

Tumorigenesis and Neoplastic Progression

MAPK/ERK-Dependent Translation Factor Hyperactivation and Dysregulated Laminin $\gamma 2$ Expression in Oral Dysplasia and Squamous Cell Carcinoma

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Lesions displaying a variety of dysplastic changes precede invasive oral and epidermal squamous cell carcinoma (SCC); however, there are no histopathological criteria for either confirming or staging premalignancy. SCCs and dysplasias frequently contain cells that abnormally express the $\gamma 2$ subunit of laminin-332. We developed cell culture models to investigate $\gamma 2$ dysregulation. Normal human keratinocytes displayed density-dependent repression of $\gamma 2$, whereas premalignant keratinocytes and SCC cells overexpressed $\gamma 2$ and secreted laminin assembly intermediates. Neoplastic cells had hyperactive EGFR/MAPK(ERK) signaling coordinate with overexpressed $\gamma 2$, and EGFR and MEK inhibitors normalized $\gamma 2$ expression. Keratinocytes engineered to express HPV16 E6 or activated mutant HRAS, cRAF1, or MEK1 lost density repression of $\gamma 2$ and shared with neoplastic cells signaling abnormalities downstream of ERK, including increased phosphorylation of S6 and eIF4 translation factors. Notably, qPCR results revealed that $\gamma 2$ overexpression was not accompanied by increased $\gamma 2$ mRNA levels, consistent with ERK-dependent, eIF4B-mediated translation initiation of the stem-looped, 5'-untranslated region of $\gamma 2$ mRNA in neoplastic cells. Inhibitors of MEK, but not of TORC1/2, blocked S6 and eIF4B phosphorylation and $\gamma 2$ overexpression. Immunostaining of oral dysplasias identified $\gamma 2$ overexpression occurring within fields of basal cells that had elevated p-S6 levels. These results reveal a causal relationship between ERK-dependent translation factor activation and laminin $\gamma 2$ dysregulation and identify new markers of preinvasive neoplastic change during progression to SCC. (*Am J Pathol* 2012, 180:2462–2478; <http://dx.doi.org/10.1016/j.ajpath.2012.02.028>)

Squamous cell carcinoma (SCC) is a major cancer that arises from stratified epithelia, including the oral epithelium, epidermis, and the epithelia covering the oropharynx, esophagus, tracheobronchus, bladder, vulva, and cervix. Among attributes shared by the normal basal cells of these epithelia are synthesis of the basement membrane protein laminin-332 (Lam332) (previously known as laminin 5) and control of their cell division by EGF.^{1,2} Approximately 300,000 new cases of SCC per year occur in the United States, resulting annually in ~100,000 deaths.^{3,4} Depending on the tissue of origin, the carcinogenic instigator is chronic exposure to UV radiation (epidermis), tobacco (oral and lung), industrial or environmental carcinogens (lung and bladder), or infection with papillomaviruses HPV16/18 (cervix, some vulva, some oropharynx).^{5–8} Abnormal hyperactivity of the EGFR/ERK and the PI3K/AKT/mTOR signaling pathways are typical characteristics of advanced SCC.^{9–11} If not excised at an early stage, SCCs infiltrate deeply and spread rapidly by colonizing regional lymph nodes, such that they can no longer be completely surgically resected. SCCs of all tissue origins have proven refractory to nonsurgical treatment regimens, including small-molecule kinase inhibitors.^{12–15} Annually in the United States there are 20,000 new cases of oral SCC and 40,000 head and neck SCCs (combined total for oral and oropharynx SCCs). Five-year survival after diagnosis of head and neck SCC is ~50%, which has remained unchanged for many decades, regardless of treatment protocols.^{3,16}

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Visual accessibility of the oral cavity has led to the identification of potential precursor lesions to SCC. Such lesions appear more white, more red, or more scaly than normal epithelium. The incidence of oral lesions is high, at approximately 1% to 3% in adults.¹⁷ Some of these lesions eventually disappear, whereas others progress to invasive SCC. It is estimated that, in the United States and Europe, oral dysplasias progress to SCC at a rate of 2% to 6% per year.^{17–20} Microscopically, many of these lesions show signs of dysplasia, including abnormal cell and nuclear shape and delay of suprabasal differentiation.^{21,22} There remains much uncertainty about the criteria for a diagnosis of premalignant dysplasia and whether any feature of dysplastic change predicts future progression to SCC,^{20,23,24} so the need to identify prognostic biomarkers is urgent.

In an earlier study,²⁵ we detected coordinate induction of p16^{INK4A} and overexpression of laminin γ 2 (Lam γ 2) in some oral and epidermal dysplasias, occurring at greater frequency in dysplasias of higher degrees of atypia and at margins of invasive SCC. Lam γ 2 and the two partner subunits laminin α 3 and laminin β 3 form Lam332. After intracellular assembly of the three subunits, normal basal epithelial cells secrete the Lam332 trimer, proteolytically process it, and deposit it into the underlying basal lamina, where it serves as an essential component of hemidesmosomes, conferring stable, polarized epithelial cell adhesion.² After tissue development is complete, basal keratinocytes stop synthesizing immunohistochemically detectable amounts of Lam γ 2.^{25,26} Invading carcinoma cells, however, frequently reinitiate high rates of Lam γ 2 synthesis.^{27–29} Because ab-

normal Lam γ 2 expression begins in premalignant lesions,²⁵ its immunohistologic detection has potential as a prognostic marker for lesions at high risk for progression to SCC. Determining the mechanism of neoplastic Lam γ 2 dysregulation could identify ancillary prognostic markers and potential targets for early-intervention drug therapy.

We have identified a human cell culture model system that recapitulates the *in vivo* situation of Lam γ 2 repression by normal keratinocytes and overexpression by premalignant and malignant keratinocytes. Using this system, we have identified EGFR/MAPK(ERK)-pathway hyperactivation as the driver of Lam γ 2 overexpression by neoplastic cells, correlated with increased phosphorylation and activation of the translation factors S6 and eIF4B. Our data reveal that neoplastic Lam γ 2 expression is the result of increased translation, not transcription. We find that in oral dysplasias *in vivo*, Lam γ 2 overexpression occurs strictly within fields of basal cells that have abnormally increased levels of p-S6. These results serve to define a new, immunohistologically detectable stage of neoplastic progression in preinvasive dysplasias.

Materials and Methods

Cells and Cell Culture

The cell lines used are characterized in Table 1 and in the articles cited therein.^{1,30–39} Derivation and use of these cell lines were IRB-approved. The premalignant oral ke-

Table 1. Human Cell Lines Used in This Study

Cell line	Donor sex (age)*	Special characteristics	References†
Epidermal keratinocyte			
Strain N	M (0)	Normal	1, 30–33
N/HPV16E6E7	M (0)	Immortal; p53- and pRb-deficient	1, 31
N/HPV16E6	M (0)	All E6 functions, including p53-deficiency	
N/HPV16E6 (JH26 mutant)	M (0)	p53 normal but has other E6 functions	
N/HPV16E7	M (0)	All E7 functions, including Rb-deficiency	30
N/p53DD/cdk4R24C	M (0)	p53-deficient and p16 ^{INK4A} -resistant	34
N/TERT1	M (0)	Immortal; loss of ability to express p16 ^{INK4A} and p14 ^{ARF}	1, 30, 31
N/TERT1/EGFR Δ (E746-A750)	M (0)	Immortal; EGF-independent for growth	
N/TERT1/HRAS (G12V)	M (0)	Immortal; EGF-independent for growth; Lam γ 2 overexpressing	
N/TERT1/cRAF1 (22W)	M (0)	Immortal; EGF-independent for growth; Lam γ 2 overexpressing	
N/TERT1/MEK1 (DD)	M (0)	Immortal; EGF-independent for growth; Lam γ 2 overexpressing	
G5Ep	M (0)	Normal	35
O5Ep	M (0)	Normal	
Epidermal SCC			
SCC-13	F (56)	p16- and p53-deficient; immortal; tumorigenic	31–33, 36, 37
Oral keratinocyte			
OKF6	M (57)	Normal	30
Dysplastic oral keratinocyte			
POE9n	M (65)	Homozygous deletion of p16 ^{INK4A} and p14 ^{ARF} ; p53-deficient, nontumorigenic	30, 31
D17	ND (ND)	p16 ^{INK4A} -deficient; normal p53; nontumorigenic	38, 39
Oral SCC			
SCC-15	M (55)	p16- and p53-deficient; immortal; tumorigenic	32, 36, 37
SCC-25	M (74)	p16- and p53-deficient; immortal; tumorigenic	32, 36, 37
SCC-68	M (ND)	p16- and p53-deficient; immortal; tumorigenic	
SCC-71	M (80)	p16- and p53-deficient; immortal; tumorigenic	31, 36

*Ages are given in years; 0 indicates newborn.

†References for derivation and characterization of previously published cell lines.

F, female; M, male; ND, no data.

ratinoocyte line D17 was provided by Dr. E. Kenneth Parkinson (Queen Mary University of London, UK). Cells were cultured in keratinocyte serum-free medium (Ksfm) (Invitrogen; Life Technologies, Carlsbad, CA) with 25 $\mu\text{g}/\text{mL}$ bovine pituitary extract, 0.2 ng/mL epidermal growth factor (EGF), and CaCl_2 to a final Ca^{2+} concentration of 0.4 mmol/L, as described previously.³⁰ To maintain healthy confluent cultures, after cultures reached ~40% confluence in Ksfm they were refed daily with 1:1 medium (1:1 vol/vol Ca^{2+} -free Dulbecco's modified Eagle's medium from Invitrogen with Ksfm and supplemented as described above for Ksfm alone), as described previously.¹ To avoid variation among cell lines with respect to stratification and differentiation, cultures approaching confluence were fed with 1:1 medium with 0.1 mmol/L CaCl_2 . To assess EGF-dependent growth, 2000 cells per 9-cm² well were plated with or without EGF, refed every 2 to 3 days, and counted 6 to 8 days later. Average growth rate in terms of population doublings (PD) per day was calculated as $\log_2(\text{number of cells obtained at subculture}/\text{number of cells plated})/\text{number of days cultured}$.

Protein Extracts and Conditioned Medium Samples

To analyze laminin protein expression in cells, cultures were suspended with trypsin/EDTA and lysed in protein extraction buffer consisting of 20 mmol/L Tris-HCl (pH 7.3), 2% SDS, EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN), and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Alternatively, cultures were rinsed twice with PBS, drained, and lysed in protein extraction buffer to obtain both intracellular proteins and proteins that had been deposited onto the culture dish (matrix proteins). Extracts were sonically disrupted and stored at -80°C . The BCA protein assay (Thermo Scientific) was used to measure protein concentrations. For phosphoprotein analysis, cultures were lysed in reducing Laemmli SDS sample buffer.⁴⁰ To analyze secreted laminin, conditioned medium of cultures was collected 24 hours after refeeding, passed through 0.2- μm filters, and stored at -80°C . After conditioned medium harvest, cultures were trypsinized and their proteins were extracted and measured to normalize loading volumes of conditioned medium samples for comparative Western blot analysis.

Western Blotting

Protein (20 μg per lane) in Laemmli sample buffer was separated by SDS-PAGE under reducing or nonreducing conditions on 4% to 20% gradient gels (Bio-Rad Laboratories, Hercules, CA), blotted to polyvinylidene difluoride membranes (Bio-Rad Laboratories), blocked for 1 hour with 5% nonfat milk in Tris-buffered saline with 0.05% polysorbate 20 (Tween 20), incubated overnight with primary antibody, rinsed, and incubated for 1 hour with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:2000) (Dako, Glostrup, Denmark) at room temperature. Blots

were developed using ECL (Roche) or SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) and exposed to HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ).

Some Lam γ 2 Western blots were analyzed densitometrically using ImageJ software version 1.45 (NIH, Bethesda, MD; <http://rsbweb.nih.gov/ij>). Two different film exposure times were analyzed. The total intensity of each Lam γ 2 band was normalized to the actin band intensity of the same extract and film exposure time.

Antibodies

The murine monoclonal antibodies used were laminin γ 2 (clone D4B5²⁶; Chemicon, Billerica MA), laminin α 3 (clone 12C4,⁴¹ provided by Jonathan Jones, Northwestern University, Chicago, IL), laminin β 3 (clone 17) and c-myc (clone 9E10; BD Transduction Laboratories, Franklin Lakes, NJ), p53 (clone DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), EGFR (clone 1F4; Cell Signaling Technology, Danvers, MA), and FLAG (clone M2; Sigma-Aldrich, St. Louis, MO). The rabbit monoclonal antibodies used were p-eIF4E(Ser209) (clone EP2151Y) from EMD Millipore (Billerica, MA) and p-AKT(Ser473) (clone D9E), p-S6(ser235/236) (clone 91B2), and p-EGFR(Tyr1068) (clone D7A5) from Cell Signaling Technology. The rabbit polyclonal antibodies used were β -actin (A-2066; Sigma-Aldrich), p-eIF4B(Ser422) (Abcam, Cambridge, MA), and ERK1/2, p-ERK1/2(Thr202/Tyr204), and p-4E-BP1(Ser65) (Cell Signaling Technology).

Signaling Pathway Inhibitors

The TGF β receptor I kinase inhibitor TRI (discovered independently as Biogen HTS466284 and Lilly LY364947) was used at 1 $\mu\text{mol}/\text{L}$ (EMD Millipore Chemicals, Gibbstown, NJ).^{31,42} The EGFR inhibitor gefitinib^{43,44} (provided by Pasi Janne, Dana-Farber Cancer Institute) was used at 1 $\mu\text{mol}/\text{L}$. The MEK inhibitors AZD6244⁴⁵ (Selleck Chemicals, Houston, TX) and U0126⁴⁶ (Cell Signaling Technology) were used at 100 nmol/L and 1 $\mu\text{mol}/\text{L}$, respectively. The mTORC1/2 inhibitor Ku-0063794^{47,48} (Chemdea, Ridgewood, NJ) was used at 500 nmol/L. The ERK inhibitor CAY10561⁴⁹ (Cayman Chemical, Ann Arbor, MI) and the RSK inhibitor BI-D1870⁵⁰ (Symansis, Auckland, NZ) were each used at 1 $\mu\text{mol}/\text{L}$. The translation inhibitor cycloheximide (Sigma-Aldrich) was used at 10 $\mu\text{g}/\text{mL}$ and the protein transport inhibitor brefeldin A (BD Biosciences, San Jose, CA) at 1 $\mu\text{g}/\text{mL}$. Inhibitors were added to the medium at plating and each refeeding from 1000 \times concentrated solutions in dimethyl sulfoxide, stored frozen between uses.

Immunofluorescence Staining

Cultures were rinsed, fixed, stored in 100% methanol at -20°C , and air-dried before use. After 15 minutes of blocking in PBS with 10% goat serum (Invitrogen), primary antibodies diluted in PBS with 0.1% BSA were applied for 1 hour at room temperature. Cultures then were rinsed with PBS, incubated with fluorescent-labeled sec-

ondary antibodies (Invitrogen) for 1 hour, rinsed, and coverslip-mounted with ProLong Gold antifade mounting medium with DAPI (Invitrogen). Cells were examined under an inverted Nikon Eclipse Ti fluorescence microscope fitted with an RT-slider SPOT camera (SPOT Imaging Solutions, Sterling Heights, MI).

Tissue Samples, Immunohistochemical Staining, and Quantification

Selection and diagnosis of a set of 26 formalin-fixed, paraffin-embedded, lateral tongue tissue specimens from the University of Connecticut Oral Pathology archival collection was performed with IRB approval by an expert oral pathologist (E.N.). The cohort included normal epithelium, benign warts, dysplasias, and microinvasive to advanced SCCs.

Paraffin sections (5 μ m thick) on slides were warmed to 37°C for 30 minutes, then deparaffinized and hydrated through xylene, ethanol, and PBS; to better preserve antigens, slides were not baked at 50°C after sectioning, as is a common practice of histopathology labs.²⁵ Antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6.0) autoclaved for 15 minutes. Endogenous peroxidase was inactivated by incubating slides in 0.3% H₂O₂ in methanol for 10 minutes. After a PBS rinse, slides were blocked with PBS with 10% horse serum (Invitrogen) for 20 minutes, incubated with primary antibody for 1 hour at room temperature, biotinylated secondary antibody (1:100) for 1 hour, and avidin/biotin/peroxidase complex (ABC) reagent (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 45 minutes, with three PBS rinses between each step. Slides were incubated with Vector Red (Vector Laboratories) for 1 to 5 minutes, the reaction was stopped by rinsing in water, and slides were coverslip-mounted with Fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL). Slides were examined under a Nikon Eclipse TE2000-S microscope and were photographed as described above.

Twenty-one dysplasia specimens for which sequential sections were stained for Lam γ 2 and p-S6 were quantified for percentage basal p-S6 and Lam γ 2 expression. The total length of dysplastic areas in each specimen was determined by examining an adjacent H&E-stained slide, and the proportion of the dysplastic region containing p-S6⁺ and Lam γ 2⁺ basal cells was determined.

Retroviral Vectors and Transduction

Retroviral vector plasmids pBABE.puro/EGFR Δ (E746-A750)-FLAG, [EGFR Δ (746–750) retroviral vector was provided by Dr. Pasi Janne (Dana Farber Cancer Institute, Boston)],⁴³ pBABE.puro/cRAF-1(22w),^{51,52} pLHCX/MEK1(S218D,S222D) [MEK1(DD)],^{53,54} pBABE.puro/HRAS(G12V),^{55,56} pL(HPV16E6)SN [HPV16E6 retroviral vector was provided by Dr. Denise Galloway (Fred Hutchinson Cancer Center, Seattle)],⁵⁷ and pL(E6(JH26))SN^{58–60} [HPV16E6(JH26) retroviral vector was provided by Drs. Karl Munger and Peter Howley (Harvard Medical School, Boston)] were transfected into Phoenix packaging cells⁶¹

using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI). Retroviral supernatants were collected in 1:1 medium (as above) at 8- to 16-hour intervals from 1 to 3 days after transfection, passed through 0.45- μ m pore filters, and stored at –80°C. Cells plated 1 day previously at 10⁵ cells/9 cm² well in Ksfm were transduced for 6 to 7 hours with retroviral supernatants containing 2 μ g/mL polybrene (Sigma-Aldrich), as described previously.³⁰ Transduced cells were subcultured the next day into p100 tissue culture dishes with Ksfm containing 15 μ g/mL hygromycin, 1 μ g/mL puromycin, or 0.2 mg/mL G418 and drug-selected for 5 to 7 days to obtain pure populations of stable transductants.

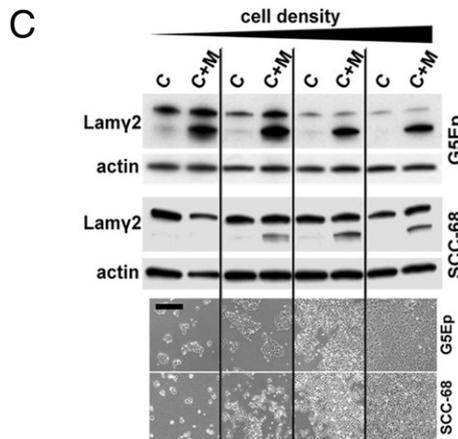
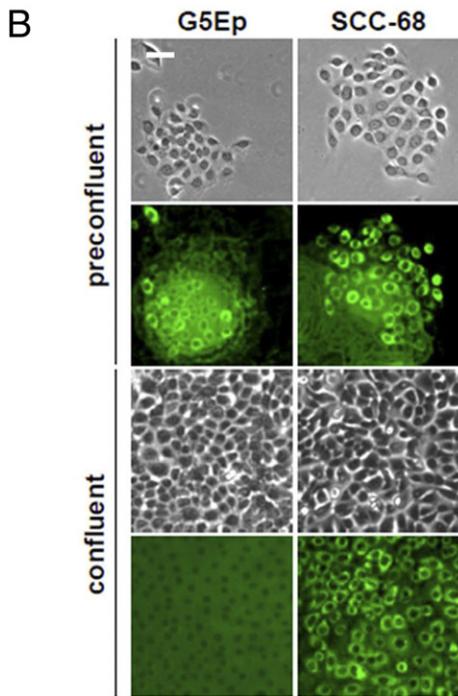
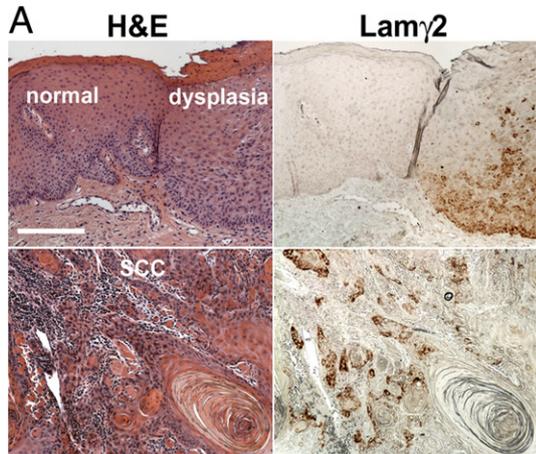
Quantitative PCR

Total RNA was isolated from cells using an RNeasy Plus mini kit (Qiagen, Valencia, CA) and cDNA synthesized from 500 ng total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). mRNA levels were quantified by quantitative real-time PCR (qPCR) using Applied Biosystems (ABI) FAST SYBR Green PCR master mix (Life Technologies, Foster City, CA) on an ABI StepONE Plus instrument (40 cycles of 95°C for 15 seconds and 58°C for 30 seconds). Amplicons were as follows: laminin γ 2 (*LAMC2* gene), forward 5'-CTCTGCTTCTCGCTCCTCC-3' and reverse 5'-TCTGTGAAGTTCCCAGATCAA-3'; laminin α 3 (*LAMA3*), forward 5'-TCAGCACATATTATCTGGGAGG-3' and reverse 5'-AAATTTTTCATGCAGCCTCG-3'; laminin β 3 (*LAMB3*), forward 5'-CCAAGCCTGAGACCTACTGC-3' and reverse 5'-GCCACATTCTCTACTCGGTGA-3'; and *GAPDH*, forward 5'-GAGCCTCAAGATCATCAGCA-3' and reverse 5'-ACAGTCTTCTGGGTGGCAGT-3'). Relative expression was calculated using the $\Delta\Delta C_T$ method, normalizing values to GAPDH within each sample; standard error of the mean was calculated from the results of triplicate aliquots.

Results

A Human Cell Culture Model of Neoplastic Laminin γ 2 Overexpression

Our earlier study that found abnormal Lam γ 2 expression in oral and epidermal dysplasias²⁵ extended previous reports that Lam γ 2 is frequently expressed by cells at the invading fronts of SCC and certain other carcinomas.^{27–29} Examples of such Lam γ 2 immunostaining patterns are shown in Figure 1A. To investigate the mechanism of neoplastic Lam γ 2 overexpression, we sought to identify culture conditions under which normal primary keratinocytes would cease, but SCC cells would continue, expressing Lam γ 2. Cell lines used are characterized in Table 1. At low cell density, both normal keratinocytes (G5Ep) and SCC cells (SCC-68) synthesized Lam γ 2 (intracellular staining) and secreted this protein onto the culture dish (extracellular staining) (Figure 1B). Substratum-deposited Lam γ 2 remained stably adherent to the culture dish for many days after secretion, as evidenced by the equal staining intensity of Lam γ 2 ad-



adjacent to and at a distance from the current position of cells (Figure 1B). (Note that during proliferation in culture keratinocytes migrate randomly over short distances, with the extent of this movement over the course of many days detectable by immunostaining for the Lam332 that the cells deposit on the substratum.³¹)

As normal keratinocytes grew to higher density, they began to reduce Lamγ2 synthesis and at confluence no longer expressed this protein at detectable levels (Figure 1, B and C). In contrast, SCC cells expressed Lamγ2 at the same levels independent of density (Figure 1, B and C). Lamγ2 previously deposited by cells onto the culture dish remained adherent after trypsin/EDTA-detachment of cells but was solubilized together with intracellular protein by direct addition of SDS lysis buffer to cultures (compare detached cell extracts with scraped extracts in Figure 1C). The scraped extract contained both intracellular, unprocessed (155 kDa) and extracellular, proteolytically processed (105 kDa) forms of Lamγ2. Western blot analysis of the conditioned medium of confluent N/E6E7 cultures showed an increased rate of Lamγ2 synthesis and secretion, similar to that of SCC cells (Figure 1D).

To determine whether different rates of intracellular Lamγ2 protein turnover contribute to the apparent differences in Lamγ2 expression between normal keratinocytes and SCC cells, we examined intracellular Lamγ2 stability using cycloheximide. Because Lamγ2 is normally secreted, we treated cells with brefeldin A, an inhibitor of endoplasmic reticulum-to-Golgi transport, to retain the protein inside cells. Treatment with brefeldin A alone increased intracellular Lamγ2, as expected (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Normal and SCC cells treated with brefeldin A and cycloheximide showed little or no decrease in Lamγ2 over 6 hours, with no indication of a shorter half-life of the protein in normal cells (see Supplemental Figure S1 at

Figure 1. Laminin $\gamma 2$ overexpression by neoplastic epithelial cells *in vivo* and at high cell density in culture. **A:** H&E-stained and Lam $\gamma 2$ -immunostained sections of the margin between normal oral epithelium and a pre-malignant dysplasia (top panels) and an invasive oral SCC (bottom panels). Note absence of Lam $\gamma 2$ expression in normal epithelium, in contrast to focal expression of Lam $\gamma 2$ by basal cells in the dysplastic lesion and in the SCC. Scale bar = 250 μm . **B:** Preconfluent and confluent cultures of normal primary keratinocytes (G5Ep) and SCC cells (SCC-68) showing phase-contrast images and the same (preconfluent) or similar (confluent) fields immunostained for Lam $\gamma 2$ (green fluorescence). Note substantial intracellular (endoplasmic reticulum/Golgi) Lam $\gamma 2$ content and deposition onto the culture dish by preconfluent normal keratinocytes and SCC cells, whereas only the SCC cells continued to express immunodetectable Lam $\gamma 2$ at confluence. Scale bar = 50 μm . **C:** Western blot analysis of Lam $\gamma 2$ expression by G5Ep and SCC-68 cells as a function of cell density. Protein extracts of replicate cultures were harvested at cell densities indicated by the phase contrast microscopic images shown beneath (Scale bar = 500 μm), either as trypsin/EDTA-detached cell extracts (C) or scraped extracts (ie, total cell plus extracellular matrix material) (C+M). The upper band in blots is the 155-kDa unprocessed form and the lower band is the 105-kDa postsecretion processed form of Lam $\gamma 2$. Note reduction of Lam $\gamma 2$ expression by normal keratinocytes with increasing cell density, in contrast to density-independent expression by SCC cells. **D:** Harvests of conditioned medium at 24 hours from confluent cultures of normal keratinocytes (G5Ep), premalignant keratinocytes (N/E6E7 and POE9n), and SCC cells (SCC-13, SCC-68) separated on a reducing gel, Western blotted, and probed for Lam $\gamma 2$. Note increased production rates of Lam $\gamma 2$ by premalignant and SCC cells, and also Lam $\gamma 2$ processing deficiencies in POE9n, SCC-13, and SCC-68.

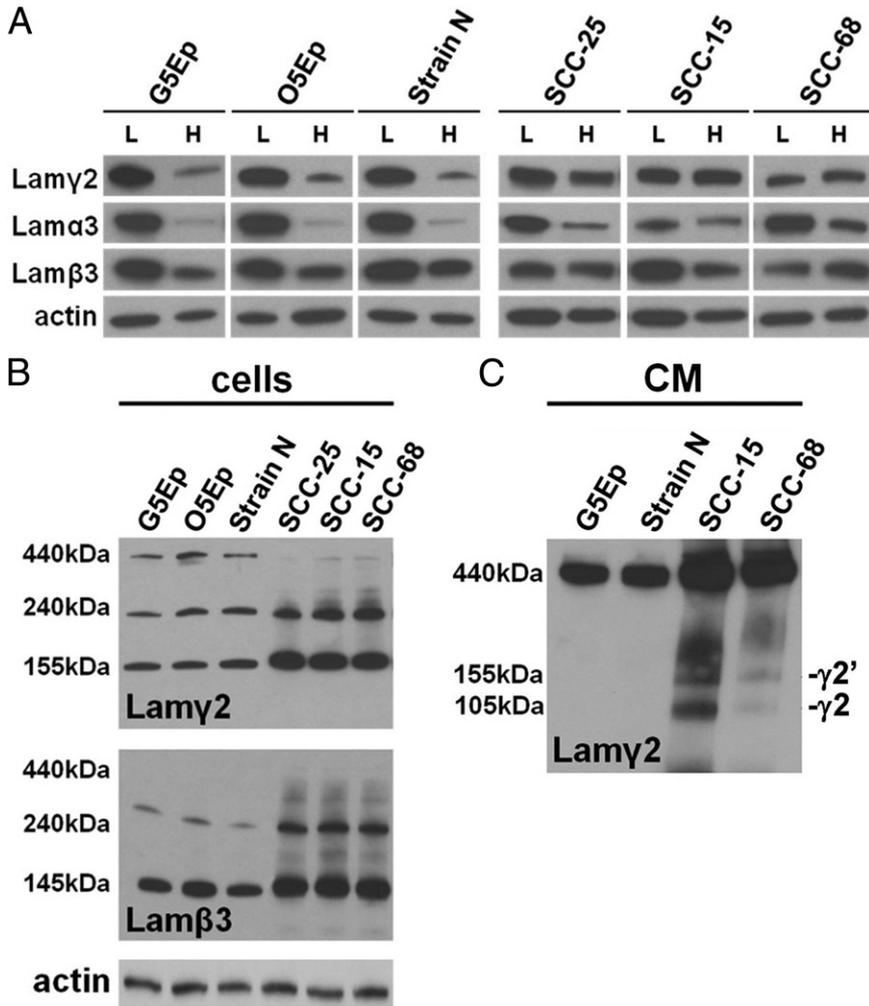


Figure 2. Unbalanced Lam332 subunit expression by SCC cells and secretion of abnormal assembly intermediates. **A:** Western blot analysis of the three Lam332 subunits in low-density (L) versus high-density (H) cultures of normal keratinocytes (G5Ep, O5Ep, strain N) and SCC cells (SCC-25, SCC-15, SCC-68). Note strong density repression of Lam γ 2 by normal keratinocytes and lack of it by SCC cells. **B:** Nonreducing gels of cell extracts of confluent normal keratinocyte and SCC cultures Western blotted for Lam γ 2 (upper gel) and Lam β 3 (lower gel). Note that SCCs have little or no intracellular Lam332 trimer, compared with normal keratinocytes, and that the Lam β 3 antibody does not bind well to Lam β 3 after assembly into the Lam332 trimer in normal cells. **C:** Nonreducing gels of conditioned medium from confluent normal keratinocyte and SCC cultures Western blotted for Lam γ 2, disclosing relative proportions of normal Lam332 trimer, β 3 γ 2 dimer, and Lam γ 2 monomer. Note secretion by SCC cells of Lam γ 2 monomer in processed (105 kDa; γ 2) and unprocessed (155 kDa; γ 2') forms.

<http://ajp.amjpathol.org>). In contrast, levels of MYC, a protein known to have a short half-life, decreased progressively in normal keratinocytes and SCC cells with time after cycloheximide treatment (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). We concluded that Lam γ 2 overexpression is the result of increased rates of synthesis of this protein by SCC cells.

Unbalanced Expression of the Three Laminin-332 Chains Results in Secretion of Laminin γ 2 Monomers by SCC Cells

We next examined expression of the other two Lam332 subunits, Lam α 3 and Lam β 3, to determine whether their synthesis is differentially regulated in normal keratinocytes and SCC cells. Normal keratinocytes (G5Ep, O5Ep, and strain N) at high cell density greatly reduced Lam α 3 expression, as well as Lam γ 2 expression, and slightly reduced Lam β 3 expression, compared with low density cells (Figure 2A). SCC cells showed density-related changes in Lam α 3 and Lam β 3 expression similar to that of normal keratinocytes, but continued to express Lam γ 2 at high levels at confluence (Figure 2A). The consequence of excess Lam γ 2 expression in SCC cells

was revealed by nonreducing Western blots, which resolve laminin monomers from the disulfide-bonded heterodimer assembly intermediates and the ultimate Lam332 trimer.⁶² Cell extracts of confluent normal keratinocytes contained Lam γ 2 and Lam β 3 monomers and the ~240 kDa Lam β 3 γ 2 assembly intermediate, in addition to fully formed ~440 kDa Lam332 trimer (Figure 2B). In contrast, SCC cells contained mostly Lam γ 2 and Lam β 3 monomers and heterodimers, with little trimer (Figure 2B), presumably the result of unbalanced subunit expression (Figure 2A). Analysis of conditioned medium (Figure 2C) revealed that normal keratinocytes secreted Lam γ 2 exclusively assembled into Lam332 trimers, whereas a fraction of the total Lam γ 2 in SCC conditioned medium was in the form of monomers, both unprocessed (155 kDa) and processed (105 kDa).⁶³

Abnormal Laminin γ 2 Regulation Begins Early in Neoplastic Progression as a Characteristic of Premalignant Keratinocytes

In vivo, Lam γ 2 overexpression begins in regions of preinvasive dysplasias²⁵ (Figure 1A). We therefore asked whether cells cultured from dysplasias display

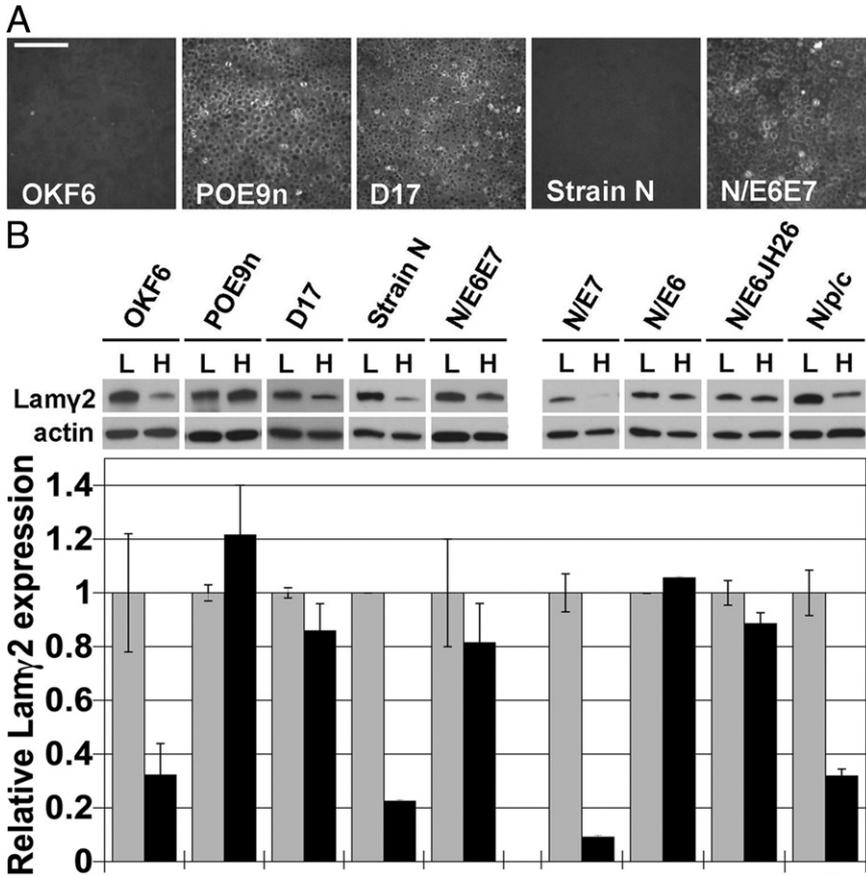


Figure 3. Abnormal laminin γ 2 expression by premalignant keratinocytes. **A:** Lam γ 2 immunofluorescence-stained confluent cultures of normal oral keratinocyte line OKF6, premalignant dysplastic oral keratinocyte lines POE9n and D17, normal epidermal keratinocyte line strain N, and strain N transduced to express the HPV16 E6 and E7 oncoproteins (N/E6E7). Note continued Lam γ 2 expression at confluence by POE9n, D17, and N/E6E7 in contrast to repression of Lam γ 2 under this condition by normal keratinocytes. Scale bar = 200 μ m. **B: Left:** Lam γ 2 Western blots of low-density (L) and high-density (H) cultures of the same cell lines, confirming lack of γ 2 repression at confluence by POE9n, D17, and N/E6E7. **Right:** Lam γ 2 Western blots of N/E7, N/E6, N/E6JH26, and of strain N transduced to express a dominant-negative mutant p53 and a p16^{INK4A}-resistant mutant cdk4 (N/p/c), showing that E6 alone can dysregulate Lam γ 2, and that functional loss of p53 is not involved in this dysregulation. Densitometric quantification of each band is shown below the blots. The Lam γ 2 level (normalized to its actin band) of the pre-confluent culture of each cell line is set as 1 (gray bars), with the relative Lam γ 2 level of the confluent culture shown for each cell line (black bars). Error bars indicate the results of quantifying scans of two exposures of each gel. The error bars show the results of the scans of two different exposure times of the same Western blot. The gray and black bars show the average of these two scans.

abnormal Lam γ 2 expression as a stable characteristic, as is the case for SCC cells. The POE9n and D17 cell lines cultured from human oral dysplasias^{30,38,39} proved to maintain Lam γ 2 expression at confluence, in contrast to the normal keratinocytes OKF6 and strain N (Figure 3, A and B).

In certain stratified epithelia (ie, cervix, tonsil, and vulva), early neoplastic development is sometimes linked to infection with human papillomavirus (HPV) type 16 or 18, leading to integration of viral genomic DNA and expression of their E6 and E7 oncogenes.⁵⁻⁸ Normal primary keratinocytes (strain N) transduced to express HPV16 E6 and E7 (the N/E6E7 cell line) maintained Lam γ 2 expression at confluence, similar to SCC cells (Figures 1 and 2) and POE9n and D17 cells (Figure 3, A and B).

Important activities of the HPV16 oncoproteins are to target p53 for degradation (accomplished by the E6 oncoprotein⁶⁰) and to inactivate pRB and thereby confer resistance to the CDK4 inhibitor p16^{INK4A} (accomplished by E7⁶⁴). We wished to determine whether loss of p53-dependent and pRB/p16^{INK4A}-dependent growth control achieved by genetic alterations not involving viral oncogenes, as is the case for most oral and epidermal SCCs, would result in loss of normal Lam γ 2 regulation. We therefore assessed the Lam γ 2 expression characteristics of strain N keratinocytes engineered to express a dominant-negative mutant p53 and a p16-insensitive mutant cdk4 (N/p/c cells³⁴) and found that N/p/c cells

reduced Lam γ 2 expression at confluence similar to normal primary keratinocytes. This finding demonstrated that activities of E6 and/or E7 other than disabling p53 or pRB/p16^{INK4A} functions are responsible for Lam γ 2 dysregulation. Comparing the properties of strain N cells engineered to express either E7 or E6 alone, we found that E6 alone caused loss of Lam γ 2 regulation (Figure 3B). Finally, examining the effects of expressing E6(JH26), a mutant of E6 that lacks p53-targeting activity but retains E6 transcriptional transactivation activity,^{58,60} we found that an activity of E6 other than p53 degradation is responsible for Lam γ 2 dysregulation (Figure 3B; see also Supplemental Figure S2 available at <http://ajp.amjpathol.org>). Notably, E6 activates transcription of a variety of genes, including *hTERT*^{65,66} and *EGFR*,^{67,68} and has been reported recently to activate mTOR.⁶⁹

Hyperactivity of Signaling Kinases Downstream of EGFR Correlates with Lamin γ 2 Overexpression

Hyperactivity of the EGFR/(MAPK)ERK and PI3K/AKT/mTOR pathways are frequent abnormalities of advanced oral and oropharyngeal SCC.⁹⁻¹¹ We sought to identify signaling alterations in these two pathways (Figure 4A) in confluent cultures of premalignant keratinocytes and SCCs that correlate with Lam γ 2 dysregulation. EGFR overexpression, accompanied by increased levels of the

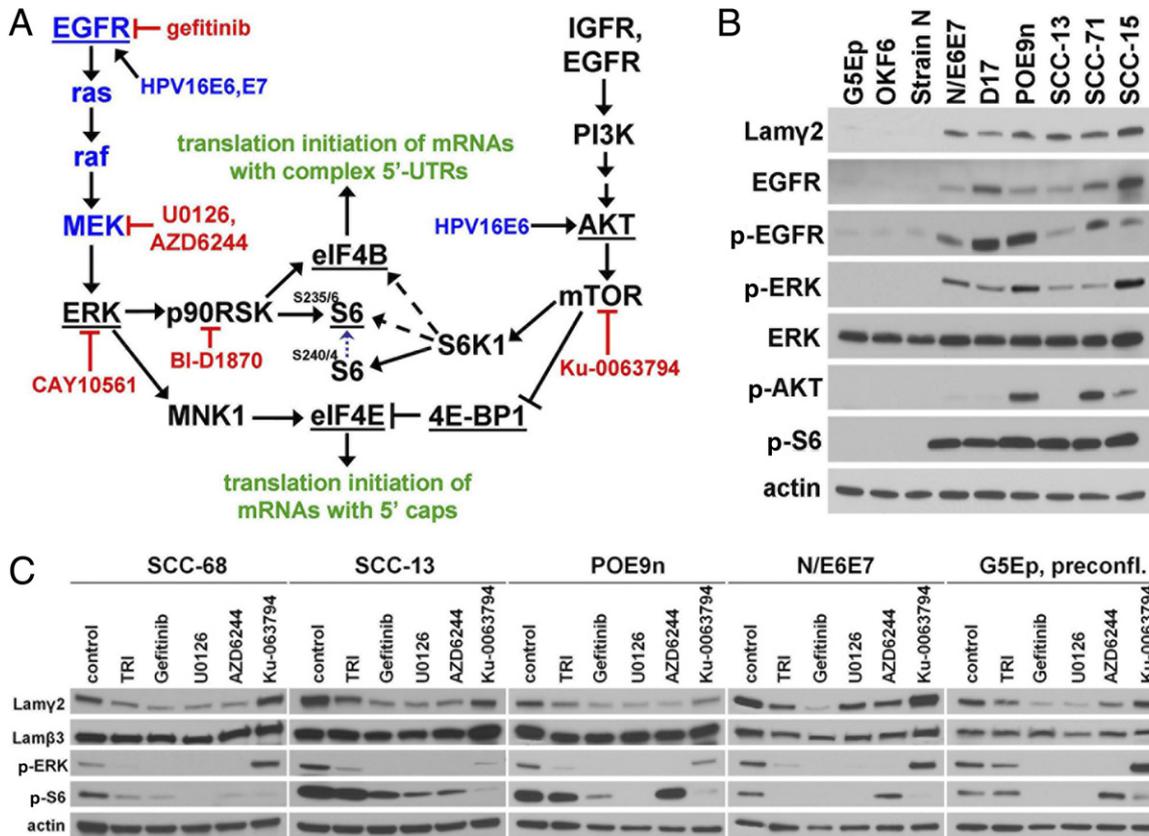


Figure 4. Neoplastic cells that lack density-dependent Lamγ2 repression display EGFR/ERK hyperactivation. **A:** The EGFR/ERK and PI3K/AKT/mTOR kinase cascade signaling pathways and their downstream targets analyzed in this study. **Arrows** indicate activation; **T-bars** indicate inhibition. **Dashed arrows** indicate a less important role for S6K1 in activating these targets. Note that S6K1 must first phosphorylate S6 at ser(240/244) before either RSK or S6K1 can phosphorylate S6 at ser(235/236). Underlining indicates proteins that were analyzed for phosphorylation/activation by Western blotting. For some experiments, some proteins (shown in blue) were expressed or hyperactivated by engineering normal keratinocytes. Inhibitors that specifically block the associated kinase are shown in red. **B:** Confluent cultures of normal (G5Ep, OKF6, strain N) and premalignant (N/E6E7, POE9n, D17) keratinocytes and SCC cells (SCC-13, SCC-71, SCC-15) analyzed by Western blotting for Lamγ2 and selected phosphoproteins. Note consistent p-EGFR, p-ERK, and p-S6 increases in all of the Lamγ2-overexpressing cell lines. **C:** Effects of kinase inhibitors on Lamγ2 and Lamβ3 expression and on signaling molecules. Confluent cultures of SCC-68, SCC-13, POE9n, and N/E6E7 and preconfluent cultures of G5Ep were treated for 2 days with kinase inhibitors and then were analyzed by Western blotting. TRI is the TGFβ receptor I inhibitor, gefitinib is an EGFR inhibitor, U0126 and AZD6244 are MEK inhibitors, and Ku-0063794 is a TORC1/2 inhibitor. Note reduction of Lamγ2 expression, but not of Lamβ3 expression, by all kinase inhibitors tested except Ku-0063794. Lamγ2 reduction correlated consistently with decreased p-ERK.

activated p-(Y1068) form of EGFR, was common to all cell lines that overexpressed Lamγ2 (Figure 4B). Levels of p-ERK, a downstream target of EGFR activation mediated by RAS, RAF, and MEK, also were elevated, compared with normal cells, in all of the Lamγ2-overexpressing neoplastic cell lines, although not proportional to p-EGFR levels (Figure 4B).

We also analyzed the phosphorylation states of AKT, a signaling mediator in the PI3K/mTOR pathway, and of S6, a downstream target of both EGFR/ERK and PI3K/mTOR pathways. p-AKT was elevated in only three of the six Lamγ2 dysregulated cell lines tested, but p-S6 was substantially elevated in all of them (Figure 4B). The p-S6 antibody we used recognizes p-S6(ser235/236), which can be phosphorylated either by the ERK-activated RSK kinase or by the TORC1-activated S6 kinase (S6K1).⁷⁰ We interpreted the lack of correlation between p-AKT and p-S6 levels as indicating that S6 phosphorylation in Lamγ2-overexpressing cells results from EGFR/ERK pathway hyperactivity.

We next assessed the effects of small-molecule inhibitors of pathway kinases on Lamγ2 overexpression by

POE9n, NE6E7, and two SCC cell lines. The EGFR inhibitor gefitinib and the MEK inhibitors U0126 and AZD6244 substantially reduced p-ERK levels and Lamγ2 expression (Figure 4C). Gefitinib and U0126 greatly reduced p-S6 levels in all four lines, although AZD6244 did not affect S6 activation in POE9n and N/E6E7 (Figure 4C). These results indicate that ERK-dependent RSK is the major activator of S6. The TORC1/2 inhibitor Ku-0063794 greatly reduced p-S6 levels, but did not change Lamγ2 expression, revealing that S6 activation is not essential for Lamγ2 overexpression (Figure 4C). S6K1 is thought to phosphorylate S6 on ser235/236 and on ser240/244, whereas the ERK-activated RSK phosphorylates S6 only on ser235/236.^{71,72} Prior phosphorylation of S6 on ser240/244 by S6K1 increases the ability of RSK to phosphorylate S6 on ser235/236.⁶⁷ The reduction of p-S6(ser235/236) levels by Ku-0063794 we observed is consistent with those studies.

We also tested the effects of a TGFβ receptor I kinase inhibitor, TRI, because the *LAMC2* gene, which encodes Lamγ2, is transcriptionally activated by TGFβ.⁷³ As expected, TRI reduced Lamγ2 expression in all cell lines

(Figure 4C). None of the kinase inhibitors we tested reduced Lam β 3 expression (Figure 4C), consistent with the lack of correlation between Lam γ 2 and Lam β 3 expression in pre-confluent and confluent normal keratinocytes and SCC cells (Figure 2A).

Normal keratinocytes transiently overexpress Lam γ 2 during wound re-epithelialization *in vivo* (see Supplemental Figure S3B at <http://ajp.amjpathol.org>).^{31,74,75} The effects of inhibitors on Lam γ 2 expression and p-ERK and p-S6 levels in pre-confluent normal keratinocytes were the same as for confluent premalignant and SCC cells (Figure 4C), demonstrating that Lam γ 2 expression is ERK-dependent in both normal and neoplastic settings.

Laminin γ 2 Expression in Neoplastic Cells Is Regulated at the Level of Translation and Correlates with the Activation States of Translation Initiation Factors

ERK activation initially was found to have effects in the nucleus, activating several transcriptional regulators, including Elk-1.⁷⁶ We used qPCR to investigate whether the reduction of Lam γ 2 protein expression caused by EGFR and MEK inhibition (Figure 4C) is the result of reduced *LAMC2* gene transcription. Confluent SCC cultures treated with EGFR or MEK inhibitors showed no substantial change

in Lam γ 2 mRNA levels, compared with untreated controls (Figure 5A). TRI reduced Lam γ 2 mRNA levels to 40% of control levels (Figure 5A), consistent with TRI effects on Lam γ 2 protein expression (Figure 4C) and confirming the role of TGF β signaling in Lam γ 2 transcription.⁷³

We next examined the density dependence of Lam γ 2 mRNA levels. Lam γ 2 mRNA levels in normal keratinocytes at confluence were 25% to 40% of that of pre-confluent cells (Figure 5B), consistent with transcription or mRNA stability playing a significant role in normal density-related Lam γ 2 repression. However, Lam γ 2 mRNA levels in premalignant and SCC cell lines did not change between pre-confluence and confluence and were typically lower than those of normal keratinocytes at confluence (Figure 5B). We concluded that Lam γ 2 mRNA is translated much more efficiently in neoplastic cells than in normal cells at high cell density. In contrast, Lam α 3 mRNA decreased at confluence in both normal keratinocytes and SCC cells, and Lam β 3 mRNA slightly decreased in normal keratinocytes and increased in SCC cells at confluence (see Supplemental Figure S4A at <http://ajp.amjpathol.org>). This confirmed that the mechanism regulating expression of Lam γ 2 is different from the mechanism or mechanisms regulating expression of the other Lam332 subunits.

Translation initiation is the rate-limiting step for protein synthesis. Efficient initiation requires translation initiation

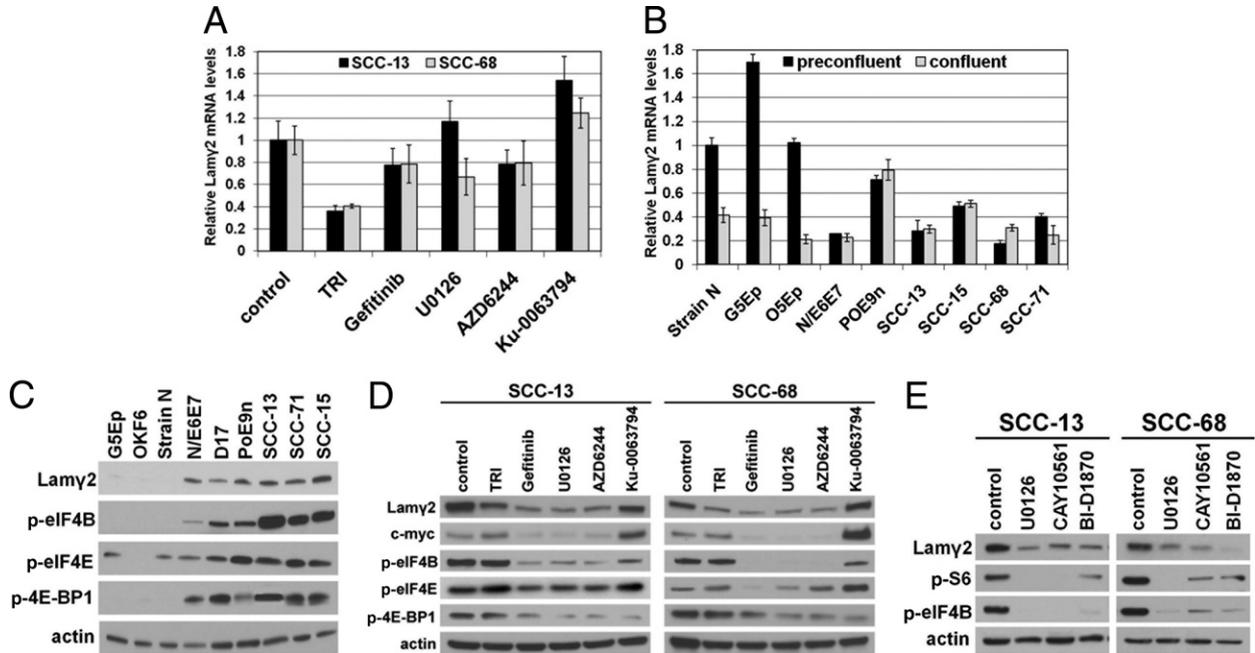


Figure 5. Lam γ 2 overexpression by neoplastic cells is unrelated to Lam γ 2 mRNA levels, but correlates with ERK-dependent activation of translation initiation factors. **A:** Effects of kinase inhibitors on Lam γ 2 mRNA levels in SCC cells. Confluent cultures were treated for 2 days with the inhibitors TRI, gefitinib, U0126, AZD6244, and Ku-0063794 and then were analyzed by qPCR for Lam γ 2 mRNA, with results for each cell line internally normalized to GAPDH mRNA levels. Note the decrease in Lam γ 2 mRNA levels resulting from treatment with TRI, but not with the EGFR inhibitor gefitinib or the MEK inhibitors U0126 and AZD6244, which block Lam γ 2 protein synthesis in confluent SCC cultures. **B:** qPCR comparison of Lam γ 2 mRNA levels in pre-confluent and confluent cultures of normal and premalignant keratinocytes and SCC cells. Results shown are relative to Lam γ 2 mRNA levels in pre-confluent cultures of strain N keratinocytes, set at an arbitrary unit of 1. Note lower levels of Lam γ 2 mRNA in neoplastic cells than in normal keratinocytes and absence of cell density-dependent mRNA decreases in neoplastic cells, compared with normal keratinocytes. **C:** Western blot analysis of activated forms of translation initiation factors in normal and premalignant keratinocytes and SCC cells. Note increased levels of p-eIF4B, p-eIF4E, and p-4E-BP1 in premalignant keratinocytes and SCC cells, compared with normal keratinocytes. **D:** Western blot analysis of effects of 2-day treatments of confluent SCC cultures with kinase inhibitors on levels of Lam γ 2, c-myc, and phosphorylated states of translation initiation factors. Note strong reduction of Lam γ 2, c-myc, and p-eIF4B levels in response to the EGFR inhibitor gefitinib and the MEK inhibitors U0126 and AZD6244, in contrast to small or no effect of the TORC1/2 inhibitor Ku-0063794. **E:** Western blot analysis of the effects on levels of Lam γ 2 and p-eIF4B of 2-day treatments of confluent SCC cultures with ERK and RSK inhibitors, compared with U0126 treatment. Note that ERK and RSK inhibitors substantially reduced Lam γ 2 and p-eIF4B levels.

factors of the eIF4 family, the activities of which are regulated by the EGFR/ERK and PI3K/mTOR signaling pathways^{71,77,78} (Figure 4A). We asked whether Lam γ 2-overexpressing neoplastic cells contain higher levels of the phosphorylated active forms of any of these factors, compared with normal cells. Western blot analysis of confluent cultures revealed higher levels of p-eIF4B, p-eIF4E, and p-4E-BP1 in neoplastic cells than in normal keratinocytes (Figure 5C). The EGFR kinase inhibitor gefitinib and the MEK inhibitors U0126 and AZD6244 greatly reduced p-eIF4B and p-4E-BP1 levels and had a lesser effect on p-eIF4E levels in SCC cells (Figure 5D). The TORC1/2 inhibitor Ku-0063794 greatly reduced p-4E-BP1 levels, slightly reduced p-eIF4B levels, and had no effect on p-eIF4E levels (Figure 5D). As expected, the TGFBR1 kinase inhibitor TRI had no effect on levels of any of these phosphoproteins (Figure 5D).

The effects of these inhibitors on c-myc expression proved to be the same as on Lam γ 2 expression (Figure 5D). Expression of c-myc is regulated at the level of translation by eIF4B/eIF4A, which aids translation initiation of mRNAs having very long 5'-UTR sequences that form stem-loop secondary structures.^{78,79} The Lam γ 2 5'-UTR proved to have such a long and complex structure, similar to that of c-myc and much longer than those of Lam α 3 and Lam β 3 mRNAs (see Supplemental Figure S4B at <http://ajp.amjpathol.org>).

We extended our analysis by testing the effects of the ERK1/2 inhibitor CAY10561⁴⁹ and of the RSK inhibitor BI-D1870.⁵⁰ These inhibitors reduced Lam γ 2 expression associated with substantially reduced levels of p-S6 and p-eIF4B (Figure 5E). These results are consistent with ERK/RSK activation of p-eIF4B-dependent Lam γ 2 mRNA translation as the mechanism of Lam γ 2 protein overexpression in neoplastic keratinocytes.

Hyperactive RAS, RAF, or MEK Signaling Is Sufficient to Cause Abnormal Laminin γ 2 Expression

The above experiments clearly demonstrated that EGFR/ERK/RSK activity is essential for Lam γ 2 overexpression in neoplastic cells. We wished to determine whether activation of the EGFR/ERK signaling pathway in normal keratinocytes is sufficient to cause loss of density-dependent Lam γ 2 regulation. Our earlier study had revealed that primary keratinocytes expressing activated HRAS grow well in the fibroblast feeder layer culture system, but undergo growth arrest if switched to a semidefined keratinocyte medium,⁸⁰ and strain N and G5Ep primary keratinocyte lines transduced to express HRAS(G12V) proved unable to proliferate in Ksfm medium. Constitutively active mutant HRAS has been reported to induce p16^{INK4A}-enforced senescence in normal cells under some conditions.^{56,81} For the following experiments, therefore, we used our TERT-immortalized normal keratinocyte line, N/TERT1,³⁰ which is unable to express p16^{INK4A} or p14^{ARF} but displays normal Lam γ 2 repression at confluence (Figure 6A).

N/TERT-1 cells transduced to express constitutively active mutant forms of either EGFR, HRAS, cRAF1, or

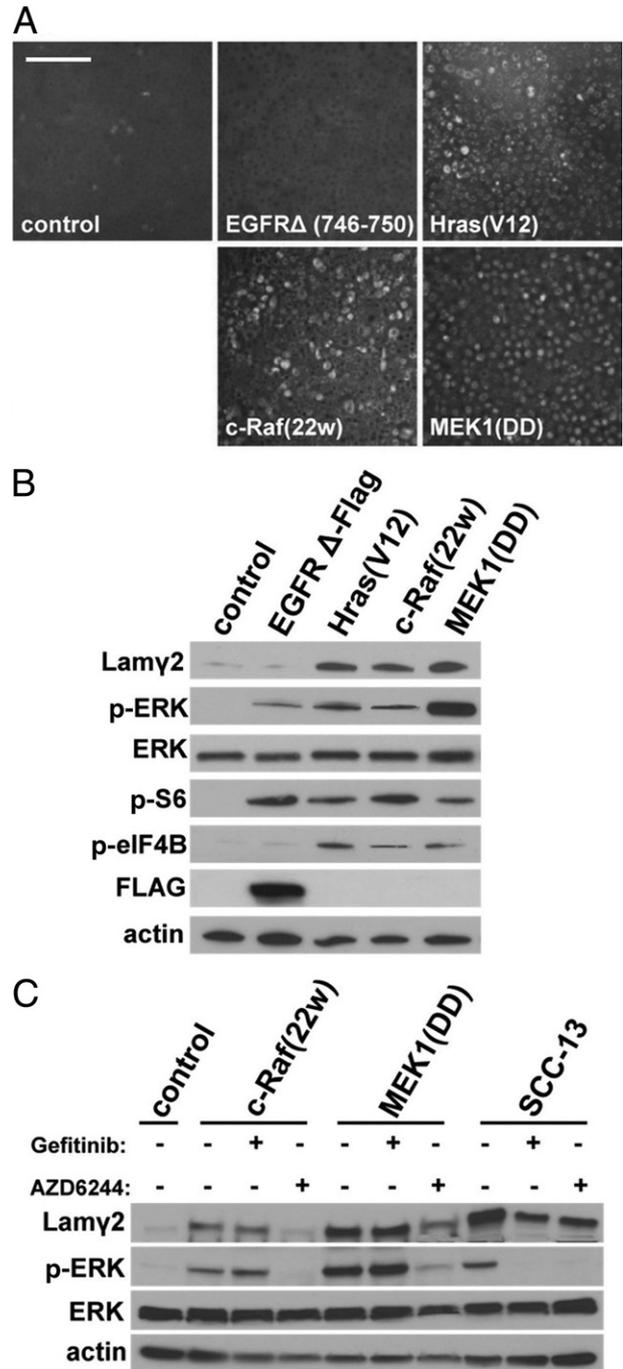


Figure 6. Hyperactivation of signaling pathway kinases upstream of ERK results in Lam γ 2 dysregulation in normal keratinocytes. **A:** Lam γ 2 immunostaining of confluent cultures of N/TERT-1 keratinocytes engineered to express constitutively active HRAS(G12V), cRAF1(22w), or MEK1(DD), and EGFR Δ (E746-A750)-FLAG. Scale bar = 200 μ m. **B:** Western blot analysis of Lam γ 2, signaling pathway phosphoproteins, and activation state of translation initiation factors of the same cell lines. Control indicates N/TERT-1 cells. Note that expression of constitutively active HRAS, cRAF1, or MEK1 increased p-ERK, p-S6, and p-eIF4B levels, whereas constitutively active EGFR slightly increased levels of p-ERK, robustly increased p-S6, but did not increase p-eIF4B and did not cause Lam γ 2 overexpression. **C:** Western blot analysis of effects of EGFR and MEK inhibitors on Lam γ 2 and p-ERK levels in control N/TERT-1 cells, cRAF1(22w) and MEK1(DD) transductants, and SCC-13 cells. Note that Lam γ 2 overexpression and increased p-ERK levels in keratinocytes expressing activated mutant cRAF1 or MEK are normalized by treatment with the MEK inhibitor AZD6244, but not with the EGFR kinase inhibitor gefitinib.

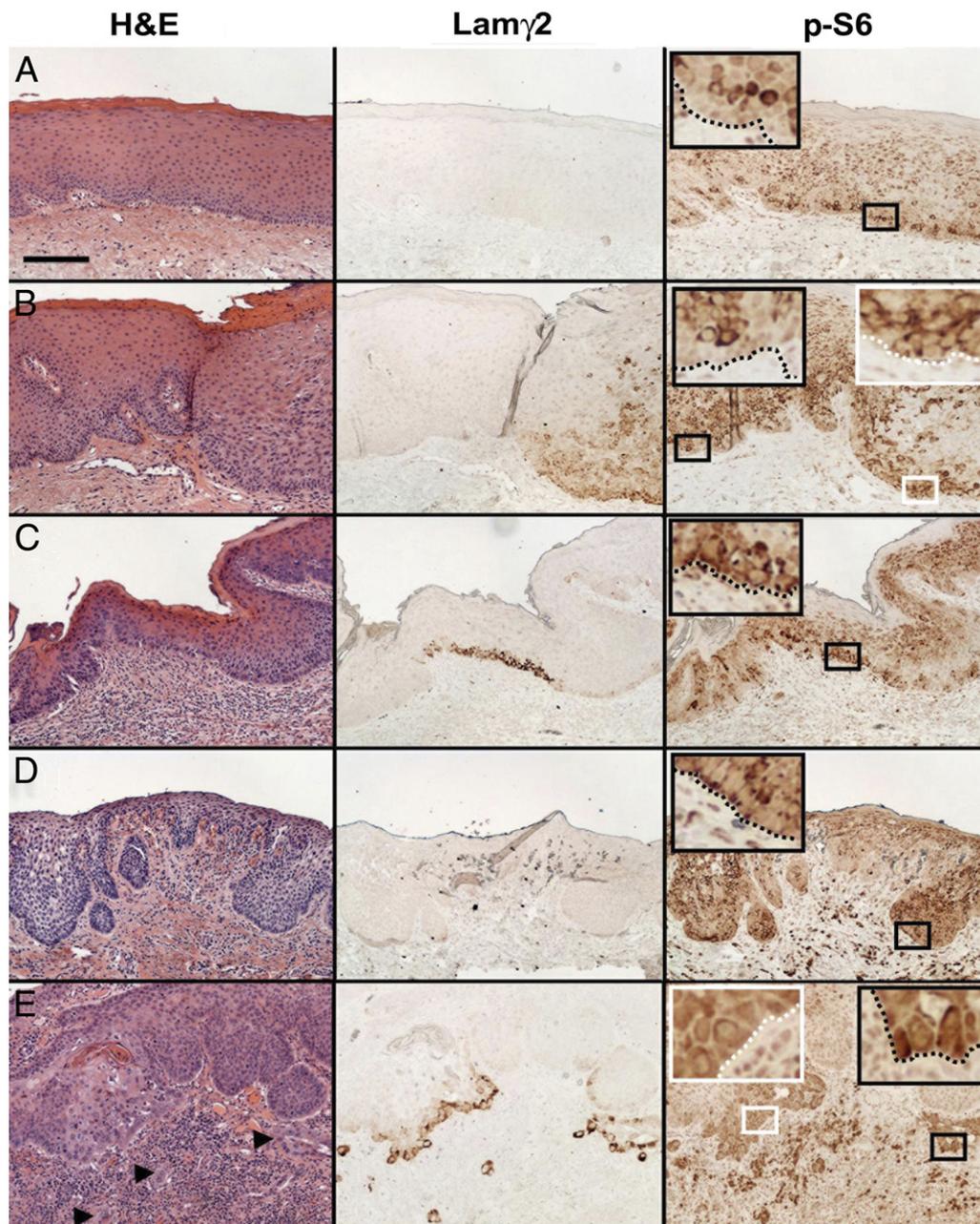


Figure 7. Immunohistologic detection of Lam γ 2 overexpression *in vivo* and its association with increased p-S6 and p-eIF4B levels in basal cells of oral premalignant dysplasias. In normal human oral mucosal epithelium (**A**), oral mucosal dysplasias (**B–D**), and a microinvasive oral SCC arising from a dysplasia (**E**), sections were stained with H&E and immunostained for Lam γ 2 and p-S6. Enlargements of the areas demarcated by small rectangles are shown as corresponding **insets**, with the basal lamina traced by **dotted lines**. Scale bar = 200 μ m. **A:** Normal oral epithelium showing no Lam γ 2 and only suprabasal p-S6 staining. **B:** Margin between normal epithelium (**left half**) and dysplasia (**right half**), showing no Lam γ 2 and p-S6 confined to suprabasal cells in the normal region, contrasting with Lam γ 2 and basal cell p-S6 in the dysplastic region. **C:** Dysplasia in which not all basal cells are p-S6⁺, not all p-S6⁺ cells overexpress Lam γ 2, and the intensity of Lam γ 2 immunostaining varies greatly. **D:** Region of a dysplasia in which many basal cells are p-S6⁺, but none express Lam γ 2. **E:** Dysplasia overlying microinvasive SCC (indicated by **arrowheads** in the H&E-stained panel), showing variable Lam γ 2 and basal p-S6 staining in the dysplasia and strong Lam γ 2 and basal p-S6 staining in areas of microinvasive SCC.

MEK1 grew rapidly and through serial passages in Ksfm and proved to be independent of EGF for optimal growth (see Supplemental Figure S5 at <http://ajp.amjpathol.org>). This EGF independence was expected from the mutant ras result of our previous study⁸⁰ and confirmed the biological activity of all four EGFR signaling pathway mutants in keratinocytes. Immunostaining and Western blot analysis of confluent cultures revealed that the constitutively active mutant forms of HRAS, cRAF1, and MEK1,

but not of EGFR, conferred loss of density-dependent Lam γ 2 repression (Figure 6, A and C). We analyzed the mutant cRAF1 and MEK1 transductants further by assessing the effects of kinase inhibitors. As expected, p-ERK levels and Lam γ 2 expression in the cRAF1(22w) and MEK1(DD) transductants were unaffected by gefitinib inhibition of the upstream EGFR kinase, but were reduced by AZD6244 inhibition of MEK (Figure 6C). Both inhibitors reduced Lam γ 2 and p-ERK levels in SCC-13

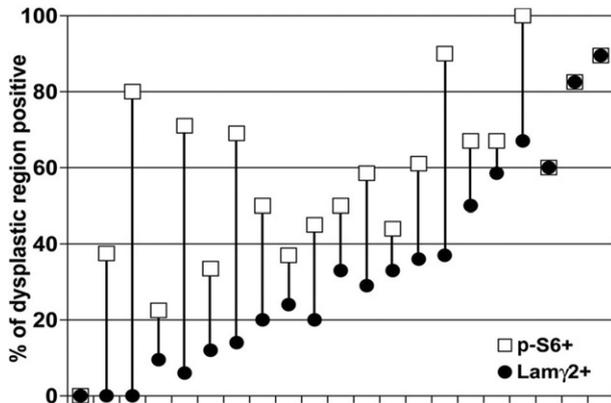


Figure 8. Association of Lam γ 2 overexpression with increased basal cell p-S6 levels in dysplastic lesions. Twenty-one oral lesions with dysplasia were evaluated microscopically for the fraction of the dysplastic region of each that contained p-S6⁺ and Lam γ 2⁺ basal cells. Specimens were charted in order of increasing percentage of Lam γ 2⁺ and p-S6⁺, to better show the range of immunostaining patterns.

(Figures 6C and 4C), consistent with an essential role of EGFR hyperactivity for ERK activation and consequent Lam γ 2 overexpression in this SCC cell line.

Having established that expression of either HRAS(G12V) or cRAF1(22w) or MEK1(DD) is sufficient to cause Lam γ 2 overexpression, we asked whether such oncogene-driven ERK hyperactivation results in activation of the downstream translation factors that we had found (as described above) to correlate closely with ERK hyperactivity. Levels of p-eIF4B and p-S6 proved to be elevated in the mutant HRAS, cRAF1, and MEK1 transductants (Figure 6B). The mutant EGFR transductant, which showed normal density repression of Lam γ 2, displayed a rather low level of p-ERK activation and no increase in p-eIF4B. Its p-S6 levels, however, were increased to the same degree as in the mutant HRAS, cRAF1, and MEK1 transductants (Figure 6B), suggesting that lower levels of ERK activation are sufficient to produce maximum p-S6. These results demonstrate that ERK hyperactivation is sufficient to instigate neoplastic Lam γ 2 overexpression and provide further correlative evidence that translation initiation factor activation produces this response.

Basal Cells in Oral Dysplasias That Overexpress Laminin γ 2 Have Elevated Levels of p-S6

Finally, we asked whether the abnormal activation of ERK, translation factors, and S6 that we had identified in premalignant dysplastic and SCC cells in culture is detectable *in vivo* and correlates with Lam γ 2 overexpression. We used immunostaining to examine a set of oral dysplastic lesions, some of which included regions of normal epithelium as well as SCC. Lam γ 2 was detectable in basal cells of some regions in 23/26 dysplasias and 8/8 invasive SCCs, but was undetectable in normal oral epithelium (Figure 7) or in two benign hyperplastic warts (see Supplemental Figure S3A at <http://ajp.amjpathol.org>). Intensity of Lam γ 2 immunostaining varied among and within specimens of dysplasia, without apparent relation to any morphological features of the dysplasias (Figure 7). The

p-ERK, p-eIF4B, p-eIF4E, and p-4E-BP1 antibodies yielded weak uniform staining or scattered suprabasal cell nuclear staining and did not identify any differences among normal, dysplastic, and SCC specimens (data not shown).

In contrast, the p-S6 antibody identified clear differences between normal and neoplastic epithelium, associated with Lam γ 2 overexpression. In normal epithelium and benign warts, p-S6 immunostaining was always confined to suprabasal cell layers (Figure 7; see also Supplemental Figure S3A at <http://ajp.amjpathol.org>). In contrast, discontinuous basal cell p-S6 staining appeared in 20% to 100% of the dysplastic regions of 20/21 specimens evaluated quantitatively (Figures 7 and 8). Lam γ 2 immunostaining was always associated with basal p-S6 immunostaining, in a specific way. Regions containing Lam γ 2⁺ cells always were within regions that contained p-S6⁺ basal cells. However, only a subfraction of the basal p-S6⁺ regions also contained Lam γ 2⁺ basal cells, ranging from zero to 100% (Figures 7 and 8).

As reported previously,^{31,74,75} normal keratinocytes at the edge of wounds or ulcers expressed Lam γ 2 (see Supplemental Figure S3B at <http://ajp.amjpathol.org>). These regions also showed basal p-S6 immunostaining (see Supplemental Figure S3B at <http://ajp.amjpathol.org>), consistent with our finding that normal keratinocytes in pre-confluent cultures, which express Lam γ 2, have ERK-dependent increased p-S6 (Figure 4C). The dysplasias we examined sometimes contained regions of ulceration or microerosion, in which the epithelium did not completely cover the underlying connective tissue. The Lam γ 2/PS-6 immunostaining patterns of ulcerated regions of dysplasia (see Supplemental Figure S3C at <http://ajp.amjpathol.org>) resembled that of a wound edge in normal epithelium, with Lam γ 2 and basal cell p-S6 immunostaining. To avoid possible wound responses in our quantitative immunostaining analysis (Figure 8), we did not score regions of dysplasias within ~20 cells of an ulcer margin. We concluded from our analysis that immunohistologically detectable increased p-S6 levels in basal cells of dysplasias precede and accompany Lam γ 2 overexpression *in vivo*, consistent with a causal relationship between ERK-dependent activation of translation factors and Lam γ 2 overexpression.

Discussion

The present study extends our earlier study identifying Lam γ 2 overexpression in many premalignant oral and epidermal dysplasias,²⁵ which had followed studies by others finding Lam γ 2 overexpression in advanced SCCs.^{28,29} Here, we have investigated the mechanism of neoplastic Lam γ 2 overexpression using a set of normal primary and premalignant human keratinocyte and SCC lines that recapitulate in culture this difference in Lam γ 2 regulation between normal and neoplastic epithelial cells. ERK hyperactivity proved to be necessary and sufficient to cause Lam γ 2 overexpression, strongly correlating with ERK-dependent phosphorylation and activation of the translation factors S6 and eIF4B. Consistent with this finding, neoplastic Lam γ 2 overexpression in culture

proved to be at the level of translation, not transcription. Our cell culture findings were confirmed by immunostaining analysis of clinical material, which revealed increased p-S6 preceding and accompanying Lam γ 2 overexpression in the basal cell layer of oral dysplasias. Most importantly, the present study identifies EGFR/ERK hyperactivity and consequent Lam γ 2 overexpression as a rather early step during progression to SCC, occurring in some regions of preinvasive dysplasias.

Laminin γ 2 Overexpression as an Indicator of ERK Hyperactivity

Hyperactivity of the EGFR/ERK^{82–84} and PI3K/AKT/mTOR^{11,85,86} signaling pathways is a frequent feature of oral and oropharyngeal SCC. We found increased levels of EGFR and of its activated, phosphorylated tyr1068 state in all of the SCC and premalignant cell lines we tested. However, we found increased p-AKT levels in only some SCC lines, consistent with a previous study,⁸⁵ and no increase of p-AKT in the premalignant lines. Small-molecule inhibitors of the EGFR and MEK kinases blocked Lam γ 2 overexpression, but an mTORC1/2 inhibitor did not, supporting the conclusion that hyperactive EGFR/ERK signaling is the cause of dysregulated Lam γ 2 expression. Reinforcing this conclusion was our finding that ERK hyperactivity achieved by engineering normal keratinocytes to express constitutively active HRAS, cRAF1, or MEK1 was sufficient to cause Lam γ 2 overexpression. Activating *HRAS* gene mutations are found in ~5% of human oral and oropharyngeal SCCs,⁸⁶ and amplifications of *KRAS* and *ERK1(MAPK3)* are occasionally detected also,⁸⁴ but EGFR overexpression is the most frequent instigator of ERK hyperactivity in oral and oropharyngeal SCCs.^{82–84}

Engineered expression of a constitutively active EGFR deletion mutant [EGFR Δ (E746-A750)], cloned from a tracheobronchial SCC,⁴³ did not yield Lam γ 2 overexpression, but resulted in a modest increase of p-ERK levels and EGF independence for growth. This finding suggests that ERK stimulation above a certain threshold is required for the Lam γ 2 phenotype, a hypothesis testable in future experiments examining the effects of other activating EGFR mutations. The E6 and E7 viral oncoproteins of HPV16 and HPV18 initiate neoplastic progression in a high percentage of tonsil/oropharynx SCCs, but are rarely involved in oral cavity and epidermal SCC.⁶ Consistent with reports that HPV16 E6, potentiated by E7, increases EGFR levels and activation in keratinocytes,^{67,68} we found that primary keratinocytes engineered to express E6 and E7 displayed hyperactive EGFR and ERK and overexpressed Lam γ 2.

The same system responsible for Lam γ 2 overexpression in dysplasias and SCCs proved to be essential for Lam γ 2 expression in normal keratinocytes in preconfluent cultures, when they are expressing high levels of Lam γ 2. Under this condition, the EGFR/ERK pathway was active and S6 was phosphorylated in normal cells (as expected, because the cells are grown with EGF as a mitogen), and EGFR and MEK inhibitors repressed Lam γ 2 expression. This cell culture finding is a manifes-

tation of the *in vivo* wound re-epithelialization response, because we found that basal keratinocytes at normal wound edges have increased Lam γ 2 and p-S6 levels.

Neoplastic Overexpression of Laminin γ 2 and eIF4B-Dependent Regulation of mRNA Translation

Consistent with a previous report of selective overexpression of the Lam γ 2 subunit of Lam332 in carcinoma cells *in vivo*,²⁷ confluent cultures of premalignant keratinocytes and SCC cells displayed ERK hyperactivity-dependent Lam γ 2 overexpression, whereas regulation of their Lam α 3 and Lam β 3 expression was normal. In the process of normal Lam332 assembly, β 3 γ 2 dimer formation necessarily precedes the addition of Lam α 3 and ultimate secretion of Lam332 trimer.⁶² Lam γ 2 overexpression resulted in abnormal secretion of Lam γ 2 monomers, with most of the Lam γ 2 molecules remaining in the unprocessed 155-kDa form. Basal epithelial cells adhere to normally processed mature Lam332 by interaction of α 6 β 4 integrin to the C-terminus of the Lam α 3 chain.^{2,41} We reported previously that Lam332 trimers having unprocessed Lam γ 2 subunits induce sustained directional motility in keratinocytes.³¹ Whether autocrine or paracrine action of secreted Lam γ 2 monomer or Lam332 with unprocessed Lam γ 2 produces any neoplastic phenotype *in vivo* remains to be determined.

We were surprised to find from qPCR analysis that neoplastic Lam γ 2 overexpression is not the result of increased mRNA levels. Transcription of the genes encoding the three subunits of Lam332 is known to require TGF β pathway signaling,⁷³ such that a TGF β RI kinase inhibitor greatly inhibits Lam γ 2 expression at both the protein level³¹ and also the mRNA level (present study). ERK activates transcriptional regulators in the nucleus,⁷⁶ so we had expected that Lam γ 2 gene expression might be ERK-dependent. However, ERK activates the signaling kinases RSK and MNK1 which, in turn, activate the ribosomal protein S6 and several subunits of the cytoplasmic eIF4 complex required for translation initiation of some mRNAs.^{77,78} eIF4E permits translation of mRNAs that have the common 5' cap (also known as the m⁷G or 7-methylguanosine cap). eIF4E activity requires inactivation of the eIF4E inhibitor 4E-BP1 by TORC1-catalyzed phosphorylation.⁸⁷ In our experiments, the TORC1/2 inhibitor Ku-0063794 blocked 4E-BP1 phosphorylation, but had no effect on Lam γ 2 synthesis, ruling out an essential role for eIF4E. Another protein of the eIF4 complex is eIF4B, an essential activator of the eIF4A helicase, which unwinds and opens mRNAs that have long 5'-UTRs with a densely hydrogen-bonded stem-loop structure. eIF4B must be phosphorylated to be active, and our kinase inhibitor experiments revealed that this is accomplished primarily by the ERK-dependent RSK kinase. In our experiments, p-eIF4B levels proved to correlate most closely with the level of Lam γ 2 expression. MYC mRNA has a long and highly structured 5'-UTR and requires eIF4B activation for translation.⁷⁹ The 5'-UTR of Lam γ 2 mRNA has a similar length and structure, and our results clearly implicate eIF4B-dependent translation initiation as the determining factor for Lam γ 2

expression. This hypothesis can be tested by determining whether the Lam γ 2 5'-UTR imposes ERK/RSK-dependent translation control on reporter constructs.

S6 Phosphorylation as a Biomarker of ERK Hyperactivity in Neoplastic Keratinocytes

S6 is a component of the 40S ribosome subunit, and its phosphorylation at ser235/236 appears to be important under conditions that require very high rates of protein synthesis and to ensure normal cell size.⁸⁸ The high level of p-S6(ser235/236) present in all of the SCC and premalignant lines at confluence indicates that S6 kinases are active in these cells. The S6(ser235/236) phosphorylation, essential for S6 activity, can be accomplished either by TORC1-dependent S6K1 or by ERK-dependent RSK.⁷⁰ The relative importance of ERK-dependent and TORC1-dependent kinases on S6 phosphorylation and activation has been incompletely studied and may vary among different cell types and circumstances. In a study of HeLa cells, most S6(ser235/236) phosphorylation was ERK-dependent.⁷² Little or no S6(ser235/236) phosphorylation occurred in keratinocytes and SCC cells when ERK activity was blocked by a MEK inhibitor, indicating that in these cells the critical ser235/236 phosphorylation is ERK-dependent. The ability of RSK to accomplish the ser235/236 phosphorylation is greatly enhanced by prior S6K1-catalyzed ser240/244 phosphorylation of S6.^{71,72} Treatment with the TORC1/2 inhibitor Ku-0063794 resulted in complete absence of p-S6(ser234/235), consistent with the precondition of TORC1-dependent S6K1 phosphorylation at ser(240/244). The absence of an effect of Ku-0063794 on Lam γ 2 overexpression shows that p-S6(ser235/236) levels are not limiting for Lam γ 2 synthesis. Despite being nonessential for Lam γ 2 overexpression, high levels of p-S6(235/236) always accompanied it in our cell culture systems in the absence of kinase inhibitors. We found that p-S6(235/236) was the most useful biomarker of signaling pathway hyperactivity associated with Lam γ 2 overexpression *in vivo*.

Immunodetection of Abnormal p-S6 and Laminin γ 2 in Clinical Specimens of Oral Dysplasia

This initial analysis of signaling protein phosphorylation status during oral epithelial neoplastic progression *in vivo* has revealed the potential power of immunohistochemical staining for detecting signaling abnormalities that may have diagnostic or prognostic value, as well as the limitations. Cytoplasmic content of the secreted protein Lam γ 2 and the phosphorylated versions of signaling mediators are excellent candidates for biomarkers of neoplastic progression, because they indicate the current or very recent physiological state of individual cells at the time of fixation. Clinical and experimental utility depends, of course, on reliable detectability. Phosphorylations of signaling pathway proteins are subject to continual turnover, owing to their susceptibility to intracellular phosphatases.^{89,90} Current procedures for handling of biopsy

and surgical specimens and for formalin fixation may not consistently preserve the phosphorylated states of some proteins. Several immunohistochemical analyses of signaling pathway activation in tissue microarrays of oropharyngeal SCCs have produced inconclusive results, owing to inconsistent, weak, and/or predominantly differentiated cell staining for phosphoproteins and the challenge of finding suitable criteria for quantifying the results.^{91,92} As an integral ribosomal protein, S6 is much more abundant, and its phosphorylated form may also be more stable, than that of eIF4B and mediators of the EGFR/ERK/RSK pathway.

p-S6 immunostaining proved to be intense, preserved in all archival pathology specimens we tested, and showing clear differences between normal or hyperplastic versus dysplastic or SCC tissue. p-S6 was readily detectable in suprabasal cell layers of normal stratified epithelia, but not in the basal cell layer. In contrast, periodic or continuous basal cell p-S6 was detectable in portions of all but one of the dysplasias examined, ranging from ~20% to 100% of the length of dysplastic regions, consistent with a previous study.⁹³ We found that Lam γ 2 overexpression was consistently confined to a subfraction, ranging from zero to 100%, of the basal p-S6⁺ regions. There are at least two explanations for this finding. First, levels of p-S6 (as a surrogate for ERK hyperactivity and consequent eIF4B activity) may fluctuate substantially *in vivo* and may need to remain elevated continuously for a sufficiently long time for enough Lam γ 2 translation to occur to be detectable. Second, increased p-S6(ser235/236) levels may in some cases result from increased AKT/mTOR activity instead of increased ERK/RSK activity, but only the latter will result in increased Lam γ 2 expression. We did not detect basal cell p-AKT by immunostaining, which could have identified a role for AKT/mTOR hyperactivity in p-S6⁺/Lam γ 2⁻ regions.

The present study demonstrates that ERK hyperactivity and consequent Lam γ 2 overexpression begins relatively early, in the preinvasive stages of oral epithelial neoplastic progression. These results provide the rationale for larger studies to seek correlations between p-S6/Lam γ 2 immunostaining patterns in dysplasias and future progression (and its time frame) to invasive SCC. Such studies would aim to identify a specific pattern or extent of p-S6 and Lam γ 2 staining that correlates with future SCC, thus providing a definitive prognosis of dysplastic lesions. Our results also support the idea of early EGFR or MEK kinase inhibitor therapy for patients whose oral lesions have a high probability of progressing to invasive SCC but cannot be completely resected surgically without compromising function of the tongue. Clinical studies to date using targeted kinase inhibitors systemically for treatment of advanced SCC have yielded modest or temporary responses and serious side effects.^{12,94} Preinvasive lesions at high risk of malignant progression, and of relatively small total mass, may be more amenable to complete cure by local and/or short-term kinase inhibitor treatment.

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