A Keratinocyte Hypermotility/Growth-Arrest Response Involving Laminin 5 and p16^{INK4A} Activated in Wound Healing and Senescence

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Keratinocytes become migratory to heal wounds, during early neoplastic invasion, and when undergoing telomere-unrelated senescence in culture. All three settings are associated with expression of the cell cycle inhibitor p16^{INK4A} (p16) and of the basement membrane protein laminin 5 (LN5). We have investigated cause-and-effect relationships among laminin 5, p16, hypermotility, and growth arrest. Plating primary human keratinocytes on the γ 2 precursor form of laminin 5 (LN5') immediately induced directional hypermotility at ~125 μ m/hour, followed by p16 expression and growth arrest. Cells deficient in p16 and either p14^{ARF} or p53 became hypermotile in response to LN5' but did not arrest growth. Plating on LN5' triggered smad nuclear translocation, and all LN5' effects were blocked by a transforming growth factor (TGF) β receptor I (TGF β RI) kinase inhibitor. In contrast, plating cells on collagen I triggered a TGF β RI kinase-independent hypermotility unaccompanied by smad translocation or growth arrest. Plating on control surfaces with TGF β induced hypermotility after a 1-day lag time and growth arrest by a p16-independent mechanism. Keratinocytes serially cultured with TGF β RI kinase inhibitor exhibited an extended lifespan, and immortalization was facilitated following transduction to express the catalytic subunit of telomerase (TERT). These results reveal fundamental features of a keratinocyte hypermotility/growth-arrest response that is activated in wound healing, tumor suppression, and during serial culture. (*Am J Pathol* 2006, 168:1821–1837; DOI: 10.2353/ajpath.2006.051027)

The molecular mechanisms of keratinocyte motility have long been of interest in the contexts of re-epithelialization in wound healing and of invasiveness in squamous cell carcinoma. In the normal steady-state condition of stratified squamous epithelia, keratinocytes are not motile. They adhere to a basement membrane containing laminin 5 (LN5),^{1,2} a protein trimer of α 3, β 3, and γ 2 chains that normally undergoes proteolytic processing of the α 3 and γ 2 chains soon after secretion by the keratinocytes.^{3–5} Basal keratinocytes adhere via α 6 β 4 integrin to mature fully processed LN5, which initiates the formation of stable, anchoring hemidesmosomes.⁶ Keratinocytes in the migrating tongue of re-epithelializing oral and epidermal wounds greatly increase their expression of LN5 and of the LN5-binding α 3 β 1 integrin.^{7–11} It has been proposed that ligation to unprocessed, precursor forms of LN5 via α 3 β 1 integrin promotes keratinocyte motility in wounds.^{10–13}

LN5 expression is also greatly increased in the cells at the invading fronts of some epithelial cancers.^{14–16} Our recent investigation of neoplastic progression in epidermis and oral mucosa revealed p16^{INK4A} (p16) expression in the same cells that express LN5, namely, cells in transition from premalignancy to early invasive cancer.¹⁷ p16 is an inhibitor of the cyclin D1-dependent kinases cdk4 and cdk6.^{18,19} p16 protein is not expressed during normal stratified squamous epithelial renewal or differentiation,^{17,20,21} yet a consistent feature of invasive squa-

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Table 1. Human Keratinocyte Cell Lines

Cell line name	Donor age, sex	Known p16, p14 ^{ARF} , or p53 abnormalities	Replicative lifespan	Reference
Human epidermal origin				
Strain N	Newborn, M	None	~55 PDs	30
N/cdk4R	Newborn, M	p16 resistant	~60 PDs	29
N/p53DD	Newborn, M	Functionally p53 deficient	~60 PDs	29
N/p53DD/cdk4R	Newborn, M	Functionally p53 deficient and p16 resistant	~95 PDs (extended)	29
N/Id1	Newborn, M	Delayed p16 and p14 ^{ARF} expression	~74 PDs (extended)	N. Radoja, S. Brown, and J. Rheinwald, unpublished data J. Benwood and J. Rheinwald, unpublished data
N/E6E7	Newborn, M	Functionally p53 deficient and p16 resistant	Immortal	
N/TERT-1	Newborn, M	Expresses no p16 or p14 ^{ARF} ; normal p53	Immortal	
N/TERT-2G	Newborn, M	Reduced frequency of p16 induction	Immortal	28
SCC-13	56, F (tumor)	p16(–/–), p53(–/–)	Immortal	30
Human oral mucosal origin				
OKF4	28, M	None	~32 PDs	75
OKF4/TERT-1F	28, M	None	Immortal	29
OKF6/TERT-2	57, M	HD of p16/p14 ^{ARF} locus	Immortal	28
POE-9n	65, M	HD of p16/p14 ^{ARF} locus; p53(–/–)	~85 PDs (extended)	28
SCC-71	80, M (tumor)	p16(–/–), p53(–/–)	Immortal	76

M, male; F, female; HD, homozygous deletion.

mous cell carcinoma is mutational loss of ability to express functional p16.²² Our recent study identified the point during neoplastic progression to squamous cell carcinoma (SCC) at which p16 becomes expressed.¹⁷

It has long been noted that the cells leading re-epithelialization to close a wound are less mitotically active than those behind them (reviewed in Ref. ²³). We have conjectured that keratinocytes confronting an abnormal substratum, such as the stromal interface of premalignant dysplasias and the margin of wounds, activate a mechanism that coordinately increases motility and arrests growth.¹⁷ Reports of increased p16 at the leading edge of oral epithelial wounds²⁴ and of an inverse relation between p16 and Ki-67 immunostaining in cells at the invading fronts of basal cell carcinoma²⁵ provide further evidence of p16-dependent growth inhibition in wound and neoplastic migratory settings. Primary human keratinocytes grown in culture are subject to a telomere status-independent senescence arrest mechanism enforced by p16, which determines the limit of their replicative lifespan and acts as a barrier to TERT immortalization.^{26–29} Immunocytochemical analysis of late-passage keratinocyte cultures has revealed that cells that have begun to express p16 also express greatly increased levels of LN5, accompanied by directional hypermotility (dHM).¹⁷ This result led us to look for an *in vivo* situation in which normal keratinocytes co-express these proteins and are migratory.

Here, we report LN5/p16 co-expression by keratinocytes at the migrating front of healing wounds *in vivo* and in culture models. We have identified experimental conditions in which a LN5/p16-related hypermotility/growth arrest (HM/GA) response is induced acutely and syn-

chronously in primary human keratinocytes. We report the fundamental features of this mechanism, in which adhesion to a form of the laminin 5 trimer in which the $\gamma 2$ subunit remains in its uncleaved precursor form (LN5') induces directional hypermotility followed by induction of p16 expression and growth arrest. Our studies have revealed dependence of both LN5'-induced HM/GA and the keratinocyte senescence mechanism on activity of the TGF β receptor I kinase.

Materials and Methods

Wound Tissue Specimens and Immunohistochemical Analysis

Fifteen formalin-fixed, paraffin-embedded skin specimens of decubitus ulcers were selected from the pathology archives at Brigham and Women's Hospital, including eight that were chronically nonhealing and seven that were in the process of progressive re-epithelialization. Wounds from immunocompromised patients and diabetics were excluded. The diagnoses were confirmed by two board-certified dermatopathologists (A.J.F.L. and T.B.).

Cell Lines and Cell Culture

The derivation and properties of the cell lines used (Table 1) have been described previously.^{28–30} Keratinocytes were cultured in keratinocyte serum-free medium³¹ (GIBCO/Invitrogen, Carlsbad, CA), supplemented as described previously²⁹ with 25 μ g/ml bovine pituitary ex-

tract, 0.2 ng/ml epidermal growth factor (EGF), and 0.4 mmol/L CaCl_2 (fully supplemented keratinocyte serum-free medium [K-sfm]). To generate dense cultures, cells were grown to ~40% confluence in K-sfm and then refed with 1:1 medium, a 1:1 (vol:vol) mixture of Ca^{2+} -free Dulbecco's modified Eagle's medium (DMEM) (GIBCO/Invitrogen) and K-sfm, supplemented with bovine pituitary extract, EGF, and 0.4 mmol/L CaCl_2 , which permits formation of healthy, confluent, stratified cultures. For some experiments, keratinocytes were cultured using the feeder/FAD system,^{32,33} in which keratinocytes are co-cultured with mitomycin-treated 3T3J2 feeder cells in serum- and growth factor-supplemented DMEM/F12 medium.²⁹ Replicative lifespans of keratinocyte cell lines were calculated as cumulative populative doublings (PDs), summing the $\log_2$ (no. of cells at subculture/no. of cells plated) for all passages until cell numbers no longer increased. The rat bladder carcinoma cell line 804G³⁴ was cultured in DMEM and 10% newborn calf serum (Hyclone).

Purification of Recombinant Human Laminin 5

293 cells were sequentially transfected with pcDNA3.1 plasmids expressing the complete coding sequences of each of the three chains of LN5. The $\beta 3$ subunit sequence contained a C-terminal 6-His tag. Each plasmid expressed a different drug-resistance gene to permit selection. A triple-transfected clone of 293 expressing high levels of all three chains was clonally isolated, grown to confluence, and then switched to serum-free medium. Two-day conditioned medium (CM) was loaded onto a His-Bind column (Novagen), eluted with 60 mmol/L imidazole, dialyzed against 10 mmol/L Tris-HCl (pH 7.5), and concentrated using Vivaspin 15R protein concentrators. All three LN5 chains were present in similar concentrations by Coomassie Blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and all three were detectable by Western blotting, with the $\alpha 3$ chain being in its cleaved, mature form (data not shown) and $\gamma 2$ chain in its precursor, uncleaved 155-kD form (see Figure 2C). Because the recombinant LN5 was isolated by its ability to bind to the 6-His affinity column via the 6-His tag on the $\beta 3$ subunit, this ensured that all $\gamma 2$ precursor in the preparations we used was assembled into the LN5 trimer. CM from mock-transfected 293 cells bound to and eluted from the column, concentrated to the same extent, and used to coat culture dishes proved to induce no hypermotility or growth inhibition (data not shown).

Extracellular Matrix Proteins

Culture dishes and wells were coated with solutions of purified extracellular matrix (ECM) proteins: recombinant human laminin 5, $\gamma 2$ precursor form (LN5') at 0.4 $\mu\text{g}/\text{ml}$ (the minimum concentration that produced a maximum hypermotility response) and collagen I of human (Invitrogen), bovine (Organogenesis, Stoughton, MA), and rat (BD Biosciences, Franklin Lakes, NJ) origin at 100

$\mu\text{g}/\text{ml}$. For some experiments, dishes were coated with 100 $\mu\text{g}/\text{ml}$ human plasma fibronectin (Invitrogen), human IgG (Sigma-Aldrich, St. Louis, MO), human plasminogen (Calbiochem, San Diego, CA), or bovine serum albumin (Sigma-Aldrich); these concentrations equaled or slightly exceeded those present in 10% serum.

For some experiments, CM from the rat bladder carcinoma cell line 804G³⁴ was used as a source of the $\gamma 2$ precursor form of LN5.^{35,36} 804G cells were grown to confluence, refed with fresh DMEM/F12 and 10% calf serum, and returned to the incubator for ~8 hours. The CM was sterilized with a 0.2- μm filter (Nalgene Corp., Rochester, NY) and stored at -20°C until use.

Hypermotility and Growth Inhibition Assays

Six-well tissue culture plates (Costar, Acton, MA) were pretreated by incubation with 804G CM or solutions of purified ECM proteins dissolved in 10% calf serum-supplemented DMEM/F12 or serum-supplemented phosphate-buffered saline (PBS) for 30 minutes at 37°C and then rinsed three times with PBS and once with K-sfm before plating cells in K-sfm. For some experiments, cells were plated on untreated dishes in K-sfm and recombinant human TGF $\beta 1$ (PeproTech, Rocky Hill, NJ), recombinant human EGF (Invitrogen), or recombinant human hepatocyte growth factor (HGF) (R & D Systems, Minneapolis, MN). These factors were diluted into the medium from 100 $\times$ or 1000 $\times$ concentrated stock solutions in 0.1% bovine serum albumin/PBS.

To measure directional hypermotility (dHM), wells plated with 500 cells were fixed ~42 hours later in 10% formalin/PBS for 30 minutes and immunostained with a LN5 $\gamma 2$ chain-specific antibody. Fifteen to 20 adjoining fields were photographed using a 2 $\times$ objective, composite pictures were assembled, and LN5 track lengths of at least 30 cells were measured. To assess growth inhibition, wells plated with 2000 cells were grown for 7 to 9 days with refeeding on days 3, 5, and 7, trypsinized, and counted with a hemacytometer; and the average growth rate was calculated as $\log_2$ (no. of cells counted/no. of cells plated)/no. of days = population doublings/day. Average (mean) migration path length and degree of growth inhibition (as measured by PDs/day growth rate) were compared between control and experimental conditions.

The TGF β receptor I kinase inhibitor [3-(pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole (TRI) (Lilly designation HTS466284; Biogen designation LY364947)^{37,38} (Calbiochem) was used at 1 $\mu\text{mol}/\text{L}$ and added to the medium at the time of plating and at each refeeding from 1000 $\times$ concentrated stock solutions in dimethylsulfoxide. For serial culture experiments, TRI was added at 0.5 $\mu\text{mol}/\text{L}$ at the time of plating and at subsequent refedings but was removed 2 or 3 days before each subculture.

Time-Lapse Photography

Cells were inoculated into a temperature-controlled chamber (Mike's Machine Company, Boston, MA) as-

sembled with a 25-mm-diameter plastic coverslip (Thermonox; Nalge/Nunc, Rochester, NY), which had been precoated by a 30-minute incubation with 10% calf serum-supplemented DMEM containing 0.4 $\mu\text{g/ml}$ recombinant LN5'. A Zeiss IM35 inverted microscope equipped with a Uniblitz Model VMM-D1 Shutter Driver (Vincent Associates, Rochester, NY) and a Spot Insight QE camera was used to capture images through a 10 $\times$ phase objective and 10 $\times$ eyepiece. Images collected at 5-minute intervals using Spot version 4.0.4 software were converted into avi movie files and examined to measure migration rates of individual cells.

Culture Wound Models

Scratch Wound

One-day postconfluent keratinocyte cultures were prepared by growing cells to approximately one-third confluence in K-sfm and refeeding the final 2 days with 1:1 medium (described above). Linear "wounds" ~ 2 mm wide were made in 1-day postconfluent cultures by scraping the point of a 1-ml Eppendorf pipette tip across the cell layer, after which cultures were returned to the incubator for 40 hours until fixation and immunostaining. For some experiments, drug inhibitors were added to the medium at the time of wounding, and relative rates of closure were compared with no drug controls daily for 4 days.

Organ-Cultured Human Skin Wound

One-square centimeter pieces of recently resected human newborn foreskin were rinsed in medium to remove residual blood components and ~ 2 -mm-diameter wounds were made through the epidermis and partially into the dermis with curved iris scissors. The wounded pieces were submerged in 1:1 medium and placed in the incubator for 2 days, after which they were fixed, sectioned, and immunostained.

Antibodies

The p16^{INK4A}-specific, murine monoclonal antibody (mAb) G175-405 (Pharmingen, San Diego, CA) was used at 2 $\mu\text{g/ml}$ for immunostaining. The p16^{INK4A}-specific, murine mAb JC1 (provided by E. Harlow, Massachusetts General Hospital, and J. Koh, University of Vermont) was used at 1:25 dilution for Western blotting. The bromodeoxyuridine (BrdU)-specific rat monoclonal antibody BU175 (ICR1) (Accurate Chemical & Scientific Corp., Westbury, NY) was used at 1:50 dilution for immunostaining. The human LN5 $\gamma 2$ chain-specific murine mAb D4B5³⁹ (Chemicon, Temecula, CA) was used at 10 $\mu\text{g/ml}$ for immunostaining and Western blots (note that D4B5 does not recognize rat LN5 $\gamma 2$, so it readily detected LN5 deposited by keratinocytes plated on 804G CM-coated surfaces). The LN5 $\gamma 2$ chain-specific rabbit polyclonal antibody J20⁴ was used at 1:40 dilution to immunostain tracks of cells

migrating on recombinant human LN5', instead of D4B5, because J20 stained the human LN5'-coated surface less intensely than the LN5 deposited by cells, making tracks easier to see. Note that both D4B5 and J20 recognize epitopes present on both precursor and mature forms of the $\gamma 2$ chain of LN5. Double immunofluorescence experiments using the mouse D4B5 and the rabbit J20 antibodies showed that the antibodies completely colocalized in cells, in the "pads" surrounding nonmigratory cells, and in the tracks left behind by migrating cells. The smad2/3-specific murine mAb (Clone 18) (Pharmingen) was used at 1:100 dilution for immunostaining to detect nuclear translocation.⁴⁰

Immunocytochemistry and Immunohistochemistry

Cultured cells were fixed in fresh 4% paraformaldehyde, permeabilized with Triton X-100, and immunostained using avidin-biotin-complex peroxidase or by sequential staining with rat BrdU antibody visualized by peroxidase/Nova Red and mouse p16 antibody visualized by alkaline phosphatase/Vector Blue (Vector Labs, Burlingame, CA), as described previously.^{17,29} Paraffin sections (5 μm) of wound tissue were cut, deparaffinized, subjected to antigen retrieval, and immunostained using avidin-biotin-complex peroxidase as described previously.¹⁷ Images were captured on a NIKON E600 Microscope with a SPOT2 digital camera using SPOTcam v.3.5.5 software (Digital Instruments, Inc., Buffalo, NY).

Western Blotting

To analyze p16^{INK4A} expression, keratinocytes were plated on untreated or on LN5'- or collagen I-precoated dishes at 1.5×10^5 cells per p100 dish and grown for 3 days. Cultures were trypsinized, rinsed in PBS, lysed in 20 mmol/L Tris buffer (pH 7.3), 2% SDS, and EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN), and sonically disrupted. An aliquot (30 μg as determined by Bio-Rad assay) of protein from each extract was separated by SDS-PAGE with precast 4 to 20% gradient gels (Bio-Rad). To analyze LN5 $\gamma 2$ expression and processing status, cells were plated at 10^5 cells per p100 dish and grown for 6 days with or without -1 ng/ml TGF β for the final 2 days, and the final 1-day CM was collected. Cells from TGF β -treated and untreated cultures were counted, and volumes of CM normalized for cell number were separated by SDS-PAGE as described above. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA), and specific proteins were detected with murine mAbs against p16^{INK4A} (JC-1; provided by E. Harlow), the $\gamma 2$ chain of LN5 (D4B5; Chemicon), and β -actin (A-2066; Sigma), followed by peroxidase-conjugated secondary antibody (Southern Biotechnologies, Birmingham, AL) and chemiluminescence reagent (ECL; Amersham Corp., Piscataway, NJ).

Figure 1. Laminin 5/p16^{INK4A} co-expression in keratinocytes at the leading edge of wounds *in vivo* and in culture. **A–C:** Hematoxylin and eosin-stained sections and adjacent sections immunoperoxidase-stained for p16^{INK4A} and for laminin 5. **A:** Migrating front of an *in vivo* human skin wound (asterisk shows fibrin scab over unhealed area of wound). **B:** Recently re-epithelialized area of a decubitus skin ulcer. **C:** Recently re-epithelialized area of an experimental organ-cultured skin wound 2 days after wounding (arrow shows border between unwounded and wounded areas). Note that consistent with our earlier report¹⁷ and those of other investigators,^{14,42,43} we did not detect the $\gamma 2$ chain of LN5 in the basement membrane underlying normal, unwounded epithelium, despite its well-established presence there,^{1,2,39} but we detected abundant $\gamma 2$ in the cytoplasm of keratinocytes in the migrating fronts and recently re-epithelialized areas of wounds. **B** and **C** reprinted from Natarajan et al⁴¹ with permission. **D:** Scratch wound in a confluent culture of the TERT-immortalized epidermal keratinocyte line N/TERT-2G double immunofluorescence stained 2 days after wounding. Bar = 100 μ m.

Results

Coordinate p16 and Laminin 5 Expression in Keratinocytes during Wound Healing

As reviewed in the introduction, LN5 and p16 are co-expressed by premalignant/early invasive keratinocytes, and LN5 has been found to be expressed by keratinocytes at the migrating front of wounds. We therefore

examined 15 human skin wounds immunohistochemically for LN5/p16 co-expression, seeking to confirm initial findings we have reported recently.⁴¹ Eight wounds were chronically nonhealing, and seven were healthy and in the process of re-epithelialization. Thirteen of the wounds showed co-expression in keratinocytes at the wound margin (Figure 1A) and in migrating bridges recently formed on the wound bed (Figure 1B). Similar to several

previous reports,^{14,42,43} our immunostaining procedure did not detect the $\gamma 2$ subunit assembled into the mature three-chain trimer in normal basement membrane. The other two wounds showed LN5 immunostaining of cells at the margin without detectable p16. We concluded that LN5/p16 co-expression is a typical response of wound-edge keratinocytes.

These results did not provide insight into the timing of expression, so we sought to recapitulate an acute skin wound in organ culture. Daniels et al⁴³ had reported LN5 expression by corneal keratinocytes migrating over abraded corneal surfaces in organ culture. We made ~3-mm-diameter partial-thickness wounds in ~1- to 2-cm² fragments of freshly resected human skin and incubated these at 37°C submerged in 1:1 medium. Two days later, immunohistochemical staining detected consistent p16/LN5 co-expression in keratinocytes at the migrating front and recently re-epithelialized areas overlying the wound (Figure 1C). Neither of these proteins were detected immunohistochemically 1 day after wounding, at which time keratinocyte migration had not yet begun (data not shown). We previously reported LN5/p16 co-expression by the leading band of cells closing scratch wounds made in confluent cultures of strain N primary human epidermal keratinocytes.¹⁷ A TERT-immortalized derivative of strain N, N/TERT-2G, which continues to produce p16⁺, growth-arrested cells at low frequency during serial passage²⁸ (Table 1), also co-expressed LN5 and p16 in the leading band of cells closing scratch wounds (Figure 1D). We concluded from the above results that LN5/p16 co-expression is a hallmark of the keratinocyte wound response, occurring within 2 days after wounding. The scratch wound result indicated that such induction does not require interaction with other cell types, so the mechanisms of LN5/p16 induction can be studied in pure keratinocyte cultures.

Directional Hypermotility Induced in Cells Plated on a Precursor Form of Laminin 5

p16-expressing senescent keratinocytes in culture express increased LN5, and many of these p16⁺/LN5^{hi} cells exhibit directional hypermotility (dHM).¹⁷ We sought to determine whether LN5/p16 co-expression could be induced in early-passage keratinocytes and whether this would be accompanied by dHM. Previous studies^{10,11} have suggested that keratinocytes convert, on stimuli such as wounding, to a migratory state involving $\alpha 3 \beta 1$ integrin and secreted precursor LN5. We therefore decided to study the effect of plating keratinocytes on culture dishes coated with a precursor form of LN5.

Our first experiments used as a source of precursor LN5 the CM from 804G cells, which secrete large amounts of LN5 with processed $\alpha 3$ but unprocessed $\gamma 2$ chain (LN5').^{35,36,44} We compared the behavior of early-passage primary human keratinocytes plated on untreated dishes, dishes precoated with 804G CM, and

Figure 2. Directional hypermotility induced by plating keratinocytes at low density on surfaces precoated with the $\gamma 2$ precursor form of laminin 5. **A** and **B**: LN5 immunostaining 2 days after plating normal primary human epidermal keratinocytes (strain N) on control dishes (**A**) or on dishes precoated with 2 $\mu\text{g/ml}$ recombinant LN5' dissolved in 10% calf serum-supplemented DMEM (**B**). **Insets** show homogenous LN5 deposition around nonmigratory cells (**A**) and stuttered pattern behind directionally hypermotile cells (**B**). **C**: Western blot using the laminin 5 $\gamma 2$ chain-specific antibody D4B5. **Lane 1**, CM from control strain N culture; **lane 2**, CM from strain N culture treated with 1 ng/ml TGF β 2 days before harvesting; and **lanes 3 and 4**, 0.05 and 2 μg purified recombinant human LN5, $\gamma 2$ precursor form (see Materials and Methods). Note the increase in total $\gamma 2$ chain and its precursor form in the medium of TGF β -treated cells and that the $\gamma 2$ chain is almost exclusively in the precursor form in the recombinant LN5 preparation. **D**: Scatterplot showing lengths of migration tracks of individual strain N cells plated ~42 hours previously on dishes precoated with the indicated proteins. Alb, bovine serum albumin; Pln, human plasminogen; IgG, human immunoglobulin type G. The vertical bar in each set of points shows the average track length of all cells measured. **E**: Motility rates (micrometers/hour) determined by time-lapse microphotography of individual strain N keratinocytes plated on a surface precoated with LN5' and serum (Supplemental Videos S1, S2, and S3 at <http://ajp.amjpatbol.org>). Points represent the distance traveled by individual cells during successive 2-hour intervals, beginning immediately after attachment. Some cells entered or left the field of view during the recording period, permitting measurement of their migration histories during a portion of the entire period shown in the graph. **E** reprinted from *J Invest Dermatol Symp Proc* 2005, 10:72–85, with permission.

Figure 3. Laminin 5 precursor-induced hypermotility is accompanied by p16^{INK4A}-dependent growth inhibition. **A:** Comparison of growth and directional hypermotility of strain N keratinocytes plated on dishes precoated with different ECM molecules. **B:** Induction of p16 expression and growth arrest in keratinocytes plated on dishes coated with LN5'. **Graph** shows relative percentages of p16- and BrdU-positive and -negative cells, detected by double-label immunocytochemical staining, 3 days after plating on dishes precoated with 804G CM, recombinant human LN5', or human collagen I or on untreated dishes with 0.3 ng/ml TGFβ added to the medium. BrdU was added to the medium of all cultures for the final 1 day before fixation. Double-label immunocytochemical staining, using mouse anti-p16 and rat anti-BrdU antibodies, disclosed the percent noncycling (BrdU-negative) cells and whether those cells were p16⁺. **C:** Uncoupling of LN5'-induced dHM from growth arrest in keratinocytes deficient in p16 expression or function and in either p14^{ARF} or p53 expression or function. N early, early-passage strain N; N late, late-passage, near-senescent strain N. Cell lines deficient in p16 and in p14^{ARF} or p53 expression by normal and p16-deficient keratinocytes plated on control or LN5'-coated surfaces. **Lanes 1 and 2**, strain N (mid-lifespan); **lanes 3 and 4**, N/TERT-2G; **lane 5**, strain N (near-senescent); **lanes 6 and 7**, N/TERT-1; **lane 8**, N/p53DD/cdk4R (extended lifespan phase); and **lane 9**, N/Id1 (extended lifespan phase). Cells were plated on control (–) or LN5'-coated (+) dishes as indicated below lanes.

dishes precoated with fresh 10% serum/DMEM. Immunostaining ~42 hours later with the D4B5 antibody that recognizes the γ2 chain of human but not rat LN5 revealed that cells plated on control dishes had deposited small pads of LN5 beneath and around them and that very few had undergone any net migration (Figure 2A). In contrast, cells plated on 804G CM-coated dishes became directionally hypermotile, as revealed by tracks of secreted LN5 (Figure 2B). To confirm that this motility was induced by the LN5' in 804G CM, we coated dishes with 0.4 μg/ml purified recombinant human LN5' (Figure 2C) dissolved in 10% serum/DMEM. This proved to induce dHM to a similar extent as 804G CM (Figures 2D and 3A). Experiments showed that 0.2 μg/ml LN5' induced near-maximum dHM but that average path lengths were shorter with 0.1 μg/ml (data not shown). Time-lapse photography revealed that cells plated on control dishes attached, spread, and underwent shape changes and local movement without net migration (Supplemental

Video S1 at <http://ajp.amjpathol.org>). In contrast, as we reported recently,⁴¹ cells plated on a LN5'-coated surface began to migrate rapidly as soon as they attached and spread (Figure 2E; Supplemental Video S2 at <http://ajp.amjpathol.org>), suggesting that a change in neither gene expression nor protein synthesis is necessary to initiate dHM. Time-lapse measurements of distances traveled by individual cells disclosed rather constant rates of motility of ~125 μm/hour during the first 8 hours after plating (Figure 2E; Supplemental Video S3 at <http://ajp.amjpathol.org>).

Hypermotility Induced by Precursor Laminin 5 and by Collagen I Requires a Serum Cofactor

As described previously,^{13,45,46} dishes coated with collagen I also induced dHM with migration rates similar to that induced by LN5' (Figures 2D and 3A). For the experiments described above, we dissolved ECM proteins in 10% serum-supplemented DMEM to permit a controlled comparison with 804G CM. We found that dishes coated with purified recombinant human LN5' dissolved in K-sfm medium (which contains 25 μg/ml bovine pituitary extract) or in PBS failed to induce dHM (Figure 2D). Dishes coated with LN5' dissolved in 10% serum/PBS (Figure 2D) or sequentially with 10% serum/PBS and LN5' in either order (data not shown) produced a maximum dHM response. Collagen I also was a poor inducer of dHM when coated on dishes in the absence of serum (Figure 2D), consistent with an earlier report.⁴⁷ K-sfm medium with bovine pituitary extract proved not to suffice as a cofactor for LN5'- or collagen I-induced dHM. Seeking to identify the serum cofactor for LN5'-induced dHM, we tested four major protein components of serum at their approximate concentrations in 10% serum. Neither fibronectin, serum albumin, plasminogen, nor IgG could replace 10% serum to synergize with LN5' and induce dHM (Figure 2D). Importantly, precoating dishes with 10% serum alone or with 10% serum and collagen I produced no growth inhibition, thereby excluding the possibility that a substratum-binding factor in serum produces a growth inhibitory effect on cells independent from synergistic action with LN5'.

Hypermotility Stimulated by Precursor Laminin 5 Is Followed by p16 Expression and Growth Arrest

We next asked whether plating keratinocytes on LN5' induces p16 expression and growth arrest. As shown in Figure 3A, cell growth was markedly inhibited on LN5'-coated but not on collagen I-coated dishes. We performed a BrdU/p16 double-immunostaining analysis to determine whether LN5'-induced growth arrest is associated with p16 expression. As shown in Figure 3B, by 3 days after plating on 804G CM- or recombinant LN5'-coated dishes, >70% of the cells were noncycling, in contrast to only 20 to 25% of control or collagen I-plated cells. Nearly all of the nondividing

cells were p16⁺. These immunostaining results were confirmed by Western blotting (Figure 3D). We concluded that p16 expression and growth arrest results from keratinocyte engagement with LN5' and, from the collagen I result, that hypermotility per se does not induce p16 expression or growth arrest.

p16- and p14^{ARF}/p53-Deficient Keratinocytes Become Hypermotile but Evade Growth Arrest in Response to Engagement with Precursor Laminin 5

The above results were consistent with the possibility that LN5' induces a growth-arrest response identical to that experienced by keratinocytes undergoing p16-related senescence during serial passage.^{28,29} We therefore asked whether LN5'-induced arrest depends on functional p16 and also contains a p14^{ARF}-instigated, p53-dependent component, as we had determined for keratinocyte senescence.^{28,29} We tested the dHM and growth inhibition responses to LN5' of epidermal and oral keratinocyte lines deficient in expression or function of p16, p14^{ARF}, and/or p53. The set of cell lines that we examined (Table 1) included squamous cell carcinoma- and oral dysplasia-derived cell lines as well as primary keratinocytes engineered to express the p16-insensitive cdk4^{R24C} mutant (which resists p16-dependent arrest), the dominant-negative p53^{DD} mutant (which evades p14^{ARF}- and p53-dependent arrest), HPV16E6E7 (which evades p16-/Rb-dependent and p14^{ARF}/p53-dependent arrest), and Id1 (which delays onset of p16 expression during serial passage).⁴⁸ We also examined several TERT-immortalized keratinocyte lines that varied in the frequency with which cells undergo spontaneous p16/p14^{ARF} induction under normal culture conditions. As shown in Figure 3C, all cell lines tested responded to plating on LN5' with dHM, but cell lines that were deficient in p16 and in either p14^{ARF} or p53 were resistant to LN5'-induced growth inhibition. N/TERT-1, which fails to express any detectable p16 or p14^{ARF} protein during serial culture,²⁸ displayed substantial resistance to LN5'-induced growth inhibition. In contrast, N/TERT-2G, which produces some p16⁺, growth-arrested cells during serial culture,²⁸ was sensitive to LN5'-induced growth inhibition. Western blot analysis revealed that N/TERT-2G was induced to express p16 in response to plating on LN5', in the same way as its normal primary parent line strain N, but not that of N/TERT-1 (Figure 3D), consistent with the growth inhibition responses of these lines (Figure 3C). We concluded that acute growth arrest induced by LN5' engagement is enforced by a dual p16- and p14^{ARF}/p53-dependent mechanism, similar to that activated in keratinocyte senescence. We also concluded that neither ability to express nor to be growth inhibited by p16 or p14^{ARF} are preconditions for dHM.

Strain N cells in their final passage ("N late") contained a subpopulation of constitutively dHM cells (Figure 3C). Some of the single transductant N/cdk4R and N/p53DD cells and of the double-transductant N/p53DD/cdk4R

cells were constitutively motile when examined at ~45 PDs, near the end of the lifespan of the strain N parent line. N/Id1 cells had become constitutively hypermotile by the time they were examined in their extended lifespan period. These results were consistent with our initial observations¹⁷ and support the conclusion that spontaneous activation of a dHM response precedes the end of keratinocyte replicative lifespan during serial culture.

TGF β Receptor Dependence of the Precursor Laminin 5-Induced HM/GA Response

The experiments described above demonstrated that both dHM and p16 expression ensue from adhesion to LN5' but provided no clue as to the mechanism. We based our initial approach to this question on previous findings of increased TGF β in keratinocytes at the leading edge of wounds^{49,50} and the report that TGF β induces increased expression of completely unprocessed (ie, α 3pre, γ 2pre) LN5 in cultured keratinocytes accompanied by increased transwell migration.⁵¹ TGF β is a potent growth inhibitor of primary human keratinocytes.^{52,53} Cells plated at low density on control dishes in medium supplemented with TGF β showed growth inhibition and hypermotility responses that increased over the range of 0.03 to 0.3 ng/ml (Figure 4A). Measuring track lengths at different times after plating with TGF β showed that the induced dHM began after a delay of ~1 day (Figure 4B), consistent with a requirement for a change in gene expression and new protein synthesis in response to TGF β before cells exhibit dHM and in clear contrast to the immediate dHM displayed by plating cells on LN5' or collagen I (Figures 4B and 2E). Analysis of medium harvested from strain N cultures revealed that TGF β treatment resulted in increased LN5 secretion with a substantial proportion remaining in the γ 2 precursor form (Figure 2C).

Two other polypeptide factors, EGF and HGF, which had been reported to be keratinocyte motility factors by the criteria of increased transwell migration or colony scattering,⁵⁴ did not induce dHM in our system, even at high concentrations (Figure 4A). EGF receptor activation has been found to result in phosphorylation of the β 4 integrin, and this event appears necessary for keratinocytes to disassemble α 6 β 4 integrin-dependent hemidesmosomes and permit them to respond to motility signals.⁵⁵ Our control medium already contains EGF at 0.2 ng/ml, optimal for proliferation, so hemidesmosome-like structures may not form in this system to potentially restrict induction of directional hypermotility by LN5' or TGF β .

The mechanism of TGF β signaling is more complex than that of EGF and many other polypeptide growth factors and cytokines in that the transmembrane receptor that binds TGF β (TGF β RII) is not the kinase responsible for activating downstream cytoplasmic mediators. Instead, on binding of TGF β , TGF β RII associates with and activates the kinase domain of TGF β receptor I (TGF β RI), which then phosphorylates smad2 and -3, TAK1 (upstream of p38MAPK), and rhoA (upstream of

Figure 4. Effects of TGFβ on keratinocyte growth and directional hypermotility. **A:** Dose response of growth inhibition and induction of directional hypermotility in normal primary keratinocytes by TGFβ compared with EGF and HGF. Migration tracks were measured 2 days after plating. **B:** Lag period of directional hypermotility response to TGFβ, compared with the immediate hypermotility induced by plating on recombinant human LN5' or on collagen I. Cultures were fixed and immunostained for LN5 to permit measurement of track lengths at times indicated on the vertical axis. **Inset** shows typical migration tracks, revealed by LN5 immunostaining, of strain N cells 3 days after TGFβ treatment. **C:** Comparative sensitivity to growth inhibition by LN5' and TGFβ of normal keratinocytes, keratinocytes deficient in p16 and p14ARF/p53 control, and SCC-derived cell lines (see Table 1 for cell line descriptions). In this set of experiments, 804G CM was used as the source of LN5' (L5g2pre in **inset** legend), and TGFβ was used at 0.3 ng/ml.

p160ROCK), thereby activating three distinct signal pathways.^{56–60} TGFβ inhibits growth by multiple mechanisms that appear not to be identical in all cell types, including induction of p15^{INK4B} expression, transcriptional repression of myc, and cdc25-dependent inactivation of cdk2.^{59–61} Consistent with such findings of p16^{INK4A}-independent mechanisms in other cell types, we found that TGFβ-treated keratinocytes plated on control surfaces growth-arrested without inducing p16 expression, in contrast to the p16 induction in cells that growth-arrested in response to plating on LN5' (Figure 3B). Additionally, the N/TERT-1, N/p53DD/cdk4R, and OKF6/TERT-2 cell lines, although resistant to LN5'-induced growth inhibition (Figure 3C), remained sensitive to TGFβ growth inhibition (Figure 4C). As we reported previously,⁵³ the two SCC cell lines that we tested were able to grow nearly as rapidly in the presence as in the absence of TGFβ. We concluded from these results that TGFβ, in contrast to LN5', inhibits keratinocyte growth by mechanisms that are not dependent on p16 or p14^{ARF}/p53 function, consistent with studies in other cell systems.^{59–61}

To extend our comparison of TGFβ- and LN5'-induced responses, we asked whether smad activation, the best-characterized TGFβ signaling step,⁵⁸ ensues from cell adhesion to LN5'. TGFβ binding to the TGFβRII receptor results in activation of the TGFβRI kinase, which phosphorylates cytoplasmic smad2 and -3, permitting these proteins to translocate to the nucleus and act as coactivators and corepressors of transcription factors to regulate expression of TGFβ-responsive genes.^{57,58} As shown in Figure 5, A and B, TGFβ treatment of keratinocytes on control surfaces caused a majority of the cells to translocate smad2/3 to the nucleus.

We next asked whether TGFβRI activation accompanies and is required for LN5'-induced hypermotility and growth arrest. Plating cells on LN5' proved to trigger smad2/3 translocation within 1 hour (Figure 5, A and B). Addition of TRI, a specific inhibitor of TGFβRI kinase,^{37,38} at 1 μg/ml inhibited both TGFβ- and LN5'-induced smad2/3 nuclear translocation, growth inhibition, and dHM (Figure 5, B and C). The spontaneous dHM displayed by a subpopulation of keratinocytes in mid-lifespan cultures, which we previously reported to be composed of p16⁺, senescent keratinocytes,¹⁷ was also inhibited by TRI (Figure 5, B and C). Keratinocyte migration to re-epithelialize scratch wounds also proved to be dependent on TGFβ receptor activity. As shown in Figure 5F, under control conditions, re-epithelialization was complete 2 days after wounding but was delayed by 2 days in the presence of TRI. We concluded that TGFβRI activation is an essential step in LN5'-induced signaling leading to dHM and growth arrest, is essential for the dHM displayed by keratinocytes as they become senescent in culture under control conditions, and also contributes significantly to the migratory ability of keratinocytes in mass populations that respond to wounding. On the other hand, the experiments described above demonstrate that keratinocytes undergo an immediate hypermotility response to plating on LN5' and that loss of p16/

Figure 5. Activation of TGFβRI kinase signaling by precursor laminin 5. **A:** TGFβ receptor-dependent smad nuclear translocation in response to TGFβ or to plating on LN5'. Panels show smad2/3 immunocytochemical staining of strain N keratinocytes showing the normal cytoplasmic location of smad2/3 under control conditions, nuclear translocation after treatment either with 1 ng/ml TGFβ or plating on LN5'-coated dishes, and prevention of smad translocation in response to 1 μmol/L TGFβRI kinase inhibitor TRI. **B:** Blocking of TGFβ- and LN5'-induced smad 2/3 nuclear translocation and rescue of growth inhibition by 1 μmol/L TRI. **C:** Blocking of TGFβ- and LN5'-induced but not collagen I-induced directional hypermotility by 1 μmol/L TRI. **D** and **E:** Collagen I-induced hypermotility and LN5'-induced growth-arrest mechanisms function independent of one another when cells engage both ECM proteins simultaneously. Growth-arrest (**D**) and hypermotility (**E**) responses of strain N keratinocytes plated on control untreated dishes or on dishes precoated with both LN5' and collagen I in medium + or - TRI. **F:** Impairment of scratch wound migration by the TGFβ receptor I inhibitor. Pictures show extent of re-epithelialization 2 days after scratch wounding a confluent culture of strain N keratinocytes in the absence (**left**) or presence (**right**) of 1 μmol/L TRI. **Dashed lines** show position and width of original scratch wound.

p14^{ARF} expression or function is sufficient to confer resistance to growth arrest by LN5'—quite distinct from the delayed hypermotility response to TGFβ and the p16/p14^{ARF}-independent mechanism by which TGFβ induces growth arrest. Therefore, we also concluded that TGFβRI

becomes activated from cell engagement with LN5' by a mechanism different from TGFβ-instigated, presumably TGFβRII-dependent, TGFβRI activation. The similarities and differences between LN5'- and TGFβ-induced growth arrest and dHM are summarized in Table 2.

Table 2. Summary: Dependence on Proteins and Factors for Induction of Growth Arrest and Hypermotility under Different Conditions

	LN5 γ 2 Precursor		Collagen I		TGF β		Senescence*	
	Growth arrest	Hypermotility	Growth arrest	Hypermotility	Growth arrest	Hypermotility	Growth arrest	Hypermotility
p16 ^{INK4A}	Req.	Ind.	NA	Ind.	Ind.	Ind.	Req.	Ind.
p14 ^{ARF}	Req.	Ind.	NA	Ind.	Ind.	Ind.	Req.	Ind.
P53	Req.	Ind.	NA	Ind.	Ind.	Ind.	Req.	Ind.
Serum cofactor	Req.	Req.	NA	Ind.	Ind.	Ind.	Ind.	Ind.
TGF β RI	Req.	Req.	NA	Ind.	Req.	Req.	Req.	Req.
smad2/3 nuclear translocation [†]		+		–		+		n.d.

*Senescence refers to the telomere-unrelated senescence arrest undergone by keratinocytes during serial culture on untreated culture dishes.

[†]+ and – indicate whether this occurs in cells under each of the conditions.

Ind., independent; Req., required; NA, not applicable (because growth arrest or hypermotility do not occur under that condition); n.d., not determined.

As described above, hypermotility induced by collagen I proved not to be associated with induction of p16 or growth arrest. We therefore we asked whether TGF β RI-dependent smad2/3 translocation occurs in response to engagement with collagen I and whether it is essential for collagen I-induced dHM. As shown in Figure 5, B and C, plating cells on collagen I did not trigger smad translocation, and TRI had no effect on collagen I-induced dHM. We concluded that TGF β RI activation does not occur and is not required for dHM induced by collagen I.

In vivo, at the edge of a wound or at the stromal interface of neoplastic invasion, keratinocytes are likely to engage both their own secreted LN5' and stromal collagen I at the same time. We therefore asked whether cells in culture can activate and sustain LN5'- and collagen I-induced motility mechanisms simultaneously or whether one supercedes the other if both ECM molecules are available to cells. We plated cells on dishes precoated with a 10% serum/PBS solution of 0.4 μ g/ml recombinant LN5' and 100 μ g/ml collagen I (concentrations of each ECM molecule that induce maximum dHM when used individually). The presence of collagen I did not prevent or mitigate the growth inhibitory effects of LN5' (Figure 5D). TRI, which blocks the dHM response of cells plated on LN5' alone (Figure 5C), did not prevent dHM of cells plated on LN5' and collagen I (Figure 5E). We concluded that the signal pathways by which dHM is induced by these two ECM proteins are distinct and that interference with the LN5' signal pathway does not compromise the mechanisms by which cells respond to collagen I. Furthermore, we concluded that activation of the non-growth-arrest-related dHM by collagen I does not prevent activation of the p16-enforced growth-arrest mechanism if the cells are also engaging LN5'.

TGF β RI-Dependent Modulation of the Keratinocyte Senescence Mechanism

As described above, LN5'-induced HM/GA and p16-related keratinocyte senescence have several similar features, consistent with the possibility that they are identical mechanisms. We investigated this further by testing the hypothesis that blocking TGF β RI kinase activity by main-

taining primary keratinocytes continuously in the presence of TRI during serially passage on control surfaces in K-sfm medium would delay senescence and extend their lifespan. The addition of 1 μ mol/L TRI to the medium at the time of plating early- to mid-passage strain N cells on control surfaces resulted in most of the cells forming symmetric, tightly clustered colonies (Figure 6A, c) without inhibiting their growth rate during 7 to 9 days of growth in that passage (Figure 5, B and D). However, consistent with a report that very low concentrations of TGF β can improve the survival and expansion potential of keratinocyte stem cells cultured from human epidermis in a defined medium,⁶² we found that mid-lifespan primary keratinocytes did not tolerate continuous exposure to 1 μ mol/L TRI for more than two passages and that they replated better if TRI was removed several days before subculture. We therefore adopted the protocol of plating cells in K-sfm and 0.5 μ mol/L TRI and refeeding without TRI 2 or 3 days before each subculture. In the experiment shown in Figure 6, A and B, strain N cells were serially passaged with or without TRI beginning at mid-lifespan (sixth passage; 30 cumulative PDs). The cells subsequently grew at the same rate under both conditions until several passages before the normal end of the culture lifespan and had similar proportions of p16⁺ cells, as determined by immunocytochemical staining. By the ninth passage, the control cultures began to show signs of senescence, marked by a reduced population doubling rate, asymmetric and fragmented colonies, and an increase in the percentage of large, p16⁺ cells (34%) (Figure 6A, a and b). In contrast, the +TRI cultures continued to divide rapidly, to form colonies typically of compact and symmetric morphology reminiscent of early-passage cells (Figure 6A, c), and to produce p16⁺ cells at low frequency (6%) (Figure 6A, d). By the end of the 11th passage, –TRI cultures contained 42% p16⁺ cells and had shown no net population growth from the previous passage (ie, were senescent). The 11th passage +TRI cultures finally began to exhibit substantial colony fragmentation and a slower population doubling time, and 30% of the cells were p16⁺. By the end of the 12th passage, the +TRI cultures contained 47% p16⁺ cells and in their next subculture, produced no increase in cell

Figure 6. Delay of keratinocyte senescence and facilitation of TERT immortalization by serial culture with a TGF β RI kinase inhibitor. **A:** Typical colony morphology (**a** and **c**) and p16 expression patterns (**b** and **d**) of strain N keratinocytes at the end of their ninth weekly passage (lifespan curve in **b**), after ~49 cumulative PDs. Control cells (**a** and **b**) and cells cultured with TRI since sixth passage (**c** and **d**). **B:** Replicative lifespan curves of the normal primary human epidermal keratinocyte line strain N growing in K-sfm medium in the absence (closed circles) or presence (open triangles) of 1 μ mol/L TGF β RI kinase inhibitor TRI beginning at sixth passage. Asterisks indicate senescence. **C:** Replicative lifespan curves of OKF4/TERT-1F, generated by transducing the normal primary human oral mucosal keratinocyte line OKF4, growing in the feeder/FAD system (black closed circles), to express TERT at the passage indicated by downward bold arrow. OKF4/TERT was serially passaged subsequently in the feeder/FAD system (red closed squares). Some OKF4/TERT cells were switched at the ninth passage (indicated by arrow) to K-sfm medium (red open squares) or to K-sfm and TRI (red open triangles). Some OKF4/TERT cells that had been serially passaged in K-sfm and TRI were switched to K-sfm without TRI (red open diamonds) at the 15th passage (indicated by dashed arrow). Asterisks indicate senescence. In the figure legend inset, F indicates the feeder/FAD system and G indicates GIBCO K-sfm medium.

number over plating density (ie, were senescent). In this experiment, the cells cultured with TRI grew for two additional passages and an additional 8 PDs (ie, ~250-fold greater population expansion than cells cultured under control conditions) before senescing at 58 PDs. In a separate experiment, strain N cells cultured with TRI beginning with the seventh passage grew for an extended lifespan of 9 PDs compared with cells passaged in parallel without TRI. Another primary keratinocyte line, OKF4, which has a shorter lifespan than strain N, grew for 5 extra PDs (from 37 to 42 PDs) when TRI was added at the seventh passage, near the end of its normal lifespan.

Growth of primary and telomerase (TERT)-transduced keratinocytes in the feeder/FAD system^{32,33} can delay,⁶³ but not prevent,²⁹ the onset of p16 expression. TERT-transduced OKF4 cells give rise to immortalized lines more readily when maintained in the feeder/FAD system than when cultured in K-sfm²⁹ (Figure 6C). We asked whether growth in K-sfm supplemented with TRI could replace growth in the feeder/FAD system to facilitate the arising of immortalized cells within the OKF4/TERT-transduced population. In the experiment shown in Figure 6C, OKF4/TERT cells that were switched at their ninth passage, five passages after transduction, from the feeder/FAD system to K-sfm senesced. However, if switched to K-sfm and TRI, they continued to divide as an immortalized line with nearly the same growth rate as when maintained in the feeder/FAD system without TRI. The immortalized phenotype of this line proved not to be dependent permanently on TRI, as indicated by the continued progressive growth, albeit at a slightly slower population growth rate, when TRI was removed at the 15th passage. Because we had found previously for many TERT-immortalized keratinocyte lines,²⁸ the immortalized OKF4/TERT line continued to generate some p16-expressing, growth arrested cells during serial culture, while maintaining a majority population of proliferating cells. We concluded from our analysis of the effects of TRI on the lifespan of primary keratinocytes that suppression of TGF β RI kinase activity delays induction of p16 expression and senescence. Our results also suggest that delay of p16 induction in TERT-transduced keratinocytes by serial passage with TRI increases the probability that a variant impaired in p16 induction by any of several mechanisms²⁸ will arise within the population, with the ability to divide as an immortalized cell line independent of TRI. The similarities between HM/GA induced by plating early-passage keratinocytes on LN5' and p16-enforced senescence during serial passage (summarized in Table 2) were consistent with the possibility that the responsible cell signaling mechanisms are identical.

Discussion

These experiments describe the coordinate induction of hypermotility and growth arrest in keratinocytes in response to engagement with a precursor form of LN5. This hypermotility/growth-arrest (HM/GA) response is activated during wound repair, early neoplastic invasion, and serial cultivation and is marked by increased LN5 syn-

thesis and secretion and by p16^{INK4A} expression. The HM/GA response of keratinocytes in culture to LN5' is distinct from the response to collagen I, which stimulates dHM independent of TGF β RI kinase and does not lead to p16 expression or growth arrest. Connective tissue collagen I is likely exposed by partial thickness wounds or during malignant invasion and becomes accessible to keratinocytes. Previous studies have used as a model for keratinocyte migration in these *in vivo* settings the behavior of cells plated on collagen I-coated dishes. This assumes that to induce a migratory response, keratinocytes must change the ECM protein to which they engage from basement membrane LN5 to collagen I. Our studies showed that the presence of collagen I is not necessary for a dHM response and that collagen I alone does not induce p16 expression, which we found to be a consistent feature of keratinocytes at the migrating front of wounds. LN5 was first implicated as playing a role in keratinocyte motility with the observation that a form of LN5 secreted by some tumor cell lines had "scatter factor" activity on some cell lines.⁶⁴ Our study has demonstrated that, because keratinocytes become hypermotile and express p16 when plated on LN5' alone, similar to their *in vivo* responses during wound healing and early invasion, signaling mechanisms activated by autocrine LN5' are likely to be important for their migratory behavior *in vivo*. Keratinocytes engage both collagen I and LN5' in wound and invasion settings *in vivo*. Our study showed that cells plated on LN5' and collagen I together become hypermotile and growth-arrested and that the growth arrest instigated by the LN5' can be prevented by inhibiting TGF β RI kinase activity without impairing the hypermotility instigated by collagen I. This result indicates a distinction between the mechanisms by which LN5' and collagen I trigger and sustain dHM and that these mechanisms operate independently of one another when both are stimulated in the same cell.

In sparse culture under control conditions, keratinocytes are not truly sessile but move back and forth and rotate without undergoing net migration (Figure 2; Supplemental Video S1 at <http://ajp.amjpathol.org>). We have used the term "directional motility,"¹⁷ and here, we use "directional hypermotility" to describe the very different form of cell motility: rapid and sustained migration, relevant to the wound response. Frank and Carter¹³ have called this type of motility "processive migration." Either term is suitably descriptive but should be reserved for cells that sustain dHM behavior for many hours. Our experiments have revealed that directional hypermotility can be sustained for many days without any apparent chemotactic gradient. Many previous studies have used surrogate assays aimed at detecting and quantitating changes in keratinocyte motility, including transwell migration, colony scattering, cell movement at the margins of densely plated cells, and areas of clearing of colloidal particles. Assays should be able to identify and accurately quantitate dHM, which is relevant to wound healing, and to distinguish it from random, nondirectional movement. Our results have demonstrated the value of LN5 track-length measurements or time-lapse photography to accomplish this.

The K-sfm culture medium that we used, which promotes maximum proliferation rates on control surfaces without the cells exhibiting dHM, contains EGF. A recent report provided experimental evidence for $\alpha 6 \beta 4$ integrin and EGF dependence of keratinocyte movement as individual cells in low-calcium medium into a scratch wound or across a transwell filter.⁶⁵ An activated EGF receptor, and consequent phosphorylation of $\beta 4$ integrin, has been reported to be necessary for cells to destabilize the interaction between $\alpha 6 \beta 4$ integrin and laminin 5⁶⁶ and disassemble hemidesmosomes.⁵⁵ This may be a necessary precondition for keratinocyte motility, but our experiments have shown that it is insufficient by itself to provoke dHM. We suspect, based on previous studies,^{10–13} that LN5' engages $\alpha 3 \beta 1$ integrin, but the cell surface molecules essential for initiating the HM/GA response remain to be identified.

The *in vivo*, organ culture, and scratch wound results that we have presented provide the rationale for the powerful experimental system we used of low-density plating under conditions that immediately and rather synchronously induce the dHM and growth-arrest response. The complete mechanism by which a keratinocytes initiates and sustains dHM remains to be determined but should lend itself to solution using this assay because differential effects of engagement to specific ECM proteins can be detected and measured. Our results clearly show that the signals that trigger dHM and determine whether cells remain proliferative or growth arrest are very different depending on whether cells initially contact collagen I or LN5', despite the fact that the cells continue to deposit endogenously synthesized LN5 beneath them in both situations and during their normal, nonmigratory situation. Antibody blocking experiments suggest that keratinocytes require function of the LN5-binding $\alpha 3 \beta 1$ integrin for migration regardless of the ECM molecule on which they are plated.⁴⁵ dHM may require reorganization of LN5 beneath cells, perhaps aided by $\alpha 3 \beta 1$ integrin,⁶⁷ and may involve proteolytic processing of $\gamma 2$ precursor to the mature 105-kd form or to smaller forms, as has been proposed for LN5-stimulated motility of some cell lines.⁴⁴ Our available sources of pure recombinant LN5, and the natural source in 804G CM, are both the $\alpha 3$ -processed, $\gamma 2$ -precursor (LN5') form. Because TGF β induces dHM and TGF β -treated cells are induced to secrete more total and $\gamma 2$ -precursor LN5, the latter may be an essential structural feature for inducing HM/GA. We do not know whether mature LN5 might have the same effect, however, especially if present in excess or in a form not organized as it is in normal basement membrane or as deposited beneath nonmotile cells in culture.⁶⁷ Our study has clearly demonstrated that unprocessed $\alpha 3$ chain is not essential for LN5 to induce HM/GA.

A substratum-bound serum cofactor was required for LN5' to induce HM/GA and also substantially enhanced migration on collagen I. Henry et al⁴⁷ found that serum enhanced motility on collagen I in a system in which keratinocytes are incubated in serum-containing medium during the time motility is measured, and Li et al^{46,68} have described augmenting effects of soluble growth factors for maximum motility on collagen I, compared with motil-

ity in the absence of any protein factors. In our system, EGF and other mitogens and proteins present in bovine pituitary extract were always present at concentrations optimal for proliferation, so the substratum-bound serum cofactor that we detected is unlikely to be the same factor detected in those previous studies.^{46,47,68} Although migrating keratinocytes in wounds increase expression of the fibronectin receptor $\alpha 5 \beta 1$ integrin,⁵⁰ possibly induced by autocrine or paracrine TGF β ,^{49,50,69} neither fibronectin alone or as a potential cofactor for LN5' could induce dHM. The serum cofactor may help initiate plasma membrane polarization in our single cell plating assay but was not necessary either during proliferation to confluence or after wounding for keratinocytes at the edge of scratch wounds to induce LN5/p16 and migrate. In the scratch wound system, a signal to establish a polarized lamellipodium may be unnecessary because the wound itself generates membrane asymmetry. The substratum-bound serum cofactor was not growth inhibitory to cells plated in the absence of LN5'. Furthermore, the growth arrest induced by LN5' aided by the serum cofactor could be evaded by disabling p16 and p14^{ARF}/p53 control, unlike the growth arrest induced by TGF β . Thus, the substratum-bound serum cofactor for LN5'-induced growth arrest and dHM is neither TGF β itself, an inducer of TGF β expression, nor a protease that could activate latent TGF β present in cells or serum, and its identity remains to be determined. Interestingly, no serum cofactor was required for TGF β -induced dHM or for the dHM displayed spontaneously by late-passage keratinocytes, consistent with the possibility that keratinocytes begin producing their own cofactor under these circumstances.

Our experiments have shown that the HM/GA response is a two-step process in which hypermotility begins immediately in response to LN5' engagement, followed later by p16 expression and growth arrest. Neither p16 expression nor growth arrest proved to be essential to the hypermotility response. SCC cells, normal keratinocytes engineered to resist the inhibitory effects of p16 and p14^{ARF}, and some TERT-immortalized keratinocyte lines that acquired loss of p16 and p14^{ARF} expression still become hypermotile in response to LN5' but are not growth inhibited. *In vivo*, the HM/GA response is not only activated in wounding but also acts as a tumor suppressor mechanism. Severely dysplastic and microinvasive premalignant keratinocytes are triggered to express p16 and increased LN5 in response to confronting a compromised basement membrane.¹⁷ The results described here are consistent with a model in which persistent activation of the HM/GA response exerts selection pressure for p16/p14^{ARF}-deficient variants able to remain proliferative and hypermotile in this setting, such that they become invasive as squamous cell carcinoma.

Previous studies detected increased TGF β at the leading edge of healing skin wounds.^{49,50} Our study produced several independent pieces of evidence that TGF β RI kinase activity is essential for the HM/GA response: cells plated on LN5' undergo smad2/3 nuclear translocation, and LN5'-induced dHM and growth arrest are both dependent on TGF β RI kinase activity. In con-

trast, collagen I does not induce smad2/3 nuclear translocation, and inhibition of TGF β RI kinase does not prevent collagen I-induced dHM. The TGF β RI kinase inhibitor that we used has been demonstrated to be highly specific for this kinase⁷⁰ and in our experiments, was sufficiently selective and discriminatory in its action to identify similarities between TGF β and LN5' signaling, to identify differences between those and collagen I signaling, and to not impair any kinase responsible for mitogenic signaling in cells plated on control dishes. Our results disclosed that activation of TGF β RI is not essential to dHM per se, because its activity is not essential for collagen I-induced dHM, but that it is an essential element of the signal pathways that lie between cell engagement with LN5' and ultimate dHM and growth arrest. A recent report found p38MAPK phosphorylation in an SV40-immortalized, Lam5-deficient keratinocyte line in response to trypsin detachment, with p38MAPK dephosphorylation immediately on re-adhesion to collagen I- or LN5-coated surfaces, before spreading occurs.⁷¹ Because p38MAPK phosphorylation lies downstream of TGF β RI activation, we would predict that p38MAPK phosphorylation would ensue at some point after adhesion or spreading on LN5' but not on collagen I.

Some elements of the signal pathways responsible for LN5'-induced dHM are different from those of TGF β -induced dHM, despite the dependence of both on TGF β RI activity. In contrast to the immediate dHM induced by plating cells on LN5', TGF β induced dHM after a delay of ~1 day (Figure 4B), consistent with a requirement for a change in gene expression and new protein synthesis before cells exhibit dHM in response to TGF β . The LN5 tracks (Figure 4B) were similar to those deposited by cells plated on LN5'-coated dishes. TGF β induces increased production and secretion of LN5⁵¹ with substantial proportions remaining as the $\gamma 2$ precursor form (Figure 2C). This suggests the possibility that TGF β -induced hypermotility is an autocrine response to increased extracellular LN5', consistent with the immediate hypermotility response to exogenously supplied LN5' in our experimental system. It is important to note that the substantial lag period before cells become motile in response to addition of TGF β to the medium precludes the possibility that LN5' induces or activates TGF β to initiate an autocrine TGF β response. LN5'-binding integrins may interact physically with and activate the kinase domain of TGF β RI in the absence of any TGF β binding to TGF β RII, the better known activator of TGF β RI. Recent studies have identified interaction between the LN5 and LN5' receptor $\alpha 3 \beta 1$ integrin and tetraspanin CD151⁷² and a wound re-epithelialization defect in CD151 knockout mice.⁷³ Those results provide the rationale for investigating changes in complex formation among $\alpha 3 \beta 1$ integrin, CD151, and TGF β RI in response to cell engagement with LN5'.

Despite common smad nuclear translocation responses and common requirements for TGF β RI activity, the ultimate enforcers of growth inhibition resulting from adhesion to LN5' are different from those resulting from exposure to TGF β . Loss of p16 and either p14^{ARF} or p53 expression/function made cells resistant to LN5'-induced

growth arrest but did not confer resistance to TGF β -induced arrest. The mechanisms by which TGF β inhibit cell growth are multifaceted, differ among cell types, and may vary within a single cell type, depending on whether sustained or transient smad-dependent signaling occurs.⁵⁷ In some cell lines, TGF β induces p15^{INK4B} expression, which displaces p27^{kip1} from a noninhibitory site on cdk4 and permits p27^{kip1} to bind and inhibit cdk2.⁶¹ In other cell lines, induction of p21^{cip1} and repression of myc have been reported to be the predominant mechanism of inhibition.⁵⁹ p160ROCK-dependent phosphorylation and inhibition of cdc25 to reduce cdk2 activity is the major growth inhibitory mechanism of TGF β in yet another cell system.⁶⁰ The essential enforcers of TGF β -induced growth arrest in primary human keratinocytes remain to be determined. Our results are consistent with the possibility that LN5'-instigated TGF β RI activation results in different relative levels or time periods of stimulation of the smad, p38MAPK, and p160ROCK signal pathways than does TGF β RI activation that results from TGF β binding to TGF β RII. Such differences could determine whether p16 expression or another growth-arrest mechanism is induced. We are currently investigating the roles of smad and non-smad signal pathway activation in LN5'- and TGF β -induced hypermotility and growth arrest, using both kinase-specific drug inhibitors and siRNA approaches.

We suspect that we first detected the HM/GA response as it becomes activated stochastically in normal keratinocytes during serial culture, resulting in p16- and p14^{ARF}-dependent senescence.^{28,29} There are several independent lines of evidence supporting identity between the telomere status-independent, senescence/immortalization barrier mechanism and HM/GA. Both p16 and p14^{ARF}/p53 enforce the growth arrest in the two situations: keratinocytes must become p16 deficient and either p14^{ARF} or p53 deficient to evade senescence²⁹ or LN5'-induced growth arrest. Both LN5'-induced growth arrest and senescence are preceded by increased LN5 synthesis and hypermotility¹⁷ (this report), and both HM/GA and p16-related senescence with its accompanying hypermotility are blocked by inhibition of TGF β RI kinase activity. Keratinocytes in serial culture on control surfaces may gradually increase production of LN5 and/or lose their ability to process secreted LN5 quickly enough to prevent engagement with the γ 2 precursor form and consequently instigate the HM/GA response. Considering that TGF β stimulates synthesis and secretion of unprocessed LN5⁵¹ (this report), blocking the TGF β RI kinase may keep LN5 synthesis under control and avoid the "spontaneous" HM/GA activation during serial passage that results in senescence arrest.^{17,28,29} Our finding that TRI extends keratinocyte lifespan and facilitates TERT immortalization has implications for optimizing culture systems to maximize expansion potential of human keratinocytes in culture for research and therapeutic purposes. A recent microarray analysis of gene expression profiles of cultured keratinocytes⁷⁴ reported that a set of genes that might be expected to be related to keratinocyte motility, includ-

ing those encoding the three LN5 subunits, the α 2 and α 5 integrins, MMP9, MMP10, and urokinase, were expressed at higher levels by cells cultured in K-sfm medium versus the feeder system and, therefore, might be responsible for the hypermotility of senescent keratinocytes we had reported previously.¹⁷ One study (Guo Z, Jensen R, Boches S, Gullans S, and Rheinwald J, unpublished data) found no such differences in expression levels of these genes between early-passage cells cultured in the two conditions but instead has found that such genes are expressed at higher levels by keratinocytes cultured in K-sfm at the end of their replicative lifespan, when most cells in the population have induced p16 expression. Thus, different culture conditions do not directly alter the pattern of expression of motility-related genes, a conclusion supported, of course, by our findings reported here that most keratinocytes in early-to-mid-passage cultures growing in K-sfm medium on untreated culture dishes are not hypermotile.

There are several potential clinical applications of our results. Our earlier study¹⁷ suggested that immunohistochemical detection of LN5/p16 co-expression may be helpful in more accurately diagnosing the risk of progression of dysplastic oral epithelial and epidermal lesions. Our analysis of a small set of decubitus ulcers (pressure sores) of the skin found no difference between wounds diagnosed as chronically nonhealing versus those that showed evidence of successful healing. It would be interesting to expand the study to include ulcers and regions at high risk of blistering in junctional epidermolysis bullosa disorders to determine whether LN5/p16 co-expression in areas other than the migrating front of healing areas is predictive of instability and future erosion. Excessive or prolonged p16 induction in wounds could curtail proliferative potential of the cells that re-epithelialize denuded areas. Screening drug libraries for compounds that block LN5'- or TGF β -induced keratinocyte dHM or growth arrest in culture, without inhibiting growth or differentiation under control conditions, should identify candidates for several potential therapeutic applications. Drugs that specifically inhibit LN5'- or TGF β -induced growth arrest without impairing the dHM response may promote healing of chronic ulcers. Drugs that selectively inhibit LN5'-induced dHM without impairing the growth-arrest response may suppress invasive behavior of cells in dysplastic skin, oral, and cervical lesions. Both the conventional culture and organ culture experimental systems we have described here will be useful for such preclinical studies.

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