Targeting a Tumor-Specific Laminin Domain Critical for Human Carcinogenesis

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Abstract

Laminin-332 is critical for squamous cell carcinoma (SCC) tumorigenesis, but targeting it for cancer therapy has been unachievable due to key role of laminin-332 in promoting tissue integrity. Here, we show that a portion of laminin-332, termed G45, which is proteolytically removed and absent in normal tissues, is prominently expressed in most human SCC tumors and plays an important role in human SCC tumorigenesis. Primary human keratinocytes lacking G45 (ΔG45) showed alterations of basal receptor organization, impaired matrix deposition, and increased migration. After SCC transformation, the absence of G45 domain in ΔG45 cells was associated with deficient extracellular signal-regulated kinase and phosphotidylinositol 3-kinase (PI3K) pathway activation, impaired invasion, deficient metalloproteinase activity, and absent tumorgenicity in vivo. Expression of G45 or activated PI3K subunit in ΔG45 cells reversed these abnormalities. G45 antibody treatment induced SCC apoptosis, decreased SCC tumor proliferation, and markedly impaired human SCC tumorigenesis in vivo without affecting normal tissue adhesion. These results show a remarkable selectivity of expression and function for laminin-332 G45 in human SCC tumorigenesis and implicate it as a specific target for anticancer therapy. [Cancer Res 2008;68(8):2885–94]

Introduction

Squamous cell carcinoma (SCC) is a prevalent (1) invasive neoplasm arising in many tissues and causing significant morbidity and mortality. SCC is the most common cancer capable of metastasis and is second in frequency only to basal cell carcinoma. The incidence of SCC seems to be rising and is more frequently affecting younger individuals (2). SCC tumors can show a high risk of recurrence, and those derived from mucosal sites often invade neighboring tissues and can also metastasize to the lymph nodes, lung, and other distant sites. Chemotherapy for SCC has not been shown to significantly affect long-term survival, and most patients with advanced disease die despite currently available therapies (3). Results from the use of epidermal growth factor receptor inhibitory agents in clinical trials of advanced head and neck SCC in combination with conventional chemotherapy have been only modestly encouraging (4). All of these factors have led to the search for new and more specific agents in the treatment of SCC.

Laminins are a family of trimeric extracellular glycoproteins associated with the basement membrane zone (BMZ; ref. 5). Laminins interact with cell surface receptors and other BMZ components to provide cells with an interface to communicate with their surrounding extracellular environment (6). Laminin-332, a large molecule consisting of α3, β3, and γ2 chains (7), shows widespread expression in many epithelial tissues, as well as in the tumor microenvironment of many carcinomas (8). In particular, several recent findings suggest that laminin-332 plays a crucial role in human SCC progression. For example, laminin-332 is required for tumorigenesis in a well-characterized in vivo model of human SCC (8, 9). In addition, laminin-332 expression in SCC tumors arising from a number of tissues (10) correlates both with tumor invasiveness and patient prognosis (11, 12). Soluble and insoluble laminin-332, furthermore, have been observed to induce the phosphotidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase pathways, which are known to mediate carcinogenesis (13).

Due to its critical role in SCC tumorigenesis, laminin-332 would represent an excellent candidate as an antitumor target were it not for the equally critical role of laminin-332 in maintaining epithelial-mesenchymal cohesion across a broad range of normal tissues (14). For example, in the inherited disorder Herlitz's junctional epidermolysis bullosa (JEB), absence of laminin-332 expression due to laminin-332 gene mutations leads to widespread blistering and erosions and usually death during infancy (15). Therefore, any anticancer strategy targeting laminin-332 would need to address how to selectively disrupt protumorigenic function of laminin-332 without affecting its procohesive function in normal tissues.

Towards this end, we focused on the α3 chain of laminin-332, which undergoes proteolytic cleavage shortly after secretion. This proteolytic event, which takes place in the large COOH terminal globular (G) domain near the junction of the third and fourth epidermal growth factor (EGF)–like repeats termed G3 and G4 (Fig. 1A), converts the laminin α3 chain from a 200-kDa precursor to a 165-kDa processed product. The 37-kDa precursor region of the laminin α3 chain, containing two EGF-like repeats G4 and G5 (G45), is removed during processing and, as a result, is absent in normal mature tissues (16, 17). Any postnatal expression of unprocessed laminin α3 chain/G45 is only detectable transiently at healing wound edges (17). There are many parallels between the process of wound healing and tumorigenesis, including active synthesis of BMZ components, proliferation, and cell migration. Because of these parallels and the absence of unprocessed laminin...
α3 in normal mature tissues, we focused on the role of the laminin α3 G45 in human SCC. In this study, we show that, whereas G45 is undetectable in normal mature tissues, it shows widespread expression in human SCC tumors, where it plays an important role in SCC tumorigenesis. Furthermore, we show that laminin α3 G45 can be selectively targeted in vivo by antibodies to inhibit human SCC tumorigenesis without disrupting normal tissues.

Materials and Methods

Cell lines. Primary human keratinocytes isolated from normal skin and a patient with junctional dystrophic epidermolysis bullosa lacking laminin-332 expression due to LAMA3 mutations (18) were cultured in a 1:1 mix of defined keratinocyte serum-free medium (SFM; Life Technologies) and Medium 154 (Cascade Biologics) at 37°C in a humidified 5% CO2 incubator. Modified human 293 PHOENIX cells (gift from Dr. G. Nolan) were cultured in DMEM supplemented with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 10% CO2 incubator.

Complementary DNA constructs. Human laminin α3 chain (ref. 19; Genbank NM227.2) is physiologically processed at residues 1337 to 1338, according to NH2 terminal sequencing studies (20). As there are no known mutations in laminin-332 α3 G45 domain in JEB patients (21), three cDNAs encoding HuLAMA3 were produced: the first one comprising the 200-kDa full-length α3 wild-type (WT) chain, coding for residues 1 to 1713; the second comprised the 165-kDa α3 chain truncated at the physiologic processing site comprising residues 1 to 1337 (DG45); and the third comprised the 37-kDa cleaved G45 (G45), residues 1338 to 1713. These were generated by PCR, verified by direct sequence analysis, and cloned into the retroviral vector backbone LZRS (22) containing the encephalomyocarditis virus–IRES and blasticidin-resistance sequences (23) and a GATEWAY (Invitrogen) destination site (pLZRS-GATEWAY). The BM40 signal sequence was incorporated upstream and in-frame of the mutant HuLAMA3 for directing expression. Retroviral expression vectors encoding either activated Ha-Ras, IεBα (9), or activated PI3K p110-CAAX (24) have been previously characterized. Amphotropic retroviral supernatant production and retroviral keratinocyte transduction were performed as described (23).

Figure 1. Laminin-332 G45 is present in human SCC tumors but absent in normal skin. A, schematic diagram of laminin-332 α3, β3, and γ2 chains, highlighting α3 chain domain structure (black), proteolytic cleavage, and epitopes for BM165 mAb and G45 pAb. B, immunofluorescence microscopic analysis of frozen sections of human SCC (top) and neonatal skin (bottom) using BM165 mAb (green) and G45 pAb (red). Merged images with nuclear Hoechst staining (blue) are shown (right). Images are representative of four frozen SCC and skin samples tested. Scale bar, 50 μm. C, results of analysis of 75 cases of paraffin-embedded human cutaneous SCC using G45 pAb by immunohistochemical analysis. Top, left, number and percentage of samples which showed negative, moderate, or strong expression. Bottom and right, representative examples of moderate/strong expression and negative skin control. Scale bar, 50 μm. D, left, representative samples of 56 cases of paraffin-embedded human extracutaneous SCC from various tissues using G45 pAb by immunohistochemical analysis showing moderate to strong expression. Scale bar, 50 μm. Right, results of extracutaneous SCC tissue survey, showing tissues of origin, and intensity of staining for G45 pAb antibody.
WT and ΔG45 cDNA forward primer, 5′-AAAAAGGCTAGCATGGTTGCTTTATATA-3′; c3Wt cDNA reverse primer, 5′-CCCCGGGCAGCGCCGCTTACAG-3′. Representative images from JEBnull activation of keratinocytes after pulsing with 10 ng/mL EGF for 2 min has been described previously (28). Briefly, assays were performed exactly as previously described (27). Assaying ERK phosphorylation, near-confluent cells were extracted in radioimmunoprecipitation assay buffer (RIPA) with protease and phosphatase inhibitors. The signal-regulated kinase (ERK) and phosphorylated Akt (Ser473; Cell Signaling), anti-α6 integrin mAb G03H (Chemicon), Ki67 mAb (LabVision), paxillin mAb (BD Biosciences PharMingen), phalloidin pAb (Invitrogen), and β-actin mAb (Sigma).

Protein analysis. Laminin-332 depletion among WT, ΔG45, and ΔG45 + G45 cells were studied over a 24-h period. Proteins from whole-cell lysates, conditioned media, and matrix deposition were extracted and quantified by immunoblot, as previously described (26). K140, an anti-laminin 3 chain mouse monoclonal antibody (mAb) BM165 (14) and G45 rabbit polyclonal antibody (pAb; ref. 25), anti–laminin-332 rabbit pAb and laminin 3 chain mouse mAb K140 (16, 26) were previously characterized. We commercially obtained anti–phosphorylated extracellular signal-regulated kinase (ERK) and phosphorylated Akt (Ser74; Cell Signaling), anti-α6 integrin mAb G03H (Chemicon), Ki67 mAb (LabVision), paxillin mAb (BD Biosciences PharMingen), phalloidin pAb (Invitrogen), and β-actin mAb (Sigma).

Immunfluorescence microscopy. Confocal analysis of cell adhesion proteins was performed exactly as previously described (27). Assaying ERK activation of keratinocytes after pulsing with 10 ng/mL EGF for 2 min has been described previously (28). Briefly, representative images from JEBnull keratinocytes expressing the indicated laminin α3 constructs were visualized by immunofluorescence microscopy using phosphorylated ERK antibody. Nuclear localization of phosphorylated ERK was calculated through densitometry. For ERK phosphorylation, Phos–labeled (or Phos–labeled + PI3K) transformed cells were growth factor starved for 24 h and then stimulated with 10 ng/mL EGF for 2 min before lysis in RIPA buffer for immunoblot analysis. Densitometric analysis was shown as phosphorylated with ERK1/2 normalized to untreated WT controls. NIH ImageJ software was used for densitometric analysis.

Immunohistochemistry. For immunoperoxidase staining, 5 μm paraffin sections of SCC tissue microarrays from skin (SK802) and multiple organs (BC00019) obtained from U.S. Biomax, Inc., were deparaffinized, rehydrated, and antigen unmasking by boiling in 50 mmol/L Tris-HCl (pH 9.5) for 15 min. Sections were then incubated with G45 rabbit pAb followed by biotin-conjugated secondary and 3,3’-diaminobenzidine detection (Vector). Tissues were counterstained with hematoxylin. G45 staining was graded by two independent blinded observers according to the percentage of number of tumor cells positive with staining: >75% (strong), 25% to 75% (moderate), and <25% (negative). For immunofluorescence, 3-μm cryosections were incubated with antibodies listed above and Hoescht-counterstained. Images were taken using a Zeiss Axioscan-100 microscope.

Cell adhesion, migration, and invasion assays. G45 adhesion studies were carried out by coating purified LG4/5 fragment (10 μg/mL) overnight at 4°C; ref. 25). Ras/l-Boxtransformed normal primary human keratinocytes were detached in PBS containing 10 mM EDTA and rinsed in serum-free medium. After washing with PBS and saturation of the wells with 1% bovine serum albumin (BSA), the cell adhesion assays were performed in serum-free medium, as described (29). Adhesion was determined after fixation with 1% glutaraldehyde in PBS and staining with 0.1% crystal violet by absorbance at 570 nm using a MR5000 ELISA reader. A blank value corresponding to BSA-coated wells was subtracted. Adhesion inhibition with G4/5 antibody (20 μg/mL) or heparin (10 μg/mL; Sigma) took place 60 min before, as well as during, the cell adhesion experiment.

Cell detachment assays were carried out as described (30). Briefly, 2 × 104 cells/cm2 were incubated for 24 h at 30% confluence. Detached cells were quantified at increasing time intervals after incubation in a 1:7 dilution of trypsin/EDTA in PBS (BioWhittaker). Each adhesion/detachment experiment was performed in triplicate. Detached monolayer scratch assays (27) were performed by plating 106 cells into 60-mm tissue culture plates and incubating cells in SFM for 24 h. Media was changed to SFM without additives for 16 h. Fresh mitomycin-C (Sigma) was added at 10 μg/mL, and cells were incubated 3 h on ice. Cells were washed twice with SFM/ WA and scratched with a 1-mm cell scraper. Plates were washed thrice with SFM/ WA and marked areas photographed using a Zeiss Axiovert 25 microscope (50× magnification). Migration was quantified by calculating percentage change in the area between migrating cell sheets using NIH image software and more than three repeats per data point.

The in vitro invasion assays (31) were performed as previously described; briefly, assays were performed in triplicate using chambers containing a polycarbonate membrane with eight micron pores, coated with Matrigel (Becton Dickinson). After 24 h, invasive cells in the bottom chamber were lysed and quantified using CyQuant GR dye. Invasion index was quantified relative to percentage invasion by JEBnull keratinocytes.

Zymography. One million keratinocytes were starved for 24 h and incubated in SFM. Conditioned media were recovered and concentrated 80×. Samples were dissolved in nonreducing sample buffer (6% glycerol, 1% SDS, and 0.004% bromophenol blue), incubated in 37°C water bath for 10 min, and loaded on a 10% gelatin gel for detection of matrix metalloproteinase 2 (MMP2) and MMP9 and 12% casein gel for detection of MMP1 (Invitrogen). The gel was run in Tris/glycine buffer for 2 h and then incubated in 2.5% Triton X-100 solution for 15 min twice to remove SDS. To detect MMP activity, the gel was incubated in reaction buffer containing 50 mol/L Tris-HCl (pH 7.4), 0.2 mol/L NaCl, 5 mol/L CaCl2, and 1 μmol/L ZnCl2 overnight at 37°C. Protease activity was detected as translucent area in a Coomassie blue–stained gel. The scanned results of gels were calculated using NIH ImageJ software.

Tumorigenicity assay. Tumorigenicity assay was performed as previously described (9). Briefly, keratinocytes were inoculated with LZRS-IREs-Blasticidin/H-Ras or LZRS-IREs-Blasticidin/b-BeCM retroviral titer. Gene transfer was verified by immunoblotting of cell lysates. One million Ras/ l-BeCM-transformed cells suspended in 200 μL Matrigel (Beckton-Dickson) were injected s.c. to the dorsal flank of 6-wk-old nude mice; five mice were used per each condition. Tumors were measured on a weekly basis and analyzed at the end of 4 wk. All animal studies were conducted in accordance with protocols approved by Stanford Animal Use Committee. In some experiments, affinity purified G45 pAb, affinity purified mAb K140, or control rabbit IgG (Sigma) were injected i.p. on a weekly basis at a dose of 500 μg/mouse/wk, which has been previously shown to maintain high circulating antibody levels (9, 31). Apoptotic tumor cells were detected using Roche’s in situ cell detection kit. Proliferating SCC cells were detected with Ki67 and 4′,6-diamidino-2-phenylindole immunofluorescent staining. Proliferation and apoptosis were quantified as the ratio of staining of nuclear Ki67 and TUNEL, respectively, to total nuclear staining. NIH image software was used to quantify the subset of apoptotic or proliferating tumor cells in five representative low-power fields on each tumor.

Results

Widespread accumulation of laminin-332 G45 in human SCC tumors. In an effort to determine whether unprocessed laminin α3 G45 domain was accumulated in human SCC tumors, we examined frozen sections of four normal skin and four cutaneous SCC specimens obtained by Moh’s surgery using immunofluorescence microscopy. Using an antibody specific to...
the G45 domain and an antibody (BM165) which recognized the processed laminin-332 trimer (Fig. 1A), we found that G45, while consistently undetectable in normal skin, was abundantly present and showed colocalization with mAb BM165 in human cutaneous SCC tumors from each of the four patient samples tested (Fig. 1B).

In a more extensive survey of 75 cutaneous (Fig. 1C) and 56 extracutaneous (Fig. 1D) paraffin-embedded SCC tumors, over 75% showed moderate to strong G45 expression. All G45-positive tumors also stained positively with total laminin-332 pAb, whereas all but one of the G45 negative tumors were also negative for total laminin-332 expression (not shown). Thus, G45 domain accumulation correlated well with total laminin-332 expression in SCC tumors.

**Laminin-332 G45 domain facilitates the organization and function of matrix receptor complexes.** To investigate laminin-332 G45 in SCC, we produced three laminin-332 α3 chain constructs (Fig. 2A), a full-length WT chain, a mutant lacking G45 (ΔG45), and G45 (G45) itself. These constructs were stably expressed in laminin-332 null keratinocytes derived from a patient with JEB (JEBnull) with underlying LAMA3 gene mutations (18). As will be shown below, G45 separately expressed from the rest of the laminin-332 molecule (ΔG45 + G45) performed many of the same functions, albeit less efficiently (detailed below), as G45 synthesized as part of the laminin-332 molecule (WT). JEBnull keratinocytes expressing G45 without the ΔG45 construct, like untransduced JEBnull keratinocytes, did not adhere to culture surfaces and were not analyzed further in vitro.

ΔG45 JEBnull cells synthesized, assembled, and secreted trimeric laminin-332 as shown by nonreduced immunoblot analysis (Fig. 2B, left) and the ΔG45 chain was of equivalent apparent molecular weight compared with processed WT α3 chain (α3p), as shown by reduced immunoblot (Fig. 2B, center). The G45 construct was expressed in ΔG45 cells at levels similar to WT cells, as clearly seen in conditioned cell medium (Fig. 2B, right), as well as cell lysate (not shown). We compared laminin-332 in these cells isolated from culture medium or extracted from culture matrix, as previously described (26), using actin from cell lysates as a control. Normally, keratinocytes deposit more laminin-332 into their matrix than they secrete into their media, but ΔG45 cells secreted more laminin-332 into medium than matrix (Fig. 2C), consistent with previous observations (17). G45 synthesis in ΔG45 cells led to the majority of laminin-332 being deposited into matrix, indicating that G45 promoted laminin-332 deposition.

This was further examined by confocal microscopy (Fig. 3A). Analysis of laminin-332 antibody staining confirmed that the absence of G45 in ΔG45 cells correlated with a reduction in deposited laminin-332, which was again improved with the expression of G45 in ΔG45 cells (Fig. 3A, top). Additional effects of G45 on basally located cell receptor complexes were also noted. WT cells showed characteristic peripheral focal adhesions containing paxillin and central stable adhesions (32) containing α6β4 integrin (ref. 33; Fig. 3A, second panel). However stable adhesions in ΔG45 cells were abnormally peripheral, adjacent to focal adhesions (Fig. 3A, third panel). G45 expression in ΔG45 cells restored some

![Figure 2. Laminin-332 G45 expression and function in human keratinocytes. A, schematic of the laminin α3 cDNA constructs used in this study. WT, WT full-length laminin α3 construct; ΔG45, laminin α3 chain truncated at amino acid 1337 (major processing site; ref. 20); G45, portion of the laminin α3 chain G domain removed during processing. B, retroviral expression of laminin α3 cDNA constructs in JEBnull keratinocytes. Left, nonreduced immunoblot of conditioned keratinocyte medium using laminin-332 pAb, position of laminin-332 trimer, and molecular weight markers (kDa; left). NK, normal human keratinocytes. Center, reduced immunoblot of extracted keratinocyte matrix using laminin-332 pAb, positions of molecular weight markers (kDa; left) and positions of individual laminin-332 chains (right). Right, nonreduced immunoblot analysis of conditioned keratinocyte matrix using G45 pAb, position of G45 (left). C, quantification of cell layer (Matrix + Lysate) and conditioned media fractions from laminin α3 null keratinocytes expressing the indicated laminin α3 constructs by immunoblot using anti-laminin β3 antibody K140 or anti-actin antibody followed by densitometric analysis showed as integrated density of the ratio of laminin β3 to actin bands. Position of laminin β3 band (right).](https://cancerres.aacrjournals.org/)
stable adhesions to their normal central location. G45 colocalized with α6β4 integrin in stable adhesions in WT and ΔG45 cells (Fig. 3A, bottom), suggesting interaction of G45 with stable adhesions. In accordance with the analysis of stable adhesion formation, ΔG45 cells showed increased sensitivity to trypsin-induced detachment (Fig. 3B), which was corrected with G45 expression, suggesting that G45 induced laminin-332 deposition and stable adhesion formation led to increased stable cell adhesion. Previous studies have shown an inverse relationship between the rate of keratinocyte migration and laminin-332 deposition (34). In accordance with this, ΔG45 keratinocytes, with decreased laminin-332 deposition, migrated faster into scratches placed in confluent monolayers than WT cells with normal laminin-332 deposition (Fig. 3C). Expression of G45 construct in ΔG45 cells partially reversed these effects. We next determined whether these adhesion and migration abnormalities and these changes in extracellular matrix deposition and organization correlated with changes in tumorigenic potential.

Laminin-332 G45 promotes tumor invasion and metalloproteinase activity. SCC tumorigenesis was examined by retroviral transduction of primary human keratinocytes with oncogenic Ras and the nuclear factor-κB inhibitor IκBα, which produces transformed cells that generate human epidermal tumors indistinguishable from human SCC upon transfer to immunodeficient mice (9). After Ras/IκBα transformation, ΔG45 cells showed impaired invasion into Matrigel compared with WT cells which was partially corrected through G45 retroviral transduction (Fig. 4A). Carcinoma invasion has been linked to metalloproteinase activity, and ΔG45 cells showed a striking deficiency of MMP-9 and MMP-1, which have been associated with SCC invasion (ref. 35; Fig. 4B). Metalloproteinase deficiencies in ΔG45 cells were reversed through expression of G45, although not to the levels of WT cells. This deficient MMP expression explains why, despite their increased migration in the untransformed state, transformed ΔG45 cells invaded Matrigel more poorly than transformed WT cells.

Activation of PI3K and ERK pathways by laminin-332 G45. PI3K pathway activation is critical for SCC invasion (36) and ΔG45 cells showed decreased AKT phosphorylation, suggesting that G45 was essential in promoting PI3K pathway activation (Fig. 4C). G45 expressed as part of the laminin-332 molecule was more efficient at promoting AKT phosphorylation compared with G45

Figure 3. Laminin-332 G45 influences matrix receptor organization and function. A, effects of G45 on keratinocyte matrix deposition and adhesion complex formation. Keratinocytes expressing the indicated α3 chain constructs were analyzed by triple-label confocal microscopy. Color of secondary antibodies and staining are designated by the color of the text listing the primary antibody. FA, focal adhesion; SA, stable adhesion. Scale bar, 10 μm. B, G45 promotes resistance to trypsin dissociation. Established keratinocyte cultures expressing the indicated laminin α3 constructs were subjected to diluted trypsin at the indicated intervals and percentage of cells dissociated was quantified. Columns, results of triplicate experiments; error bars, SD. *, P < 0.05 compared with WT cells, #, P < 0.05 compared with ΔG45 cells. C, G45 modulates keratinocyte migration during in vitro wound healing assay. Confluent monolayers of keratinocytes expressing indicated laminin constructs were tested for their ability to migrate into 1-mm scratches over the course of 24 h and percentage of closure of scratch was quantified. Columns, results of triplicate experiments; error bars, SD. *, P < 0.05 compared with WT cells, #, P < 0.01 compared with ΔG45 cells.
expressed separate from laminin-332 (ΔG45 + G45). We also found an impairment of ERK activation in transformed ΔG45 cells, which was restored partially through overexpression of G45 but fully through overexpression of activated PI3K p110 catalytic subunit (Fig. 4D). In addition, ΔG45 cells showed defective nuclear translocation of activated ERK, which was corrected partially through G45 expression but fully through activated p110 expression (Fig. 4D).

**Laminin-332 G45 is required for in vivo Ras-driven SCC tumorigenesis.** We next examined G45 in human SCC tumorigenesis in vivo. After transfer to immunodeficient mice, transformed ΔG45 cells showed strikingly impaired tumorigenesis (Fig. 5, top row). This was slightly improved when G45 was expressed in ΔG45 cells and tumor growth was more fully restored in ΔG45 cells through activated p110 expression. Transformed JEBnull cells overexpressing G45 alone produced no detectable tumors 4 weeks after injection, similar to what has been previously shown for transformed JEBnull cells alone (ref. 9; not shown).

Whereas invasion into underlying muscle was consistently noted in tumors expressing WT laminin α3 chain, ΔG45 tumors showed a conspicuous invasive defect (Fig. 5A, second row). However, invasion into muscle was noted in ΔG45 cell tumors after expression of G45 or activated p110 subunit. Widespread apoptosis was present in ΔG45 tumors (Fig. 5A, third row and B). G45 expression diminished, and p110 expression completely inhibited apoptosis in ΔG45 tumors. ΔG45 tumors showed deficient proliferation, which was modestly increased with G45 domain expression and fully restored to WT levels with p110 expression (Fig. 5A, fourth row and C). Expression of G45 promoted laminin-332 deposition in the tumors, as did activated p110 subunit expression (Fig. 5A, fifth row), suggesting a possible link between G45 function, PI3K pathway activation, and laminin-332 deposition during SCC tumorigenesis.

**Laminin-332 G45 antibody disrupted SCC tumorigenesis in vivo without affecting normal tissue integrity.** Over the course of 4 wk of treatment, G45 antibodies dramatically inhibited in vivo human SCC tumorigenesis (Fig. 6A). G45 antibody–treated tumors produced both inhibition of proliferation and pronounced apoptosis compared with control antibody–treated tumors (Fig. 6B) similar to the G45 genetic deletion experiments described above. Affinity purified G45 antibody was shown to specifically inhibit transformed keratinocyte adhesion to recombinant G45 protein (Fig. 6C). Interestingly heparin also disrupted this interaction, suggesting that the heparan-binding properties of G45 were involved in its cellular interactions. Given its inhibitory effects on SCC tumor growth, we recognized the potential of the G45 antibody as an anticancer agent and looked for possible toxic
side effects of the antibodies in a survey of normal tissues. Interestingly, although the G45 pAb specifically recognized native murine laminin-332, as shown by nonreduced immunoblot analysis of conditioned mouse keratinocyte culture medium (Fig. 6C), we found no blistering epithelial-mesenchymal separation or other morphologic abnormalities observed in laminin-332–expressing tissues of mice treated with G45 antibodies, even after 4 weeks of antibody injections (Fig. 6D). Transmission electron microscopy of mouse skin after 4 weeks of antibody treatment showed no vesiculation or BMZ abnormalities (Fig. 6D).

**Discussion**

This study clearly indicates, through genetic and antibody-mediated inhibition, a key role for laminin-332 G45 in SCC tumorigenesis. One of the striking aspects of this study was the stark contrast between undetectable G45 in normal mature tissues and prevalent accumulation of G45 in a wide array of SCCs. Over 75% of both cutaneous and noncutaneous SCCs expressed laminin-332 and G45. Laminin-332 G45 was present in nearly 100% (all but one) of human SCC tumors positive for total laminin-332 expression. As has been previously shown in clinical studies, laminin-332 expression correlates with carcinoma invasiveness and a poor prognosis in SCC patients. As G45 persistence also correlates closely with laminin-332 expression in SCC tumors but is absent in normal tissues, G45, through additional clinical correlative studies, may prove to be an extremely useful marker to identify invasive SCC tumors. Also, as this study only looked at tumors at the carcinoma stage, the question of whether the initiation of detectable G45 coincides with invasion or begins at an earlier stage of *in situ* or premalignant carcinoma development.

**Figure 5.** G45 promotes human SCC tumorigenesis *in vivo*. A, representative photos in the top show a lack of tumor growth in cells lacking G45 expression (ΔG45, top), but a partial restoration when G45 was separately expressed (ΔG45 + G45), and a near-complete restoration of tumorigenesis when activated p110 (ΔG45 + PI3K). Histologic invasion of underlying muscle (second panel) was absent in tumors lacking G45 expression (arrow). Apoptosis was greatly increased in tumors lacking G45 (third panel) as shown by TUNEL assay of frozen tumor sections. Inset, Hoechst nuclear stain. Proliferation was greatly reduced in tumors lacking G45 (fourth panel) shown by Ki67 antibody analysis of frozen tumor sections. Inset, Hoechst nuclear stain. Laminin-332 deposition (fifth panel) was greatly reduced in the absence of G45 subunit, but was partially rescued by expression of G45 and fully rescued by activated p110 (PI3K) expression as shown by immunofluorescence microscopy of frozen tumor sections using laminin-332 pAb. G45 construct produced levels of G45 expression in tumor sections equivalent to WT construct as assessed by immunofluorescence microscopy of frozen tumor sections using G45 pAb (bottom). Scale bar, 100 μm. B, G45 promotes proliferation and protection from apoptosis. TUNEL assay and Ki67 staining as assessed by immunofluorescence microscopy of frozen tumor sections was quantified as a percentage of staining of total nuclei. Error bars, SD. *, P < 0.05 compared with WT cells; #, P < 0.05 compared with ΔG45 cells. C, G45 promotes proliferation and protection from apoptosis. TUNEL assay and Ki67 staining as assessed by immunofluorescence microscopy of frozen tumor sections was quantified as a percentage of staining of total nuclei. Error bars, SD. *, P < 0.05 compared with WT cells; #, P < 0.05 compared with ΔG45 cells.
remains to be tested. Whereas laminin-332 is not extractable from tissue without the use of proteases (16), which complicates the ability to analyze tumor samples by immunoblot analysis, it is possible that the G45 domain may persist in a soluble state after cleavage. Thus, clinical studies examining serologic detection of the G45 domain in carcinoma patients as a potential diagnostic or prognostic tool would be a worthwhile future consideration.

Although laminin-332 G45 is undetectable in mature tissues, it is transiently detectable at the leading edges of wounds (17). SCC invasion shares similarities with wound healing, as both are processes of epithelial proliferation and extension, which require the active synthesis and deposition of new BMZ components. These two processes differ in significant ways, however. In wounds, laminin-332 G45 becomes undetectable after closure, when synthesis of new BMZ components subsides and full processing and BMZ assembly is completed. With SCC tumor invasion, the synthesis of BMZ components is not regulated by a closure event, such as in wound healing, and thus, the synthesis of BMZ components continues, without allowing for processing and maturation of the BMZ to occur to completion. This may account for the poor ultrastructural BMZ organization in invasive carcinomas compared with normal tissues (37).

Why G45 accumulates at SCCs and at the leading edges of healing wounds may simply be a reflection of higher levels of total laminin-332 expression. One possibility is that laminin-332 is produced in wounds and tumors at a rate exceeding the ability of the processing enzyme to remove the G45 domain, resulting in a steady-state persistence of unprocessed laminin-332. The enzymes which process laminin α3 chain include plasmin (38) and the C-proteinase family of enzymes, especially mammalian tolloid and bone morphogenic protein 1 (26, 39). Alternatively, there may be other mechanisms that control the rate of laminin-332 G45 processing, such as the tissue plasminogen proteolytic cascade (38). In addition, a group of enhancer proteins, which modulate C-proteinase activity, has also been described, and while one, termed PCPE-1, has not been shown to influence laminin-332

Figure 6. Laminin α3 G45 pAb inhibited human SCC tumorigenesis without disrupting normal epithelial adhesion. A, G45 pAb blocked human tumorigenesis. Left, representative photos of Ras/I−/−-transformed SCC tumors in immunodeficient mice after 4 wk of treatment with G45 pAb or control IgG. Right, quantification of tumor growth during weekly tumor volume measurements in mice treated with G45 pAb, laminin γ2 mAb K140, or control IgG. B, after 4 wk of G45 pAb antibody injection, proliferation was reduced in tumors as shown by analysis of frozen tumor sections using immunofluorescence microscopy and Ki67 antibody. Left, proliferation was significantly reduced and apoptosis was greatly increased with G45 pAb treatment, as shown by Ki67 staining and TUNEL assay of frozen tumor sections; insets, Hoechst nuclear stain. Scale bar, 100 μm. Right, quantification expressed as number of cells with Ki67 and TUNEL staining, respectively, as a percentage of total nuclei. All error bars, SD. *, P < 0.05 compared with control antibody. C, left, Ras/I−/−-transformed normal human epidermal keratinocytes (NHEK) were plated on dishes coated with recombinant laminin α3 G45 domain in the presence of 20 μg/mL control or affinity purified G45 IgG or heparin for 1 h, and then attached cells were analyzed by colorimetry and quantified as percentage of control. Right, laminin α3 G45 pAb reacted with native mouse laminin-332 as shown by nonreduced immunoblot of conditioned mouse keratinocyte medium using laminin-332 pAb (pLam), G45 pAb (G45), or nonimmune rabbit IgG (control). *, P < 0.01 compared with control. D, top, representative micrographs of G45 antibody–treated mouse tissues known to express laminin-332 show no evidence of epithelial detachment or other histologic defects. Scale bar, 200 μm. Bottom, transmission electron microscopy of antibody-treated mouse skin revealed no vesiculation or disruption of BMZ ultrastructure in representative samples. Left bar, 5 μm; right bar, 100 nm.
processing, other members of this family, including PCP-2, remain to be evaluated (40). As the G45 seems to have potent protumorigenic effects, factors that influence its proteolytic removal may have important bearing on SCC tumor progression.

Major protumorigenic effects of G45 include its ability to enhance laminin-332 deposition in SCC tumors. It is possible that G45 promotes laminin-332 deposition in SCC directly by anchoring the laminin-332 molecule to the matrix during the process of BMZ assembly. Laminin-332 G45 domain (41) has heparin-binding properties (42) and may have the ability to bind with extracellular heparin sulfate proteoglycan BMZ components, such as perlecan (43) or dystroglycan (44). However, even in the absence of laminin, laminin-332 deposition was shown to be restored to near WT levels in SCC tumors through activation of the PI3K pathway. This suggests that, rather than acting by anchoring, G45 likely promotes laminin-332 deposition by a signaling mechanism, perhaps induced through interaction with another cell surface receptor. As we showed our tumor cell interactions with G45 could be blocked with heparin, one possible candidate that deserves further study is syndecan-1, a transmembrane heparan sulfate proteoglycan receptor which is expressed in epidermal cells and can directly bind the laminin α3 G45 domain (25). We previously showed that Ras/β1/β0 transformed keratinocytes expressing deletion of domains IV to III on the short arm of the β3 chain of laminin-332 also showed impaired tumorigenesis associated with increased apoptosis (36); however, these cells showed no significant impairment of proliferation or laminin-332 deposition. Thus, the G45 domain seems to provide unique protumorigenic functions, including laminin deposition and proliferative stimulation, which are not provided by the laminin β3 chain short arm.

While G45 is not known to directly bind α6β4 integrin, a number of observations suggest that G45 and α6β4 integrin functions in promoting tumorigenesis may be related. First, we noted that G45 and α6β4 integrin localized together in basal keratinocyte receptor complexes termed stable adhesions. While α6β4 integrin is well known to play a key role in the formation of these complexes (32), here, we also noted a role for G45 in stable adhesion formation. Specifically, the absence of G45 disrupted the localization of stable adhesion complexes, changing them from central to peripheral localization. In addition to colocalizing to and playing a role in the formation of stable adhesions, α6β4 integrin and G45 also showed interesting parallels of relevance to SCC tumorigenesis. G45 was noted for its promotion of cellular invasion and its activation of PI3K and ERK signaling pathways, leading to protection from apoptosis and increased proliferation. It is also well known that α6β4 integrin, like G45, promotes carcinoma invasion, PI3K pathway activation (45), and nuclear ERK translocation, which leads to increased proliferation (28). Furthermore, α6β4 integrin was shown to perform these functions in a laminin-dependent manner through its substrate domain. Deletion of this substrate domain led to phosphorylated ERK, which accumulated in the cytoplasm (28), similar to ΔG45 cells in our study. Thus, it is likely that α6β4 integrin and signaling from its substrate domain is involved in the function of the laminin α3 G45 domain. It should also be noted that, in our studies, G45 promoted protumorigenic functions best when it was expressed as part of the laminin α3 chain, suggesting G45 may need to be associated with the rest of the laminin-332 trimer for optimal function. It is possible that the close proximity of the G45 domain to the α6β4 integrin (G1-G3) binding site on the unprocessed laminin α3 chain (Fig. 1A) plays a role in this process.

G45 domain clearly modulates the expression of MMP-9 and MMP-1, two metalloproteinases known to play important roles in carcinoma invasion. Our observation extends earlier findings of the role of G45 domain in up-regulating MMP-1 (46) and MMP-9 in keratinocytes to squamous carcinoma cells (47). These results are consistent with other studies showing the role of extracellular matrix molecules changing MMP expression and activities (48). Overexpression of PI3K has been previously shown to promote MMP-9 expression in carcinoma cells (49, 50), and it is possible that the ability of G45 to promote activation of the PI3K pathway may be related to its function in promoting MMP-9 activity in human SCC.

While proadhesive function of G45 may play a role in vivo, either in healing wound edges or SCC tumors during periods of active laminin-332 synthesis, G45 is not required for maintenance of normal tissue cohesion, as no epithelial-mesenchymal disruption was seen in normal tissues repeatedly treated with G45 inhibitory antibodies. This is not surprising, as G45 is completely proteolytically removed from normal mature tissues (16, 17). Overall, its striking protumorigenic activity, its prevalent accumulation in human SCC tumors, and its absence and lack of function in normal developed tissues collectively make G45 an attractive anticancer target.

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