Blue-stained gel. (**D**) RT-PCR (RT) and western blot (WB) analysis of vimentin mRNA and protein levels in stable K8-knockdown clones and a vector control clone. (**E**) Histogram (means±s.d. for three independent experiments) showing the total number of colonies formed per plate by the indicated clones. *P<0.05. (**F**) Tumorigenicity of K8-knockdown cells in nude mice. Tumour growth is plotted against time. Results are means±s.d. for two independent experiments performed in triplicate (n=6 animals injected for each clone). Note decreased tumorigenicity in K8-knockdown clones. *P<0.05.

(shRNAK8.2-C1) were subjected to two-dimensional PAGE (Fig. 3A) followed by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF-MS). Four different protein spots that were altered in K8-knockdown cells compared with the vector control cells were identified as the actin-bundling protein, fascin, K1 and two isoforms of K8, respectively (data not shown). Downregulation of fascin was confirmed by western blot analysis (Fig. 3B). Furthermore, confocal microscopy analysis showed a reduction in the fascin level (by ~35%) at the cell surface in K8-knockdown cells (Fig. 3C).

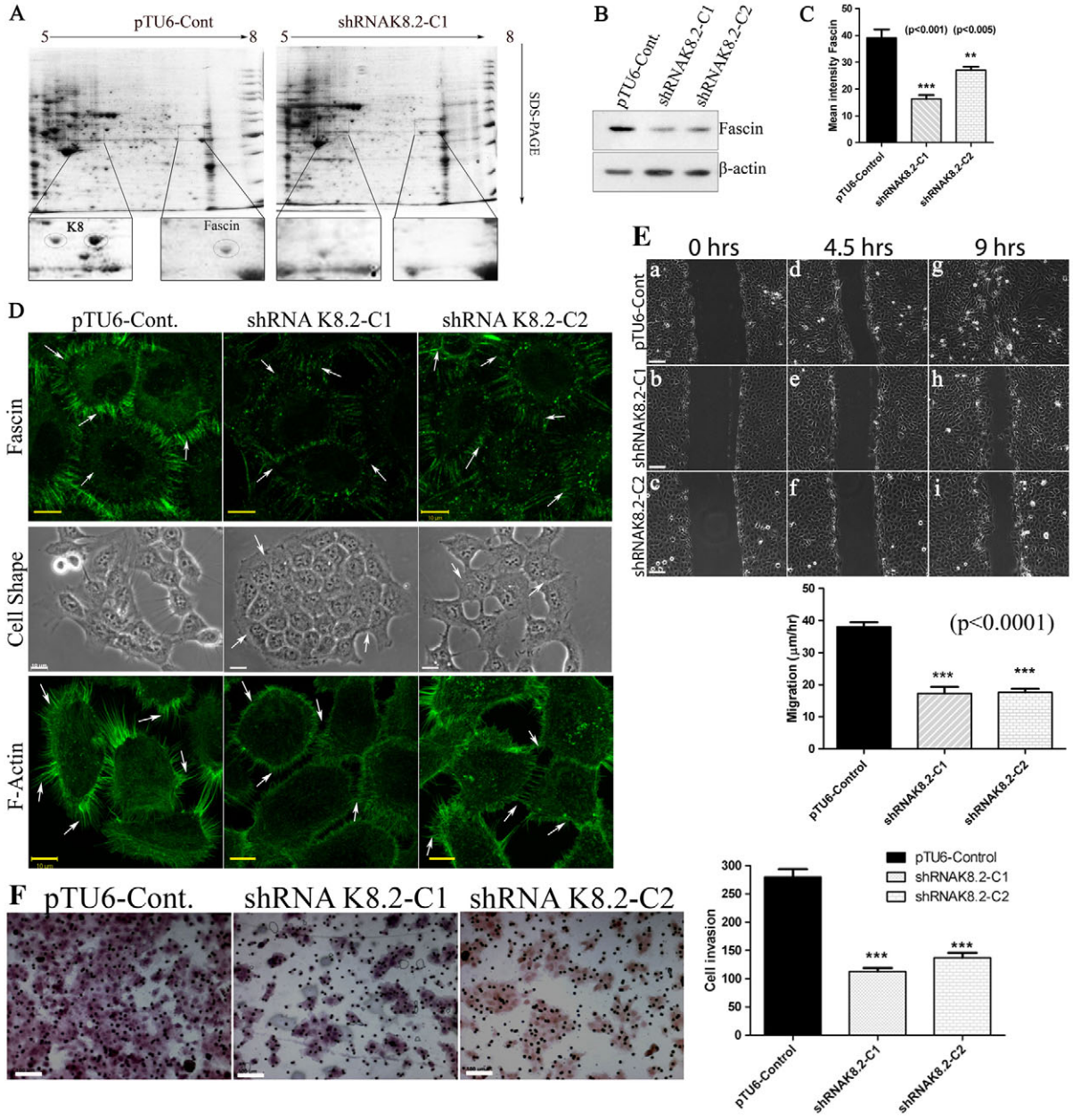

Fig. 3. K8 knockdown leads to alterations in actin cytoskeleton, migration and invasiveness of the cells. (**A**) Colloidal-gold-stained two-dimensional PAGE showing differential protein spots in K8-knockdown (shRNAK8.2-C1) clone as compared with that in vector control clone (pTU6-Cont.). MS analysis identified these spots as fascin, K1 and two isoforms of K8. (**B**,**C**) Western blot analysis (B) and mean fluorescence intensity (+s.d.; three independent experiments) of fascin (C) in the indicated clones. ***P<0.001; **P<0.05. (**D**) Cell morphology (shape) and confocal analysis of fascin and F-actin in vector control or K8-knockdown clones (shRNAK8.2-C1 and shRNAK8.2-C2). Scale bar: 10 μm. (**E**) Wound healing assay of vector control and K8-knockdown clones. Phase-contrast images of wound closure at 0 hours (a–c), 4.5 hours (d–f) and 9 hours (g–i) of the indicated clones. Scale bar: 100 μm. Migration rate was calculated by AxioVision software. The graph shows means+s.d. for three independent experiments ***P<0.0001. (**F**) Boyden chamber invasion assay of vector control and K8-knockdown clones. Membrane filter inserts were coated with Matrigel and cells were seeded in the upper chamber. After 20 hours of incubation, the cells that invaded through the membrane were stained with haematoxylin and eosin, and images were captured using inverted microscopy. Representative fields of view for each clone are shown. Cell invasion was quantified by counting cells in six random fields (using Metamorph software). Scale bar: 100 μm. The graph gives means+s.d. for three independent experiments. ***P<0.005.

K8 loss leads to alterations in cell shape, actin organization, as well as reduced cell migration and cell invasion

To check the effect of K8 knockdown on cell shape and actin reorganization, phase-contrast microscopy and phalloidin staining followed by confocal microscopy were performed. K8-knockdown cells had less filamentous-actin-based structures, such as cell protrusions and microspike formation, as analysed by FITC–phalloidin staining (Fig. 3D). In addition, analysis of cell morphology by live phase-contrast microscopy revealed a more symmetric contracted epithelial appearance in K8-knockdown cells (Fig. 3D) compared with that of control cells. To determine whether the alteration in fascin levels and actin morphology led to changes in cell migration, scratch wound healing assays were performed. K8-knockdown cells showed an ~50% reduction in cell migration rate as compared with vector control cells (Fig. 3E). Furthermore, the invasive potential of K8-knockdown cells and vector control cells was determined by Boyden chamber Matrigel invasion assays. K8-knockdown cells were found to be less invasive compared with vector control cells (Fig. 3F). These results together correlate well with the decrease in tumorigenic potential of the cells and the reduction in fascin and F-actin levels in response to K8 knockdown.

K8 depletion leads to a reduction in α6 and β4 integrin expression levels

Co-immunoprecipitation and colocalization experiments were conducted to check whether K8 directly interacts with fascin. It was found that there was no direct interaction between K8 and fascin (supplementary material Fig. S2A,B). These results indicate that K8 regulates fascin expression indirectly through some other mechanism. Recent reports suggest that keratin filaments can modulate β4 integrin signalling in keratinocytes, and β4-integrin-mediated signalling has been shown to modulate fascin levels (Chen et al., 2009). To test the hypothesis that these integrins are involved, the levels of α6 and β4 integrin were determined by western blot analysis. The levels of both β4 and α6 integrins were reduced in K8-knockdown cells when compared with vector control cells (Fig. 4A). Importantly, cell surface expression of β4 integrin was also decreased by ~30% in the K8-knockdown cells compared with vector control cells, as analysed by flow cytometry (Fig. 4B). We did not find any substantial difference in the levels of mRNA expression of β4 integrin and α6 integrin (Fig. 4D), suggesting that the regulation of β4 integrin expression might be at the post-transcriptional level. We also checked the cell surface expression of β1 integrin in K8-knockdown cells and found no substantial change compared with that in vector control cells (Fig. 4C).

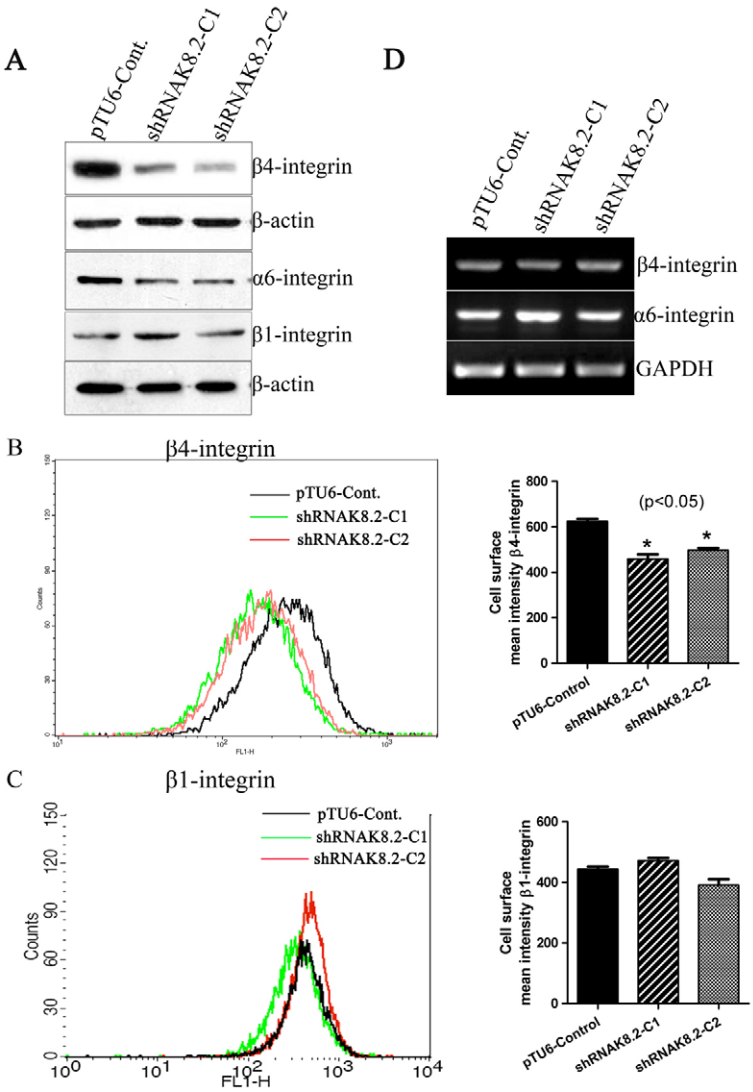

Fig. 4. Downregulation of β4 integrin in K8-knockdown cells. (**A**) Western blot analysis of stable K8-knockdown clones (shRNAK8.2-C1 and shRNAK8.2-C2) and a vector control (pTU6-Cont.) clone with antibodies to α6 integrin and β4 integrin. β-actin was used as an internal loading control. (**B**,**C**) Histograms [mean fluorescence intensity (+s.d.) for three independent experiments; right-hand panels] showing cell surface intensity of β4 and β1 integrin in stable K8-knockdown and vector control cells, as analysed by flow cytometry (left-hand panels). **P*<0.05. (**D**) RT-PCR analysis of genes encoding α6 and β4 integrin in stable K8-knockdown clones and a vector control clone. *GAPDH* was used as an internal control.

K8 loss leads to reduction in β4-integrin-associated signalling molecules

To determine whether K8 modulates α6β4 integrin signalling in oral tumour cells, western blot analysis was performed to determine the levels of some important molecules downstream of the α6β4 integrin signalling pathway, such as FAK, Shc and ERK1/2. A substantial decrease was observed in the levels of phosphorylation of FAK in the K8-knockdown cells (Fig. 5A) and there was also reduced phosphorylation of Shc (Fig. 5B) compared with that in the vector control cells. We also observed a considerable reduction in phosphorylation of ERK1/2 in K8-knockdown cells compared with that in the vector control cells (Fig. 5A). The reduced phosphorylation of FAK, Shc and ERK1/2 correlates with the downregulation of β4 integrin upon K8 knockdown. These results together suggest that a downregulation of α6β4 integrin signalling leads to a reduction in the activation of FAK, Shc and ERK in K8-knockdown cells.

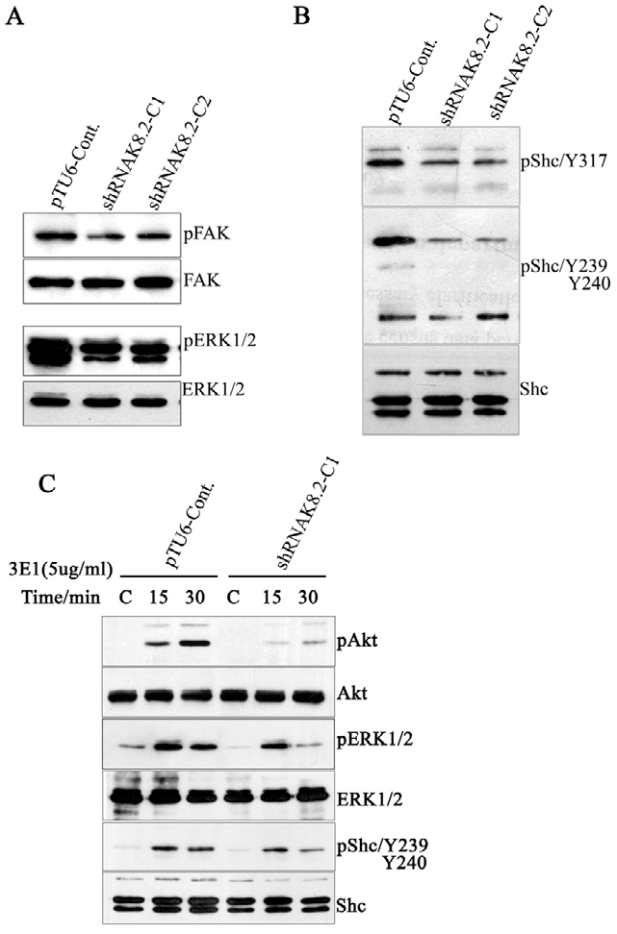

Fig. 5. Loss of K8 leads to downregulation of β4-integrin-mediated signalling. (**A**,**B**) Western blot analysis of stable K8-knockdown clones (shRNAK8.2-C1 and shRNAK8.2-C2) and a vector control (pTU6-Cont.) clone with antibodies to phosphorylated forms of FAK, Shc and ERK1/2. Total FAK, Shc and ERK1/2 were used as internal loading controls, respectively. (**C**) K8-knockdown and vector control cells were stimulated with 5 μg/ml 3E1 or mouse IgG (C) for 15 or 30 minutes after 18 hours of serum starvation. Subsequently protein extracts were analysed by western blotting using antibodies against phosphorylated forms of Akt, ERK1/2 and Shc. Total Akt, ERK1/2 and Shc were used as loading controls.

K8-knockdown cells showed reduction in activation of β4 integrin signalling upon ligation of the 3E1 antibody

In order to verify the effect of K8 depletion on β4 integrin signalling, activation of its downstream effectors in response to ligation of β4 integrin by the activating antibody 3E1 was studied. The time-dependent phosphorylation of Akt (PKB), Shc and ERK1/2 in response to 3E1 was analysed in overnight serum-starved K8-knockdown and vector control cells by western blot analysis. The activation of Akt, Shc and ERK1/2 was substantially reduced in K8 knockdown cells as compared with vector control cells upon 3E1 ligation (Fig. 5C). These results again confirmed that depletion of K8 led to downregulation of β4 integrin signalling in these cells.

K8 knockdown had no effect on EGFR phosphorylation

The association of α6β4 integrin and EGFR signalling pathways has been previously reported in tumour progression (Guo et al., 2006); therefore phosphorylation of EGFR was determined in K8-knockdown cells by western blot analysis. There was no significant decrease in the levels of phosphorylation on the tyrosine 1045 and tyrosine 1068 residues in EGFR in the K8-knockdown clones compared with that in vector control clones (supplementary material Fig. S2C), indicating that downregulation of α6β4 integrin signalling upon K8-knockdown is independent of EGFR signalling in OSCC cells.

Rescue of K8-knockdown phenotypes by re-expression of shRNA-resistant K8

To determine that the phenotypes associated with K8 knockdown were not due to off-target effects of the shRNA, a rescue experiment was performed using the construct K8GFP-RR which was designed to be resistant to the shRNAK8.2. First, K8GFP-RR resistance to shRNAK8.2 was confirmed by co-transfecting these constructs into HEK-293 cells followed by western blot analysis (supplementary material Fig. S3). Upon transient transfection of K8GFP-RR in the K8-knockdown clone shRNAK8-C1, protein levels of α6 integrin, β4 integrin, fascin, activated FAK and activated ERK1/2 were substantially increased when compared with the levels in pEGFP-transfected cells (Fig. 6A,B). To confirm that the phenotypic changes observed in the K8-knockdown cells were not due to off-target effects from the shRNA, stable clones were selected from shRNAK8-C1 cells after transfection of the constructs K8GFP-RR (designated R3, R4 and R7) and pEGFP (designated G4 and G8). K8 expression and filament formation were confirmed in the rescue clones by western blot analysis and confocal microscopy (Fig. 6C,D). K8GFP-RR stable clones demonstrated an increase in the number of colonies formed in soft agar (Fig. 6F), the tumour volume in SCID mice (Fig. 6E), migration in wound healing assays (Fig. 6G,H) and cell invasion through Matrigel compared with the pEGFP-expressing shRNAK8-C1 cell clone (data not shown). These results together confirmed that molecular alterations and phenotypic changes observed upon K8 knockdown are specific to the depletion of K8 levels.

Fascin overexpression rescued the cell-motility-associated phenotype in K8-knockdown cells

To determine whether the phenotype associated with reduced cell migration in K8-knockdown cells was due to downregulation of the fascin level, an exogenous GFP-tagged fascin and pEGFP vector alone were transfected in the K8-knockdown clone shRNAK8-C1. Two clones containing GFP-tagged fascin (C1-

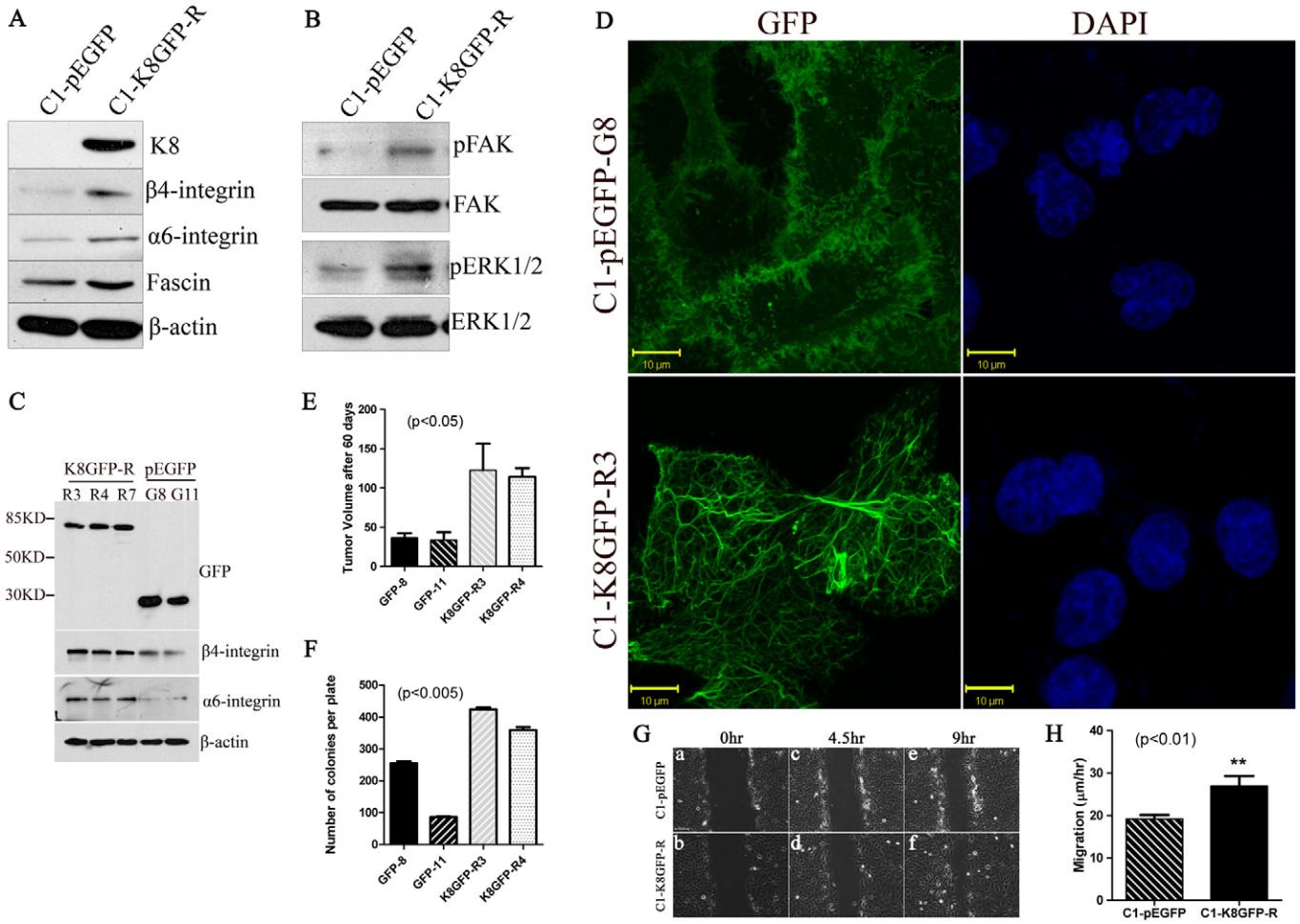

Fig. 6. Rescue of molecular and phenotypic changes in K8-knockdown cells. (**A**,**B**) Western blot analysis of shRNAK8.2-C1 cells transfected with shRNA-resistant GFP-tagged K8 (C1-K8GFP-R) and pEGFP vector control (C1-pEGFP) with antibodies to K8, β4 integrin, α6 integrin, fascin, phosphorylated FAK and phosphorylated ERK1/2. β-actin, FAK and ERK1/2 were used to validate equal loading. (**C**) Western blot analysis of stable K8GFP-R clones (R3, R4 and R7) and pEGFP clones (G8 and G11) derived from the K8-knockdown clone shRNAK8.2-C1 cells with antibodies to GFP, α6 integrin and β4 integrin. β-actin was used as a loading control. (**D**) Representative confocal images of GFP-tagged K8 and GFP of stable K8GFP-R and pEGFP clones. DAPI was used for nuclear staining. Scale bars: 10 μm. (**E**) Tumour volumes (mm^3; means+s.d. for five animals) in SCID mice were measured 60 days post injection. $P<0.05$. (**F**) The total number of colonies formed in soft agar per plate by the clones was counted. Means+s.d. for three independent experiments are shown. $P<0.005$. (**G**) Wound closure of K8GFP-R3 and pEGFP-G8 cells at different time points, as indicated. (**H**) Migration rate was calculated by using Axio Vision software. The graph shows mean+s.d. for three independent experiments. $^{**}P<0.01$.

Fascin-1 and C1-Fascin-2) and one clone transfected with pEGFP alone (C1-pEGFP) were selected and overexpression of the proteins was confirmed by western blot analysis (Fig. 7A). Furthermore, the localization (cell surface expression) of fascin was also analysed by confocal microscopy. Exogenous fascin was localized in the cytoplasm and in the cell membrane microspikes (Fig. 7B). To determine the effect of increased fascin levels on actin polymerization, F-actin was analysed by confocal microscopy using phalloidin staining. The fascin-transfected stable clone C1-Fascin-1 demonstrated an increase in F-actin levels accompanied with increased microspike and cell protrusion formation compared with control cells transfected with pEGFP vector (Fig. 7C). These cells also showed an increase in cell migration in wound healing assays compared with that in the pEGFP-expressing shRNAK8-C1 cells (Fig. 7D). In addition, we also checked the levels of K8 and β4 integrin in these cells by western blot analysis. We did not find any substantial difference in levels of K8 and β4 integrin in fascin-transfected cells compared with that in vector control cells (Fig. 7A). These results confirm that the alterations in actin organization and reduction in cell migration observed upon K8-knockdown are due to the downregulation of the levels of fascin.

In summary, K8-knockdown cells show a reduction in tumorigenic potential, cell motility and invasive ability. These cells demonstrated alterations in actin cytoskeleton and downregulation of the actin-bundling protein fascin. Fascin overexpression rescued the cell-motility-associated phenotype in K8-knockdown cells. In addition, β4 integrin signalling and its associated downstream molecules were found to be downregulated in these cells. Rescue experiments, by re-expression of shRNA-resistant K8, resulted in reversal of the phenotypic and molecular alteration seen in K8-knockdown cells.

Discussion

Alterations in the keratin expression pattern have been shown in different pathological conditions including cancer (Moll et al., 2008). The simple epithelial keratins, K8 and K18 are aberrantly

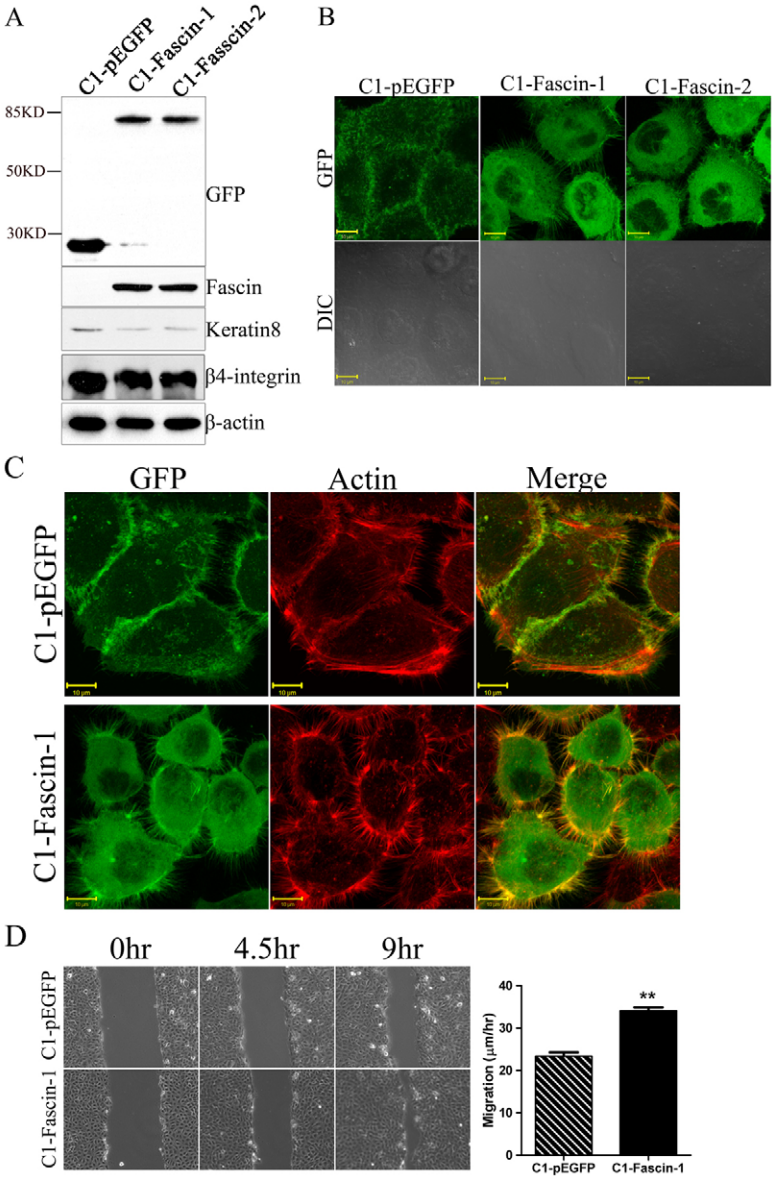

Fig. 7. Rescue of cell migration and its associated changes by overexpression of fascin in K8-knockdown cells. (**A**) Western blot analysis of K8-knockdown clone shRNAK8.2-C1 overexpressing fascin–GFP (C1-Fascin-1 and C1-Fascin-2) or transfected with empty vector pEGFP (C1-pEGFP) with antibodies to GFP, fascin, K8 and β4 integrin. β-actin was used to validate equal loading. (**B**) Representative confocal images of GFP-tagged fascin (C1-Fascin-1 and C1-Fascin-2) and pEGFP (in C1-pEGFP) are shown. DIC images were taken to visualize the morphology of the cells. Scale bars: 10 µm. (**C**) Representative confocal images of actin polymerization and the colocalization of F-actin with fascin–GFP in the indicated clones. Scale bars: 10 µm. (**D**) Wound closure of C1-Fascin-1 and C1-pEGFP at different time points, as indicated. The data are means+s.d. for three independent experiments. $^{**}P<0.05$.

expressed in majority of the carcinomas (Moll et al., 2008), including OSCC (Vaidya et al., 1996), and their expression has been correlated with aggressiveness of the tumour and poor prognosis (Fillies et al., 2007). Aberrant expression of this keratin pair at the invasive front of OSCC has also been correlated with invasion of these tumours (Schaafsma et al., 1993). Previously, we have demonstrated that K8 overexpression, and its filament formation with K18, induces neoplastic transformation of the stratified epithelial cell line derived from human foetal buccal mucosa (Raul et al., 2004). These findings were further supported by transgenic studies (Casanova et al., 2004).

In the present study, we have investigated the effects of K8 loss on tumorigenicity of OSCC and further tried to understand the molecular basis of K8- and K18-mediated neoplastic progression in OSCC. We generated K8-knockdown AW13516 cells (derived from a tumour of the tongue) using shRNA constructs (Fig. 1). Loss of K8 resulted in a decrease in cell migration and a substantial reduction in tumorigenic potential, both in vitro (anchorage independent growth; Fig. 2E) and in vivo (tumour formation in nude mice; Fig. 2F). Furthermore, re-expression of shRNA-resistant K8 could rescue this phenotype. Similar results for K8 downregulation have been found in HCT116 cells by the Dalal laboratory (Nileema Khapare, S.T.K., Lalit Sehgal, Rashmi Priya, Mugdha Sawant, Prajakta Gosavi, Neha Gupta, H.A., Madhura Karkhanis, Nishigandha Naik, M.M.V. and S.N.D., unpublished results). These observations are in agreement with earlier reports (Casanova et al., 2004; Raul et al., 2004) and suggest that the K8 and K18 pair contributes to the tumorigenicity of the OSCC cells.

K8-knockdown OSCC cells had a >90% inhibition of K8 levels and a 60% reduction in the levels of its normal partner K18 (Fig. 1C). Because K18 was still able to form some filaments, it is possible that K18 pairs with another type II keratin in these cells. Keratins are expressed in specific pairs and alterations in the levels of one keratin have been shown to have effect on its corresponding partner. For example, in a K8-knockout mouse model, K18 levels are significantly downregulated (Baribault et al., 1993). Here, no change in mRNA levels of K18 was observed after K8 knockdown (Fig. 1B). This indicates that the reduction in K18 protein levels is

due to a decrease in its stability. Moreover, shRNA-mediated knockdown of K8 did not inhibit the expression of other keratins, including K5 and K14 (Fig. 2A), demonstrating the specificity of our K8 shRNA, which is especially important given that the keratins share a great deal of homology at the sequence level (Schweizer et al., 2006). The fact that no substantial change was observed in other keratin levels indicates that the phenotypic changes observed are due to the loss of K8 and K18 only.

Next, we wanted to understand the molecular basis of K8- and K18-mediated neoplastic progression of OSCC cells. We hypothesized that suppression of K8 would alter the expression pattern of some of the proteins associated with it. Our proteomics analysis revealed that K8 depletion led to reduction in the levels of the cell-motility-associated protein fascin (Fig. 3A,B). Fascin is a globular actin cross-linking protein that is widely expressed in mesenchymal and neuronal cells and is low or absent in adult epithelia (Adams, 2004; Yamashiro et al., 1998). It is upregulated in many human carcinomas, including OSCC, and an increased level of fascin correlates with the clinical aggressiveness of tumours and poor patient survival (Hashimoto et al., 2005). Here, downregulation of fascin could be further correlated with alterations in cell morphology, such as reductions in cell membrane projections accompanied with increased cell–cell contacts (Fig. 3D). In addition, fascin loss in K8-knockdown cells was reflected in a reduction in F-actin-based structures, such as microspikes and filopodia formation (Fig. 3D). It has been shown previously that there is a partial loss of F-actin in the distal colon of K8-null mice (Toivola et al., 2004). In addition, K8-depleted cells had a reduction in the amount of cell migration in a scratch wound healing assay (Fig. 3E). Khapare et al. have also reported alterations in actin organization and cell migration upon K8 knockdown in a simple epithelial cell line (Nileema Khapare, S.T.K., Lalit Sehgal, Rashmi Priya, Mugdha Sawant, Prajakta Gosavi, Neha Gupta, H.A., Madhura Karkhanis, Nishigandha Naik, M.M.V. and S.N.D., unpublished results). Fascin overexpression was able to restore the F-actin-based structures and cell migratory ability of K8-depleted cells (Fig. 7). Previously, it has been shown that fascin activity is essential to filopodial dynamics (Kureishy et al., 2002; Vignjevic et al., 2006), and downregulation of fascin using RNA interference (RNAi) in oesophageal carcinoma cells results in reduced cellular motility and invasive properties (Hashimoto et al., 2006; Xie et al., 2005). Recently, Yamashiro et al. demonstrated that overexpression of K8 and K18 in cutaneous SCC (cSCC) cells led to an increase in their invasive potential, which also correlated with increased K8 and K18 levels in cSCC tumour tissues (Yamashiro et al., 2010). These observations support our results and together suggest that K8 and K18 promote cell motility and invasiveness of cells by regulating fascin-mediated actin polymerization in OSCC cells.

Alterations in keratins levels are often associated with changes in vimentin expression in tumour and tumour-derived cell lines (Hendrix et al., 1992; Paccione et al., 2008; Ramaekers et al., 1983). Our results indicated that there was no substantial change in vimentin expression upon K8 knockdown (Fig. 2D). Both K8 and K18 filaments and vimentin filaments have independently been shown to promote tumour cell motility and invasion in different cell types (Chu et al., 1993; Chu et al., 1996; Hendrix et al., 1992; Hendrix et al., 1996). Thus, it is possible that K8 and K18 filaments and vimentin filaments independently regulate cell motility and invasion by different mechanisms.

Co-immunoprecipitation (data not shown) and colocalization (supplementary material Fig. S2) results ruled out the possibility of direct interaction between K8 and fascin, thus indicating that K8 controls fascin expression indirectly through some other mechanism. It has been shown that fascin levels increase in response to β4 integrin overexpression in breast cancer cells (Chen et al., 2009). Importantly, keratins are associated with β4 integrin through plectin or dystonin at hemidesmosomes (Borradori and Sonnenberg, 1996). Studies on dystonin-null mice have shown that detachment of keratin cytoskeleton from hemidesmosomes (β4 integrin) reduces the ability of epidermal cells to exhibit migratory behaviour (Guo et al., 1995). Recently, it has also been demonstrated that alterations in keratin filaments, such as in epidermolysis bullosa simplex (EBS) or upon chemically induced aggregation, result in changes in β4-integrin-mediated signalling (Kippenberger et al., 2010). Here, we observed reduction in both total and cell surface levels of β4 integrin upon K8 knockdown (Fig. 4A–C). The levels of β4 integrin were restored upon re-expression of K8. In addition, we did not find any substantial difference in the levels of mRNA expression of β4 integrin (Fig. 4D), suggesting that there is post-transcriptional regulation of β4 integrin expression. β4 integrin downregulation upon K8 knockdown also correlated with the decrease in tumorigenicity, cell migration and invasiveness of the K8-knockdown cells. β4 integrin is known to be upregulated in SCC (Giancotti, 2007), and suppression of β4 integrin expression by RNAi results in a reduction in the tumorigenic potential, both in vitro and in vivo, in breast carcinoma cells (Lipscomb et al., 2005). Importantly, α6β4 integrin expression was also found to be high in OSCC (Mendez et al., 2002) and correlated with aggressiveness of the tumours (Wolf and Carey, 1992). We have made similar observations in OSCC (unpublished data). Here, we did not observe any change in total and cell surface levels of β1 integrin (Fig. 4C), possibly because of the different cell type used in the study. β1 integrin is known to promote cell motility in a FAK-dependent manner in epithelial cells (Guo and Giancotti, 2004). Recently, Bordeleau et al., have reported that K8 and K18 regulate β1 integrin signalling through protein kinase C (PKC) and thereby modulate cell migration in hepatocytes (Bordeleau et al., 2010). These results were support the idea that K8 and K18 might promote neoplastic progression of OSCC through β4 integrin.

We were further able to demonstrate that K8 loss leads to a decrease in phosphorylation of downstream signalling associated with β4 integrin, such as that mediated by FAK, Shc and ERK1/2 (Fig. 5A,B). FAK has been shown to promote cell survival and cell migration in epithelial cells (Frisch et al., 1996). Similarly, ERK1/2 becomes activated directly by integrins through several FAK-dependent pathways and its activation is known to regulate invasion and migration in carcinomas (Frisch et al., 1996; Hood and Cheresh, 2002; Keely et al., 1998; Sieg et al., 1999). Ligation of the laminin-binding α6β4 integrin in primary human keratinocytes has been shown to cause tyrosine phosphorylation of Shc and stimulation of the MAPKs ERK and JNK. By contrast, ligation of the laminin- and collagen-binding integrins α3β1 and α2β1 do not trigger these events (Mainiero et al., 1997). Importantly, we show here that re-expression of shRNA-resistant K8 rescued the molecular changes associated with β4-integrin-mediated signalling (Fig. 6). Furthermore, activation with the 3E1 antibody resulted in reduced activation of downstream effector molecules of β4 integrin signalling, such as that of Akt, ERK1/2 and Shc, in K8-depleted cells (Fig. 5C), indicating that β4 integrin signalling was compromised as result of K8 loss. Crosstalk between EGFR and β4 integrin signalling (Guo et al., 2006) and association of EGFR

with K8 and K18 filaments has been previously reported (Huang et al., 2010). Our results show that the alterations observed in β4-integrin-mediated signalling upon K8 depletion are independent of EGFR signalling (supplementary material Fig. S2C). Taken together these results suggest that K8 and K18 promote the cell migration and neoplastic progression by modulating β4-integrin-mediated signalling in OSCC.

In summary, K8 depletion in OSCC cells results in decreased tumorigenicity, cell migration and invasion accompanied with changes in fascin levels and actin reorganization. Furthermore, we could also demonstrate downregulation of α6β4 integrin levels and its associated signalling. On the basis of these results, we conclude that K8 and K18 promote cell motility and tumour progression, possibly by deregulating β4 integrin signalling in OSCC. Some of the questions, such as how K8 and K18 modulate β4 integrin signalling and its downstream events, are still unanswered. It will be interesting to look at the role of intermediate proteins, such as plectin and dystonin, in K8-mediated modulation of β4 integrin signalling. Thus, the present study provides important insights into the understanding of keratin-mediated signalling during tumour progression and identified K8 and K18 as important regulators of integrin-mediated signalling. Intervention strategies to inhibit K8- and K18-mediated integrin signalling in SCC might lead to the development of novel therapeutic targets for SCC.

Materials and Methods

Plasmids, mutagenesis and constructs

To generate the shRNA vector constructs, four 21–23 nucleotide long sequences were selected from the cDNA sequence of K8 (GenBank accession number NM_002273.3) (supplementary material Table S1). The selected sequences were cloned into pTU6 PURO and then validated by co-transfecting them with GFP-tagged K8 as described previously (Kundu et al., 2008) (the K8–GFP construct was a gift from Sushant Kashayap, John Hopkins, USA, and fascin–GFP was gift from Josephine C. Adams, Lerner Research Institute, Ohio, USA). The shRNA-resistant GFP-tagged K8 construct was generated by introducing a silent mutation at the target sequence for the shRNA using the QuikChange site-directed mutagenesis kit (Stratagene). Oligonucleotides (5′-GGAGGCATCACCGCTGTGACGGTCAA-CCAGAGC-3′) were synthesized according to manufacturer's protocol (Sigma). The resulting mutation was verified by DNA sequencing.

Cell lines, selection of stable clones and stimulation of β4 integrin signalling

The cell line AW13516, derived from a tongue SCC (Tatake et al., 1990), and HEK-293 cells (ATCC) were cultured in IMDM and DMEM (Gibco), respectively, supplemented with 10% fetal calf serum (FCS; Hyclone) and antibiotics, at 37°C and under a 5% CO_2 atmosphere. To generate stable clones in AW13516 cells, 2 μg of plasmid DNA was transfected with the liposome-based FuGENE HD transfection reagent (Roche) and stable clones were selected in medium containing 0.5 μg/ml puromycin (Sigma) or 1000 μg/ml G418 sulphate (Sigma). In order to study the activation of β4 integrin signalling, cells were serum-starved for 18 hours and consecutively treated with 5 μg/ml of 3E1 antibody (5 μg/ml mouse-IgG-treated cells were used as a control). After 15 or 30 minutes of treatment, proteins were extracted and used for western blot analysis.

Antibodies and reagents

Antibodies against the following proteins were used: keratin 8, keratin 18, β-actin (Sigma), keratin 5 (Novocastra), keratin 14 (ABD Serotec), β4 integrin (Santa Cruz Biotechnology), α6 integrin, FAK, phosphorylated FAK, phosphorylated JNK1/2, JNK1/2, phosphorylated Shc, Shc, phosphorylated EGFR and EGFR (Cell Signaling), fascin, vimentin, phosphorylated ERK1/2, and ERK1/2 (Abcam), β4 integrin 3E1 and β1 integrin (Millipore). Secondary antibodies were: HRP-conjugated anti-mouse-IgG and anti-rabbit-IgG (Amersham), and Alexa-Fluor-488-conjugated anti-mouse-IgG and Alexa-Fluor-568-conjugated anti-rabbit-IgG (Molecular Probes). FITC-conjugated phalloidin (Sigma) was used to analyse F-actin according to the manufacturer's instructions.

Keratin extraction, western blotting and immunofluorescence staining

Keratins were extracted using a high-salt extraction buffer as described previously (Achtstaetter et al., 1986). Western blotting and immunofluorescence staining was performed as described previously (Raul et al., 2004). The mean fluorescence intensity was measured in arbitrary units using LSM10 software.

RT-PCR

RNA was isolated with the TRI reagent (Sigma) and RT-PCR was conducted using RevertAid First Strand cDNA synthesis Kit (Fermentas) according to manufacturer's protocol. The primers and PCR conditions are shown in supplementary material Table S2 (Lo et al., 2001; Ma et al., 2006; Schoenfeld et al., 2010).

Tumorigenicity, invasion and wound-healing assays

Soft agar assays and assessment of tumour formation in nude or SCID mice was performed as described previously (Raul et al., 2004). Five animals were used for each group. 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. The ellipsoid volume formula 1/2×L×W×H was used to calculate the tumour volume. Wound-healing assays were performed as described previously (Kundu et al., 2008). The invasiveness was determined by Boyden chamber invasion assays. 50 μl Matrigel (1 mg/ml; BD Biosciences) was applied to 8-μm-pore-size polycarbonate membrane filters and the bottom chamber was filled with 0.6 ml of IMDM medium with 10% FCS. 1×10^5 cells were seeded in the chamber in serum-free medium, and then incubated for 18 hours at 37°C. The membranes containing invaded cells were fixed with methanol and stained with haematoxylin and eosin. The invasiveness was quantified by counting ten random fields under a light microscope. Data obtained from three separate chambers are shown as mean values.

Two-dimensional gel electrophoresis and mass spectroscopy

Two-dimensional gel electrophoresis of whole cell lysates were performed using 8-cm (pH 3–8) IPG strips (Bio-Rad). Differentially expressed spots observed on Colloidal Coomassie stained two-dimensional gels were excised and washed with destainer (200 mM ammonium bicarbonate in 40% acetonitrile). The gel pieces were dried under vacuum and digested in 20 μg/ml trypsin. Peptides were dissolved in 1% TFA and subjected to MALDI–TOF-MS.

Flow cytometry for cell surface staining

The cells were grown for 48 hours and were harvested using accutase (Sigma). 1×10^6 cells were incubated with mouse anti-β4- (clone 450-9D, Serotec) and anti-β1-integrin (clone 6S6, Millipore) monoclonal antibodies for 45 minutes. After incubation with primary antibodies, cells were washed three times with FACS buffer (1% FCS and 0.02% sodium azide in 1× PBS). The cells were then incubated with Alexa-Fluor-486-conjugated anti-mouse-IgG secondary antibody (Molecular Probes) for 45 minutes. Cells were fixed with 1% paraformaldehyde and then analysed with a FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer. The mean fluorescence intensity was measured in arbitrary units using LSM10 software. The mean fluorescence intensity was measured in arbitrary units Msiy CellQuest software.

Statistical analysis

Two groups of data were compared by performing a *t*-test statistical analysis with the histograms generated using Graphpad Prism5 software. $P<0.05$ was considered statistically significant.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/12/????/DC1

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Table S1. shRNA oligonucleotides directed against Keratin 8

S.No.	shRNAs	TARGET SITE	SEQUENCE
1	shRNAK8.1	124bp–144bp	5′CCGGCAGCAGCAACTTTCGCGGTAAGTTCTCTACCGCGAAAGTTGCTGCTGCCTTTTTTC 3′ and 5′TCGAGAAAAAAGGCAGCAGCAACTTTCGCGGTAGAGAACTTACCGCGAAAGTTGCTGCTG 3′
2	shRNAK8.2	184bp–205bp	5′CCGGCATCACCGCAGTTACGGTCAAAGTTCTCTTGACCGTAACTGCGGTGATGCCTTTTTTC 3′ and 5′TCGAGAAAAAAGGCATCACCGCAGTTACGGTCAAGAGAACTTTGACCGTAACTGCGGTGATG 3′
3	shRNAK8.3	1024bp–1044bp	5′CCGGAGAGCTGGCCATTAAGGATAAGTTCTCTATCCTTAATGGCCAGCTCTCCTTTTTTC 3′ and 5′TCGAGAAAAAAGGAGAGCTGGCCATTAAGGATAGAGAACTTATCCTTAATGGCCAGCTCT 3′
4	shRNAK8.4	5’UTR K8	5′CCGGATCTCCGCCTGGTTCGGCCAAGTTCTCTGGCCGAACCAGGCGGAGATCCTTTTTTC 3′ and 5′TCGAGAAAAAAGGATCTCCGCCTGGTTCGGCCAGAGAACTTGGCCGAACCAGGCGGAGAT 3′

Table S2. RT-PCR details (primer sequence and PCR conditions)

S.No	Gene	Sequence	Annealing temprature(°C)	Product Size (bp)	PCR Cycles	Ref.
1	Keratin8 For	5′AGATGAACCGGAACATCAGC 3′	56°C	390	28	
	Keratin8 Rev	5′TCCAGCAGCTTCCTGTAGGT 3′				
2	Keratin18 For	5′TGAGACGTACAGTCCAGTCCTT 3′	55°C	114	28	
	Keratin18 Rev	5′GCTCCATCTGTAGGGCGTAG 3′				
3	β4-integrin For	5′GCGACTATGAGATGAAGGTG 3′	58°C	704	28	Lo et al., 2001
	β4-integrin Rev	5′GTGAGTTGTAGTCCCGTGTG 3′				
4	α6-integrin For	5′CTAACGGAGTCTCACAACTC 3′	60°C	843	25	Lo et al., 2001
	α6-integrin Rev	5′ACTCTGAAATCAGTCCTCAG 3′				
5	GAPDH For	5′GAAGGTGAAGGTCGGAGTC 3′	58°C	126	27	Ma et al., 2006
	GAPDH Rev	5′GAAGATGGTGATGGGATTTC 3′				
6	Vimentin For	5′GTCAGCAATATGAAAGTGTGGC 3′	54°C	258	27	Schoenfeld et al., 2010
	Vimentin Rev	5′ GGTAGTTAGCAGCTTCAACGG 3′				