Switch from $\alpha v\beta 5$ to $\alpha v\beta 6$ integrin expression protects squamous cell carcinomas from anoikis

Sam M. Janes and Fiona M. Watt

Keratinocyte Laboratory, CR-UK London Research Institute, London WC2A 3PX, England, UK

Stratified squamous epithelia express the $\alpha v\beta 5$ integrin, but in squamous cell carcinomas (SCCs) there is down-regulation of $\alpha v\beta 5$ and up-regulation of $\alpha v\beta 6$. To investigate the significance of this finding, we transduced an $\alpha v$-negative human SCC line with retroviral vectors encoding $\alpha v$ integrins. $\alpha v\beta 5$-expressing cells underwent suspension-induced apoptosis (anoikis), whereas $\alpha v$-negative cells and cells expressing $\alpha v\beta 6$ did not. Resistance to anoikis correlated with PKB/Akt activation in suspension, but not with changes in PTEN or p110$\alpha$ PI3 kinase levels. Anoikis was induced in parental and $\alpha v\beta 6$-expressing cells by inhibiting PI3 kinase. Conversely, activation of Akt or inhibition of caspases in $\alpha v\beta 5$-expressing cells suppressed anoikis. Caspase inhibition resulted in increased phospho-Akt, placing caspase activation upstream of decreased Akt activation. Anoikis required the cytoplasmic domain of $\beta 5$ and was independent of the death receptor pathway. These results suggest that down-regulation of $\alpha v\beta 5$ through up-regulation of $\alpha v\beta 6$ may protect SCCs from anoikis by activating an Akt survival signal.

Introduction

Extracellular matrix receptors of the integrin family regulate the growth, differentiation, and tissue assembly of keratinocytes in stratified squamous epithelia such as the epidermis and the lining of the oral cavity (van der Flier and Sonnenberg, 2001; Watt, 2002). In normal stratified squamous epithelia, integrin expression is largely confined to the basal, proliferative layer of cells attached to the underlying basement membrane; expression is lost as cells move through the suprabasal layers and undergo terminal differentiation. The most abundant integrins in stratified squamous epithelia are $\alpha 2\beta 1$ (collagen receptor), $\alpha 3\beta 1$ (laminin receptor), and $\alpha 6\beta 4$ (laminin receptor; Watt, 2002). Other integrins, such as $\alpha v\beta 5$ (vitronectin receptor), are expressed at lower levels.

Integrin expression is frequently perturbed in squamous cell carcinomas (SCCs), which are tumors of stratified squamous epithelia (Mercurio and Rabinovitz, 2001; Watt, 2002). One such change is an up-regulation of $\alpha v\beta 6$. In normal epidermis, $\alpha v$ forms a heterodimer exclusively with $\beta 5$ (Pasqualini et al., 1993). In hyperproliferative stratified squamous epithelia, both $\alpha v\beta 5$ and $\alpha v\beta 6$ are expressed (Breuss et al., 1995; Haapasalmi et al., 1996). In SCCs, $\alpha v\beta 6$ is up-regulated, and this is often correlated with a down-regulation of $\alpha v\beta 5$ expression (Jones et al., 1997; Regezi et al., 2002). The down-regulation of $\alpha v\beta 5$ in association with expression of $\alpha v\beta 6$ probably reflects a hierarchy in the preference of $\alpha v$ to heterodimerize with different $\beta$ subunits (Koistinen and Heino, 2002).

There are several ways in which up-regulation of the $\alpha v\beta 6$ integrin can affect keratinocyte behavior. $\alpha v\beta 6$ binds the latency-associated peptide derived from latent TGF$\beta$ and activates TGF$\beta$ (Munger et al., 1999). Expression of $\alpha v\beta 6$ increases epithelial cell motility and invasion (Thomas et al., 2001b; Ramos et al., 2002), stimulates cell proliferation (Ahmed et al., 2002), and inhibits fibronectin matrix assembly (Ramos et al., 2002). The effects on growth and invasion may reflect the abilities of $\alpha v\beta 6$ to activate Erk2 MAPK (Ahmed et al., 2002) and to up-regulate or activate matrix metalloproteinases (Agrez et al., 1999; Thomas et al., 2001a; Ramos et al., 2002).

We have previously described a cell line that provides an excellent model system for studying the significance of different $\alpha v$ integrins in SCCs. The H357 cell line is derived from a human SCC of the tongue and lacks expression of $\alpha v$ integrins (Sugiyama et al., 1993). When the $\alpha v$ subunit is transfected into H357 cells and $\alpha v$-positive clones selected by multiple rounds of FACS®, $\alpha v$ is expressed on the cell surface as $\alpha v\beta 5$. Expression of $\alpha v\beta 5$ is correlated with inhibition of anchorage-independent growth and induction of terminal differentiation in H357 cells (Jones et al., 1996).

Abbreviations used in this paper: dn, dominant-negative; ER, estrogen receptor; OHT, 4-hydroxytamoxifen; SCC, squamous cell carcinoma.
the present experiments, we have uncovered a previously unrecognized function of αvβ5 in promoting suspension-induced apoptosis, known as anoikis (Frisch and Francis, 1994), and show that when αvβ5 is replaced at the cell surface with αvβ6, anoikis is prevented. These observations suggest that up-regulation of αvβ6 may confer a survival advantage on cells in SCCs.

Results
Transduction of H357 cells with αv integrin subunit results in surface expression of functional αvβ5
In previous experiments, the αv integrin subunit was introduced into H357 cells by transfection followed by multiple rounds of FACS® and clonal selection (Jones et al., 1996). To avoid such intense selection pressure, we transduced H357 cells with a retroviral vector encoding αv (Levy et al., 1998). As controls, H357 cells were transduced with the empty retroviral vector or with a vector encoding the α4 integrin subunit, which is not expressed in normal stratified squamous epithelia or SCCs (Watt, 2002).

Cell surface expression of αv and α4 was examined by flow cytometry (Fig. 1). As reported previously (Sugiyama et al., 1993), parental H357 cells did not express detectable surface levels of αv (Fig. 1 a). Transduction with the αv retroviral vector resulted in higher cell surface levels of the integrin subunit (Fig. 1 b) than achieved previously by transfection and clonal selection (Fig. 1 c). As expected, parental cells did not express the α4β1 integrin (Fig. 1 d). The level of cell surface expression of the α4 subunit after retroviral transduction (Fig. 1, e and f) was the same whether or not the cells were also transduced with the chick β1 subunit (Fig. 1 f), indicating that the endogenous pool of β1 subunits was not limiting. Introduction of the αv or α4 integrin subunits had no effect on cell surface levels of any of the endogenous β1 integrins (Fig. 1, g–i, and not depicted) nor on levels of α6β4 (Fig. 1, j–l).

When H357 cells are transduced with αv and clonally selected the integrin is expressed as a heterodimer with the β5 subunit (Jones et al., 1996). To examine heterodimer formation in H357 cells transduced with the αv retrovirus, cells were labeled with heterodimer-specific antibod-
ies and analyzed by flow cytometry (Fig. 1, m–r) using parental H357 cells as negative controls (Fig. 1, m–o). There was no detectable labeling of αv-transduced cells with antibodies to αvβ3 (Fig. 1 p) or αvβ6 (Fig. 1 q). However, αvβ5 was readily detected (Fig. 1 r). We conclude that when αv is introduced into H357 cells, whether by transfection and clonal selection (Jones et al., 1996) or by retroviral infection (Fig. 1), it is expressed as the αvβ5 heterodimer.

To examine whether or not the αvβ5 heterodimer expressed by retrovirally transduced H357 cells was functional, adhesion assays on vitronectin were performed. Up to 90% of H357 cells expressing αvβ5 plated on 20 μg/ml vitronectin (b–d and g) or fibronectin (e and f) for 30 min, double labeled with phalloidin (red fluorescence) and antibodies to αvβ5 (b–d), αv (e), or β1 (f and g; green fluorescence). Panel d is the merged image of b and c. Bars, 2 μm. (b–g) H357 cells expressing αvβ5 plated on 20 μg/ml vitronectin (b–d and g) or fibronectin (e and f) for 30 min, double labeled with phalloidin (red fluorescence) and antibodies to αvβ5 (b–d), αv (e), or β1 (f and g; green fluorescence). Panel d is the merged image of b and c. Bars, 2 μm.

αvβ5 expression inhibits anchorage-independent growth without inducing terminal differentiation of H357 cells

Clones of H357 cells transfected with αv were unable to form colonies in soft agar (Jones et al., 1996). To examine if retroviral transduction with αv had the same effect on a polyclonal population, cells were suspended in soft agar for 3 wk, after which time the number of colonies was scored. Cells transduced with αv showed a marked reduction in colony formation compared with parental cells and cells transduced with the empty vector (Fig. 2 h and Table I).

Table 1. Percentage of colony-forming efficiency (CFE) of H357 cells in soft agar

| Experiment | H357 parental expression | H357 expressing empty vector | H357 expressing αvβ5 |
|------------|--------------------------|-----------------------------|---------------------|
| Experiment 1 | 25.4 ± 3.0               | 20.3 ± 2.2                  | 0.2 ± 0.1           |
| Experiment 2 | 18.7 ± 1.2               | 16.2 ± 1.4                  | 0.1 ± 0.1           |
| Experiment 3 | 25.2 ± 2.8               | 24.6 ± 1.6                  | 0.0 ± 0.1           |

Three separate experiments are shown. Data are means and SDs of triplicate dishes.
In clones of H357 cells transfected with \( \alpha v \), suppression of anchorage-independent growth correlates with an increased ability to undergo terminal differentiation, as measured by expression of involucrin (Jones et al., 1996). Terminal differentiation of retrovirally transduced H357 cells was examined by Western blotting, immunofluorescence microscopy, and flow cytometry with antibodies to involucrin and two additional markers of terminal differentiation, transglutaminase 1 and cornifin (Fig. 2 and not depicted).

Neither transduction with \( \alpha v \) nor with \( \alpha 4 \) increased the proportion of terminally differentiated cells (Fig. 2, i–k and m–o). As reported previously (Jones et al., 1996), H357 cells had a much lower proportion of differentiated cells (<0.5%) than primary human keratinocytes (Fig. 2, l and p). Whereas suspension induces terminal differentiation of primary keratinocytes, this was not the case for H357 cells, irrespective of whether or not they expressed \( \alpha v \) (Jones et al., 1996; unpublished data).

**\( \alpha v\beta 5 \) induces anoikis of H357 cells**

Next, we investigated whether or not expression of \( \alpha v\beta 5 \) induced anoikis (Frisch and Francis, 1994). We determined the proportion of cells with a sub-G1 DNA content, as a measure of the number of apoptotic cells (Frisch, 1999b). We compared parental H357 cells, cells transduced with the empty retroviral vector, cells transduced with \( \alpha v \), and cells transduced with \( \alpha 4 \).

Typical flow cytometry profiles are shown in Fig. 3 a, and data pooled from six experiments are shown in Fig. 3 b. The proportion of cells with a sub-G1 DNA content was <3% in freshly harvested cells. In parental cells and cells transduced with \( \alpha 4 \) or empty vector, the proportion increased to almost 10% by 48 h and 20% by 72 h in suspension. Cells transduced with \( \alpha v \) had a similar level of apoptosis to controls after 24 h in suspension, but by 48 h the proportion of apoptotic cells had increased to almost 30%, and to over 50% by 72 h. The increased apoptosis of H357 cells transduced with \( \alpha v \) compared with controls was statistically significant (\( P = 0.008 \) at 72 h; \( t \) test).

The results were confirmed using two other methods to quantitate apoptosis, TUNEL (unpublished data) and microscopic evaluation of nuclear morphology in cells labeled with Hoechst 33258. The percentage of cells with condensed, fragmented nuclei and the SEM of three separate experiments (each counting 200 cells) were as follows. At time 0, 1.7 ± 0.1% of H357 cells expressing \( \alpha 4 \) were apoptotic, and this increased to 14.6 ± 1.7% after 72 h in suspension. At time 0, 1.5 ± 0.1% H357 cells expressing \( \alpha v \) were apoptotic, a level that was similar to cells expressing \( \alpha 4 \). However, after 72 h in suspension this level had risen to 64.2 ± 3.2%, fourfold higher than in \( \alpha 4 \)-expressing cells.

Anoikis is inhibited by integrin ligation of suspended cells or by treatment with caspase inhibitors (Frisch, 1999b). Therefore, we examined the effects of these treatments on H357 cells expressing \( \alpha v\beta 5 \) (Fig. 3 c). The cells were held in suspension for 48 or 72 h in medium alone (control) or containing DMSO (caspase inhibitor solvent), the pan caspase inhibitor z-VAD–fmk (100 \( \mu \)M), vitronectin (100 \( \mu \)g/ml; \( \alpha v \) ligand), or the \( \alpha v \) function-blocking antibody 13C2 (100 \( \mu \)g/ml; Davies et al., 1989). At both time points, z-VAD-fmk and vitronectin substantially reduced the proportion of apoptotic cells. 13C2 also had an inhibitory effect, albeit slightly smaller.

We conclude that unligated \( \alpha v\beta 5 \) expression in H357 cells leads to an increase in suspension-induced apoptosis and that this is the likely mechanism by which anchorage-independent growth is inhibited.

**Effect of overexpressing \( \alpha v \) in cells with normal endogenous levels of \( \alpha v\beta 5 \)**

To investigate whether or not \( \alpha v\beta 5 \)-induced anoikis was specific to H357 cells or a more general phenomenon, we transduced a second SCC line, SCC4, and primary human keratinocytes with the \( \alpha v \) or \( \alpha 4 \) retroviral vectors (Fig. 3, d–g). SCC4 has a similar level of endogenous \( \alpha v \) integrins to primary keratinocytes (Fig. 3, d and f; Levy et al., 2000), but is heterozygous for an activating \( \beta 1 \) integrin mutation (Evans et al., 2003). The levels of \( \alpha v \) expression achieved by retroviral transduction were higher than the endogenous levels (Fig. 3, d and f) and equivalent to that of \( \alpha v \)-transduced H357 cells (Fig. 1 b).

Fig. 3 h shows pooled data from three experiments in which the proportion of apoptotic SCC4 cells was compared immediately after detachment from the culture dish (0 h) or after suspension for 24 or 48 h. At both 24 and 48 h, the proportion of apoptotic cells was significantly higher in cells transduced with \( \alpha v \) than in parental SCC4 or SCC4 transduced with the \( \alpha 4 \) retroviral vector (\( P < 0.05 \)).

It has previously been reported that when primary human keratinocytes are placed in suspension they undergo terminal differentiation as opposed to anoikis (Gandarillas et al., 1999). Consistent with those observations, the proportion of primary keratinocytes in suspension with a sub-G1 DNA content did not exceed 15%, even after 48 h (Fig. 3 i). The proportion of apoptotic cells was not significantly different when parental keratinocytes were compared with keratinocytes transduced with \( \alpha 4 \) or \( \alpha v \) retroviral vectors (Fig. 3 i). The proportion of cells that underwent terminal differentiation was also unaffected by overexpression of \( \alpha v \) or \( \alpha 4 \) (unpublished data).

We conclude that in the SCC lines examined elevated expression of \( \alpha v \) stimulated anoikis independent of whether or not the parental cells expressed \( \alpha v \). However, overexpression of \( \alpha v \) in primary keratinocytes was not sufficient to induce anoikis.

**Role of PI3 kinase activation in anoikis**

Signaling pathways known to protect cells from anoikis include FAK activation leading to Erk MAPK phosphorylation and PI3 kinase activation resulting in activation of PKB/Akt (Frisch et al., 1996). There is conflicting evidence regarding c-Jun kinase and p38 MAPK (Frisch et al., 1996; Khwaja and Downward, 1997). To examine the role of these pathways in anoikis of H357 cells, we added the PI3 kinase inhibitor LY294002, the MEK1/2 inhibitor UO126, and the p38MAPK inhibitor SB203580 to H357 cells transduced with \( \alpha v \) or \( \alpha 4 \) and measured the proportion of apoptotic cells after 72 h in suspension (Fig. 4 a). z-VAD-fmk was added as
Figure 3. Expression of αvβ5 in SCC cells induces anoikis. (a) DNA content of H357 cells (parental or transduced with αv or α4) after 0, 24, 48, or 72 h in suspension. Regions corresponding to sub-G1, G1, S, and G2+M are indicated. (b) Percentage of anoikis was determined as the percentage of cells with sub-G1 DNA content. SEM of six experiments are shown, comparing parental H357 cells to those expressing empty vector (pBabe puro), α4, or αv. (c) H357 cells transduced with αv suspended in medium alone (Cont) or supplemented DMSO, z-VAD-fmk (Ci) at 100 μM, 100 μg/ml vitronectin (VN), or 100 μg/ml 13C2 anti-αv. Means and SDs of three experiments are shown. (d–g) Flow cytometry profiles of SCC4 and primary human keratinocytes (strain km) labeled with secondary antibody alone (dashed lines) or antibodies to αv or α4 integrins. Gray lines, parental cells; black lines, cells transduced with αv (d and f) or α4 (e and g). (h and i) Percentage of anoikis (sub-G1 DNA) of parental SCC4 cells and primary keratinocytes (km) or cells transduced with αv or α4 integrin subunits. SEM of three experiments are shown.
with an antibody to phosphoAkt 473 or actin (loading control).

The proapoptotic effect of αvβ5 is not shared with αvβ6 and is mediated by the β5 cytoplasmic domain

Because up-regulation of αvβ6 is a feature of many SCCs, we investigated whether or not this integrin, like αvβ5, promoted anoikis. H357 cells transduced with the αv retroviral vector were infected with a second vector that encoded the β6 integrin subunit. Introduction of β6 had no effect on cell surface levels of αv (Fig. 5 a; compare Fig. 1 b). However, cells no longer expressed surface αvβ5 (detected with P1F6 antibody); instead all of the αv on the cell surface was now in a heterodimer with the β6 subunit (detected with 10D5 antibody; Fig. 5 a).

The relative levels of integrins in H357 cells were also compared by Western blotting (Fig. 5, c–e). As expected, there was no difference in β1 integrin levels between parental H357 cells and cells expressing αvβ5 or αvβ6 (Fig. 5 c). The αv subunit was not detected in parental cells and was expressed at similar levels in cells transduced with αv (αvβ5-expressing cells) or with αv and β6 (Fig. 5 c). There was no detectable β6 protein in parental H357 cells; β6 was weakly detectable in cells transduced with αv alone and was strongly expressed in cells transduced with both αv and β6 (Fig. 5 d). The β5 protein was detectable in all three cell populations, but was more abundant in cells transduced with αv than parental cells or cells transduced with αv and β6 (Fig. 5 e). The β5 protein band in Western blots of parental and αvβ6 cells was less diffuse and migrated slightly faster than in cells transduced with αv, which is consistent with intracellular accumulation and incomplete glycosylation (Fig. 5 e).

In contrast to cells expressing αvβ5, cells expressing αvβ6 did not undergo anoikis and had the same sub-G1 DNA profiles as parental H357 cells (Fig. 5 f) and cells transduced with the αv integrin subunit (Fig. 5 g). Whereas cells expressing αvβ5 did not activate Akt in suspension, cells expressing αvβ6 did activate Akt (Fig. 5 h) and the kinetics were similar to parental cells and cells expressing αv (Fig. 4 b and not depicted). Akt activation in αvβ6-expressing cells was blocked by treatment with the PI3 kinase inhibitor LY294002 (Fig. 5 h).

To investigate whether or not the cytoplasmic domain of the β5 subunit conferred sensitivity to anoikis, we constructed a chimeric integrin subunit, consisting of the β6 extracellular and transmembrane domains and the β5 cytoplasmic domain (β6X5C). H357 cells were doubly infected with αv and β6X5C retroviral vectors, and cells were examined by flow cytometry with an αvβ6-specific antibody (Fig. 5 b). Cells transduced with β6X5C alone did not express the chimera on the cell surface, confirming that it only heterodimerized with αv (unpublished data).

Cells were placed in suspension for 72 h, and the proportion of apoptotic cells was compared in parental H357 cells and cells expressing αvβ5, αvβ6, or αvβ6X5C. The results of one experiment are shown in Fig. 5 f, and data pooled from three separate experiments are shown in Fig. 5 g. H357 cells expressing αvβ6X5C were as susceptible to anoikis as cells expressing αvβ5. We conclude that the cytoplasmic domain of β5 mediates the proapoptotic effect of αvβ5.

The ability of H357 cells to avoid anoikis again correlated with their ability to activate Akt in suspension (Fig. 5 i). Parental cells and cells expressing αvβ6 activated Akt, whereas cells transduced with αv (expressed on the cell surface as αvβ5) or αvβX6C5 did not.

**Activation of Akt can overcome the proapoptotic effect of αvβ5**

If αvβ5 expression triggers anoikis by preventing activation of Akt, then constitutive activation of Akt should allow αvβ5-
expressing cells to survive in suspension. Two Akt constructs were introduced into H357 cells. The first construct, M+Akt:ER*, has a myristoylation targeting sequence fused to the NH2 terminus of a constitutively active form of Akt lacking the PH domain (myrAkt del4-129). At the COOH terminus is a modified form of the hormone-binding domain of the mouse estrogen receptor (ER) that binds 4-hydroxytamoxifen (OHT) but is refractory to estrogen. In response to OHT, M+Akt:ER* is rapidly recruited to the plasma membrane where it is activated by local PI3 kinases. The second con-
struct, A2M+Akt:ER*, acts as an inactive control because the myristoylated glycine at the second amino acid position has been converted to alanine to eliminate the membrane-targeting function (Kohn et al., 1998). M+Akt:ER* and A2M+ Akt:ER* were expressed in retroviral vectors and introduced into parental and integrin-expressing H357 cells. Adherent cultures were treated with OHT for 1 h, lysed, and examined by Western blotting. An antibody to the mutant ER detected expression of the Akt constructs at equal levels in all cell populations (Fig. 6 a). The level of endogenous serine 473–phosphorylated Akt was also the same in all cells (Fig. 6 a). However, only cells expressing M+Akt:ER* had a second band detected by anti-phospho-Akt, which migrated at 97 kD, the size of the AktER fusion protein (Fig. 6 a). H357 parental cells and cells expressing αv or α4 were transduced with the active or inactive Akt constructs and placed in suspension for 72 h in the presence of OHT (Fig. 6 b and c). Parental H357 cells and α4-expressing cells had a low level of anoikis that was not influenced by Akt activation. H357 cells expressing αvβ5 and the inactive Akt construct underwent anoikis, and the proportion of apoptotic cells was equivalent to that seen in cells expressing αvβ5 alone (Fig. 6 c). H357 cells expressing active Akt were protected from anoikis and had the same low level of apoptotic cells as H357 parental cells and cells expressing αv integrin (αvMyr and αvA2, respectively) and treated with OHT (1 μM) for 1 h. The antibody to phospho-Akt detects endogenous phospho-Akt in all cells (bottom band), and phosphorylated M’Akt:ER in cells expressing that construct. (bottom) Probed with anti-ER. (b and c) Anoikis of H357 cells transduced with M’Akt:ER* (Myr) or A2Akt:ER* (A2) alone (parent) or in combination with αv or α4 integrins. Cells were suspended for 72 h in the presence of OHT. (c) SEM of three experiments. (d) Western blot of H357αvMyr or αvA2 suspended for 72 h with 0.1 μM OHT, probed with anti-phosphoAkt473 (top), total Akt (middle), and ER (bottom). (top panel) Anti-phosphoAkt recognizes two bands, corresponding to endogenous Akt (bottom band) and to phosphorylated M’Akt:ER (top band).

**Figure 6. Akt activation protects against anoikis.** (a) Western blot of adherent H357 cells transduced with M’Akt:ER* or A2Akt:ER* alone (parMyr and parA2, respectively) or in combination with αv integrin (αvMyr and αvA2, respectively) and treated with OHT (1 μM) for 1 h. (top) The antibody to phospho-Akt detects endogenous phospho-Akt in all cells (bottom band), and phosphorylated M’Akt:ER in cells expressing that construct. (bottom) Probed with anti-ER. (b and c) Anoikis of H357 cells transduced with M’Akt:ER* (Myr) or A2Akt:ER* (A2) alone (parent) or in combination with αv or α4 integrins. Cells were suspended for 72 h in the presence of OHT. (c) SEM of three experiments. (d) Western blot of H357αvMyr or αvA2 suspended for 72 h with 0.1 μM OHT, probed with anti-phosphoAkt473 (top), total Akt (middle), and ER (bottom). (top panel) Anti-phosphoAkt recognizes two bands, corresponding to endogenous Akt (bottom band) and to phosphorylated M’Akt:ER (top band).

αvβ5-induced anoikis of H357 cells is not mediated by activation of the death receptor pathway

Apoptosis can be triggered by intrinsic or extrinsic pathways. The intrinsic pathway involves alterations in mitochondrial homeostasis and activation of the apotosome;
the extrinsic pathway occurs through ligand binding of death domain surface receptors such as Fas (Grossmann, 2002). Experiments on MDCK cells, derived from a simple epithelium, have shown that anoikis is induced by the extrinsic pathway, involving death receptors and their associated adaptor molecules (Frisch, 1999a; Rytömaa et al., 1999). MDCK cell anoikis can be blocked by a dominant-negative (dn) FADD (FAS associated death domain protein) mutant (dNFADD), which cannot activate caspase 8. Caspase 8 can also be stimulated by a mitochondrially driven feedback mechanism (intrinsic pathway) involving caspase 9 (Rytömaa et al., 1999).

Suspension experiments were performed in the presence of the general caspase inhibitor z-VAD-fmk (Fig. 4 a and Fig. 7 a), the caspase 8 specific inhibitor z-IETD-fmk, and the caspase 9 inhibitor z-LEHD-fmk (Fig. 7 a). All three drugs inhibited apoptosis of \( \alpha v \) expressing cells to the same extent (Fig. 7 a and b). This finding suggested that the intrinsic pathway was involved in anoikis.

To determine whether or not anoikis was indeed death receptor independent, we introduced dNFADD into \( \alpha v \)-expressing H357 cells. The dNFADD construct, tagged with eGFP, was subcloned into pBabe puro and used to infect H357 cells. A high level of expression of dNFADD in over 90% of cells was achieved (Fig. 7 c). Suspension experiments were performed comparing parental H357 cells, \( \alpha v \)-expressing H357 cells alone, and \( \alpha v \)-expressing H357 cells in combination with dNFADD. Expression of dNFADD had no effect on anoikis of \( \alpha v \)-expressing H357 cells (Fig. 7 d). As a positive control, dNFADD was also introduced into MDCK cells; it inhibited anoikis even more effectively than z-VAD-fmk (Fig. 7 e). This finding suggests that FADD does not play a role in \( \alpha v \)-mediated anoikis.

**Inhibition of caspase 8 increases Akt activity**

We investigated potential mechanisms by which Akt phosphorylation was down-regulated when \( \alpha v \)-expressing H357 cells were held in suspension for 72 h. PTEN is a negative reg-
The Journal of Cell Biology

428

FMK, z-IETD-FMK, z-LEHD-FMK, or DMSO. Amount of protein loaded blots of H357 cells transduced with b). Actin (a) and Erk MAPK (b) were loading controls. (c) Western and probed with antibodies to PTEN (a) or PI3 kinase (p110 

Discussion

When the αv integrin subunit was introduced into αv-negative H357 cells it formed a functional heterodimer on the cell surface with the β5 subunit. Expression of αvβ5 induced anoikis, which could be blocked by integrin ligation or caspase inhibition but not by blocking FADD death receptor signaling. The effect was not specific for H357 cells because a second SCC line was also stimulated to undergo anoikis by overexpression of αv. Anoikis was dependent on the β5 cytoplasmic domain and was not observed in cells expressing αvβ6. Anoikis of parental H357 cells was induced with a P13 kinase inhibitor and correlated with a failure of αvβ5-expressing cells to activate Akt in suspension. Resistance to anoikis was achieved by constitutive activation of Akt or caspase inhibition. Caspase 8 inhibition resulted in increased phosphoAkt levels, placing caspase 8 upstream of Akt. We conclude that αvβ5 confers susceptibility to anoikis in SCC cells and that this is overridden when β5 is removed from the cell surface through loss of αv or up-regulation of the β6 integrin subunit. Up-regulation of β6 is a common feature of human SCCs, and we propose that this is a means of allowing growth of tumor cells in the absence of a basement membrane. This is a novel way in which up-regulation of αvβ6 contributes to cancer progression.

The ability of integrin ligation to suppress apoptosis is well established, and several different mechanisms have been reported, reflecting a degree of cell type and integrin specificity (for review see Jost et al., 2001; Miranti and Brugge, 2002). Ligation of integrins can induce antiapoptotic proteins, such as Bcl-2 (Matter and Ruoslahti, 2001), or suppress proapoptotic proteins, such as Bax (Gilmore et al., 2000; Valetijn et al., 2003). Ligation of α5β1 or αvβ3 increases Bcl-2 expression through NFκB activation (Scatena et al., 1998). Integrin activation of FAK leads to increased expression of inhibitors of apoptosis proteins, which bind to and inhibit executioner caspases (Sonoda et al., 2000).

Previous work on anoikis has demonstrated the involvement of death receptor signaling and the subsequent activation of the extrinsic death pathway via caspase 8 activation (Frisch, 1999a; Rytömaa et al., 2000). αvβ5-mediated anoikis could not be inhibited with dnFADD, suggesting that unlike anoikis of endothelial cells and simple epithelial cells (Frisch, 1999a; Rytömaa et al., 1999; Hood et al., 2003), SCC anoikis is independent of death receptor signaling. In endothelial cells, αvβ5 ligation protects against FADD-mediated apoptosis (Hood et al., 2003), demonstrating that the same integrin can regulate apoptosis by distinct mechanisms, dependent on cellular context. αvβ5-mediated anoikis could be blocked with inhibitors of caspase 8 and 9. Caspase 8 can be activated independently of the extrinsic death pathway in cells with unligated integrins (Stupack et al., 2001). Caspase 8 cleaves Bid (a Bcl-2 family member), triggering mitochondrial cytochrome c release and activation of caspase 9 (Rytömaa et al., 2000). Caspase 9 may then lead to further cleavage and activation of caspase 8, hence amplifying the signal (Rytömaa et al., 2000).

It has previously been reported that whereas unligated β1 and β3 integrin subunits can directly activate caspase 8, β5 does not (Stupack et al., 2001). In agreement with this report, we were unable to coimmunoprecipitate αvβ5 or αvβ6 with caspase 8 in H357 cells under a variety of conditions (unpublished data). In H357 cells expressing αvβ5 it is more likely that the β5 cytoplasmic tail activates caspase 8 indirectly via effects on the cytoskeleton. In some dying cells, actin, integrins, and caspase 8 have been seen to colocalize in complexes that lack death receptors and FADD (Stupack et al., 2001), and local accumulation of caspase 8 is sufficient to trigger apoptosis. The mechanism of the association of caspase 8 with the cytoskeleton is not known, but newly described proteins Hip and Hippi, homologous to cytoskeleton linker proteins talin and myosin, have been found that include “pseudo-death effector domains” (Gervais, 2002).

In contrast to αv-negative H357 cells and cells expressing αvβ6, cells that expressed αvβ5 did not activate Akt in suspension. The pro-survival function of Akt is well established. Activated Akt can phosphorylate and inhibit several different proapoptotic proteins including BAD (Datta et al., 1997) and caspase 9 (Cardone et al., 1998). Active Akt also induces NFκB (Kane et al., 1999), which reduces the release of cyto-
chrome c from mitochondria (Kennedy et al., 1999). Akt is negatively regulated by the phosphatase PTEN, and a lack of PTEN leads to increased resistance to apoptosis (Lu et al., 1999). However, there were no differences in PTEN levels in parental H357 cells or cells expressing αvβ5 or αvβ6.

The mechanism by which unligated αvβ5 triggers anoikis requires activation of caspases 8 and 9 and suppression of Akt activity. Because there was no reduction in total Akt when αvβ5-expressing H357 cells were held in suspension, caspase-dependent cleavage of Akt (Bachelder et al., 2001) does not appear to be involved. Nevertheless, inhibition of caspase 8 did lead to an increase in phospho-Akt, placing caspase 8 upstream of the suppression of Akt activation. Inhibition of caspase 8 did not increase phospho-Akt, leading us to speculate that Akt is upstream of caspase 9 (Cardone et al., 1998).

One striking difference between primary human keratinocytes and SCC cells is that primary keratinocytes undergo terminal differentiation and not anoikis when held in suspension (Gandarillas et al., 1999). Moreover, when unligated integrins are expressed in the suprabasal layers of the epidermis, apoptosis is not stimulated (Carroll et al., 1995). When αv was overexpressed in primary human keratinocytes they retained the ability to terminally differentiate and did not undergo anoikis. It seems possible that the selection pressure placed on clones of αv-transfected H357 cells described previously selected for rare cells that had the ability to terminally differentiate rather than apoptosis (Jones et al., 1996).

The similarities between apoptosis and keratinocyte terminal differentiation have previously been discussed and include inhibition of suspension-induced differentiation by integrin ligation (Levy et al., 2000) and by caspase inhibitors (Allombert-Blaise et al., 2003). Akt activation plays a role in driving the later stages of keratinocyte terminal differentiation (Janes et al., 2004). We speculate that by preventing Akt activation, αvβ5 acts as a fail-safe device, triggering anoikis in SCC cells that have lost the ability to differentiate. Resistance to anoikis can potentially enhance epithelial neoplasia by allowing cells to proliferate in the absence of attachment to extracellular matrix and to leave their normal environment and travel to distant body sites, thereby forming metastases. Up-regulation of αvβ6 is a novel mechanism by which tumor cells can avoid anoikis.

Materials and methods

Cell culture
Human epidermal keratinocytes (strain km, passage 2-6) were cultured on a feeder layer of J2 3T3 cells in FAD medium (1 part Ham's F12, 3 parts DME, and 180 μM adenine) supplemented with 10% FCS and HICE medium (0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml cholera enterotoxin, and 10 ng/ml EGF), essentially as described previously (Levy et al., 1999). The H357 (Sugiyama et al., 1993; a gift from S. Prime, University of Bristol, Bristol, UK) and SCC4 (Evans et al., 2003) cell lines were cultured in FAD, 10% FCS, and HICE without a feeder layer. J2-3T3 cells and puro-resistant J2-3T3 cells (J2-puro; Levy et al., 1998) were cultured in DME and 10% donor calf serum. 2.5 μg/ml puromycin was added to the medium of J2-puro cells. MDCK cells and retroviral packaging cells were cultured in DME and 10% FCS.

Retroviral vectors
To generate αvβ5 puro, human αv cDNA (provided by J. Loftus, Scripps Research Institute, La Jolla, CA) was blunt-end inserted into Snabl cut, shrimp alkaline phosphatase–treated pBabe puro vector (Levy et al., 1998) using T4 ligase. αvβ5 puro and β6pBabe puro were gifts from J. Marshall and I. Hart (Cancer Research UK, London, UK; Thomas et al., 2001b). M’Akt:ER* and AM2: Akt:ER (Kohn et al., 1998; Mirza et al., 2000) in retrovirus vector pWZLneo were gifts of L.M. Martins and J. Downward (Cancer Research UK, London, UK). eGFP-dNFAFD:AU1 was exogenously expressed from pCAG-fdNFAFD:AU1 (gift of J. Downward; Hydema et al., 1999) with Nil and BamHI, blunt and inserted into Snabl cut, shrimp alkaline phosphatase–treated pBabe puro using T4 ligase.

The chimeric β6β5 integrin subunit (β6X5C) was constructed as follows. The β6 extracellular and transmembrane domain was made by PCR using β6pBabe puro as a template. The primers used had the BamHI and EcoRI sites added and were as follows: 5’-GGGATCCTCCACATGCGGAT- TGAACCTGTTCCCTGTCTTT-3’; 3’-GGATTCTTTCGATGACACTGAGTACGACAAACCTCCATAGA. The resulting β6 cytoplasmic domain-deleted PCR product (β6ΔC) was cloned into pCR-4-TOPO, cut with EcoRI and BamHI, and ligated into pBabe puro. The cytoplasmic domain of β5 was based as part of an I.M.A.G.E. clone (7160-k21; Geneservice) and generated by PCR using primers that had an EcoRI site at the 5’ prime end and SalI at the 3’ end. The primers were as follows: 5’-GGGATCCTCGTCTTGCACACTCATTCAAGCACGAGAGGATTT-3’; 3’-GAGTTCGACCTACGCACTGGGCGTCTTGAGGTTTGTG. The confirmed sequence was cut using EcoRI and SalI and ligated into β6ΔC pBabe puro puro giving the β6X5C chimeric molecule, but with an additional EcoRI site. This site was removed using Mung Bean Nuclease. The cDNA was then religated, bringing the chimeric integrin subunit correctly into frame.

Retroviral transduction
Ecotropic Phoenix packaging cells were transiently transfected with retroviral vectors, and virus-containing supernatants were used to infect AM12 packaging cells, as described previously (Legg et al., 2003). 60-85% of AM12 cells transduced with retrovirally encoded integrin subunits had high surface expression of the integrins following puromycin selection; these cells were selected by FACS® to achieve high expression in >95% of the population (Levy et al., 1998). Stably transduced AM12 cells were cultured with 2.5 μg/ml puromycin or, in the case of the Akt retroviral vectors, 2 mg/ml G418 (Geneticin; GibCO BRL).

SCC cells and primary keratinocytes were transduced with retroviral vectors either by culture with AM12 cells (Levy et al., 1998) or by incubation with AM12 supernatant (Legg et al., 2003). 70-85% of epithelial cells had high surface expression of the retrovirally encoded integrin subunits after puro selection; these cells were further enriched by FACS® as described for AM12 cells.

When cells were transduced with two retroviral vectors, selection was either with puromycin and FACS® or with puromycin and neomycin. SCC and primary keratinocytes transduced with retroviral vectors were cultured with 1 μg/ml puromycin and/or 2 mg/ml G418, as appropriate.

Antibodies
The following mAbs to human integrins were used: HAS4 (α2β1), VM-2 (α3β1), 7.2R (α4; a gift from J. Marshall and I. Hart), 13C2 (αv subunit), P1W7 (αv subunit), P1D6 (α5β1; Chemicon), goH3 (α6 integrins), P1F6 (αvβ5), AB1932 (αvβ3; Chemicon), 23C6 (αvβ3), 10D5 (αvβ6; Chemicon), MPM410 (α6 integrins), P5D2 (β1 integrins), and 3E1 (β4 integrins). We used JG22 to detect chick β1 integrins. Actin was detected with AC40 (Sigma-Aldrich), transglutaminase 1 with B.C1 (a gift of R. Rice, University of California, Davis, Davis, CA), and involucrin with SY5. An antibody directed against involucrin was purchased from Molecular Probes, and HRP-conjugated species-specific secondary antibodies conjugated to Alexa 488 or Alexa 594 were purchased from Molecular Probes, and HRP-conjugated secondary antibodies were purchased from Amersham Biosciences.

FACS®, flow cytometry, and immunofluorescence staining
Live cells were labeled with antibodies diluted in PBS/AB and sorted into FAD, 10% FCS, and HICE medium using a FACSVantage® machine (Becton Dickinson), as described previously (Levy et al., 1998). Intracellular transglutaminase I expression was examined by flow cytometry of PFA-fixed, saponin-permeabilized cells, as described previously (Gandarillas et al., 1999), using a FACScan® machine (Becton Dickinson).
DNA content of ethanol-fixed cells stained with 5μg/ml propidium iodide in the presence of RNase A (50 μg/ml) was determined with a FACS-Calibur flow cytometer.

Focal adhesions were visualized in cells fixed for 10 min with 4% paraformaldehyde in PBS and 0.1% Triton X-100, as described previously (Levy et al., 2000). Cells were examined at RT with a confocal laser scanning microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) and software (Carl Zeiss MicroImaging, Inc.) using a 40/NA 1.2 objective (Carl Zeiss MicroImaging, Inc.).

Adhesion assays
Vitronectin was purified from human plasma (Cheresh and Sprio, 1987) and coated onto 96-well bacterial plates (Evans et al., 2003). Cells were labeled with 5 μM of CellTracker dye (Molecular Probes), harvested with trypsin/EDTA, and resuspended at 4 x 10^5 cells/ml in TBS containing 1 mM Mn^2+; 100 μl of cell suspension was added per well and incubated at 37°C for 30 min. The number of adherent cells was quantitated as described previously (Evans et al., 2003).

Anchorage-independent growth and suspension culture
Anchorage independent growth in soft agar was evaluated as described previously (Jones et al., 1996). 5 x 10^3 cells per 35-mm dish in 0.3% agar were cultured for 3 wk, and then stained with 1 mg/ml of nitroblue tetrazolium (Sigma-Aldrich), and U0126 (10 μM; Sigma-Aldrich), LY294002 (50 μM; Promega). Cells were stained for 4 h, 150 mM NaCl, 0.1% NP-40, 0.1% SDS, 0.5% deoxycholate, 5 mM EDTA, and 1% Triton X-100, with protease and phosphatase inhibitor cocktails (Sigma-Aldrich; Hobbs and Watt, 2003). Soluble proteins were resolved by SDS-PAGE on 10% Laemmli gels and transferred onto Immobilon PVDF membranes (Millipore). Antibody labeling was performed as described previously using a chemiluminescence kit (Western Lightning; NEN Life Science Products) for detection (Hobbs and Watt, 2003).

Western blotting
Cells were lysed on ice in a modified RIPA buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 5 mM EDTA, and 1% Triton X-100, with protease and phosphatase inhibitor cocktails (Sigma-Aldrich; Hobbs and Watt, 2003). Soluble proteins were resolved by SDS-PAGE on 10% Laemmli gels and transferred onto Immobilon PVDF membranes (Millipore). Antibody labeling was performed as described previously using a chemiluminescence kit (Western Lightning; NEN Life Science Products) for detection (Hobbs and Watt, 2003).

References
Agrez, M., X. Gu, J. Turton, C. Meldrum, J. Niu, T. Antalis, and E.W. Howard. 1999. The αvβ6 integrin induces gelatinase B secretion in colon cancer cells. Int. J. Cancer. 81:90–97.
Ahmed, N., J. Niu, D.J. Dorathy, X. Gu, S. Andrews, C.J. Meldrum, R.J. Scott, M.S. Baker, I.G. Macreadie, and M.V. Agrez. 2002. Direct integrin αvβ6-ERK binding: implications for tumour growth. Oncogene. 21:1370–1380.
Allomber-Blaise, C., S. Tanji, L. Mortier, H. Fauvel, M. Tual, E. Delaporte, F. Gervais, F.G., R. Singaraja, S. Xanthoudakis, C.A. Gutekunst, B.R. Leavitt, M. Piette, E.M. DeLassale, P. Formstecher, P. Marchetti, and R. Polakowska. 2002. Direct integrin αvβ6 binding: implications for tumour growth. J. Cell Biol. 158:933–943.

We thank K. Nishi, J. Downward, R. Evans, R. Hobbs, D. Owens, S. Broad, and D. Campbell for advice, reagents, and practical help. H357 cells were a kind gift from S. Prime.

S.M. Janes was the recipient of a Medical Research Council Clinical Fellowship. This work was supported by Cancer Research UK.

Submitted: 9 December 2003
Accepted: 10 June 2004

Expression of the β6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. J. Cell Sci. 108:2241–2251.
Cardone, M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, and J.C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science. 282:1318–1321.
Carroll, J.M., M.R. Romero, and F.M. Watt. 1995. Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. Cell. 83:957–968.
Cheresh, D.A., and R.C. Sprio. 1987. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J. Biol. Chem. 262:17703–17711.
Datta, S.R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M.E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 91:231–241.
Davies, J., J. Warwick, N. Totty, R. Philip, M. Helfrich, and M. Horton. 1989. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J. Cell Biol. 109:1817–1826.
Evans, R.D., V.C. Perkins, A. Henry, P.E. Stephens, M.K. Robinson, and F.M. Watt. 2003. A tumor-associated β1 integrin mutation that abrogates epithelial differentiation control. J. Cell Biol. 160:589–596.
Frisch, S.M. 1999a. Evidence for a function of death-receptor-related, death-domain-containing proteins in anokiosis. Curr. Biol. 9:1047–1049.
Frisch, S.M. 1999b. Methods for studying anokiosis. Methods Mol. Biol. 129:251–256.
Frisch, S.M., and H. Francis. 1994. Disruption of epithelial cell–matrix interactions induces anokiosis. J. Cell Biol. 124:619–626.
Frisch, S.M., K. Vuori, D. Kela, and S. Sicks. 1996. A role for Jun-N-terminal kinase in anokiosis; suppression by bcl-2 and cmrA. J. Cell Biol. 135:1377–1382.
Gandarillas, A., L.A. Goldsmith, S. Gschmeissner, I.M. Leigh, and F.M. Watt. 1999. Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes. Exp. Dermatol. 8:71–79.
Gervais, F.G., R. Singaraja, S. Xanthoulakis, C.A. Gutekunst, B.R. Levitt, M. Menet, A.S. Hackam, J. Tam, J.P. Vaillancourt, V. Houzatger, et al. 2002. Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hipp. Nat. Cell Biol. 4:95–105.
Gilmore, A.P., A.D. Metcalfe, L.H. Romer, and C.H. Streuli. 2000. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. J. Cell Biol. 149:431–446.
Grossmann, J. 2002. Molecular mechanisms of “detachment-induced apoptosis–Anokiosis”. Apoptosis. 7:247–260.
Haapasalmi, K., K. Zhang, M. Tonnesen, J. Olerud, D. Sheppard, T. Salo, R. Krohn, R.A. Clark, Y.J. Uitto, and H. Larjava. 1996. Keratinocytes in human wounds express αvβ6 integrin. J. Invest. Dermatol. 106:42–48.
Hobbs, R.M., and F.M. Watt. 2003. Regulation of interleukin-1α expression by integrins and epidermal growth factor receptor in keratinocytes from a mouse model of inflammatory skin disease. J. Biol. Chem. 278:19798–19807.
Hood, J.D., R. Frausto, W.B. Kiouss, M.A. Schwartz, and D.A. Cheresh. 2003. Differential αvβ5 integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. J. Cell Biol. 162:933–943.
Janes, S.M., T. Olsfad, D.H. Campbell, F.M. Watt, and D.M. Prowse. 2004. Transient activation of FOXN1 in keratinocytes induces a transcriptional programme that promotes terminal differentiation: contrasting roles of FOXN1 and Akt. J. Cell Sci. In press.
Jones, J., M. Sugiyama, P.M. Speight, and F.M. Watt. 1996. Restoration of αvβ5 integrin expression in neoplastic keratinocytes results in increased capacity for terminal differentiation and suppression of anchorage-independent growth. Oncogene. 12:119–126.
Jones, J., F.M. Watt, and P.M. Speight. 1997. Changes in the expression of αv integrins in oral squamous cell carcinomas. J. Oral Pathol. Med. 26:63–68.
Jost, M., T.M. Huggett, C. Kari, and U. Rodeck. 2001. Matrix-independent survival of human keratinocytes through an EGFR receptor/MAPK-kinase-dependent pathway. Mol. Biol. Cell. 12:1519–1527.
Kane, L.P., V.S. Shapiro, D. Stokoe, and A. Weiss. 1999. Induction of NF-κB by the Akt/PKB kinase. Curr. Biol. 9:601–604.
Kennedy, S.G., E.S. Kandel, T.K. Cross, and N. Hay. 1999. Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol. Cell. Biol. 19:5800–5810.
Khwaja, A., and J. Downward. 1997. Lack of correlation between activation of
Jun-NH$_2$-terminal kinase and induction of apoptosis after detachment of epithelial cells. *J. Cell Biol.* 139:1017–1023.

Kohn, A.D., A. Barthel, K.S. Kovacina, A. Bege, W. Wallach, S.A. Summers, M.J. Birnbaum, P.H. Scott, J.C. Lawrence, Jr., and R.A. Roth. 1998. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J. Biol. Chem.* 273:11937–11943.

Koistinen, P., and J. Heino. 2002. The selective regulation of integrin structure, function and distribution of pulmonary inflammation and fibrosis. *J. Biol. Chem.* 275:16309–16315.

Kohn, A.D., A. Barthel, K.S. Kovacina, A. Boge, B. Wallach, S.A. Summers, M.J. Birnbaum, P.H. Scott, J.C. Lawrence, Jr., and R.A. Roth. 1998. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J. Biol. Chem.* 273:11937–11943.

Koistinen, P., and J. Heino. 2002. The selective regulation of integrin structure, function and distribution of pulmonary inflammation and fibrosis. *J. Biol. Chem.* 275:16309–16315.

Legg, J., U.B. Jensen, S. Broad, I. Leigh, and F.M. Watt. 2003. Role of melanoma chondroitin sulphate proteoglycan in patterning stem cells in human interfollicular epidermis. *Development*. 130:6049–6063.

Levy, L., S. Broad, A.J. Zhu, J.M. Carroll, I. Khazaal, B. Peault, and F.M. Watt. 1998. Optimised retroviral infection of human epidermal keratinocytes: long-term expression of transduced integrin gene following grafting on to SCID mice. *Gene Ther.* 5:913–922.

Levy, L., S. Broad, D. Diekmann, R.D. Evans, and F.M. Watt. 2000. β1 integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms. *Mol. Biol. Cell.* 11:453–466.

Lu, Y., Y.Z. Lin, R. Lapushin, B. Cuevas, X. Fang, S.X. Yu, M.A. Davies, H. Khan, T. Furui, M. Mao, et al. 1999. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene.* 18:7034–7045.

Matter, M.L., and E. Ruoslahti. 2001. A signaling pathway from the α5β1 and αvβ3 integrins that elevates bcl-2 transcription. *J. Biol. Chem.* 276:27757–27765.

Mercurio, A.M., and I. Rahinovitz. 2001. Towards a mechanistic understanding of tumor invasion–lessons from the α6β4integrin. *Semin. Cancer Biol.* 11:129–141.

Miranti, C.K., and J.S. Brugge. 2002. Sensing the environment: a historical perspective on integrin signal transduction. *Nat. Cell Biol.* 4:E83–E90.

Mirza, A.M., A.D. Kohn, R.A. Roth, and M. McMahon. 2000. Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. *Cell Growth Differ.* 11:279–292.

Munger, J.S., X. Huang, H. Kawakatsu, M.J. Griffiths, S.L. Dalton, J. Wu, J.F. Pittert, N. Kaminiski, C. Garat, M.A. Marthay, et al. 1999. The integrin αvβ6 binds and activates latent TGF β1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell.* 96:319–328.

Pasqualini, R., J. Bedorova, S. Ye, and M.E. Hemler. 1993. A study of the structure, function and distribution of β3 integrins using novel anti-β3 monoclonal antibodies. *J. Cell Sci.* 105:101–111.

Ramos, D.M., M. But, J. Regezi, B.L. Schmidt, A. Atakilit, D. Dang, D. Ellis, R. Jordan, and X. Li. 2002. Expression of integrin β6 enhances invasive behavior in oral squamous cell carcinoma. *Matrix Biol.* 21:297–307.

Regezi, J.A., D.M. Ramos, R. Pytyla, N.P. Dekker, and R.C. Jordan. 2002. Tenascin and β6 integrin are overexpressed in floor of mouth in situ carcinomas and invasive squamous cell carcinomas. *Oral Oncol.* 38:332–336.

Ryttömaa, M., L.M. Martins, and J. Downward. 1999. Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr. Biol.* 9:1043–1046.

Ryttömaa, M., K. Lehmann, and J. Downward. 2000. Matrix detachment induces caspase-dependent cytocrome c release from mitochondrial inhibition by PKB/Akt but not Raf signalling. *Oncogene.* 19:4661–4668.

Scatena, M., M. Almeida, M.L. Chaisson, N. Fausto, R.F. Nicosia, C.M. Giachelli, P.D. Arora, J. Ma, W. Min, T. Cruz, and C.A. McElloch. 1998. NF-κB mediates αvβ3 integrin–induced endothelial cell survival. *J. Cell Biol.* 141:1083–1093.

Sonoda, Y., Y. Matsumoto, M. Funakoshi, D. Yamamoto, S.K. Hanks, and T. Kasahara. 2000. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J. Biol. Chem.* 275:16309–16315.

Stambolic, V., A. Suzuki, J.L. de la Pompa, G.M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J.M. Penninger, D.P. Siderovski, and T.W. Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.* 95:29–39.

Stupack, D.G., X.S. Puente, S. Boutsaboualoy, C.M. Storgard, and D.A. Cheshire. 2001. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J. Cell Biol.* 155:459–470.

Sugiyama, M., P.M. Speight, S.S. Prime, and F.M. Watt. 1993. Comparison of integrin expression and terminal differentiation capacity in cell lines derived from oral squamous cell carcinomas. *Carcinogenesis.* 14:2171–2176.

Thomas, G.J., M.P. Lewis, I.R. Hart, J.F. Marshall, and P.M. Speight. 2001a. αvβ6 integrin promotes invasion of squamous carcinoma cells through up-regulation of matrix metalloproteinase-9. *Int. J. Cancer.* 92:641–650.

Thomas, G.J., M.P. Lewis, S.A. Whawell, A. Russell, D. Sheppard, I.R. Hart, P.M. Speight, and J.F. Marshall. 2001b. Expression of the αvβ6 integrin promotes migration and invasion in squamous carcinoma cells. *J. Invest. Dermatol.* 117:67–73.

Toker, A., and A.C. Newton. 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PKD-2 site. *J. Biol. Chem.* 275:8271–8274.

Valentin, A.J., A.D. Molteni, A. Lamperti, and A.P. Gilmore. 2003. Spatial and temporal changes in Bax subcellular localization during anoikis. *J. Cell Biol.* 162:599–612.

van der Flier, A., and A. Sonnenberg. 2001. Function and interactions of integrins. *Cell Tissue Res.* 305:285–298.

Watt, F.M. 2002. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J.* 21:3919–3926.