Co-expression of p16\textsuperscript{INK4A} and Laminin 5 by Keratinocytes: A Wound-Healing Response Coupling Hypermotility with Growth Arrest that Goes Awry During Epithelial Neoplastic Progression


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The replicative lifespan of human keratinocytes in culture is restricted by a telomere-unrelated induction of p16\textsuperscript{INK4A} (p16) and p14\textsuperscript{ARF}. We have found that, in vivo, p16 is expressed by epidermal and oral keratinocytes at the migrating fronts of healing wounds and at the stromal interface of severely dysplastic and early invasive lesions and that such cells also invariably display increased expression of Laminin 5 (Lam5). In culture, p16 and Lam5 are coexpressed in keratinocytes at senescence, at the edges of wounds made in confluent cultures, and when cells are plated on dishes coated with the \( \gamma_2 \) precursor form of Lam5 (Lam5; Lam5;2pre). Lam5/p16 coexpression in all three in vitro settings is associated with directional hypermotility and growth arrest. Hypermotility and growth arrest are uncoupled in p16- and p14\textsuperscript{ARF}/p53-deficient keratinocytes and squamous cell carcinoma (SCC) cells; such cells become hypermotile in response to Lam5;2pre but do not grow arrest. Thus, the Lam5/p16 response is activated in normal wound healing, causing growth arrest of migratory keratinocytes that lead wound reepithelialization. This response also becomes activated at a critical stage of neoplastic progression, acting as a tumor suppressor mechanism. Rare premalignant cells that lose p16 remain motile and proliferative, thereby resulting in invasive growth as SCC.

Key words: epidermis/human cells/senescence/tumor suppressor


Squamous cell carcinoma (SCC) is a malignancy of the oral mucosal epithelium, the epidermis, and other stratified squamous epithelia. SCC arise within areas of abnormal, preinvasive cell growth (dysplasia), which may take many years to progress to invasive cancer (Bouquot et al, 1998; Mashberg and Feldman, 1998; Lee et al, 2000b). A consistent feature of invasive SCC and detectable in many premalignant dysplasias is loss, by mutation, deletion, or promoter hypermethylation, of the ability to express functional p16\textsuperscript{INK4A} (p16) (Kamb et al, 1994; Yeudall et al, 1994; Merlo et al, 1995; Shapiro et al, 1996; Califano et al, 1996; Reed et al, 1996; Olshan et al, 1997; Papadimitrakopoulou et al, 1997; Soufir et al, 2000; Poi et al, 2001; reviewed by Rocco and Sidransky, 2001). p16 is a specific inhibitor of the cyclin D1-dependent kinases cdk4 and cdk6 (Serrano et al, 1993; Parry et al, 1995) (see Fig 1). Normally, cells do not express p16 and whether cells cycle or not is controlled by cyclin D1, the expression of which is regulated by mitogen availability and cell–substratum anchorage. Cyclin D1 forms complexes with and activates cdk4 and cdk6, which phosphorylate and inactivate the Rb protein, permitting E2F-dependent transcription of genes whose products are necessary to permit the G1/S transition and initiate chromosome replication (reviewed by Sherr, 1996).

Western blot analysis of normal primary human keratinocytes in culture has identified an increase in p16 levels with serial passage, as the cells approach the end of their replicative lifespan (Loughran et al, 1996; Kiyono et al, 1998; Munro et al, 1999; Dickson et al, 2000; Lee et al, 2000a). Immunocytochemical analysis of such cultures has revealed that p16 expression occurs heterogeneously and abruptly, followed by growth arrest, and that the probability that a keratinocyte will express p16 increases steadily with each passage until all cells are p16-positive and senescent (Dickson et al, 2000; Rheinwald et al, 2002). Our studies have provided strong evidence that, if p16 expression fails to arrest growth, induction of p14\textsuperscript{ARF} expression follows soon thereafter to effect a p53-dependent, p21\textsuperscript{CIP1}-enforced arrest in either G1 or G2, as described in Rheinwald et al (2002) and summarized in the model shown in Fig 2. Keratinocytes engineered to express the catalytic subunit of telomerase, TERT, extend and stabilize their telomeres; yet, still they undergo p16-enforced senescence (Kiyono et al, 1998; Dickson et al, 2000). This fate can be modified by mutational, epigenetic, or regulatory loss/reduction of p16 expression in TERT-transfected cells, yielding immortalized lines (Kiyono et al, 1998; Dickson et al, 2000; Farwell et al, 2000; Rheinwald et al, 2002). p16-enforced keratinocyte

Abbreviations: BUdR, bromodeoxyuridine; CM, conditioned medium; \( \gamma_2 \)pre, \( \gamma_2 \) precursor form of Laminin 5; HPV, human papillomavirus; KMA, keratinocyte motility/arrest; K-sfm, keratinocyte serum-free medium; Lam5, Laminin 5; p16, p16\textsuperscript{INK4A}; PD, population doublings

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senescence is distinct from the telomere-sensitive, p53/p21cip1-dependent replicative senescence mechanism that enforces the replicative lifespan limit of human skin fibroblasts and several other cell types (Bond et al., 1994; Brown et al., 1997; Stein et al., 1999; Rheinwald et al., 2002).

Humans and mice that inherit a heterozygous or homozygous loss of function mutation in the p16 gene (Gruis et al., 1995; Sharpless et al., 2001, 2004) or that express a p16-insensitive mutant form of cdk4 (Rane et al., 2002) are predisposed to a variety of spontaneous and carcinogen-induced cancers, but they undergo normal development and form structurally and functionally normal stratified squamous epithelia. These results confirm the tumor suppressor function of p16 and are also consistent with the finding that p16 protein is not expressed as a feature of normal stratified squamous epithelia. Senescence is a stage of neoplastic progression toward SCC at which the p16 protein becomes expressed and functions as a tumor suppressor. Our recent study, summarized below, characterized p16 expression immunohistochemically in a set of normal, benign hyperplastic, pre-invasive, and invasive epithelial tissue specimens from the skin and oral mucosa (Natarajan et al., 2003). p16 proved to be expressed heterogeneously in cells of some premalignant lesions and consistently in areas of microinvasion and at superficial margins of invasive SCC.

As described below, we found (Natarajan et al., 2003) that the p16-expressing cells in such lesions also express greatly increased levels of Laminin 5 (Lam5), previously identified as a marker of invasion in many types of epithelial cancers (Pyke et al., 1995; Kainulainen et al., 1997; Lohi, 2001; Lennander et al., 2003). Lam5 is a three-chain heterotrimeric protein and an important component of the basement membrane of stratified squamous epithelia (Carter et al., 1991; Rousselle et al., 1991). The trimer of α3, β3, and γ2 chains is synthesized by keratinocytes as an M̄r ~ 460 kDa precursor form and processed by cleavage of the α3 and γ2 chains at specific sites (Fig 3) by BMP1/Tld and other proteases (Marinkovich et al., 1992; Goldfinger et al., 1998; Amano et al., 2000; Veitch et al., 2003) to an M̄r ~ 400 kDa mature form. In their normal, undisturbed state in vivo, basal keratinocytes maintain an intact, continuous layer of mature Lam5 beneath them, to which they adhere via α6β4 integrin to form stable, anchoring hemidesmosome (HD) structures. In HD, α6β4 integrin forms clusters and associates closely with the transmembrane BP180 protein; the cytoplasmic domains of these proteins form stable connections with the cytokeratin filament network, mediated by BPAG1 and plectin (Baker et al., 1996; reviewed by Jones et al., 1998; Van der Flier and Sonnenberg, 2001). Keratinocytes at the migrating front of wounds express high levels of Lam5 and display an increased expression and unpolarized distribution of α6β4, α3β1, and α2β1 integrins (Larjava et al., 1993; Figure 1).
p16-related keratinocyte senescence mechanism and immortalization barrier  Our initial investigations confirmed and extended the findings of Kiyono et al. (1998), that the expression of TERT in keratinocytes via stable retroviral transduction results in telomere maintenance but is insufficient by itself to produce an immortalized line. We found (see Dickson et al., 2000) that rare, immortalized variants frequently emerged from a TERT-transduced population following either a short and subtle, or a prolonged and obvious, “slow growth phase” (SGP), during which most of the cells underwent p16-enforced senescence (as in the example in Fig 4a of the emergence of N/TERT-1 from the primary newborn foreskin keratinocyte line strain N). In some TERT-transduced keratinocyte populations, rapidly dividing immortalized (RDI) variants emerged. Some of these were associated either with complete loss, by deletion or other mechanisms, of p16 expression. Other TERT-transduced, immortalized keratinocyte lines we generated proved to be p16-related arrest by markedly reducing the frequency with which p16-expressing, growth-arrested cells are produced during serial passage, rather than by mutantational loss or promoter silencing of the p16 locus (Dickson et al., 1999; Nguyen et al., 2000a, b). Following the initial observation by Kikkawa et al. (1994) that a form of Lam5 secreted by some carcinoma cell lines induces colony scattering, Zhang and Kramer (1996) reported that exogenously applied Lam5 promoted α3β1 integrin-dependent keratinocyte motility as measured by a Boyden chamber/transwell migration assay. Subsequent studies by Goldfinger et al. (1999); Nguyen et al. (2000a, b), and Decline and Rouselle (2001) provided evidence that reduced ligation of mature Lam5 to α6β4 integrin and increased ligation of precursor Lam5 to β1 integrins, principally α3β1, results in keratinocyte motility at the leading edge of healing wounds.

Interestingly, Lam expression is increased in p16-positive, senescent normal keratinocytes in late passage cultures, associated with a remarkable directional hypermotility (Natarajan et al., 2003). As described below, we have found that coordinate Lam5 and p16 expression occurs in normal wound healing. We have identified an experimental system for inducing this response acutely and synchronously in culture and have used this system to characterize the fundamental elements of a keratinocyte motility/growth arrest (“KMA”) mechanism, in which certain changes in cell–substratum adhesion induce directional hypermotility followed by an increase in p16 expression and growth arrest (Natarajan et al., submitted).1

Results

Figure 4
The p16NK4A-dependent barrier to human keratinocyte immortalization in culture can be bypassed by spontaneous mutations or by genetic engineering to evade p16NK4A and p14ARF/p53-dependent arrest mechanisms. Panel a summarizes the results described in Dickson et al. (2000). The inset photographs show examples of cultures immunostained for p16 at various stages in the lifespan of primary keratinocytes and TERT-transduced keratinocytes. Normal primary human keratinocytes, such as strain N depicted in this panel, can be serially passaged in suitable culture medium formulations for as many as 60 population doublings (PD) before senescence (asterisk), associated with induction of p16 expression in all cells. Cells stably transduced to express TERT at the point in their lifespan indicated by the downward red arrow maintain long telomeres but enter senescence at the same time as untransduced control cells. TERT-expressing keratinocyte populations exhibit a “slow growth phase (SGP)” of indefinite length (nine passages over 2 mo in this experiment) in which only a small subpopulation remains p16-non-expressing and proliferative, and may produce rare variants in which a second event has occurred, associated with complete loss (upper right photograph, N/TERT-1) or greatly reduced frequency (lower right photograph, N/TERT-2G) of p16 expression occurs, permitting the cells to divide rapidly and without limit as an immortalized line. Panel b summarizes the results described in Rheinwald et al. (2002). Strain N cells transduced to express either the p16-non-binding mutant cdk4R24C (cdk4R) or a dominant-negative p53 (p53ΔD) after about the same number of population doublings as the untransduced control cells, but doubly transduced (p53DD + cdk4R) cells evade senescence and divide for an additional 25–35 PD before being limited by very short telomere crisis. These cells become “directly” immortalized after transduction to express TERT, without requiring the acquisition of further complementary second events.

Interestingly, TERT-transduced keratinocytes cultured from a p53 (+/-) individual (Li-Fraumeni hereditary predisposition to cancer syndrome) proved to select for loss of the wild-type p53 allele following attenuation of p16

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expression (Dickson et al, 2000). This result was consistent with our subsequent study (Rheinwald et al, 2002), in which we genetically engineered normal keratinocytes to bypass senescence and continue dividing to short telomere crisis. Those experiments showed that, in order to evade their senescence arrest mechanism, keratinocytes require abrogation of both p16 and p53 function, accomplished by sequential retroviral transduction to express a p16 non-binding mutant form of cdk4 (cdk4R24C (Wolfel et al, 1995)) and a dominant-negative p53 (p53DD: Shaulian et al, 1992) (Fig 4b). Keratinocytes in which both p16 and p53 were missing or rendered nonfunctional proved to be directly immortalized following transduction to express TERT. Supporting this result, we found that keratinocytes cultured from a p16 (+/−) individual (familial melanoma hereditary predisposition to cancer syndrome) underwent selection for a spontaneously arising, extended lifespan variant that lost the wild-type p16 allele, dependent upon experimental p53DD expression to abrogate the other, p53-dependent component of p16-related senescence (Rheinwald et al, 2002).

Two types of experiments showed that the p16-dependent component of the dual, p16- and p14ARF-dependent keratinocyte senescence mechanism is activated first and enforces growth arrest. First, whether cultured in K-sfm or in the fibroblast feeder system, senescence arrest proved to correlate tightly with high levels of p16, identified by BUdR/p16 double immunocytochemical staining (Rheinwald et al, 2002) (Fig 5a). Second, two-channel flow cytometry, measuring DNA content by propidium iodide fluorescence and cycling vs. non-dividing state by BUdR immunofluorescence following a 24 h labeling period, showed that keratinocytes arrest with a G1 DNA content, as expected for p16-enforced arrest (Gray and Rheinwald, unpublished) (Fig 5b). This result was very different from that of senescing fibroblasts, which arrested in either G1 or G2 (in a ratio similar to their G1/G2 distribution during exponential growth) as a result of telomere erosion-triggered, p53-dependent, p21cip1-enforced arrest (data not shown).

The p53-dependent component of keratinocyte senescence proved to be activated by a signal other than telomere shortening (Rheinwald et al, 2002). Keratinocytes sequentially transduced to express cdk4R and TERT were not immortalized, but an immortalized variant arose from within this population that proved to have acquired a function-imparing mutation in p53. Furthermore, immunocytochemical staining and western blot analysis showed that, in keratinocytes engineered to resist p16 and to block p53 function, both p16 and ARF levels increased in cells just at the time when they began dividing beyond their normal replicative lifespan limit. These results support the sequential, two-stage model of the p16-enforced keratinocyte senescence arrest mechanism and immortalization barrier shown in Fig 2.

**p16 expression during neoplastic progression to squamous cell carcinoma** We next sought to determine the setting in which p16 becomes expressed in keratinocytes in vivo to function as a tumor suppressor mechanism. We examined sets of normal, benign hyperplastic, dysplastic, carcinoma in situ, and invasive SCC lesions of human oral mucosa and epidermis (Natarajan et al, 2003). Our initial hypothesis, based on our observation of increased frequency of p16 expression with serial passage of normal primary keratinocytes, was that excessive cell division in vivo, as would occur in chronic benign epithelial hyperplasia or premalignant dysplasia, would trigger p16 expression. We found, however, that benign hyperplastic lesions never contained p16-positive cells, but that p16 was expressed by cells engaged in or about to begin neoplastic
invasion through the basement membrane into superficial connective tissue (Fig 6). As expected from results published by others that the p16 locus is typically lost or silenced by promoter hypermethylation in advanced, deeply invasive SCC (reviewed by Rocco and Sidransky, 2001), p16 protein expression proved to be lost at some point between the stages of early invasive and deeply invasive SCC in both epidermal and oral epithelial neoplastic progression (Fig 6).

Coordinate p16 and Lam5 expression in neoplastic progression and normal wound healing

Interestingly, we found that the same regions and many of the same cells that expressed p16 also expressed greatly increased levels of Lam5 (Fig 7a–c). Lam5 was reported previously to be associated with regions of early invasion (e.g., Pyke et al, 1995) and with the leading edge of healing epithelial wounds (e.g., Nguyen et al, 2000a, b). We found that coordinate induction of p16 and Lam5 expression occurs in vivo in keratinocytes at the edge of skin wounds (Fig 7d–f), and is also displayed by keratinocytes at the migrating front of partial thickness wounds made in pieces of surgically resected human skin and subsequently maintained in organ culture for two days (Fig 7g–i). Lam5 and p16 also proved to be coordinately upregulated at the edge of experimental wounds made in confluent cultures of early passage keratinocytes (Natarajan et al, 2003) (Fig 7j–l). Taken together, our neoplastic and wound tissue analyses and our studies of scratch wounded, early-passage keratinocyte cultures, thereby providing a useful experimental system with which to determine the time course of activation and the temporal and causal relationships among Lam5 expression, p16 expression, hypermotility, and growth arrest. Following studies by Jones et al, described above, which concluded that the keratinocyte wound response is associated with a change in cell–substratum adhesion from (α6)β4 integrin: mature Lam5) to (α3)β1 integrin: precursor Lam5), we asked whether plating keratinocytes on culture dishes coated with precursor Lam5 would induce KMA. In our first experiments, we plated keratinocytes at a low density onto dishes that had been precoated with medium conditioned by the rat bladder carcinoma cell line 804G, known to secrete Lam5 having a processed α3 chain and unprocessed γ2 chain (Baker et al, 1996; Giannelli et al, 1997) (“Lam5γ2pre”). After 2 d at 37°C, immunostaining with a human Lam5-specific antibody revealed that keratinocytes plated on control surfaces had formed small pads of Lam5 and had undergone little or no net migration. In contrast, cells plated on 804G CM-coated dishes became hypermotile, as revealed by the tracks of Lam5 each cell left behind. Composite low-magnification images of immunostained wells were used to measure the lengths of these tracks. As indicated in the scatterplot graph in Fig 8b, the rate of migration stimulated over the 2 d period after plating averaged ~ 1500 μm per d—remarkable for these ~ 12 μm diameter cells.

Using purified recombinant human Lam5 (the α3-processed, γ2-precursor form; Lam5γ2pre), we have confirmed that coating dishes with 0.4 μg per mL of this protein dissolved in culture medium induces the same hypermotility

Hypermotility and growth arrest induced by plating cells on a precursor form of Lam5

These results led us to conduct a series of experiments to determine whether KMA can be induced synchronously in early-passage keratinocyte cultures, thereby providing a useful experimental system with which to determine the time course of activation and the temporal and causal relationships among Lam5 expression, p16 expression, hypermotility, and growth arrest. Following studies by Jones et al, described above, which concluded that the keratinocyte wound response is associated with a change in cell–substratum adhesion from (α6)β4 integrin: mature Lam5) to (α3)β1 integrin: precursor Lam5), we asked whether plating keratinocytes on culture dishes coated with precursor Lam5 would induce KMA. In our first experiments, we plated keratinocytes at a low density onto dishes that had been precoated with medium conditioned by the rat bladder carcinoma cell line 804G, known to secrete Lam5 having a processed α3 chain and unprocessed γ2 chain (Baker et al, 1996; Giannelli et al, 1997) (“Lam5γ2pre”). After 2 d at 37°C, immunostaining with a human Lam5-specific antibody revealed that keratinocytes plated on control surfaces had formed small pads of Lam5 and had undergone little or no net migration. In contrast, cells plated on 804G CM-coated dishes became hypermotile, as revealed by the tracks of Lam5 each cell left behind. Composite low-magnification images of immunostained wells were used to measure the lengths of these tracks. As indicated in the scatterplot graph in Fig 8b, the rate of migration stimulated over the 2 d period after plating averaged ~ 1500 μm per d—remarkable for these ~ 12 μm diameter cells.

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![Figure 6](image_url)

**Figure 6**

p16<sup>INK4A</sup> expression during neoplastic progression of stratified squamous epithelia toward squamous cell carcinoma (SCC) occurs at the histopathologically classified stages of severe dysplasia to early invasion. Summarizing the principal results of Natarajan et al. (2003), the panels show hematoxylin/eosin-stained sections (above) and p16 immunostained sections (below) of specimens of benign hyperplasia, severe dysplasia, carcinoma in situ (non-HPV-related), and actinic keratosis overlying a deeply invasive squamous cell carcinoma. Note absence of p16 expression in benign hyperplasia, variegated pattern of p16 expression in late-stage pre-malignant lesions, and loss of p16 expression by deeply invading, progressively growing SCC (asterisk).
response as 804G CM. We do not have available a source of purified recombinant Lam5 mature form to compare; thus, we do not know whether the \( \gamma 2 \) precursor form is essential for eliciting this response. We can conclude, however, that the \( \alpha 3 \) precursor is not essential for triggering hypermotility. Time-lapse photography has disclosed how quickly cells become hypermotile after plating on a recombinant human Lam5\( \gamma 2 \)pre-coated surface. Such experiments have shown that cells begin to migrate within 20 min of attachment and spreading on a coated substratum, suggesting that a change in gene expression or new protein synthesis is not necessary to initiate hypermotility. By measuring the distance traveled during successive 2 h intervals of filming (i.e., 0–2, 2–4, 4–6 h, etc), the behavior of individual cells could be determined. As shown in Fig 6c, cells typically maintained rather constant rates of motility, averaging about 125 \( \mu m \) per h during the first day after plating.

Figure 7
Coordinate induction of \( p16^{INK4A} \) and Laminin 5 (Lam5) expression in keratinocytes of premalignant dysplasias and in normal keratinocytes migrating during wound healing. \( p16 \) immunostaining (b, e, h, k) and Lam5 immunostaining (c, f, i, l). Panels a–c: microinvasive region of severe epithelial dysplasia. Panels d–e: edge of a healing skin wound (arrow indicates edge of migrating tongue of epithelium; asterisk indicates fibrin scab). Panels g–i: wound made in an excised piece of newborn foreskin and maintained in organ culture for 2 d before immunostaining (N indicates normal, intact epidermis; W indicates the margin of the original wound). Panels j–l: confluent culture of strain N keratinocytes scratch wounded and maintained for 2 d before immunostaining.
Hypermotility stimulated by precursor Lam5 is followed by p16 expression and growth arrest

We next asked whether cells induced to become hypermotile by plating on Lam5\textsubscript{2}pre are growth inhibited by plating cells in control and precoated wells and counting the cells 7 d later. As shown in Fig 9a, cell growth was markedly inhibited on Lam5\textsubscript{2}pre-coated surfaces. Note that plating keratinocytes on dishes coated with culture medium alone or with fibronectin dissolved in medium resulted neither in increased directional motility nor in growth inhibition. Examining the cells by immunostaining for p16 expression (Fig 9b) showed that by 1 and 4 d after plating on Lam5\textsubscript{2}pre, \( \sim 25\% - 30\% \) and \( \sim 60\% - 70\% \) of the cells were expressing p16, respectively, and by 7 d were unable to reinitiate growth when subcultured onto a control surface (Fig 9c).

Figure 8
Directional hypermotility induced in keratinocytes by plating on a culture dish pre-coated with the \( \gamma 2 \) precursor form of Laminin 5 (\( \gamma 2 \text{Lam5pre} \)). Panel a shows Lam5 immunostaining of strain N keratinocyte cultures 2 d after plating at a low density in control conditions (dishes pre-coated with fresh culture medium) or on a dish pre-treated for 30 min with medium conditioned by a confluent culture of 804G rat bladder carcinoma cells, which secrete the \( \gamma 2 \) precursor form of Lam5. Note that in control conditions, cells wander little from the point at which they first attached, as evidenced by the distribution of secreted Lam5, in contrast to the remarkable hypermotility of cells plated on a dish coated with Lam5\textsubscript{2}pre. Panel b shows migration distances of individual cells, determined by measuring path lengths from composite pictures assembled from a series of adjacent microphotographic images of the immunostained culture. Panel c shows the migration distances traveled during successive 2 h intervals by keratinocytes plated on a coverslip coated with recombinant human Lam5\textsubscript{2}pre-dissolved in fresh culture medium and tracked by time-lapse photography beginning at the time of plating. Note that most cells migrate at or near their maximum rate, typically \( \sim 150 \) um per h, as soon as they have attached to the surface.

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p16- and p14ARF/p53-deficient keratinocytes retain the hypermotility component but evade the growth arrest component of the keratinocyte motility/arrest response

The immediacy of the hypermotility response, in contrast to the slower induction of p16 and subsequent irreversibility of growth arrest, indicates that p16 expression does not induce hypermotility. Instead, some aspect of the substratum that induces hypermotility, or the act of hypermotility itself, induces p16 expression and growth arrest. This conclusion was supported by the behavior of keratinocytes deficient in p16 and either p14\text{ARF} or p53. The advanced, invasive epidermal SCC-derived cell line SCC13 and normal primary keratinocytes engineered by transduction to express cdk4\textsuperscript{R24C} and p53\textsuperscript{DD} (N/P/C)—thereby evading p16- and p14\text{ARF}/p53- dependent arrest mechanisms, responded to plating on Lam5\textsubscript{2}pre with directional hypermotility but were not growth arrested (Fig 10). Thus, they maintained the hypermotility component but evaded the growth arrest component of KMA. Interestingly, one of our TERT-immortalized lines (N/TERT-1), which had lost p16/ p14\text{ARF} expression following transduction to express TERT as a rare heritable event associated with the emergence of an immortalized line (Dickson et al, 2000; Fig 4a), also displayed a substantial resistance to Lam5\textsubscript{2}pre-induced
growth arrest (Fig 10). These results prove that neither the expression of p16 nor its ability to cause growth arrest is essential for the hypermotility response, and also suggest that the p16/p14ARF arrest mechanism that is activated asynchronously in serially passaged keratinocytes is identical to the growth arrest component of the Lam5\(^{g2}\) pre-induced, acute KMA response.

We are currently seeking to understand the signal pathways responsible for inducing the hypermotility and growth arrest components of KMA, which at present remain a “black box”. Our initial studies have used, as a point of departure, observations by Decline and Rousselle (2001) that cultured keratinocytes treated with the growth-inhibitory polypeptide factor transforming growth factor beta (TGF\(\beta\)) increase synthesis and secretion of the \(\alpha2\) pre, \(\gamma2\) pre form of Lam5 and exhibit increased motility as assessed using a porous filter trans-migration, Boyden chamber assay. We have confirmed that TGF\(\beta\) treatment induces a directional hypermotility response similar to that induced by plating cells on Lam5\(^{g2}\) pre. The p16 expression-reduced TERT-immortalized strain line N/TERT-2G gave results similar to that of strain N, in contrast to the p16/p14\(^{ARF}\)/p53-deficient lines marked by the box on the left, which exhibited a hypermotility response to Lam5\(^{g2}\) pre but were not substantially growth inhibited.

**Figure 9**
The hypermotility response of normal keratinocytes to Laminin 5 (Lam5)\(^{g2}\) precursor (Lam5\(^{g2}\) pre) is accompanied by p16\(^{INK4A}\) induction, growth inhibition, and ultimate irreversible arrest. Panel a shows the average population growth rate (during 7 d after plating) and the motility response (measured 2 d after plating) of strain N keratinocytes plated on fresh culture dishes or dishes pre-coated with serum-containing medium, fibronectin, or Lam5\(^{g2}\) pre (804G CM). Note substantial growth inhibition and hypermotility from Lam5\(^{g2}\) pre but not from fibronectin. Panel b shows induction of p16\(^{INK4A}\) protein expression, determined in individual cells by immunostaining, during the first 4 d after plating cells on surfaces coated with 804G CM or with purified human recombinant Lam5\(^{g2}\) pre. Panel c shows the colony-forming ability on fresh dishes of 1000 cells subcultured from a 7 d control culture or from a 7 d Lam5\(^{g2}\) pre-coated dish culture, revealing the irreversibility of the growth arrest ensuing from long-term adhesion to Lam5\(^{g2}\) pre.

**Figure 10**
p16 and p14\(^{ARF}\)/p53 expression and function is essential for the growth arrest component, but not the hypermotility component, of the keratinocyte motility/arrest response to \(\gamma2\) precursor form of Laminin 5 (Lam5\(^{g2}\)pre). Growth inhibition and hypermotility responses of keratinocyte cell lines plated on dishes pre-coated with Lam5\(^{g2}\) pre (804G CM). The normal primary keratinocyte line strain N at sixth passage (~ 30 population doublings (PD)/mid-lifespan) showed the normal response. Note that strain N at 13th passage (~ 50 PD, near-senescence) showed little proliferation under control conditions and significant constitutive hypermotility (as reported in Natarajan et al., 2003), which was not increased further by plating on Lam5\(^{g2}\) pre. The p16 expression-reduced TERT-immortalized strain line N/TERT-2G gave results similar to that of strain N, in contrast to the p16/p14\(^{ARF}\)/p53-deficient lines marked by the box on the left, which exhibited a hypermotility response to Lam5\(^{g2}\) pre but were not substantially growth inhibited.

**Discussion**

The experiments described here have characterized the fundamental elements of a KMA response that is activated in keratinocytes *in vivo* and in culture when they encounter certain types of abnormal substrata. In retrospect, we recognize that we first detected KMA as it is activated heterogeneously in normal keratinocytes during serial culture, where its detectable effect is to cause senescence arrest. Keratinocyte replicative potential in culture is limited by two independent mechanisms. One, common to all somatic cell types, senses progressive telomere shortening and consequent exposure of telomere overhang sequences by the cells' p53-dependent DNA damage response mechanism,
exhibit an abrupt induction of p16 INK4A expression and irreversibly arrest in G1 with increasing frequency at each passage. Considering that p16 induction is triggered in early-passage keratinocytes when plated on surfaces coated with the \( \gamma 2 \) precursor form of Lam5, keratinocytes in serial culture may gradually lose their ability to process secreted Lam5 quickly enough to prevent engagement of precursor Lam5 with their \( \alpha 3\beta 1 \) integrin and consequently instigate the KMA response.

The phenomenon of p16-related senescence in culture led us to test the hypothesis that induction of p16 expression occurs in stratified squamous epithelia in vivo as a mechanism to restrict the proliferative potential of normal keratinocyte stem cells. Our study of hyperplastic and dysplastic lesions of the epidermis and oral mucosa did not support this hypothesis but, instead, revealed that p16 expression is specifically induced in pre-malignant oral and epidermal keratinocytes that have progressed to the stage of incipient initial invasion into underlying connective tissue as SCC (Natarajan et al, 2003). The finding of coordinate p16 and Lam5 expression at sites of severe dysplasia and microinvasion, as well as in normal keratinocytes at the migrating front of healing wounds, suggests a common response by keratinocytes to these two pathologic situations. We have conjectured (Natarajan et al, 2003) that the KMA response evolved as part of the wound-healing process to re-epithelialize but that it is also activated at a specific stage of neoplastic progression. KMA is marked (1) by increased synthesis of Lam5 and (presumably) its deposition in an incompletely processed, \( \gamma 2 \) precursor form that makes the cells directionally hypermotile and (2) by subsequent p16 expression that arrests their growth. We hypothesize that sacrifice of the proliferative potential, by p16 induction, in the band of cells that are leading migration to reepithelialize a wound may enhance the directional motility of these cells so that they can more efficiently pull their still-proliferative neighbors behind them to cover the wound surface. Figure 12 illustrates our hypothetical model of how KMA is activated during epithelial neoplastic progression. Rare preneoplastic cells that evade the growth arrest component of KMA, typically by ceasing to express p16, would then be able to grow progressively as invasive SCC. Recent studies from other laboratories have reported an increase in p16 expression at the leading edge of healing oral mucosal epithelial wounds (Bartkova et al, 2003) and an inverse relation between p16 expression and Ki67 staining in cells at the invading fronts of basal cell carcinoma (Svensson et al, 2003), consistent with our findings.

Identification of the cell surface and extracellular matrix molecules that determine whether keratinocytes remain sessile or display migratory behavior has been a subject of active investigation for almost 20 years. Some of the earliest work in this field recognized that some cell types increase their motility in response to exposure to some forms of Lam5 (Kikkawa et al, 1994) and that keratinocytes migrating on a variety of extracellular matrix molecules secrete and interact with endogenously produced Lam5 (Zhang and Kramer, 1996), a conclusion that has been supported definitively by recent studies (Frank and Carter, 2004). Evidence to date best supports a model for keratinocyte wound healing-related motility (Goldfinger et al, 1999; Nguyen et al, 2000a; b; Decline and Rouselle, 2001) in which keratinocytes convert from a more sessile state favored by \( \alpha 6\beta 4 \) integrin adhesion to mature Lam5 to a more motile state favored by \( \alpha 3\beta 1 \) integrin adhesion to precursor Lam5 (or, as recently suggested (Decline et al, 2003; Okamoto...
As a squamous cell carcinoma, if a rare p16-deficient variant arises within the lesion, it can continue dividing while invading tumor suppressor to prevent progressive growth. If a rare p16-deficient expression in these cells, which results in cell cycle arrest and acts as an inhibitor of the many signaling molecules and pathways that remain to be characterized. These include the events downstream of integrin engagement by Lam5pre that lead to immediate hypermotility, the events that induce expression of p16 and p14ARF to cause growth arrest, and the events downstream of TGFβ receptor activation that result in hypermotility and growth arrest. We are currently investigating the possibility of cross-talk and shared signal pathway events between Lam5pre-induced and TGFβ-induced KMA. We have begun studies using signal pathway kinase-specific inhibitory drugs, seeking to identify signaling steps that are either unique to hypermotility or else can be modulated by concentrations of drugs that impair motility without causing an acute, p16-unrelated growth inhibition. We are aug-
menting this approach with the design and expression of specific RNAi vectors to knock down expression of candidate signal pathway proteins and assess the consequence to the hypermotility and growth arrest components of the keratinocyte motility/arrest response.

**Materials and Methods**

**Cell lines and cell culture** The derivation and properties of the cell lines used in these experiments (see Table I) have been described (Rheinwald and Beckett, 1981; Dickson et al, 2000; Rheinwald et al, 2002). Keratinocytes were cultured in the “keratinocyte serum-free medium (K-sfm)” formulation (Pirisie et al, 1987) supplied by GIBCO/Invitrogen, (Carlsbad, California) supplemented as described previously (Rheinwald et al, 2002) with 25 μg per mL bovine pituitary extract (BPE), 0.2 ng per mL EGF, and 0.4 mM CaCl2. To generate near-confluent or confluent cultures, cells were grown to about 40% confluence in K-sfm, then refed with a medium consisting of a 1:1 (vol:vol) mixture of calcium-free DMEM supplemented with 0.4 mM CaCl2. To generate near-confluent or confluent cultures, cells were cultured using the fibroblast feeder layer system (Rheinwald et al, 1984), in which keratinocytes are co-cultured with mitomycin-treated 3T3J2 cells in FAD medium, consisting of DMEM/F12 (1:1 vol/vol) medium (GIBCO, Invitrogen) + 5% calf serum (CS) (Hyclone, Logan, Utah), 10 ng per mL EGF, 0.4 μg per mL hydrocortisone (HC), 5 μg per mL insulin, 10 × 10^{-10} M cholera toxin (CT), 2 × 10^{-11} M triiodothyronine, and 1.8 × 10^{-8} M adenine. 804G cells (Izumi et al, 1981) were cultured in DMEM supplemented with 10% newborn calf serum (Hyclone).

**Antibodies and immunostaining** The human p16^{INK4A}-specific mouse monoclonal antibody G175-405 (Pharmingen, San Jose, California) was used at 2 μg per mL and the Lam5 γ2 chain-specific mouse monoclonal antibody D4B5 (Mizushima et al, 1998) (Chemicon, Temecula, California) was used at 10 μg per mL. The Lam5 γ2 chain-specific rabbit polyclonal antibody J20 (Goldfinger et al, 1998) was used at 1:40 dilution. Details of the methods for immunostaining tissue sections and cultured cells are described in Natarajan et al. (2003). Images were captured on a Nikon E600 Microscope (Nikon, Melville, New York) with a SPOT2 digital camera using SPOTcam v.3.5.5 software (Diagnostic Instruments, Sterling Heights, Michigan).

**Assay for acute KMA induction and modulation by extracellular matrix proteins and polypeptide factors** A six-well tissue culture plate (9 cm² area per well per 1.5 mL medium volume) (Costar Corning, New York) were pretreated by incubation with conditioned medium (CM) from confluent cultures of 804G cells or with solutions of purified extracellular matrix proteins dissolved in 10% calf serum-supplemented DMEM:F12 medium for 30 min at room temperature, and then were rinsed and used to plate cells in K-sfm medium. To measure directional hypermotility, wells plated with 500 cells were fixed in 4% paraformaldehyde 40 h later and immunostained with the J20 chain in its precursor, uncleaved 155 kDa form, as determined by western blotting with the D4B5 antibody (data not shown).

**Time-lapse photography** Keratinocytes were inoculated into a temperature-controlled heated chamber (Mike’s Machine Company, Boston, Massachusetts) assembled with a 25 mm diameter Nalge/Nunc Thermonox (Rochester, New York) plastic coverslip, which was pre-coated with control medium or with medium containing conditioned medium of 804G cells.

**Table I. Human keratinocyte cell lines**

<table>
<thead>
<tr>
<th>Cell type of origin</th>
<th>Cell line name</th>
<th>Donor age, sex</th>
<th>Known p16, p14ARF, and p53 genetic abnormalities</th>
<th>Replicative lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal keratinocyte</td>
<td>Strain N</td>
<td>Newborn, M</td>
<td>None</td>
<td>~ 60 PD</td>
</tr>
<tr>
<td>Epidermal keratinocyte</td>
<td>N/p53DD/cdk4R</td>
<td>Newborn, M</td>
<td>Functionally p53- and p16-deficient</td>
<td>~ 95 PD</td>
</tr>
<tr>
<td>Epidermal keratinocyte</td>
<td>N/TERT-1</td>
<td>Newborn, M</td>
<td>None (but expresses no p16 or p14ARF)</td>
<td>Immortal</td>
</tr>
<tr>
<td>Epidermal keratinocyte</td>
<td>N/TERT-2G</td>
<td>Newborn, M</td>
<td>None (but reduced p16 expression)</td>
<td>Immortal</td>
</tr>
<tr>
<td>Epidermal SCC</td>
<td>SCC-13</td>
<td>56, F</td>
<td>p16(−/−), p53(−/−)</td>
<td>Immortal</td>
</tr>
</tbody>
</table>

PD, population doublings; SCC, squamous cell carcinoma.
taining Lam5;2pre, A Zeiss (Zeiss, Göttingen, Germany) IM35 inverted microscope (Diagnostic Instruments) equipped with a Uniblitz Model VMM-D1 Shutter Driver (Vincent Associates, Rochester, New York), and a Spot Insight QE camera was used to capture images through a ×10 phase objective and ×10 eyepiece (total magnification ×100). Images collected at 5 min intervals using Spot version 4.0.4. software were converted into movies, which were examined to trace and measure the path lengths of cells.

**Culture wound models** Scratch wound assay: Linear “wounds” ~ 2 mm wide were made in 1d post-confluent keratinocyte cultures (prepared as described above) by scraping the point of a sterile Eppendorf pipette tip across the cells, after which the cultures were returned to the incubator for 40 h until fixation and immunocytochemical analysis.

Organ cultured human skin wound assay: 1 cm² pieces of recently resected human newborn foreskin were rinsed in medium to remove residual blood components and ~ 2 mm diameter wounds were made through the epidermis and partially into the dermis with the aid of a fine forceps and curved iris scissors. The wounded pieces were submerged in medium and placed in the incubator for 2 d, after which they were fixed, sectioned, immunostained, and analyzed.

It is a pleasure to recognize the contributions of current and past members of the Rheinwald lab who have contributed to this research: Zongyou Guo, Jay Omobono, Paula Hercule, Sarah Browne, Mark Dickson, Matt Ramsey, Laura Gray, Jenny Wu, and Kathleen O’Toole. We also thank Barry Alpert of Microvideo Instruments, Inc. (Avon, Massachusetts) for assisting with the design of our time-lapse microscopy system and for optimizing our digital microphotography system. The research described here has been aided in very important ways by past collaborators, including William Hahn, Robert Weinberg, David Louis, Yasu Ino, Vincent Ronfard, Fred Li, Philip McKee, Sook-Bin Woo, Christopher Crum, Hensin Tsao, Michele DeLuca, and Caterina Catricala. We wish to especially acknowledge our current collaborators Alex Lazar and Thomas Brenn, Brigham, and Women’s Hospital, for providing pathology file specimens of wounds. The human research materials were collected under appropriate, IRB-approved protocols including informed consent. This work has been supported by grants from NIDCR, NIAMS, and NIDDK.

DOI: 10.1111/j.1087-0024.2005.200415.x

Manuscript received December 29, 2004; revised July 28, 2005; accepted for publication August 9, 2005

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