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 p16INK4A (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 γ2 precursor form of laminin 5 (LN5γ) immediately induced directional hypermotility at ~125 μm/hour, followed by p16 expression and growth arrest. Cells deficient in p16 and either p14ARF or p53 became hypermotile in response to LN5 but did not arrest growth. Plating on LN5γ triggered smad nuclear translocation, and all LN5γ effects were blocked by a transforming growth factor (TGFβ) receptor I (TGFβRI) kinase inhibitor. In contrast, plating cells on collagen I triggered a TGFβRI kinase-independent hypermotility unaccompanied by smad translocation or growth arrest. Plating on control surfaces with TGFβ induced hypermotility after a 1-day lag time and growth arrest by a p16-independent mechanism. Keratinocytes serially cultured with TGFβ exhibit 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)
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 synchronously 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 γ2 subunit remains in its uncleaved precursor form (LN5H) induces directional hypermotility followed by induction of p16 expression and growth arrest. Our studies have revealed dependence of both LN5H-induced HM/GA and the keratinocyte senescence mechanism on activity of the TGFβ 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 medium (GIBCO/Invitrogen, Carlsbad, CA), supplemented as described previously with 25 μg/ml bovine pituitary ex-

<table>
<thead>
<tr>
<th>Table 1. Human Keratinocyte Cell Lines</th>
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<tr>
<td><strong>Cell line name</strong></td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Human epidermal origin</td>
</tr>
<tr>
<td>Strain N</td>
</tr>
<tr>
<td>N/cdk4R</td>
</tr>
<tr>
<td>N/p53DD</td>
</tr>
<tr>
<td>N/lit1</td>
</tr>
<tr>
<td>N/E6E7</td>
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<tr>
<td>N/TERT-1</td>
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<tr>
<td>N/TERT-2G</td>
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<tr>
<td>SCC-13</td>
</tr>
<tr>
<td>Human oral mucosal origin</td>
</tr>
<tr>
<td>OKF4</td>
</tr>
<tr>
<td>OKF4/TERT-1F</td>
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<tr>
<td>OKF6/TERT-2</td>
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<tr>
<td>POE-9n</td>
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<td>SCC-71</td>
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M, male; F, female; HD, homozygous deletion.
tract, 0.2 ng/ml epidermal growth factor (EGF), and 0.4 mmol/L CaCl₂ (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 Ca²⁺-free Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO/Invitrogen) and K-sfm, supplemented with bovine pituitary extract, EGF, and 0.4 mmol/L CaCl₂, which permits formation of healthy, confluent, stratified cultures. For some experiments, keratinocytes were cultured using the feeder/FAD system, in which keratinocytes are co-cultured with mitomycin-treated 3T3J2 feeder cells in serum- and growth factor-supplemented DMEM/F12 medium. Replicative lifespans of keratinocyte cell lines were calculated as cumulative population doublings (PDs), summing the log₂ (no. of cells at subculture/no. of cells plated) for all passages until cell numbers no longer increased. The rat bladder carcinoma cell line 804G 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 β3 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 cloned and 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-polycrylamide gel electrophoresis (SDS-PAGE) gels, and all three were detectable by Western blotting, with the α3 chain being in its cleaved, mature form (data not shown) and γ2 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 β3 subunit, this ensured that all γ2 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, γ2 precursor form (LNS’), at 0.4 µg/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 µg/ml. For some experiments, dishes were coated with 100 µg/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 804G was used as a source of the γ2 precursor form of LN5. 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 TGFβ1 (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× or 1000× 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 an LN5 γ2 chain-specific antibody. Fifteen to 20 adjoining fields were photographed using a 2× 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 log₂ (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 TGFβ receptor I kinase inhibitor [3-(pyridin-2-yl)-4-(4-quinonyl)-1H-pyrazole] (TRI) (Lilly designation HTS466284; Biogen designation LY364947) was used at 1 µmol/L and added to the medium at the time of plating and at each refeeding from 1000× concentrated stock solutions in dimethylsulfoxide. For serial culture experiments, TRI was added at 0.5 µmol/L at the time of plating and at subsequent refeedings 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-
seemed with a 25-mm-diameter plastic coverslip (Thermo-mon; Nalge/Nunc, Rochester, NY), which had been precoated by a 30-minute incubation with 10% calf serum-supplemented DMEM containing 0.4 μg/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× phase objective and 10× 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 p16NK4A-specific, murine monoclonal antibody (mAb) G175-405 (Pharmingen, San Diego, CA) was used at 2 μg/ml for immunostaining. The p16NK4A-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 y2 chain-specific murine mAb D4B5 39 (Chemicon, Temecula, CA) was used at 10 μg/ml for immunostaining and Western blots (note that D4B5 does not recognize rat LN5 y2, so it readily detected LN5 deposited by keratinocytes plated on 804G CM-coated surfaces). The LN5 y2 chain-specific rabbit polyclonal antibody J20 4 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 y2 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 1:00 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 p16NK4A expression, keratinocytes were plated on untreated or on LN5°- or collagen I-coated dishes at 1.5 × 10⁶ 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 y2 expression and processing status, cells were plated at 10⁵ cells per p100 dish and grown for 6 days with or without ~1 ng/ml TGFβ for the final 2 days, and the final 1-day CM was collected. Cells from TGFβ-treated and untreated cultures were counted, and volumes of CM normalized for cell number were separated by SDS-PAGE as described above. Proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA), and specific proteins were detected with murine mAbs against p16NK4A (JC-1; provided by E. Harlow), the y2 chain of LN5 (D4B5; Chemicon), and β-actin (A-2066; Sigma), followed by peroxidase-conjugated secondary antibody (Southern Biotehnologies, Birmingham, AL) and chemiluminescence reagent (ECL; Amersham Corp., Piscataway, NJ).
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...
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+/LN5hi cells exhibit directional hypermotility (dHM). 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 have suggested that keratinocytes convert, on stimuli such as wounding, to a migratory state involving α3β1 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 α3 but unprocessed γ2 chain (LN52). We compared the behavior of early-passage primary human keratinocytes plated on untreated dishes, dishes precoated with 804G CM, and previous reports, our immunostaining procedure did not detect the γ2 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 al 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. A TERT-immortalized derivative of strain N, N/TERT-2G, which continues to produce p16, growth-arrested cells at low frequency during serial passage (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.

Figure 2. Directional hypermotility induced by plating keratinocytes at low density on surfaces precoated with the γ2 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 μg/ml recombinant LN5 dissolved in 10% calf serum-supplemented DMEM (B). C: Insets show homogenous LN5 deposition around nonmigratory cells (A) and scattered pattern behind directionally hypermotive cells (B). C: Western blot using the laminin 5 γ2 chain-specific antibody D4B5. Lane 1, CM from control strain N culture; lane 2, CM from strain N culture treated with 1 ng/ml TGFβ 2 days before harvesting; and lanes 3 and 4, 0.05 and 2 μg purified recombinant human LN5, γ2 precursor form (see Materials and Methods). Note the increase in total γ2 chain and its precursor form in the medium of TGFβ-treated cells and that the γ2 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.amijpathol.org). Points represent the distance traveled by individual keratinocytes 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. F: reprinted from J Invest Dermatol Symp Proc 2005, 10:72–85, with permission.
dishes precoated with fresh 10% serum/DMEM. Immunostaining ~42 hours later with the D485 antibody that recognizes the y2 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).

### Keratinocyte Hypermotility/Growth-Arrest Response 1827

#### 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 non-cycling, 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 p14ARF/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 p14ARF, 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, p14ARF, 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 cdk4R24C mutant (which resists p16-dependent arrest), the dominant-negative p53DD mutant (which evades p14ARF, and p53-dependent arrest), HPV16E6E7 (which evades p16-/Rb-dependent and p14ARF/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/p14ARF 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 p14ARF or p53 were resistant to LN5-induced growth inhibition. N/TERT-1, which fails to express any detectable p16 or p14ARF 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 p14ARF/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 p14ARF 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.

**TGFβ 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 TGFβ in keratinocytes at the leading edge of wounds49,50 and the report that TGFβ induces increased expression of completely unprocessed (ie, α3pre, γ2pre) LN5 in cultured keratinocytes accompanied by increased transwell migration.51 TGFβ is a potent growth inhibitor of primary human keratinocytes.52,53 Cells plated at low density on control dishes in medium supplemented with TGFβ 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 TGFβ 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 TGFβ 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 TGFβ treatment resulted in increased LN5 secretion with a substantial proportion remaining in the γ2 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 β4 integrin, and this event appears necessary for keratinocytes to disassemble α6β4 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 TGFβ.

The mechanism of TGFβ signaling is more complex than that of EGF and many other polypeptide growth factors and cytokines in that the transmembrane receptor that binds TGFβ (TGFβRII) is not the kinase responsible for activating downstream cytoplasmic mediators. Instead, on binding of TGFβ, TGFβRII associates with and activates the kinase domain of TGFβ receptor I (TGFβRI), 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-induced hypermotility. Keratinocytes deficient in p16 and p14ARF/p53 function, consistent with studies in other cell systems.59–61 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 TGFβR 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 TGFβRII 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 TGFβR 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/
p14ARF 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/p14ARF-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-istigated, presumably TGFβRII-dependent, TGFβRI activation. The similarities and differences between LN5- and TGFβ-induced growth arrest and dHM are summarized in Table 2.

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.
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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 LNS5 and stromal collagen I at the same time. We therefore asked whether cells in culture can activate and sustain LNS5- and collagen I-induced motility mechanisms simultaneously or whether one supersedes 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
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
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-.

### TGFβRI-Dependent Modulation of the Keratinocyte Senescence Mechanism

As described above, LNS5
'-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 TGFβRI kinase activity by maintaing 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
μmol/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 TGFβ 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
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*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.

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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 can delay, but not prevent, 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-sfm (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, 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 TGFβRI 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 mechanisms 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 LNS’ 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 LNS. This hypermotility/growth-arrest (HM/GA) response is activated during wound repair, early neoplastic invasion, and serial cultivation and is marked by increased LNS syn-
thsis and secretion and by p16\(^{NK4A}\) 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\(\beta\)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.\(^{6,4}\) 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\(\beta\)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 https://ajp.amipathol.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 Carter\(^{13}\) have called this type of motility “prospective migration.” Either term is suitable 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 keratinocyte migration in these situations. Antibody blocking experiments suggest that keratinocytes require function of the LN5-binding integrin for migration regardless of the ECM molecule on which they are plated.\(^{46}\) dHM may require reorganization of LN5 beneath cells, perhaps aided by a3\(\beta\)1 integrin,\(^{6,7}\) and may involve proteolytic processing of y2 precursor to the mature 105-kd form or to smaller forms, as has been proposed for LN5-stimulated motility of some cell lines.\(^{4,4}\) Our available sources of pure recombinant LN5, and the natural source in 804G CM, are both the a3-processed, y2-precursor (LN5\(^*\)) form. Because TGF\(\beta\) induces dHM and TGF\(\beta\)-treated cells are induced to secrete more total and y2-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 nonmotive cells in culture.\(^{6,7}\) Our study has clearly demonstrated that unprocessed a3 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 al\(^{4,7}\) 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\(^{4,6,8}\) have described augmenting effects of soluble growth factors for maximum motility on collagen I, compared with motil-

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 a6\(\beta\)4 integrin and EGF dependence of keratinocyte movement as individual cells in low-calcium medium into a scratch wound or across a transwell filter.\(^{6,6}\) An activated EGF receptor, and consequent phosphorylation of \(\beta4\) integrin, has been reported to be necessary for cells to destabilize the interaction between a6\(\beta4\) integrin and laminin 5\(^{6,6}\) and disassemble hemidesmosomes.\(^{5,5}\) 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 a3\(\beta\)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 a3\(\beta\)1 integrin for migration regardless of the ECM molecule on which they are plated.\(^{46}\) dHM may require reorganization of LN5 beneath cells, perhaps aided by a3\(\beta\)1 integrin,\(^{6,7}\) and may involve proteolytic processing of y2 precursor to the mature 105-kd form or to smaller forms, as has been proposed for LN5-stimulated motility of some cell lines.\(^{4,4}\) Our available sources of pure recombinant LN5, and the natural source in 804G CM, are both the a3-processed, y2-precursor (LN5\(^*\)) form. Because TGF\(\beta\) induces dHM and TGF\(\beta\)-treated cells are induced to secrete more total and y2-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 nonmotive cells in culture.\(^{6,7}\) Our study has clearly demonstrated that unprocessed a3 chain is not essential for LN5 to induce HM/GA.

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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. Although migrating keratinocytes in wounds increase expression of the fibronectin receptor α5β1 integrin, possibly induced by autocrine or paracrine TGFβ, neither fibronectin alone nor 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 p14ARF/P19/p53 control, unlike the growth arrest induced by TGFβ. Thus, the substratum-bound serum cofactor for LN5-induced growth arrest and dHM is neither TGFβ itself, an inducer of TGFβ expression, nor a protease that could activate latent TGFβ present in cells or serum, and its identity remains to be determined. Interestingly, no serum cofactor was required for TGFβ-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 p14ARF, and some TERT-immortalized keratinocyte lines that acquired loss of p16 and p14ARF, and some TERT-immortalized keratinocyte lines in response to trypsin detachment, with p38MAPK dephosphorylation immediately on re-adhesion to collagen I- or LN5-coated surfaces, before spreading occurs. Because p38MAPK phosphorylation lies downstream of TGFβRI 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 TGFβ-induced dHM, despite the dependence of both on TGFβRI activity. In contrast to the immediate dHM induced by plating cells on LN5, TGFβ 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 TGFβ. The LN5 tracks (Figure 4B) were similar to those deposited by cells plated on LN5-coated dishes. TGFβ induces increased production and secretion of LN5 with substantial proportions remaining as the γ2 precursor form (Figure 2C). This suggests the possibility that TGFβ-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 TGFβ to the medium precludes the possibility that LN5 induces or activates TGFβ to initiate an autocrine TGFβ response. LN5-binding integrins may interact physically with and activate the kinase domain of TGFβRI in the absence of any TGFβ binding to TGFβRI, the better known activator of TGFβRI. Recent studies have identified interaction between the LN5 and LN5 receptor α3β1 integrin and tetraspans CD151 and a wound re-epithelialization defect in CD151 knockout mice. Those results provide the rationale for investigating changes in complex formation among α3β1 integrin, CD151, and TGFβRI in response to cell engagement with LN5.

Despite common smad nuclear translocation responses and common requirements for TGFβRI activity, the ultimate enforcers of growth inhibition resulting from adhesion to LN5 are different from those resulting from exposure to TGFβ. Loss of p16 and either p14ARF or p53 expression/function made cells resistant to LN5-induced
growth arrest but did not confer resistance to TGFβ-induced arrest. The mechanisms by which TGFβ 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, TGFβ induces p15INK4b expression, which displaces p27kip1 from a noninhibitory site on cdk4 and permits p27kip1 to bind and inhibit cdk2.61 In other cell lines, induction of p21cip1 and repression of myc have been reported to be the predominant mechanism of inhibition.59 p16ROCK-dependent phosphorylation and inhibition of cdc25 to reduce cdk2 activity is the major growth inhibitory mechanism of TGFβ in yet another cell system.60 The essential enforcers of TGFβ-induced growth arrest in primary human keratinocytes remain to be determined. Our results are consistent with the possibility that LN5- and TGFβRII activation results in different relative times or time periods of stimulation of the smad, p38MAPK, and p16ROCK signal pathways than does TGFβRII activation that results from TGFβ binding to TGFβRII. 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 TGFβ-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 p14ARF-dependent senescence.28,29 There are several independent lines of evidence supporting identity between the telomere status-independent, senescence/mortalization barrier mechanism and HM/GA. Both p16 and p14ARF/p53 enforce the growth arrest in the two situations: keratinocytes must become p16 deficient and either p14ARF or p53 deficient to evade senescence or LN5-induced growth arrest. Both LN5- and TGFβ-induced growth arrest and senescence are preceded by increased LN5 synthesis and hypermotility7 (this report), and both HM/GA and p16-related senescence with its accompanying hypermotility are blocked by inhibition of TGFβRII 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 γ2 precursor form and consequently instigate the HM/GA response. Considering that TGFβ stimulates synthesis and secretion of unprocessed LN51 (this report), blocking the TGFβRII 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, including those encoding the three LN5 subunits, the α2 and α5 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 hypermotive.

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 TGFβ-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 TGFβ-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.

Acknowledgments

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References


