

Visinin-like protein-1 is a potent inhibitor of cell adhesion and migration in squamous carcinoma cells

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Tumor cell invasion is a highly integrated and complex process comprising several biologically distinct functions such as cell adhesion, motility, proteolysis, etc. Visinin-like protein-1 (VILIP-1), a member of the neuronal EF-hand calcium-sensor protein family, plays a role in regulating tumor cell invasiveness of mouse squamous cell carcinoma (SCC). VILIP-1 enhances cyclic adenosine monophosphate levels through PKA induction. However, the mechanism by which VILIP-1 reduces cell invasiveness is not well understood. In this study, we show that VILIP-1 decreased cell adhesion and migration/invasiveness of highly invasive mouse SCC cells. Forced expression of VILIP-1 reduced cell adhesion to fibronectin in parallel to downregulating αv and $\alpha 5$ integrin subunit levels. VILIP-1 overexpression also led to decreased migration ability. Conversely, short hairpin RNA-mediated VILIP-1 knock-down of SCC cells' characterized by little or no invasiveness, correlated with increased adhesion to fibronectin and enhanced expression of αv and $\alpha 5$ integrin subunits together with increased cell migration. Function-blocking assays with inhibitory anti- $\alpha 5$ and anti- αv integrin antibodies showed that both subunits contributed to cell adhesion, migration, and invasiveness of highly invasive SCC cell lines. These results point to a critical role of VILIP-1 in regulating cell adhesion and migration by downregulation of fibronectin receptor expression. Decreased or absent VILIP-1 expression in SCC cell subpopulations may lead to a more advanced malignant phenotype characterized by changes in adhesive ability and increased cell motility, suggestive of a tumor suppressor function.

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Introduction

Tumor progression, through a complex process called the metastatic cascade, results in the spread of malignant cells to local and distant sites (Jin and Varner, 2004). Metastasis is the leading cause of morbidity and mortality associated with cancer. During the transition from *in situ* to invasive carcinoma, tumor cells penetrate the epithelial basement membrane and enter the underlying stroma. An initial step during tumor cell invasion is the loss of cell–cell adhesion and tumor cell attachment to the matrix. This attachment is mediated through specific glycoproteins such as laminin and fibronectin and through tumor cell plasma membrane receptors (Hood and Cheresch, 2002). After attachment, tumor cells either secrete hydrolytic enzymes or induce stromal cells to express the same or similar enzymes able to degrade the extracellular matrix (ECM). Enhanced tumor cell locomotion into the area previously modified by protein degradation is another component of this cascade. Continued invasion of the stromal matrix may take place by multiple repetitions of these steps (Woodhouse *et al.*, 1997; Kassis *et al.*, 2001).

Interactions between tumor cells and the ECM strongly influence tumor development, affecting cell proliferation, survival, and the ability of neoplastic cells to migrate beyond the original site of the primary tumor and into other tissues to form metastases. Many of these interactions are mediated by integrins, a ubiquitously expressed family of adhesion receptors. Integrins are essential for cell attachment and controlling cell migration, cell cycle progression and programmed cell death. These responses are regulated in synergy with other signal transduction pathways. At least 10 different members of the integrin family have been reported to bind fibronectin, but only some of these are expressed in epithelial cells. Integrin $\alpha 3\beta 1$, which has been shown to bind fibronectin in some cell types, localizes to epidermal basal keratinocytes (Peltonen *et al.*, 1989). Integrin $\alpha v\beta 5$ is also present in the basal layers of oral epithelium (Jones *et al.*, 1997). $\alpha 5\beta 1$ and $\alpha v\beta 6$ fibronectin/tenascin receptors are induced during wound healing (Larjava *et al.*, 1993; Haapasalmi *et al.*, 1996). *De novo* expression of $\alpha v\beta 6$ integrin has been observed in mouse squamous cell carcinomas (SCCs) (Jones *et al.*,

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1997; Bagutti *et al.*, 1998). Integrin $\alpha v \beta 1$ is a low-affinity fibronectin receptor that is expressed by cultured keratinocytes *in vitro* (Adams and Watt, 1991; Zhang *et al.*, 1993; Koivisto *et al.*, 1999) and in SCC cells. Changes in the expression of some integrins have been observed in different malignant cell types (Gong *et al.*, 1997; Jones *et al.*, 1997; Bagutti *et al.*, 1998; Felding-Habermann *et al.*, 2002). For example, highly metastatic murine tumor cells express increased levels of $\alpha v \beta 3$ (Li *et al.*, 2001), and a number of studies have shown that $\alpha v \beta 6$ expression correlates with progression of oral SCC (Thomas *et al.*, 2001a, b). However, the mechanisms leading to changes in integrin expression are not well understood.

In a previous report we have shown that overexpression of VILIP-1, a member of the visinin-recoverin neuronal calcium-sensor protein family, results in a cyclic adenosine monophosphate (cAMP)-mediated decrease of *in vivo* and *in vitro* growth and invasiveness of murine SCC cells (Mahloogi *et al.*, 2003). In the present study, we examined in four SCC cell lines, the role of VILIP-1 in the modulation of cell adhesion to ECM and cell migration/invasiveness. The results indicate that overexpression of VILIP-1 in two high-grade SCC cell lines, CC4A and CH72T3, reduced cell motility and adhesion to fibronectin and downregulated the expression of αv and $\alpha 5$ integrins. Moreover, knocking down VILIP-1 in two low-grade SCC cell lines, CC4B and CH72 cells, enhanced cell motility/invasiveness, adhesion, and specific integrin expression. These results suggest that VILIP-1, via effects on integrin expression, could be an important regulator of tumor progression.

Results

Aggressive SCC cells display increased adhesion to ECM components

Cell attachment to the matrix is an initial step during tumor invasion. This attachment is mediated through matrix glycoproteins, such as fibronectin or laminin, and integrins. In order to elucidate SCC cell behavior during cell invasion, cell adhesiveness of SCC cell lines to laminin, fibronectin and collagen IV was characterized. Fibronectin is a component of mesenchymal matrices, while laminin and collagen IV are present in the basement membrane. Cells with high affinity to fibronectin will penetrate more efficiently through mesenchymal stroma. Hence, cells with lower affinity to laminin and collagen IV but high affinity for fibronectin are likely to be more invasive than cells with opposite affinities for matrix components.

CC4A, CC4B, CH72T3 and CH72 cells of different degrees of invasiveness were analysed (Hubbard *et al.*, 1997). Adhesion of the highly invasive CC4A cells to purified fibronectin was dramatically higher than adhesion of the less invasive CC4B cells in a dose-dependent manner. The maximum adhesion was reached at a concentration between 1 and 5 $\mu\text{g}/\text{ml}$ of fibronectin

(Figure 1a). A similar result was obtained with the CH72T3/CH72 cell line pair. The more invasive cell line (CH72T3) showed increased adhesiveness to fibronectin than its less aggressive counterpart (CH72) (Figure 1b).

CC4A cells also showed an increased dose-dependent adhesion to laminin and collagen IV when compared with CC4B cells, although the difference was less significant than that observed with fibronectin (Figure 1c and e). CH72T3 cells did not show a more significant dose-dependent adhesion to laminin and collagen IV than CH72 cells (Figure 1d and f).

These studies show that cell adhesion to fibronectin, somewhat less for laminin and collagen IV, correlated well with the previously determined biological properties (Hubbard *et al.*, 1997) of these SCC tumor cells.

VILIP-1 overexpression interferes with tumor cell adhesion to fibronectin of highly invasive SCC cells

CH72T3 cells express less VILIP-1 than CH72 cells. In order to determine whether this protein has a role in diminishing cell adhesion to fibronectin, we transfected CH72T3 and CC4A cells with a plasmid expressing VILIP-1 (Figure 2a) and a dose-dependent cell adhesion assay to fibronectin was performed.

Figure 2b depicts the ability of CH72T3 VILIP-1 and CC4A VILIP-1 cells and their respective vector-alone-transfected counterparts to adhere to purified fibronectin. The lower adhesive ability of VILIP-1 transfecteds *vis a vis* the control CH72T3.cin and CC4A.cin cells was maintained through a range of fibronectin concentrations. These results suggest that VILIP-1 plays a role in reducing cell adhesion to fibronectin.

VILIP-1 gene silencing enhances cell adhesiveness of two low-grade SCC cell lines

Several laboratories have shown that short hairpin RNA (shRNA) is a potent experimental tool for gene silencing in mammalian somatic cells (Paddison *et al.*, 2002, 2004). We generated an shRNA targeted to mouse VILIP-1. Transient transfections were made and the levels of VILIP-1 protein were analysed by Western blot (Figure 3a). shRNA induced VILIP-1 gene silencing in CC4B and CH72 cell lines, resulting in VILIP-1 protein knock-down. As a control of specificity of this hairpin, we generated a plasmid expressing the same shRNA with two point mutations (shRNAVILIPmut); this plasmid did not change VILIP-1 levels (Figure 3a). We also generated stable transfectants expressing the shRNA of VILIP-1.

After selecting with G418, three clones from each cell line showing lower levels of VILIP-1 (protein level reduction > 70%) were chosen and used in experiments (Figure 3b). We used shRNAVILIP-1 to further demonstrate that VILIP-1 is involved in regulating cell adhesion to fibronectin. Knocking down VILIP-1 in CC4B and CH72 cells increased cell adhesiveness to fibronectin when compared to those cells transfected with a control plasmid (Figure 3c and d).

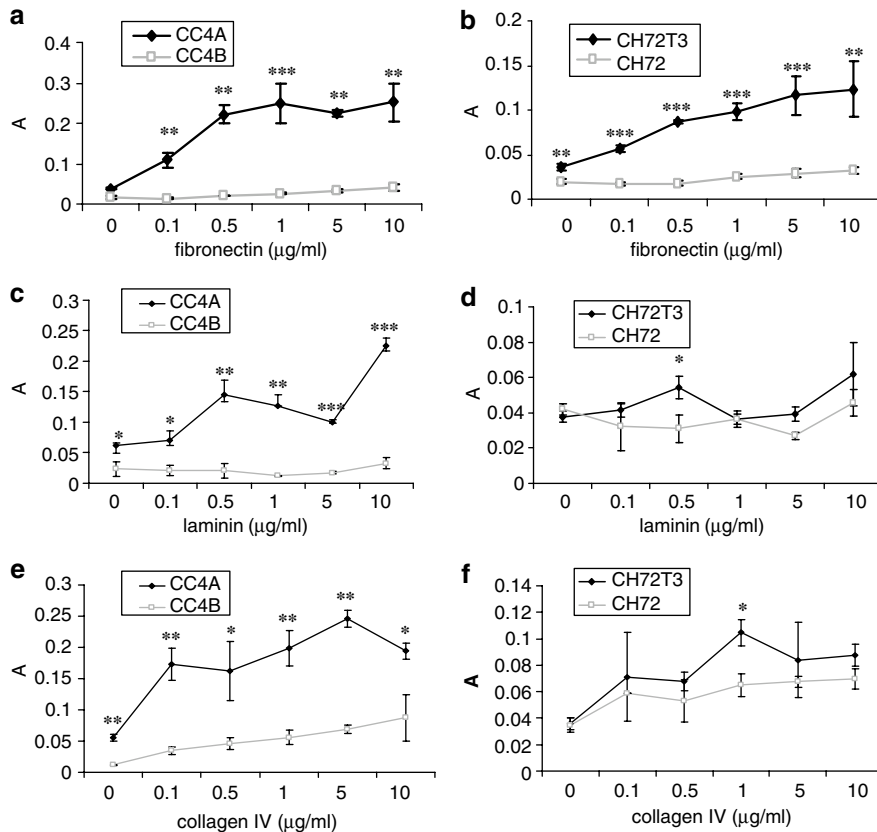


Figure 1 Dose-dependent cell adhesion to collagen IV, fibronectin and laminin. (a, c, e) Adhesion of CC4A (▲) compared to CC4B (■) cells. (b, d, f) Adhesion of CH72 (▲) compared to CH72T3 (■) cells. Multiwell plates were coated with different concentrations of collagen IV, fibronectin and laminin (x-axis). Cells were added to the coated wells and the plates were incubated at 37°C for 1 h. The attached cells (y-axis) were quantified as described in Materials and methods. Adhesion values represent mean and s.e.m. from triplicate determinations. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

SCC cells exhibiting increased adhesiveness to fibronectin have elevated levels of the integrin subunits $\alpha 5$ and αv

To search for the potential receptors involved in the interaction with fibronectin during cell adhesion, we studied the expression of two integrin subunits, αv and $\alpha 5$ integrins, which are commonly found in several fibronectin receptors.

CC4B and CH72 cells showed a significantly reduced expression of both integrin subunits than their more invasive counterparts, CC4A and CH72T3 cells, respectively (Figure 4a). To test the hypothesis of a potential link between the cellular levels of VILIP-1 and $\alpha 5$ and αv integrin expression, we used stable transfectants of CC4A and CH72T3 cells expressing VILIP-1 (Mahloogi *et al.*, 2003). Expression of $\alpha 5$ and αv integrins was evaluated by Western blot analysis. Ectopic expression of VILIP-1 resulted in a strong downregulation of $\alpha 5$ and αv integrins in both transfectants (Figure 4b). Knocking down VILIP-1 in CC4B and CH72 cells increased $\alpha 5$ and αv integrin expression when compared to cells transfected with the control plasmid (Figure 4c).

Evaluation of $\beta 1$ integrin revealed that it is highly expressed in all cells and no significant differences could be established among the different cell lines (data not

shown), suggesting that this integrin subunit may not be limiting the amount of fibronectin receptors on the cell surface.

αv and $\alpha 5$ integrin subunits mediate aggressive SCC cell adhesion to fibronectin

The interaction between fibronectin and its cellular receptors can be blocked by peptides that contain the tripeptide sequence RGD (Nagaoka *et al.*, 2003) or in the presence of blocking antibodies (Takahashi *et al.*, 1990; Yasuda *et al.*, 1995). Following the same concepts, we performed blocking assays to demonstrate the functional contribution of the $\alpha 5$ and αv integrin subunits during SCC cell adhesion to fibronectin. RGD peptide (10 µg/ml) inhibited the binding of CH72T3, CC4A, CH72shRNAVILIP or CC4BshRNA-VILIP cells to fibronectin-coated wells. No inhibition occurred with the same concentration of the control peptide RGE (Figure 4d). We then tested the effect of $\alpha 5$ or αv blocking antibodies and the combination of these antibodies on SCC cell adhesion to fibronectin. Anti- $\alpha 5$ or anti- αv antibody alone was sufficient to partially prevent cell adhesion. The inhibition of SCC cell

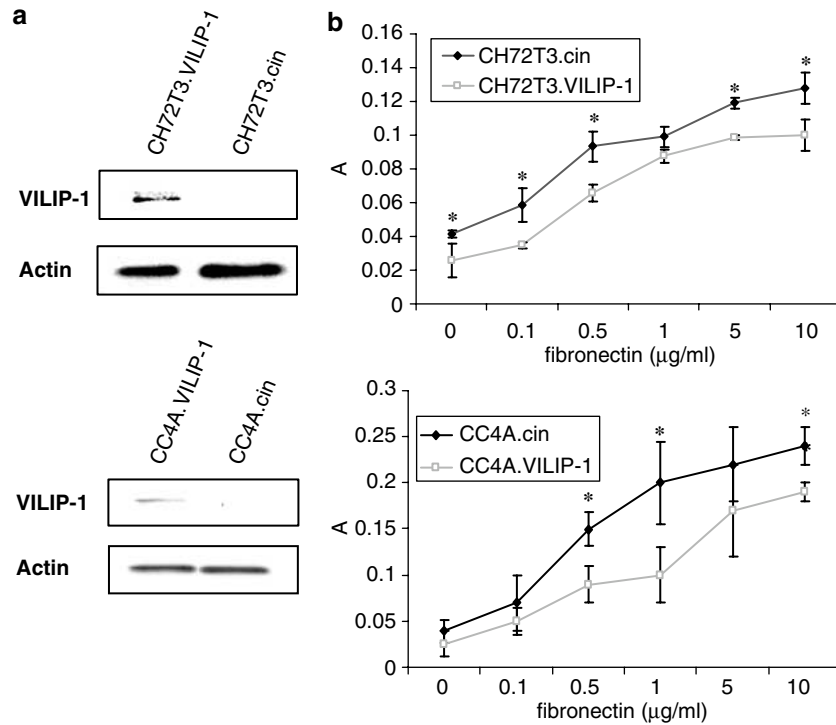


Figure 2 Dose-dependent cell adhesion to fibronectin. (a) CH72T3 and CC4A cells were transfected with a cDNA VILIP-1 plasmid as described in Materials and methods. Whole-cell extracts were prepared and analysed by Western blot analysis for VILIP-1 and actin (control). (b) Multiwell plates were coated with different concentrations of fibronectin (x-axis). Cells were added to the coated wells and the plates were incubated at 37°C for 1 h. Attached cells (y-axis) were quantified as described in Materials and methods. Adhesion values represent mean and s.e.m. from triplicate determinations. * $P < 0.05$

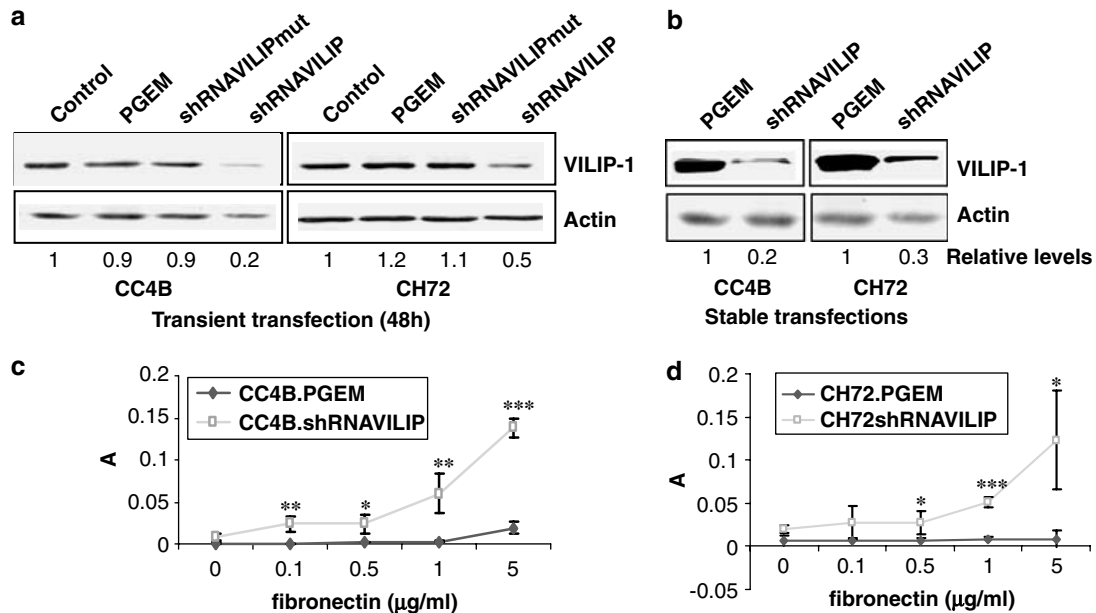


Figure 3 Adhesion to fibronectin of CC4B and CH72 cells with shRNA-mediated suppression of VILIP-1. (a) CC4B and CH72 cells were transfected with VILIP-1-specific shRNA (shRNAVILIP), a double-mutant shRNA (shRNAVILIPmut) or with an empty vector (PGEM). Cell extracts were analysed 48 h later by Western blotting for VILIP-1 and actin (control). The first lane (control) represents nontransfected cells (CC4B or CH72 cells). (b) Whole-cell extracts from stable cell lines carrying VILIP-1-specific shRNA (shRNAVILIP) and empty vector (PGEM) were prepared and evaluated by Western blot analysis of VILIP-1 and actin (control). Multiwell plates were coated with different concentrations of fibronectin. CC4B (c) and CH72 (d) shRNAVILIP- or mock-transfected cells were added to the coated wells and the plates were incubated at 37°C for 1 h. Attached cells were quantified as described in Materials and methods. Adhesion values represent mean and s.e.m. from triplicate experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

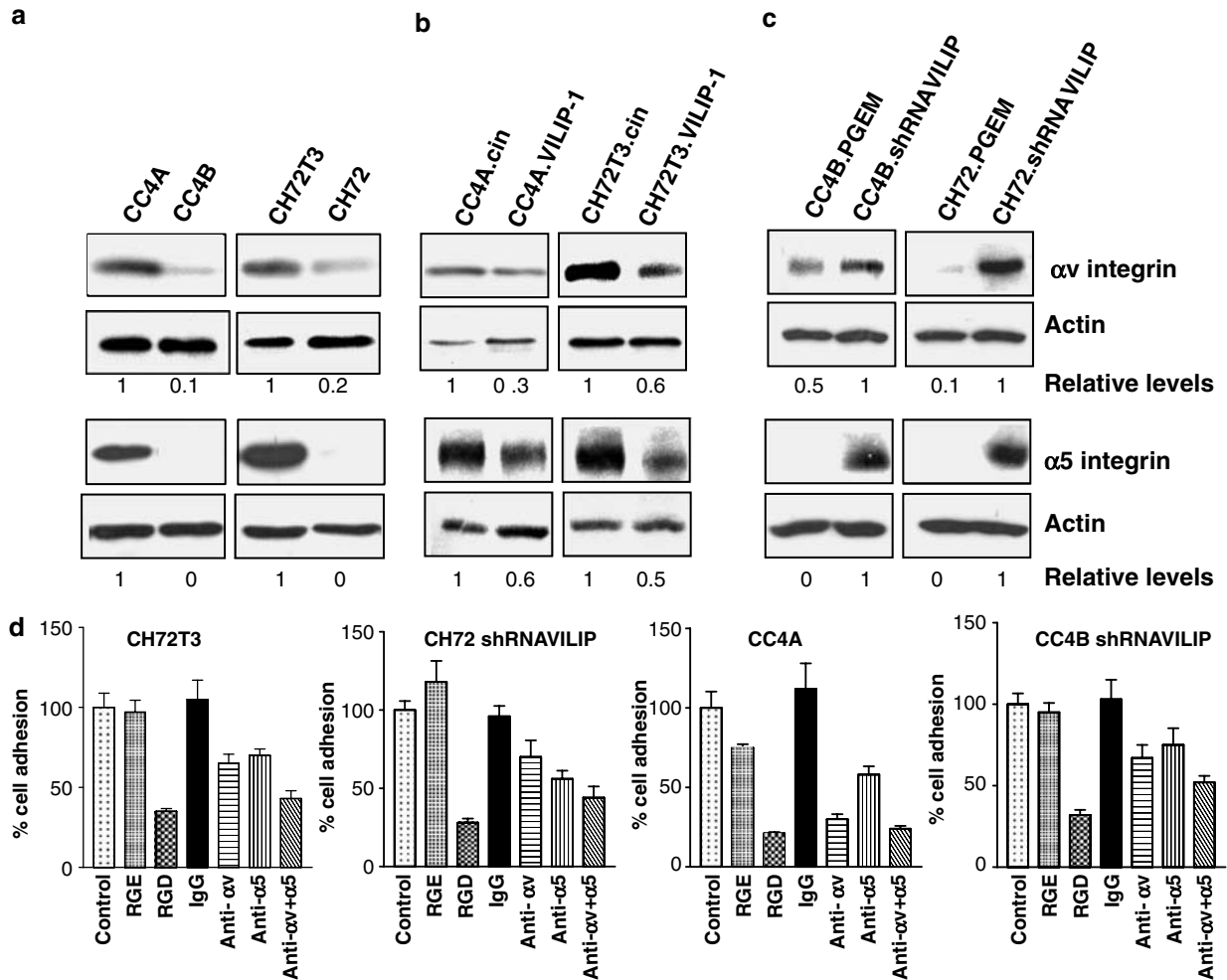


Figure 4 Integrin αv and $\alpha 5$ expression in SCC cells. (a) Whole-cell extracts from CC4A, CC4B, CH72T3 or CH72 cells were prepared and αv and $\alpha 5$ integrin expression was analysed by Western blot analysis. Note that the most aggressive cell lines, CC4A and CH72T3, exhibit higher integrin expression. (b) Western blotting for αv and $\alpha 5$ integrin in CC4A or CH72T3-transfected cells showed decreased expression of αv and $\alpha 5$ integrin expression in VILIP-1-transfected cells when compared with vector-alone-transfected cells (cin). (c) Western blot analysis of αv and $\alpha 5$ integrin in CC4B or CH72shRNA VILIP-1-transfected cells showed increased expression of αv and $\alpha 5$ integrin expression in shRNA VILIP-1-transfected cells when compared with vector-alone-transfected cells (PGEM). (d) Multiwell plates were coated with 100 μ l of fibronectin (10 μ g/ml). CH72T3, CH72shRNA VILIP-1-transfected cells, CC4A and CC4BshRNA VILIP-1-transfected cells were added to the coated wells in the presence or absence of RGE control peptide (10 μ g/ml), RGD peptide (10 μ g/ml), control rat IgG (10 μ g/ml), blocking anti- αv integrin antibody (10 μ g/ml), blocking anti- $\alpha 5$ integrin antibody (10 μ g/ml) or a combination of blocking anti- αv and anti- $\alpha 5$ antibodies (10 μ g/ml each). The plates were incubated at 37°C for 1 h. Attached cells were quantified as described in Materials and methods. Adhesion values represent mean and s.e.m. from triplicate experiments

adhesion was enhanced by using a combination of antibodies against αv and $\alpha 5$ integrins (Figure 4d).

VILIP-1 overexpression interferes with cell migration

To investigate a possible inhibitory effect of VILIP-1 on cell migration, we performed an *in vitro* migration assay. This assay showed that migration of CC4A and CH72T3 VILIP-1-transfected cells (CC4A.VILIP-1 and CH72T3.VILIP-1 cells) was considerably lower than the migratory ability of the control (empty-vector-transfected cells CC4A.cin and CH72T3.cin) (Figure 5a). This experiment supported the hypothesis that VILIP-1 had an inhibitory effect on cell migration.

This hypothesis was further advanced by an experiment in which stable transfection with VILIP-1 shRNA

of the two low-grade SCC cell lines CC4B and CH72 resulted in a strong increase of their respective migratory abilities (Figure 5b).

Taken together, these results indicate that VILIP-1 plays an important role in controlling cell migration of SCC cells.

To determine whether or not $\alpha 5$ and αv play a role in the increased motility of cells that express little or no VILIP-1, we performed migration experiments in the presence of blocking peptides or antibodies. To perform these assays, we used the highly motile cells CH72T3, CC4A, CC4BshRNA VILIP-1 and CH72shRNA VILIP-1. The presence of RGD peptide blocked the migration of all the cells analysed, while no inhibition was seen in the presence of the control peptide (RGE) (Figure 5c). Next, we studied the effect of anti- αv and anti- $\alpha 5$ integrin

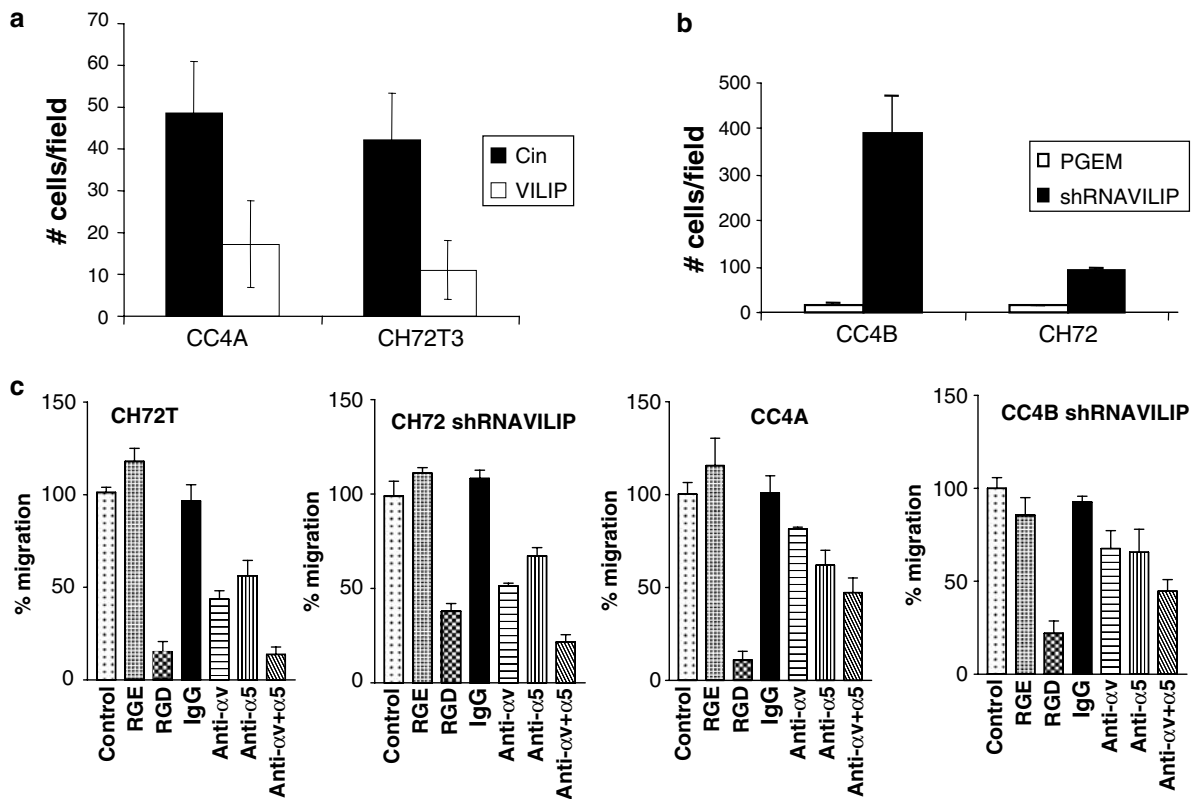


Figure 5 Cell migration assay. Cell migration was assayed using 24-well Transwell chambers as described in Materials and methods. CC4A and CH72T3 cells transfected with an empty vector (full bars) and with VILIP-1 vector (blank bars) (a) or with VILIP-1-specific shRNA (shRNAVILIP) (full bars) and empty vector (PGEM) (blank bars) (b). Cells were seeded in the upper compartment of 24-well Transwell chambers at a density of 2.5×10^5 cells/well. After 8 h incubation at 37°C , migration of cells to the lower surface of the filter was quantified. Values represent mean and s.e.m. from triplicate samples. (c) Blocking cell migration assays of CH72T3, CH72shRNAVILIP-transfected cells, CC4A and CC4BshRNAVILIP-transfected cells. Cells were seeded in the upper compartment of 24-well Transwell chambers (2.5×10^5 cells/well) in the presence or absence of RGE control peptide ($10 \mu\text{g/ml}$), RGD inhibitory peptide ($10 \mu\text{g/ml}$), control rat IgG ($10 \mu\text{g/ml}$), blocking anti- α v integrin antibody ($10 \mu\text{g/ml}$), blocking anti- α 5 integrin antibody ($10 \mu\text{g/ml}$) or a combination of blocking anti- α v and anti- α 5 integrin antibodies ($10 \mu\text{g/ml}$ each). After 8 h incubation at 37°C , migration of cells to the lower surface of the filter was quantified. Values represent mean and s.e.m. from triplicate samples

antibodies on cell migration. Anti- α 5 or anti- α v antibodies alone reduced cell migration of CH72T3 and CH72shRNAVILIP-1 cells. Conversely, the blocking reagents had no significant effect on cell migration of CC4A or CC4BshRNAVILIP-1, suggesting that these cell lines are able to switch from one fibronectin receptor to another when one of them is blocked. However, in combination, anti- α v and anti- α 5 antibodies significantly inhibited cell migration at the same concentration ($10 \mu\text{g/ml}$ each). Control rat antibody did not inhibit cell migration (Figure 5c). These experiments demonstrated that both integrin subunits, α v and α 5, are utilized for cell migration of the highly motile mouse SCC cells CH72T3, CC4A, CH72shRNAVILIP or CC4BshRNAVILIP.

Effects of integrin blocking antibodies on SCC invasion

In vitro invasion assays were performed to further investigate the role of α v and α 5 integrin subunits in the metastatic behavior of mouse SCC cells. RGD peptide and a combination of neutralizing α v and α 5 integrins were able to reduce cell invasiveness by more than 60%

in most cell lines (Figure 6), indicating that fibronectin receptors containing these α v and α 5 subunits are implicated in SCC cell invasion.

PKA inhibition increases integrin expression and cell migration

In previous studies we have shown that VILIP-1 increases cAMP levels in SCC cell lines (Mahloogi *et al.*, 2003). To examine the hypothesis that cAMP metabolism plays a role in VILIP-1 inhibition of migration and downregulation of α 5 and α v integrins, a cAMP-dependent protein kinase (PKA) inhibitor (H89) and a specific peptide inhibitor of PKA (PI) were used. Inhibition of PKA with H89 ($25 \mu\text{M}$) or PI ($1 \mu\text{M}$) in CH72 cells, which expressed high levels of VILIP-1, increased the rate of migration two- and threefold (Figure 7a). In agreement with previous findings (O'Connor *et al.*, 1998, 2000), these data indicate that PKA inhibits SCC cell migration.

Pretreatment of CH72T3 and CC4A VILIP-1-transfected cells with PI increased the levels of α 5 and α v integrin (Figure 7b and c). PI did not induce integrin

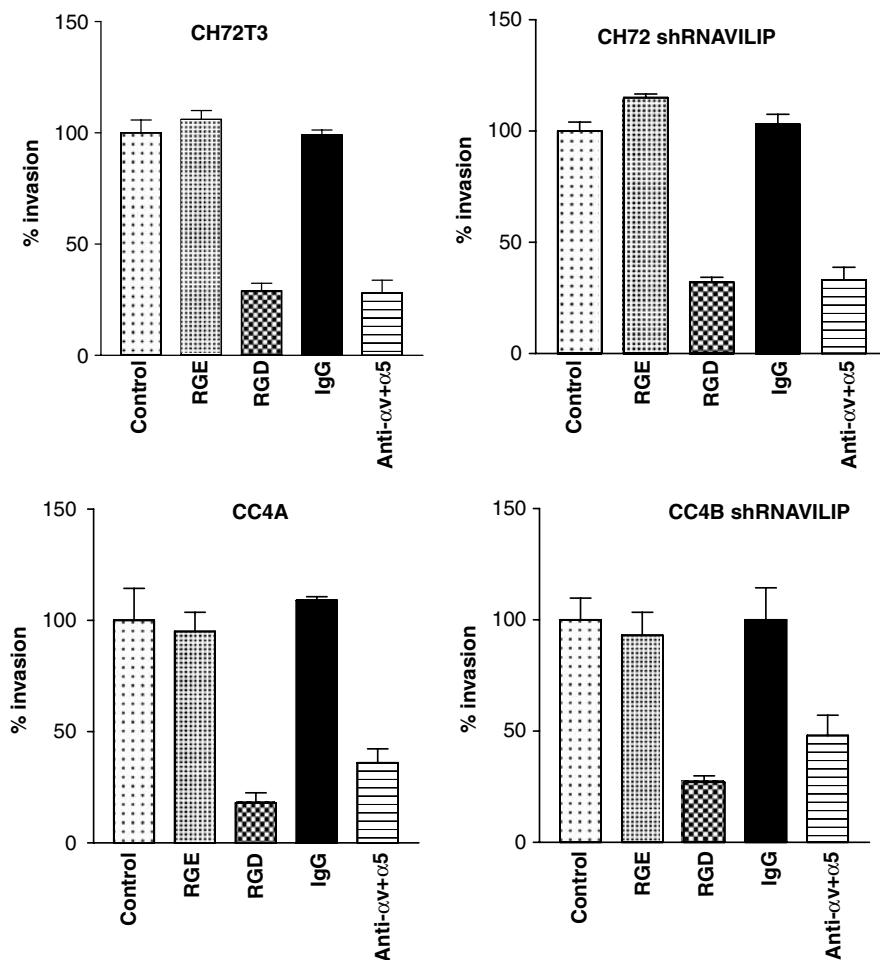


Figure 6 *In vitro* invasion assay. Histogram of the *in vitro* invasion assays performed with CH72T3, CH72shRNAVILIP-transfected cells, CC4A and CC4BshRNAVILIP-transfected cells. Cells were seeded in the upper compartment of 24-well chambers (2.5×10^5 cells/well) in the presence or absence of RGE control peptide ($10 \mu\text{g/ml}$), RGD inhibitory peptide ($10 \mu\text{g/ml}$), control rat IgG ($10 \mu\text{g/ml}$) and a combination of blocking anti- αv and anti- $\alpha 5$ integrin antibodies ($10 \mu\text{g/ml}$ each). After 24 h incubation at 37°C , invasion of cells to the lower surface of the filter was quantified. Values represent mean and s.e.m. from triplicate samples

expression in CH72T3 and CC4A empty-vector-transfected cells (data not shown). These studies suggest that the reduction of the levels of integrins αv and $\alpha 5$ by VILIP-1 might occur after the induction of cAMP and subsequent PKA activation.

Discussion

VILIP-1 is expressed in the central nervous system, where it regulates cAMP levels, cell signaling and differentiation. In a recent report, we have reported that VILIP-1 expression is lost in high-grade SCC (Mahloogi *et al.*, 2003). We have also shown that VILIP-1 plays a critical role in regulating the invasive/metastatic phenotype by decreasing cell proliferation and matrix degradation/tumor cell invasiveness through a cAMP-mediated pathway (Mahloogi *et al.*, 2003).

Metastasis is the most challenging and crucial aspect of cancer progression, and is usually associated with limited therapeutic options and decreased survival rates.

Inherent to metastasis is invasion, the process by which cells infiltrate into adjacent tissues, blood and lymph vessels and subsequently into distant organs by degrading basement membranes and ECM and disrupting tissue architecture. The process of tumor cell invasion and dissemination requires active cell migration through the ECM with the simultaneous remodeling of intercellular adhesions. In this study, we report that overexpression of VILIP-1 decreased cell migration of high-grade SCC cells. Conversely, knock-down of VILIP-1 protein by stable transfection of low-grade SCC cells with a plasmid expressing an shRNA specific for VILIP-1 resulted in increased cell motility when compared to mock-transfected cells.

Several integrins have an enhancing effect during the process of tumor cell invasion, whereas intercellular adhesion restrains invasion and promotes a more differentiated phenotype. Integrins play an important role in promoting cell proliferation, migration, and survival *in vitro* and *in vivo*, and their antagonists suppress cell migration and invasion of primary and transformed cells. For example, antagonists of integrins

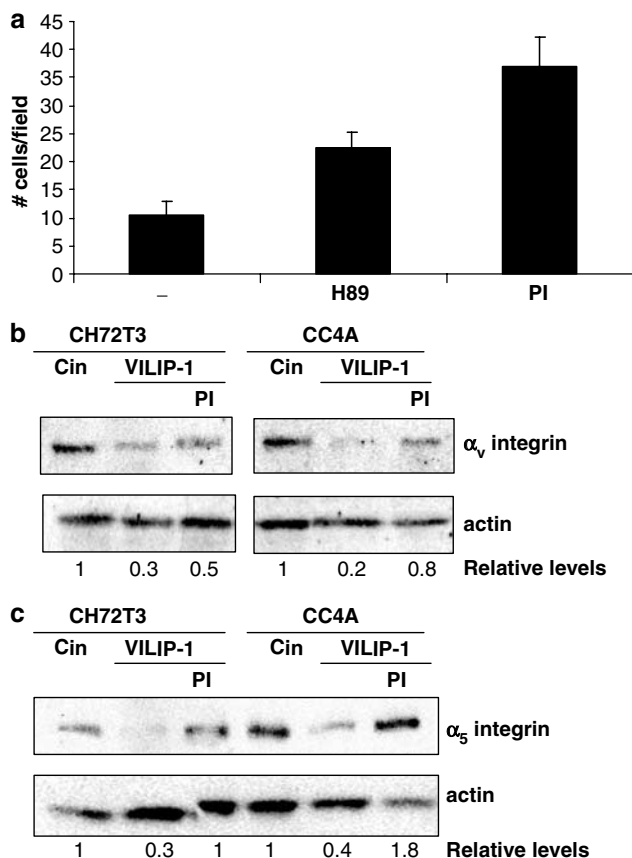


Figure 7 Effect of PKA inhibition on SCC migration and integrin expression. (a) CH72 cells were treated with two different PKA inhibitors H89 (25 μ M) or PI (1 μ M), or with vehicle alone (-) for 8 h. Cells were seeded in the upper compartment of 24-well Transwell chambers at a density of 2.5×10^5 cells/well. After 8 h incubation at 37°C in the presence or absence of the PKA inhibitors, migration of cells to the lower surface of the filter was quantified. Values represent mean and s.e.m. from triplicate samples. CH72T3 or CC4A VILIP-1-transfected cells were incubated with 1 μ M PI or vehicle for 8 h and whole-cell extracts were submitted to Western blot analysis of α_v (b) and α_5 (c) integrins. VILIP-1 protein levels were evaluated by densitometry (as described in Materials and methods) and are shown as percent change relative to untreated cin control cells

$\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ show great promise as potential inhibitors of tumor growth and metastasis as well as tumor angiogenesis (Kerr *et al.*, 2002; Jin and Varner, 2004). Many studies have shown different levels of integrin expression associated with cancer promotion. For example, an increased level of integrin $\alpha v\beta 3$ is closely associated with increased cell invasion and metastasis (Huang *et al.*, 2000; Reinmuth *et al.*, 2003; Rolli *et al.*, 2003). Expression of $\alpha 5\beta 1$ integrin is also reported in less-differentiated invasive and metastatic oral SCC tumors (Koukoulis *et al.*, 1997; Koivisto *et al.*, 2000). $\alpha v\beta 6$, $\alpha v\beta 1$ and $\alpha 5\beta 1$ integrins have been shown to act cooperatively and interchangeably in human SCC migration on fibronectin (Koivisto *et al.*, 2000). Consistent with previous results observed in human oral cancer cells, our studies demonstrate that αv and $\alpha 5$

integrins were elevated in high-grade SCC cells. Enforced expression of VILIP-1 decreased the levels of αv and $\alpha 5$ integrins in cancer cells. The decrease of fibronectin receptor components correlated with down-regulation of cell adhesion to fibronectin and cell migration.

Furthermore, experiments utilizing integrin blocking agents provided functional evidence for the role played by αv and $\alpha 5$ integrin subunits in adhesion, migration and invasion of highly aggressive SCC cell lines, supporting the participation of these proteins in the mechanism of VILIP-1-mediated repression of migration and adhesion. VILIP-1 did not influence the levels of $\beta 1$ integrin (data not shown); nevertheless, its effects on other integrin subunits should be further investigated.

PKA inhibitors blocked the inhibition of migration and adhesion by VILIP-1. A PKA-specific peptide inhibitor reverted the reduction of $\alpha 5$ and αv integrin levels by VILIP-1. Taken together, these findings along with previous work performed in the laboratory, which established VILIP-1 to increase cAMP levels, suggest that VILIP-1-mediated regulation of cAMP is a significant component of SCC progression. Previous reports have described that direct activation of PKA by forskolin or by overexpression of the catalytic/active subunit of PKA inhibits cell migration (Kim *et al.*, 2000; Mercurio and Rabinovitz, 2001; Shiraha *et al.*, 2002; Santibanez *et al.*, 2003). PKA also inhibits cell migration by disrupting the formation of stress fibers (Kikuchi *et al.*, 1997; Ma *et al.*, 1999). Hence, it is possible that VILIP-1, through the elevation of cAMP levels, regulates cell migration at different checkpoints, for example, by decreasing the amount of fibronectin receptors in the cell and modulating transduction signals into the cell that are important mediators for cell migration.

In summary, these results demonstrate that VILIP-1 is a critical negative regulator of integrin-modulated cell adhesion and migration and that its loss or reduction could have enhancing effect on the malignant phenotype, suggesting a possible tumor suppressive function.

Materials and methods

Antibodies and reagents

Rabbit anti-integrin αv subunit (AB1030) and anti- $\alpha 5$ subunit (AB1949) were purchased from Chemicon International (Harrow, UK). VILIP-1 was detected using a specific rabbit polyclonal antibody (Mahloogi *et al.*, 2003). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin was obtained from Amersham. Type IV collagen, fibronectin and laminin were from Chemicon. H89 and myristoylated PKA inhibitory peptide amide (PI) were purchased from Calbiochem (San Diego, CA, USA).

Rat anti-mouse integrin αv subunit and rat anti-mouse $\alpha 5$ were used as blocking antibodies, while rat IgG was used as control (Research Diagnostics Inc., Flanders, NJ, USA). RGD and RGE peptides (Sigma) were also used in blocking experiments.

Cell lines and cell culture

The following mouse SCC cell lines were used: CC4A, CC4B, CH72 and CH72T3 (Ruggeri *et al.*, 1991; Mahloogi *et al.*, 2003). Cells were grown in S-MEM (Sigma, St Louis, MO, USA) medium containing FCS (10%), L-glutamine (2 mM) and penicillin/streptomycin (100 µg/ml). CC4A and CC4B were derived from the same tumor. When injected subcutaneously into nude mice, CC4A gave rise to a high-grade SCC or spindle cell carcinoma (SCC IV), whereas CC4B gave rise to a well-differentiated, less-aggressive, low-grade SCC (SCCII) (Hubbard *et al.*, 1997). CH72 also gave rise to a low-grade SCC after subcutaneous inoculation. CH72T3 is a subcloned cell line obtained by *in vivo* passage of CH72 into nude mice that resulted in a high-grade SCC (Ruggeri *et al.*, 1991).

Cell transfections

Human *VILIP-1* (*VSNL1*) cDNA (Mahloogi *et al.*, 2003) was digested with *DraI* and cloned into the *SmaI* site of a pCi.Neo expression vector (Promega, Madison, WI, USA). Either 4 µg of the resulting construct or 4 µg of the vector was used to transfect CH72T3 cells by Lipofectamine reagents (Life Technologies Inc.) according to the manufacturer. At 2 days post-transfection, G418 was added at a concentration of 800 µg/ml. After 1 week of selection, cells were lysed, proteins were extracted and Western blotting was performed to screen for *VILIP-1* expression.

Cell adhesion assay

Multiwell tissue culture (96 wells; Costar) plates were coated with selected concentrations of laminin, fibronectin and collagen IV substrates by overnight absorption at 4°C. After saturation of the wells with 1% BSA (fraction V; FisherBiotech), the plates were immediately used for cell adhesion assays. A total of 3×10^5 cells/well were plated in serum-free medium plus 0.1% BSA. Cells were maintained at 37°C for 1 h and rinsed with PBS. The extent of adhesion was determined after fixation of the adherent cells with 1% glutaraldehyde in PBS by staining with 0.1% crystal violet and color reading at 570 nm with an ELISA reader. A blank value corresponding to BSA-coated wells was automatically subtracted. For all experiments, each assay point was determined in triplicate.

Migration assays

Cell migration assays were performed as previously described (Thomas *et al.*, 2001b). Briefly, cells were prepared as in adhesion assays. A total of 2.5×10^4 cells in 500 µl of serum-free S-MEM containing 0.1% BSA were added to the upper chamber of Boyden chambers (Transwell, 6.5 mm diameter, 8 µm pore filter; Costar, Cambridge, MA, USA) and allowed to migrate for 8 h at 37°C under tissue culture conditions. A 500 ml portion of S-MEM containing 10% serum was added to the bottom chamber and used as a chemoattractant. Cells were removed from the upper chamber with a cotton swab and the cells that had migrated to the lower surface of the membrane were fixed, stained with Giemsa and counted.

In vitro invasion assay

This assay was performed using BioCoat Matrigel inserts (Becton Dickinson Labware, Bedford, MA, USA) according to the manufacturer's instructions. Cells were seeded in the inserts in a serum-free medium with or without 25 or 50 µM forskolin. Inserts were transferred to wells containing S-MEM medium with 5% FBS as a chemoattractant. Because of the

relatively low invasive ability of transfected cells, the invasion chambers were incubated at 37°C, 5% CO₂ for 36 h. Invading cells were stained and counted (Mahloogi *et al.*, 2003).

Immunoblotting

Subconfluent cultures were washed with cold PBS and solubilized in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 1% sodium deoxycholate, 2 mM sodium vanadate, 50 mM sodium fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. The lysate was homogenized and centrifuged for 10 min at 4°C to remove debris, and the protein concentration was measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Lysates were subjected to a 4–12% gradient SDS-PAGE and transferred. After blocking with 5% skim milk in TBST, the membranes were incubated for an hour with the indicated primary antibodies and for 1 h with the corresponding secondary horseradish peroxidase-conjugated antibodies. Membranes were washed three times in TTBS for 15 min after each incubation step. Visualization was performed by using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). For reprobing, membranes were stripped with a solution containing 2% SDS, 100 mM 2-mercaptoethanol and 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50°C.

VILIP shRNA vectors

shRNA vectors were made according to the 'SHAG' protocol (<http://katahdin.cshl.org:9331/RNAi/html/rnai.html>). In brief, an shRNA insert was made by amplifying the template pGEM-U6 (a gift from Greg Hannon, CSHL) with the primers 5' CAC CGA TTT AGG TGA CAC TAT AG 3' and 5' AAA AAA GCA ATC CAT AGC ATA ACA TCA GCC CAC TCA AGC TTC AAT GAG CTG ATG TCG ATG CTA TGG ACT GCG GTG TTT CGT CCT TTC CAC AA 3' (*VILIP*) or a control primer bearing two point mutations in the hairpin. PCR was performed in a 50 µl volume using *Taq* polymerase and 50 pmol of each primer with the following cycling profile: 95°C for 3 min; 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; followed by one cycle of 72°C for 10 min.

The unpurified ~1.1 kb PCR product was cloned into the pENTR/D Topo vector (Invitrogen) according to the manufacturer's recommendations.

Quantification and statistical analysis

Western blots were scanned and quantified using NIH densitometry software (available on the World Wide Web at www.scioncorp.com). The migration and adhesion results are presented as mean values ± s.e.m.

Data were analysed using ANOVA and a paired Student's *t*-test to determine the level of significance between the different groups.

Abbreviations

VILIP-1, visinin like protein-1; cAMP, cyclic adenosine monophosphate; ECM, extracellular matrix; SCC, squamous cell carcinoma.

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