Differential Distribution of Laminin N-Terminus α31 Across the Ocular Surface: Implications for Corneal Wound Repair

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PURPOSE. Laminin N-terminus (LaNt) α31 is a relatively unstudied protein derived from the laminin α3 gene but structurally similar to netrins. LaNt α31 has, to date, been investigated only in two-dimensional (2D) keratinocyte culture where it influences cell migration and adhesion, processes integral to wound repair. Here we investigated LaNt α31 distribution in ocular surface epithelium, during limbal stem cell activation, and corneal wound healing.

METHODS. Human, mouse, and pig eyes, ex vivo limbal explant cultures, and alkali burn wounds were processed for immunohistochemistry with antibodies against LaNt α31 along with progenitor cell–associated proteins. LaNt α31 expression was induced via adenoviral transduction into primary epithelial cells isolated from limbal explants, and cell spreading and migration were analyzed using live imaging.

RESULTS. LaNt α31 localized to the basal layer of the conjunctival, limbal, and corneal epithelial cells. However, staining was nonuniform with apparent subpopulation enrichment, and some suprabasal reactivity was also noted. This LaNt α31 distribution largely matched that of keratin 15, epidermal growth factor receptor, and transformation-related protein 63 (p63), and displayed similar increases in expression in activated limbal explants. During active alkali burn wound repair, LaNt α31 displayed increased expression in limbal regions and loss of basal restriction within the cornea. Distribution returned to predominately basal cell restricted once the wounded epithelium matured. Cultured corneal epithelial cells expressing LaNt α31 displayed increased 2D area and reduced migration, suggesting a functional link between this protein and key wound repair activities.

CONCLUSIONS. These data place LaNt α31 in position to influence laminin-dependent processes including wound repair and stem cell activation.

Keywords: cornea, ocular surface epithelium, corneal wound healing, basement membrane, laminin

Laminin N-terminus (LaNt) α31 is member of a relatively unstudied family of proteins generated by alternative splicing from laminin-encoding genes. To date, only one study describing LaNt function has been published, which suggested a role for LaNt α31 in the regulation of epidermal keratinocyte adhesion, likely through influencing laminins. These data suggest that LaNt α31 could influence tissue remodeling. Therefore, knowledge of this protein’s distribution in healthy tissue and in disease contexts, such as during corneal repair, is key to understanding when and where it could exert its effects. In this study, we have focused on the ocular surface epithelium, where laminins are key mediators of numerous processes including regulating limbal stem cell maintenance and corneal repair processes.5–7

Laminins contribute to a wide variety of cellular behaviors; however, they are best understood for their structural roles as core components of specialized regions of extracellular matrix, termed basement membranes (BM). BM underlie and support all epithelial and endothelial sheets of cells as well as nerves and muscles (for reviews see Refs. 8–10). In addition to their structural support roles, laminins also present cell surface receptor binding sites and initiate a variety of outside-in signaling cascades. Many of these are context specific, including regulating proliferation and adhesion and influencing the ability of cells to generate the traction forces required for migration.8–10 Moreover, there is increasing evidence that specific laminins can contribute to either maintaining stem cells in an undifferentiated state, or can help drive cells down a defined differentiation pathway depending on which of the 16 different laminin family members the cells interact with.11–18

LaNt α31 is generated from the LAMA3 gene, which encodes two laminin subunits, α3a and α3b.1,19,20 Laminin α3b has not been as extensively investigated; however, laminin α3a is highly expressed in the ocular surface epithelium where it is directly implicated in conjunctival, limbal, and corneal epithelial cell function, and dysfunction is associated with a variety of ocular pathologies.5,7 Specifically, laminin α3 has been shown to be critical for epithelial integrity, demonstrated by the inherited disease junctional epidermolysis bullosa where patients with mutations in LAMA3 present with widespread epithelial fragility including effects on the corneal epithelium.21,22 Similarly, in the autoimmune disorder mucous membrane...
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METHODS

Ethical Approval

Research with human subjects followed the tenets of the Declaration of Helsinki. All experiments on human tissues were approved by the University of Liverpool and UK National Health Service Health Research Authority ethics committees (#16/EM/0090, #T0093). Specific informed consent from donor or family members was acquired before human corneal limbal rim use. All animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies

Rabbit polyclonal antibodies against human LaNt α31 were described previously.1 Rabbit polyclonal antibodies against keratin 1246 and LM332,47 and mouse monoclonal antibodies against laminin α3 (RG13),48 were kind gifts from Jonathan Jones, Washington State University, Washington, United States. Rabbit polyclonal antibodies against epidermal growth factor receptor (EGFR; 1:100, #068487; Merck Millipore, Darmstadt, Germany), transformation-related protein 63α (p63α; 1:250, #4892S; Cell Signaling Technology, Danvers, MA, USA), cytokeratin 5 (K5, 1:100 ab53121; Abcam, Cambridge, MA, USA), and mouse monoclonal antibodies against cytokeratin 15 (K15, 1:500, ab80522; Abcam) and vimentin (clone V9, 1:100; Sigma-Aldrich, Poole, UK) were used for immunohistochemistry and/or immunofluorescence microscopy. Rabbit (1:200) and mouse (1:1000) IgG controls were obtained from BD Biosciences (San Jose, CA, USA). Anti-mouse and anti-rabbit secondary antibodies for immunohistochemistry were obtained from EnVision + System-HRP kit (ready-to-use solution; Agilent, Santa Clara, CA, USA). Cy5 and fluorescein isothiocyanate–conjugated goat anti-mouse IgG and tetramethylrhodamine-conjugated goat anti-rabbit IgG were obtained from Jackson Labs (Sacramento, CA, USA). Goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680CW were obtained from LiCor Biosciences (Rugby, UK).

Mouse monoclonal antibodies against human LaNt α31 clone 3E11 were raised against a synthetic peptide corresponding to residues 437 to 451 (VLPQRSHQANFGSV; GenWay Biotech, San Diego, CA, USA) conjugated to keyhole limpet hemocyanin following the procedure previously described,37 with help from the recombinant protein production core at Northwestern University (Chicago, IL, USA). Five days following the final boost, the spleen was removed and isolated splenocytes were fused with the myeloma cell line Sp2 for the production of hybridomas.49 Hybridoma clones were selected on the basis of ELISA reactivity to the unconjugated peptide and then tested for immunohostaining reactivity against a human epidermal keratinocyte (HaCaT)50 protein extract. Selected hybridoma cells were cloned twice by limited cell dilution. The final antibodies were used for immunohistochemistry and Western blotting at 1.6 μg/mL final concentration, and immunofluorescence microscopy at 5 μg/mL.

Rabbit polyclonal antibodies were raised against CLNSDSSMFSLPRML (Eurogentec, Liège, Belgium) corresponding to mouse LaNt α31 residues 465 to 480. Antibodies were screened by ELISA against the unconjugated peptide, then by Western blotting against total protein extracts from mouse keratinocytes, and then affinity purified using the unconjugated peptide. Antigen depletion was performed by preincubating the anti-mouse LaNt α31 antibodies with the antigenic peptide (1 μM in 15 mM Na2CO3 35 mM NaHCO3) overnight at 4°C.

Tissue Processing: Immunohistochemistry

Human donor eyes obtained from the Liverpool Research Eye Bank (Liverpool, UK) were sectioned anterior-posteriorly through the pupil and the anterior segment was analyzed separately. Mouse globes from 21-week-old B6CBAF1 female mice were processed as whole globes. In each case, tissues were fixed in 10% neutral buffered formalin (Leica, Wokingham, UK) for 24 hours at the time of dissection, then processed through graded ethanol and xylene before being embedded in paraffin wax.
Sections (4 μm) were cut using a rotary microtome RM2235 (Leica), adhered to microscope slides (EnVision FLEX system, Agilent), and then dried overnight at 37°C. Sections were then dewaxed and rehydrated through a series of decreasing ethanol concentrations. Antigen retrieval was performed by microwaving sections in preheated 0.01 M citrate buffer pH 6.6 (Sigma-Aldrich) for 5 minutes. Endogenous peroxidases were blocked by treatment with 0.3% vol/vol hydrogen peroxide (15 minutes; Agilent), and then slides were blocked in 20% vol/vol goat serum (Sigma-Aldrich). Sections were then incubated with primary antibodies or isotype-matched controls at 4°C overnight (except for anti-EGFR, which was incubated at room temperature [RT] for 1 hour), followed by HRP-conjugated secondary antibodies at RT for 30 minutes (EnVision + System-HRP, Agilent). At each stage washing was performed with PBS with 0.1% Tween 20 (Sigma-Aldrich). HRP signal was detected using either 3,3′-diaminobenzidine (DAB, Agilent) or 3-amin-9-ethylcarbazole (AEC) chromogens at RT for 5 minutes. Sections were counterstained with filtered Mayer’s hematoxylin (Leica), dehydrated through a series of ascending ethanol concentrations, and then mounted using either Pertex (Histolab, Gothenburg, Sweden) for DAB or Aquatex mounting media (Merek Millipore) for AEC. Images were captured with an Olympus BX60 system microscope equipped with an Olympus DP71 digital camera and cell imaging software (Olympus, Southend-on-Sea, UK).

Scoring of immunohistochemistry staining, to evaluate LaNT ≥31 basal versus suprabasal expression, was performed by four independent observers analyzing red-green-blue (RGB) images following color deconvolution to remove hematoxylin signal using Fiji32 software (National Institutes of Health, Bethesda, MD, USA). A score of 0, 1, 2, or 3 was given to DAB-stained basal or suprabasal cells by each observer. Independent scorers had the full panel of stained sections available.

**Tissue Processing: Immunofluorescence**

Porcine anterior segments were snap frozen in liquid nitrogen before submerging in optimal cutting temperature compound (OCT) and freezing on dry ice. Human limbal rims were submerged in OCT and fast frozen on a dry ice/isopentane slurry. Tissue sections (5 μm), cut using Leica cryostat, were fixed in ice-cold acetone (Sigma-Aldrich) for 10 minutes, then incubated overnight at 4°C with primary antibodies or isotype-matched controls diluted in PBS with 5% vol/vol goat serum. Slides were washed in PBS, then incubated with secondary antibodies in PBS for 1 hour at 37°C. Slides were finally washed, then mounted in Vectashield mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA), and imaged using a Zeiss LSM880 confocal microscope (Zeiss, Jena, Germany).

**Limbal Explant Culture**

Human corneal limbal rims (n = 10), acquired from the Tissue and Eye Services at St. Paul’s Eye Unit, Royal Liverpool University Hospital, Liverpool, were isolated from the remnants of donor corneas that had been used for penetrating keratoplasty. Excess scleral, iris tissue, and corneal endothelium were carefully removed from the corneal limbal rings, leaving a ring of approximately 3-mm diameter. The rings were dissected into ~2-mm cuboidal segments for ex vivo culture. These segments were placed in the center of a well of a 12-well culture plate (Corning, Inc., Corning, NY, USA) and cultured at 37°C and 5% CO2 in limbal epithelial stem cell medium consisting of equal volumes of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B1 (all Sigma-Aldrich), and 0.4 mg/mL hydrocortisone, 10 mg/mL insulin, 20 μg/mL triiodothyronine, 40 mg/mL adenine, 50 μg/mL cholaer toxin, and 100 ng/mL epidural growth factor (Lonza, Slough, UK). Medium was changed every 2 days and epithelial outgrowth monitored daily for 7 to 14 days with a phase-contrast microscope (Nikon, Surrey, UK). Then explants displaying outgrowths were fixed and processed for immunohistochemistry, or embedded in OCT and frozen for immunofluorescence processing.

**Pig Corneal Wound Model**

Porcine eyes were obtained from local abattoirs, and anterior segments were dissected by coronal incision within 6 hours of enucleation. Extracocular tissue, iris, ciliary body, and lens were removed, leaving only intact cornea and trabecular meshwork. Corneas were washed in PBS with 200 U/mL penicillin, 200 μg/mL streptomycin, and 5 μg/mL amphotericin B1. An alkali burn epithelial wound was induced as previously described51 by using a sterile 5-mm-diameter round filter paper soaked in 0.5 M NaOH applied for 20 seconds to the central cornea. Tissues were maintained in culture for up to 72 hours following wounding, in high-glucose DMEM medium, supplemented with 10% fetal calf serum (FCS; Labtech East Sussex, UK), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B on a rotary incubator, then washed, fixed, and processed for immunohistochemistry.

**Cell Culture**

Primary corneal epithelial cells (pCEC) were isolated from limbal explants after 14 days of ex vivo culture, by TrypLE Express Enzyme (Thermo Fisher Scientific, Waltham MA, USA), then cultured in limbal epithelial culture media. Spontaneously transformed human epidermal keratinocytes HaCaT52 were cultured using DMEM (Sigma-Aldrich) supplemented with 10% FCS (LabTech) and 4 mM L-glutamine (Sigma-Aldrich).

**Adenovirus Production and Cell Transduction**

Full-length LAMA3LN1 was PCR amplified from cDNA generated from cultured human keratinocytes, then cloned into pCR2.1 (Life Technologies, Carlsbad, CA, USA) and sequence verified by DNA sequencing (DNA Sequencing and Services, University of Dundee). The native translational stop codon was converted to an Agel restriction enzyme site by site-directed mutagenesis following the manufacturer’s directions for QuikChange II XL mutagenesis kit (Agilent). The mutated LAMA3LN1 was then subcloned using KpnI and Agel (New England Biolabs, Hitchin, UK) into pENTR4 (Life Technologies) with egFP inserted into the BglII and Kpnl sites of the multiple cloning site (a kind gift from Jonathan Jones, Washington State University, WA, USA). LR recombination was used to transfer the LAMA3LN1-eGFP construct from pENTR to PAD-CMV/V5-DEST (Life Technologies) and adenoviral particles produced following the standard gateway-adapted ViralPower adenoviral expression protocol (Life Technologies).

For cell transduction, 3 × 105 pCEC were seeded in 60-mm dishes (Greiner Bio-One, Stonehouse, UK), then transduced with adenovirus in 4 μL media 24 hours after seeding. Analyses of LaNT ≥31-GFP (+LaNT ≥31), GFP (+GFP), or untreated cells were conducted 48 to 72 hours following transduction.

**Cell Migration and Cell Morphology Analyses**

For cell morphology analyses and low-density migration assays, cells were seeded at 2.5 × 104 cells/well onto uncoated 24-well
plates (Greiner Bio-One). For morphology, ×20 phase-contrast images were acquired on a Nikon TiE microscope (Nikon), and cell perimeters of individual cells were manually traced to define cell area using Fiji32 software. For low-density migration assays, cells were imaged every 2 minutes over a 2-hour period, using a ×20 objective on a Nikon TiE fluorescent microscope; then individual cells were tracked using the MTrackJ plugin of the Fiji32.

**Immunocytochemistry**

Cells (1 × 10^5) were seeded for 5 hours on uncoated glass coverslips, then fixed and extracted in ice-cold ethanol for 4 minutes and air-dried. Primary antibodies were diluted in PBS with 20% normal goat serum (Jackson Labs) and incubated at 37°C for 2 hours; coverslips were then washed extensively with PBS prior to probing for 1 hour at 37°C with Cy5-conjugated secondary antibodies diluted 1:400 in PBS. Coverslips were washed in PBS 0.05% Tween 20, then mounted with polyvinyl alcohol mounting medium with DABCO (Sigma-Aldrich). Images were obtained using Zeiss LSM510 confocal microscope (Zeiss, Cambridge, UK).

**RNA Isolation**

Paired donor anterior segments after Descemet's stripping endothelial keratoplasty (DSEK) were dissected for either central or peripheral cornea RNA isolation. The trabecular meshwork and any residual iris tissue were removed from both eyes. The ocular surface epithelium from one anterior segment was removed by scraping with a scalpel blade to generate a stroma-only sample. The central cornea region (with or without epithelium) was then excised using a 5-mm trephine, and microscissors were used to isolate the peripheral corneal tissue containing the limbus and small amounts of conjunctiva and peripheral cornea. The extracted tissue was snap-frozen in liquid nitrogen and ground with a pestle and mortar before dry ice. The samples were then thawed before adding 200 μL chloroform (Sigma-Aldrich), mixed thoroughly, and then centrifuged at 4°C for 15 minutes at 12,000g to separate phases. The upper RNA-containing phase was then added to the genomic DNA (gDNA) removal column from the Monarch Total RNA Miniprep Kit (New England Biolabs), and the manufacturer's protocol was followed to isolate total RNA.

RNA from pCEC was extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs). Briefly, 200 μL lysis buffer was added directly to the cells following removal of explanted tissue. The cells were homogenized using a cell scraper, and the lysate was collected in a microcentrifuge tube before adding equal volume of absolute ethanol (Sigma-Aldrich) to precipitate the RNA following the manufacturer's protocol to isolate total RNA. RNA quality and quantity were measured using the Agilent TapeStation 2200, with a RNA integrity number of >8 deemed acceptable for further downstream analyses.

**Reverse Transcription–Polymerase Chain Reaction**

Following RNA extractions, 40 ng total RNA was reverse transcribed (RT) using Precision nanoScript 2 Reverse Transcription kit (Primersdesign, Southampton, UK), using a combination of oligo-dt and random hexamers. Intron-spanning primer pairs (Supplementary Table S1) were designed to amplify specific LAMA3 isomir RNAs, GAPDH, and RPLO, with primers being synthesized by Sigma-Aldrich. Isoform-specific PCR was performed using RedTaq PCR Readymix (Sigma-Aldrich). RT-RNA (10 ng) was used in each reaction, with the following protocol: one cycle of 94°C for 5 minutes, 40 cycles of 94°C for 30 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, followed by a final cycle of 72°C for 5 minutes. Then products were run on a 2% agarose gel with a 100-base pair ladder (Sigma-Aldrich). PCR products were sequence validated to confirm specificity (DNA Sequencing and Services).

**RESULTS**

**LaNt α31 Displays Differential Expression Across the Human Ocular Surface Epithelium**

LaNt α31 is produced from the same gene as laminin α3, which is well characterized as being abundantly expressed in conjunctival, limbal, and corneal epithelium. To confirm expression at the transcript level, we performed isoform-specific RT-PCR using RNA extracted from human donor tissue donated after DSEK surgery, comparing expression in the central and peripheral cornea either with or without ocular surface epithelium (Supplementary Fig. S1). This confirmed the presence of LAMA3LN1 mRNA in both the central and peripheral cornea and corneal stroma (Supplementary Fig. S1A).

Having confirmed expression at the mRNA level, we next investigated LaNt α31 protein distribution in human ocular anterior segments from postmortem tissue (N = 10) using two different antibodies raised against the human form of the protein. First, we used previously characterized rabbit polyclonal antibodies raised against GST fusion protein of the entirety of the unique 54 amino acids of LaNt α31. Secondly, we raised mouse monoclonal antibodies against a 14-mer peptide within the unique region of the protein. Hybridoma clones were screened by ELISA against the unconjugated peptide, then by Western blotting against protein extracts from HaCaT immortalized epidermal keratinocytes, and subsequently against protein extracts from cells expressing a LaNt α31-GFP fusion protein (Supplementary Figs. S1B, S1C). Both antibodies recognize the same products; however, compared with the rabbit pAb, the new anti-LaNt α31 mAB gave cleaner, more consistent staining (Supplementary Figs. S1B, S1C).

Processing of limbal explants with both of these antibody preparations yielded near-identical distribution patterns, providing further validation for their shared specificity (Fig 1A; Supplementary Fig. S1D).

Immunohistochemistry staining revealed LaNt α31 expression in most cells of the basal layer of the conjunctival, corneal, and limbal epithelia, with higher overall staining observed within limbal regions (Fig. 1A; Supplementary Figs. S2, S3). Interestingly, the staining intensities were not uniform within each of the surface epithelial regions, with notable groups of cells displaying relatively higher expression compared with their neighbors (Fig. 1A; Supplementary Figs. S2, S3). We also observed suprabasal staining in addition to the expected basal localization of this protein; the frequency and relative intensity of this suprabasal staining varied between different donor eyes (Supplementary Table S2). Across the study population, conjunctival and limbal epithelium displayed similar general trends, with strong basal LaNt α31 staining and generally weak or absent staining in suprabasal cells (Supplementary Fig. S3; Supplementary Table S2). In the peripheral and central cornea, strong basal staining was observed in all samples. However, staining of suprabasal cells was more frequently present; indeed, more samples lacked the clear demarcation between basal and suprabasal staining intensities (10 of 7 of 10 cases.
LaNt α31 protein is differentially distributed across ocular anterior segment epithelium. (A) Paraffin-embedded human anterior segment serial sections processed for immunohistochemistry with mouse monoclonal anti-LaNt α31 antibodies (top and lower left) or rabbit polyclonal anti-LaNt α31 (lower right). Boxed region at top shown at higher magnification in lower parts of figure. Scale bars: 500 µm (top); 200 µm (lower). (B, C) OCT-embedded human anterior segment section processed with mouse mAb antibodies against LaNt α31 and rabbit polyclonal antibodies against LM332. (B) Low-magnification stitched image of anterior surface epithelium; scale bar: 500 µm. Yellow boxed region shown at higher magnification in (C). (C) Upper: phase-contrast image; yellow dotted line denotes the end of Bowman’s layer, the start of the corneal epithelium. Second and third
display strong suprabasal staining in the peripheral cornea compared with 4/10 in conjunctiva; Supplementary Fig. S2; Supplementary Table S2). In addition to the epithelial layers, limbal vessels also displayed positive reactivity in most cases, consistent with reports of laminin 33b expression in blood vessels. In order to obtain higher-resolution distribution data and perform co-localization studies, we next processed frozen human anterior segments for indirect immunofluorescence microscopy with antibodies against LM332 along with mouse mAbs against anti-LaNT 31 (Figs. 1B, 1C) or with the rabbit anti-LaNT 31 pAb and mouse mAbs to laminin 31 (Supplementary Fig. S1C). Consistent with paraffin section staining, this revealed enrichment of LaNT 31 expression in the limbal epithelial regions relative to cornea. With fluorescence staining, stromal reactivity was much more pronounced than with immunohistochemistry, though both approaches displayed positivity in cells surrounding limbal vessels (Fig. 1B). In addition, the fluorescence images revealed occasional immunoreactivity of dendritic-like processes possibly associated with melanocytes. Closer examination of the limbal epithelium revealed subpopulations of reactivity, with enrichment along the basal aspect of epithelial cells apical to the strongest LM332 reactivity (Fig. 1C; Supplementary Fig. S1B). Interruptions in LaNT 31 staining frequently coincided with the location of melanocytes (Fig. 1C, arrowheads).

LaNT 31 Distribution in Ocular Surface Epithelium Is Conserved Between Mouse, Pig, and Human

Next, we compared LaNT 31 distribution between species. The rabbit pAb to human LaNT 31 was raised against a large region of the protein and therefore we predicted reactivity to the porcine version of the protein. Processing frozen and paraffin-embedded pig anterior segment sections with this antibody revealed a similar overall distribution pattern to that observed in human samples. Specifically, basal cells stained throughout all epithelia types, but local variation in terms of intensities and localization was observed; limbus and conjunctival regions were more strongly stained than the central cornea (Fig. 2). Indeed, the corneal epithelial staining was comparatively weak and more restricted in distribution to the matrix-associated aspect of basal cells, whereas more general cell staining was observed in limbal and conjunctival regions. Suprabasal reactivity was also present in conjunctiva and limbus and, also consistent with human samples, stromal structures including blood vessels displayed immunoreactivity (Fig. 2).

The specific peptide used to generate the mouse monoclonal antibody is not conserved between species (Supplementary Fig. S4A). Therefore, as a further negative control validation for the new mouse mAb, we processed porcine ocular surface sections and obtained equivalent signal intensity and distribution as obtained with isotype-matched IgG control treated sections (Supplementary Fig. S4B).

As an additional tool to investigate this new protein, and as further validation for these human and pig data, we raised rabbit polyclonal antibodies against a peptide unique to the mouse form of LaNT 31. As for human and pig sections, staining was observed throughout the ocular surface epithelium, with basal cell enrichment in conjunctival, limbus, and corneal epithelium; however, we again observed suprabasal reactivity, including small regions of the cornea where no distinct basal enrichment relative to suprabasal was observed (Supplementary Fig. S4C).

LaNT 31 Distribution in Limbal Subpopulations Mirrors K15, EGFR, and p63a

The variability in LaNT 31 staining intensities across the ocular surface and, specifically, the localized enrichment in basal epithelial cells of limbal regions suggested the possibility that this differential expression was indicative of subpopulations of cells. The limbal epithelium harbors stem cell niches and populations of transit-amplifying cells; we therefore next processed serial sections with antibodies against LaNT 31 and against K15, p63a, and EGFR (Fig. 3A). Staining for each of these proteins was detected in clusters of cells in the basal regions of the limbus, and these clusters broadly correlated with regions where LaNT 31 staining was relatively enriched (Fig. 3A).

This potential correlation prompted us to next ask if the LaNT 31 expression changes during times when stem cells are active. To address this, we used an ex vivo stem cell activation model where human corneal–limbal rims obtained as surplus to requirements from corneal graft surgery were cultured for 14 days to stimulate LESC activation, as determined by presence of an epithelial outgrowth from the explant (Fig. 3B), before processing for immunohistochemistry and hematoxylin and eosin (H & E) staining (Fig. 3C).

In the explant sections, as expected, K15, p63a, and EGFR all displayed increased staining in the limbal epithelium and throughout the growing epithelial sheets, with definitive basal cell enrichment throughout the outgrowth (Fig. 3C). LaNT 31 staining was observed in near-identical patterns, with strong basal staining in the activated limbal cells and growing epithelium.

LaNT 31 Is Dynamically Redirected During Corneal Wound Repair

Increased expression of LaNT 31 following introduction of a scratch wound in two-dimensional (2D) cultured epithelial keratinocytes has previously been described.1 We therefore next wanted to determine what happens to LaNT 31 expression and distribution in a more physiologically relevant porcine ex vivo three-dimensional (3D) alkali wound model (Fig. 4A). Anterior segments were fixed at 24, 36, 48, or 72 hours after wounding, then processed for immunohistochemistry with rabbit anti-human LaNT antibodies (Figs. 4B–D). Strong LaNT 31 staining was observed across all layers of the epithelial cells surrounding the wound from 24 hours after wounding (Fig. 4B); however, staining intensity was reduced at 48 hours following wounding, by which point the wound was covered by nascent epithelium (Fig. 4B). In contrast, 72 hours following wounding, by which point the epithelium was more mature and more similar to unwounded tissue, LaNT 31 expression was again predominantly restricted to the basal layer (Fig. 4B), similar to what was observed in unwounded tissue.

Interestingly, in the limbal region, distinct populations of basal epithelial cells were highly enriched in LaNT 31 throughout the wound process (Fig. 4C). Strikingly, these populations of positive cells extended into the peripheral cornea during active repair; and in this region, those cells...
displaying high LaNt α31 expression appeared to display larger cross-sectional area compared with adjacent low-expressing basal cells (Fig. 4D). Together these data suggest a role for LaNt α31 in corneal repair and epithelial morphology and maturation.

**Induced Expression of LaNt α31 in Limbal-Derived Epithelial Cells Leads to Increased Cell Spreading and Reduced Migration Rates**

As our wound and stem cell models indicated transient and localized increases in LaNt α31 expression, we next investigated the impact that increased LaNt α31 levels have on cell behavior. To do so, we cultured pCEC from limbal explants and used an adenoviral delivery mechanism to induce LaNt α31 GFP expression (Supplementary Fig. S1B). Keratin 12 staining was used to validate the corneal epithelial lineage of isolated keratinocytes (Supplementary Fig. S5).

Consistent with the apparent increase in cell area in the peripheral cornea of pig wounds, the quantity of limbal-derived epithelial cells expressing LaNt α31 GFP 2D area was, on average, approximately twice that of GFP-expressing control cells and nontransduced pCEC (2D area + LaNt α31 27.20 ± 720 μm² versus pCEC 1230 ± 380 μm² and +GFP 1280 ± 280 μm², P < 0.05) (Figs. 5A, 5B). We next assessed single cell motility by plating the transduced cells at low density on uncoated dishes and then tracking motility over 2 hours (Fig. 5C). These analyses revealed the LaNt α31 GFP-expressing cells to display significantly reduced cell migration rates compared with controls (+LaNt α31 0.46 ± 0.14 μm/min versus pCEC 0.91 ± 0.14 μm/min and +GFP 0.77 ± 0.04 μm/min, mean values for all donors, P < 0.05) (Fig. 5D).

**DISCUSSION**

The data presented here demonstrate a differential distribution of LaNt α31 across the ocular surface epithelium that changes during corneal wound repair, and that largely mimics limbal epithelial stem/transit-amplifying cell-associated proteins in activated limbal explants. Moreover, in vitro data indicate that LaNt α31 can influence the adhesion and migration characteristics of limbal-derived corneal epithelial cells. These findings implicate LaNt α31 as a new, potentially important, player in corneal homeostasis and wound repair.
LaNt α31 is enriched in populations of cells positive for K15, EGFR, and p63α. (A) Serial sections from human anterior segments processed with antibodies against LaNt α31, or against p63α, K15, or EGFR. Scale bar: 200 μm. (B) Diagram of limbal explant culture system. Inset: phase-contrast image showing epithelial outgrowth following 14 days in culture. Magenta dashed area marks boundary of explant; yellow dashed line indicates outgrowth of epithelial cells. (C) Serial explant sections processed for H & E or with indicated antibodies against LaNt α31, p63α, K15, or EGFR. Middle and right in (C) show dashed boxed regions at higher magnification. Scale bars: 50 μm.
The best-supported model of corneal regeneration after injury involves cell proliferation in the limbus followed by migration from the limbus toward the cornea. Our finding that LaNtα31 is enriched in an activated limbus but reduced in an actively healing cornea, combined with corneal cell migration being slowed in the presence of high LaNtα31 levels, indicates that spatial and temporal control is required. Indeed, our induced expression data were somewhat surprising, as knockdown of LaNtα31 leads to reduced migration in HaCaT epidermal keratinocytes. The primary corneal-like cells used here are slightly different from HaCaT, with higher baseline migration rates; however, together these data suggest that there is an optimum LaNtα31 concentration for migration, and moreover that titration of the LaNtα31 expression level could have evolved as means of switching corneal cells between a migratory, wound healing phenotype and an adherent mature epithelium.
The shared domains between LaNt α31 and laminins predict an ability to influence BM assembly via interaction with laminins influencing the latter’s network assembly. Changes to laminin matrix organization could explain the observed influences on cell behavior. However, the LaNt α31 domain architecture also resembles that of the netrin proteins, which are best known as signaling proteins. While immunofluorescence imaging does suggest association with BM, the basal subpopulation enrichment of LaNt α31 is similar to that described for netrin-1 and netrin-4, although it is striking to note that in the mouse eye, netrin-4 has been shown to be highly expressed in the cornea but not in the limbal regions of the developing or adult eye—effectively the mirror image to LaNt α31. Netrin-4 has also been established as capable of influencing laminin interactions both in vitro and in vivo, and, as such, LaNt α31 may turn out to be a new class of laminin-derived netrins.

As one would expect from a study of a “new” protein, these findings have generated additional questions. One of the surprising findings of our study was the presence of the LaNt α31 protein in layers above the basal layer, as we would predict a protein derived from a laminin gene to be located only in BM-associated regions. This staining was observed in mouse, human, and pig sections, using three different antibody preparations, and, as such, we do not believe it to be a processing artifact. The finding of LaNt α31 expression in suprabasal cells raises questions of whether the protein continues to be translated from long-lived mRNAs, or if we are detecting protein that has been retained within the cells as they differentiate, or whether the transcript continues to be produced. What role, if any, LaNt α31 plays in the suprabasal cells will be an interesting future direction.

We also observed antibody reactivity in limbal and conjunctival stromal regions in all three species. These findings are less surprising, as the promoter that drives LaNt α31 production has been shown to be active in NIH3T3 fibroblasts, and laminin α3b protein has also been described as being expressed in the dermal microvasculature. What roles the LaNt α31 plays in these contexts is as yet unknown; however mesenchymal cells, including fibroblasts and pericytes, contribute to BM formation, and therefore the simplest explanation is that the LaNt protein from the limbal stroma is ultimately involved in establishing the epithelial BM.

Maintenance and effective repair of the ocular surface epithelium is essential to preserve vision and relies on precise regulation of a number of overlapping cellular functions, including proliferation and differentiation, adhesion and migration, and extracellular matrix remodeling. Together, the data presented here implicate the LaNt α31 protein as a player in numerous aspects of these processes. Ultimately this may be
relevant not only for wound repair but also in conditions such as recurrent corneal erosions where defective BM and cell to matrix–adhesion reassembly are associated with loss of corneal epithelial integrity.2–8

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