Identification of a Novel Family of Laminin N-terminal Alternate Splice Isoforms

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION*§

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The laminins are a family of heterotrimeric basement membrane proteins that play roles in cellular adhesion, migration, and tissue morphogenesis. Through in silico analysis of the laminin-encoding genes, we identified a novel family of alternate splice isoforms derived from the 5′-end of the LAMA3 and LAMA5 genes. These isoforms resemble the netrins in that they contain a laminin N-terminal domain followed by a short stretch of laminin-type epidermal growth factor-like repeats. We suggest the terms LaNt (laminin N terminus) α3 and LaNt α5, for the predicted protein products of these mRNAs. RT-PCR confirmed the presence of these transcripts at the mRNA level. Moreover, they exhibit differential, tissue-specific, expression profiles. To confirm the existence of LaNt α3 protein, we generated an antibody to a unique domain within the putative polypeptide. This antibody recognizes a protein at the predicted molecular mass of 64 kDa by immunoblotting. Furthermore, immunofluorescence analyses revealed a basement membrane staining in epithelial tissue for LaNt α3 and LaNt α3 localized along the substrate-associated surface of cultured keratinocytes. We have also tested the functionality LaNt α3 through RNAi-mediated knockdown. Keratinocytes exhibiting specific knockdown of LaNt α3 displayed impaired adhesion, stress resistance, and reduced ability to close scratch wounds in vitro.

The identification of a lower than initially predicted number of genes in mouse and human genomes has dramatically increased the focus on alternate splicing as an important mechanism of providing the necessary temporally and spatially restricted changes required in gene and protein expression as an organism both develops and responds to injury (1). Indeed, according to recent reports, 40–60% of human genes are alternatively spliced (2). One area where alternative splicing is thought to be of particular importance is in the extracellular matrix (ECM) (3, 4).

ECM proteins provide substrates for a range of functions including, but not limited to, structural roles in tissues such as skin, cartilage, and bone, in cell signaling, and in the apparently opposing roles of promoting cellular adhesion and migration (4, 5). Many ECM genes, including those that encode fibronectin, tropoelastin, the collagens, and proteoglycans, are large with multiple exons (4, 6–9). Alternative splicing of such genes results in the inclusion or exclusion of particular exons with the functional consequence of varying the domain composition of the ECM protein (8, 10). In addition, through alternate promoter or first exon usage or conversely through inclusion of an alternate exon or read through of an intron/exon boundary resulting in the inclusion of an in-frame stop codon, “truncated” forms of ECM proteins have been described (11–13). For example, splice site readthrough of the fibronectin exon III-Ia introduces a novel 3′-end containing a termination codon and leads to generation of a 70-kDa protein termed migration-stimulating factor (MSF) (12).

In this study, we have utilized an in silico approach to identify a family of transcripts derived by alternative splicing from the laminin extracellular matrix family. The laminins are a major family of basement membrane proteins with roles in maintenance of tissue integrity, in signaling, and development (14, 15). To date, 12 laminin-encoding genes have been identified, which, based on sequence identity, are subdivided into 5 α, 4 β, and 3 γ chains encoded by LAMA1–5, LAMB1–4, and LAMC1–3, respectively (16). Within the laminin family, the most striking alternative splicing occurs from the LAMA3 gene, where two major transcripts have been identified; LAMA3A, which consists of exons 39–76 and is initiated from an internal site in intron 38, encoding the N-terminally truncated laminin α3 protein (Fig. 1A) (13, 17). In contrast, LAMA3B contains exons 1–38 and 40–76 (i.e. skips exon 39), and encodes a full-length laminin termed α3b (Fig. 1B). Further alternate or minor isoforms have been identified in LAMC2 (18, 19), LAMA4 (20), LAMB3 (21), and LAMA2 (22).

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4 The abbreviations used are: ECM, extracellular matrix; LM, laminin; LN, laminin N-terminal domain; LaNt, laminin N terminus; LE, laminin-type epidermal growth factor-like repeat; LCC, laminin coiled-coil domain; LG, laminin globular domain; ISH, in situ hybridization; PBS, phosphate-buffered saline; EST, expressed sequence tag.
Here, we describe the identification of multiple, short, alternate splice isoforms derived from the 5′-end of the genes (LAMA3 and LAMAS5) encoding the α3 and α5 laminin subunits. Functional studies utilizing siRNA knockdown of one of these newly identified isoforms shows that it plays an important role in epidermal cell adhesion and in keratinocyte migration.

**EXPERIMENTAL PROCEDURES**

*Antibodies*—Exon9e of LAMA3 was amplified using primers; L3CprbFor 5′-GTA AGT TCC ATT TCA AGT TGG-3′ and L3CprbRev 5′-ATC CAA AAT TCAA AGA GAC TG-3′ and cloned into pCR2.1, sequence verified, and subcloned into pET4.1 (Novagen, Madison, WI). The pET41 construct was transformed into BL21(DE3)pLysS and protein expression induced by adding 1 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich) for 3 h at 37 °C. Proteins were extracted using Bug-buster (Novagen), and the fusion protein was bound to glutathione-Sepharose beads, washed extensively, and separated on a 4–12% NuPAGE bis/Tris gel. The band corresponding to GST-LAMA3exon9e was excised and used for rabbit polyclonal antibody production (Moravian Biotechnology, Czech Republic). Mouse monoclonal anti-β-actin was obtained from Sigma Aldrich; GB3, a mouse monoclonal against laminin γ2 and B1K, a mouse monoclonal against laminin β3, were obtained from Harlan Sera lab Ltd, England and Transduction Laboratories, Lexington, KY, respectively. Rabbit serum J18 against laminin 332 and mouse monoclonal R1G3 against laminin α3 were described previously (23, 24). Secondary antibodies used were purchased from DakoCytomation, Denmark and Molecular Probes, Invitrogen.

*MtCdNA Panel RT-PCR*—Primer pairs were designed to specifically amplify unique regions of the human LAMA3A, LAMA3B, LAMA3LN1, LAMA3LN2, LAMASLN1, LAMASLN2, and mouse lama3ln1 transcripts (supplemental Table S1). RT-PCR with these primers were performed alongside those for MTGPH (BD Biosciences, Franklin Lakes, NJ) on human multiple tissue cDNA panels MTC1 and MTCII (BD Biosciences) and cDNA from cultured primary human keratinocytes, HaCaT cells, and mouse keratinocytes according to standard protocols (25–27).

*In Situ Hybridization (ISH)*—A 170-bp LAMA3LN1 specific probe (exon 9e) for ISH was generated by RT-PCR from HaCaT cDNA using primers: L3CprbFor 5′-GTA AGT TTC ATT TCA AGT TGG-3′ and L3CprbRev 5′-ATC CAA AAT TCAA AGA GAC TG-3′, Promega buffer, 15 mM MgCl2, and 1 unit of Taq (Promega) with conditions of: 1 cycle of 94 °C 2 min, 38 cycles of 94 °C 15 s; 55 °C 15 s; 72 °C 20 s, 1 cycle of 72 °C 10 min. The product size was confirmed on a 2% agarose gel and cloned into pCR2.1 (Invitrogen) and subcloned into pBluescript (Stratagene). ISH was performed as previously described (28).

*Cell Culture*—HaCaT cells and mouse epidermal keratinocytes (PAM cells) were maintained in Dulbecco’s minimal Eagle’s medium supplemented with 10% fetal calf serum (Sigma, DMEM), 10,000 units of penicillin and 10 μg/ml streptomycin (Sigma) (29, 30). Human epidermal keratinocytes, immortalized with human papilloma virus genes E6 and E7, were described previously (31). The cells were maintained in defined keratinocyte serum-free medium supplemented with a 1% penicillin/streptomycin mixture (Invitrogen). All cell cultures were maintained in a 37 °C, 5% CO2 environment. Proliferation assays were performed as previously described (32). Live cell images were generated using a Zeiss Axiocvert 200 M (Carl Zeiss), digital camera (Hamamatsu), and Volocity 3DM software (Improvision, Coventry, UK).

**siRNA**—siRNA sequences were designed using a web-based design algorithm (33). siA 5′-UCA GCG AAG UCA UGA GGC U-3′, siB 5′-GUA UCU AAG CCA GGU UGU A-3′. For siRNA transfection HaCaT cells were seeded at ~8.3 × 105 cells/well of 6-well plates (Invitrogen). 24 h after plating, 100 μl of DMEM were incubated with 15 μl of RNAiFect (Qiagen) and 200 nmol of siRNA solution (Dharmacon Inc, Lafayette, CO) for 30 min at room temperature, then added dropwise to each well (2 ml total volume, siRNA final concentration, 100 μM).

**Adhesion Assay**—Cell-based adhesion assays were performed as described elsewhere (32, 34). HaCaT cells: 72-h post-transfection, cells were dissociated with trypsin, centrifuged, and washed with PBS. Cells were resuspended at 1.5 × 106 cells/ml and then plated onto 96-well dishes precoated with bovine serum albumin and allowed to adhere for 30, 60, or 360 min. Nonadherent cells were removed by PBS washes, and adherent cells were fixed with 3.7% formaldehyde and stained with crystal violet in 20% methanol (Sigma). It should be noted that by 360 min, >99% of cells had attached to substrate regardless of treatment. Crystal violet stain was eluted with 50% ethanol, 0.1 M sodium citrate, pH 4.2, and the absorbance at 540 nm was measured. The percentage of attached cells was calculated relative to the value obtained at the 360-min time point. To evaluate spreading, cells were plated at ~7.5 × 105 cells/well in 6-well plates, and images taken from computer-selected random areas 2 h after seeding. The percentage of flattened cells in the adherent population was calculated.

**Detachment Assay**—A variation of the technique described by Yuen et al. (32, 35) was used. Cells were transfected with siRNA and, 24 h later, they were trypsinized and replated. After an additional 48 h, the growth medium was removed, and wells were washed twice with PBS. They were then treated with 0.005% trypsin, 0.0004% EDTA in PBS (Invitrogen). Phase contrast images were taken every 5 min for 90 min, and the percentage of rounded cells in the population was determined.

**Migration Assay**—72 h after transfection, a scratch wound was created in a confluent monolayer of HaCaT cells with a 10-μl tip, washed twice with PBS, and the medium replaced with CO2-independent medium (Invitrogen) supplemented with 10% fetal calf serum. Images of the wound area were taken every 20 min for 36 h. At 1, 2, 3, 4, 5, 6, 9, 12, and 15 h post-scratching, the width of the wound was measured using Meta- morph software (Universal Imaging Corp., Molecular Devices, Downingtown, PA).

**SDS-PAGE/Western Blotting**—Extracts of cells in monolayer were generated by scraping washed cells directly into urea/SDS sample buffer as previously described (36). ECM preparations of cultured cells were prepared as previously described (24). Briefly, cells were lysed by treatment with 20 mM NH4OH (Sigma) for 5 min, followed by extensive PBS washes to remove cellular debris. Microscopy confirmed complete removal of cells. ECM proteins were then solubi-
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RESULTS

Immunofluorescence Analysis—Cells and tissues were prepared for indirect immunofluorescence as described previously (17, 23).

Identification of a Novel Family of Laminin Alternate Splice Isoforms—During the study of a rare variant of junctional epidermolysis bullosa (17), in silico analysis of the laminin a3-encoding gene was undertaken utilizing tools available on the University of Santa Cruz genome browser. Careful analyses of the 5′-end of the LAMA3 gene identified 2 ESTs containing readthrough of 5′ splice sites, raising the possibility that short, novel, splice isoforms are transcribed from this gene. IMAGE

RT-PCR products were designed to amplify the equivalent mouse sequence, and the presence at the message level (Fig. 2B).

A second EST was identified that also contained the first 9 LAMA3 exons with splicing occurring within exon 10 to a site within exon 18, then continuing to exon 25 (LAMA3LN2). Readthrough of the 5′ splice site of exon 25 incorporated a unique region containing a polyadenylation signal. The sequence corresponding to this mRNA (GenBank™ AK096422) contained a frameshift insertion, which, in turn, led to a termination codon in exon 19, predicting a protein of 487 amino acids (Fig. 1A). RT-PCR products derived from human epithelioid-containing tissues and keratinocytes confirmed the presence of this transcript at the mRNA level (Fig. 2A).

Analysis of the mouse and human sequences surrounding the exon 9/intron 9 splice boundary revealed perfect cross species conversation; however, in both there are three changes within the exonic sequence relative to the consensus splice site sequence suggesting a weak splice site (Fig. 1B) (37). Readthrough into mouse lama3 intron 9 also introduced an in-frame stop codon 5′ to a consensus polyadenylation signal sequence in a similar location to the human sequence and therefore predicted production of a peptide of almost identical length to the human (Fig. 1C). RT-PCR primers were designed to amplify this peptide (Fig. 1A).
both these products were spliced normally to the first 7 exons, one would predict products of 1039 amino acids and 994 amino acids, respectively, of which the final 4 amino acids were derived from exon 25e and were therefore unique.

The conserved domain architecture of the various laminin chains, coupled with the expression of fewer isoforms in lower organisms, suggested that gene duplication and rearrangement are responsible for the presence of the multiple chains. With this in mind, the 5'-end of the LN domain containing laminin α chains was analyzed in silico. No potential products were identified in LAMA1 or LAMA2; however, an analogous EST was identified that aligns to LAMA5. This EST (GenBank™

TABLE 1  mRNA expression profiling

| Transcript | LAMA3A | LAMA3B | LAMA3LN1 | LAMA3LN2a/b | LAMA5LN1 | LAMA5LN2 |
|------------|--------|--------|----------|-------------|----------|----------|
| Composition | Laminin α3a | Laminin α3b | LaNt α31 | LaNt α32a | LaNt α32b | LaNt α51 | LaNt α52 |
| Heart      | ++      | ++      | ++       | ++          | ++       | ++       |
| Brain      | ++      | ++      | ++       | ++          | ++       | ++       |
| Placenta   | +       | +       | +        | +           | +        | ++       |
| Lung       | +       | +       | +        | +           | +        | ++       |
| Liver      | +       | +       | +        | +           | +        | ++       |
| Skeletal Muscle | +     | +       | +        | +           | +        | ++       |
| Kidney     | +       | +       | +        | +           | +        | ++       |
| Pancreas   | +       | +       | +        | +           | +        | ++       |
| Spleen     | +       | +       | +        | +           | +        | ++       |
| Thymus     | +       | +       | +        | +           | +        | ++       |
| Prostate   | +       | +       | +        | +           | +        | ++       |
| Testis     | +       | +       | +        | +           | +        | ++       |
| Ovary      | +       | +       | +        | +           | +        | ++       |
| Small Intestine | + | +       | +        | +           | +        | ++       |
| Colon      | +       | +       | +        | +           | +        | ++       |
| Leukocyte  | +       | +       | +        | +           | +        | ++       |
| Keratinocyte | +      | +       | +        | +           | +        | ++       |

FIGURE 2. mRNA expression profiling. A, cDNA from the indicated tissues and cultured cells were processed for sqRT-PCR using primers specific for LAMA3A, LAMA3B, LAMA3LN1, LAMA3LN2, and LAMA5LN1. Arrowheads denote presence of some minor products, which may represent additional isoforms. B, RT-PCR of mouse keratinocyte cDNA with primers specific for mLama3ln1 results in generation of a 220 bp product. C, D, and E show LAMA3LN1 in situ hybridization in human face skin (C and D) and in a human hair follicle (E). Reactive product is pseudocolored in red, whereas the tissue was counterstained with Mayer’s hematoxylin (gray).
BC015386), had been isolated from a breast adenocarcinoma library and was predicted to encode the first 11 exons of LAMA5 spliced to an alternate 12th exon (exon 12*) including a termination codon (LAMA5LN2) (Fig. 1A). Translation of this mRNA would generate a peptide of 500 amino acids with the final eight being encoded by the alternate 12th exon. The latter would therefore be unique to this transcript. Notably, splice site boundaries at both LAMA3 exon25 and LAMA5 exon 11 also diverged from the consensus sequence (Fig. 1B).

RT-PCR primers were designed to amplify this LAMA5-derived transcript. These yielded two major products (LAMASLN1 and LAMASLN2) when tested on normal breast cDNA (Fig. 2A). Sequencing of these products revealed the smaller product to contain the expected exons 1–11 plus 12* and the larger, more abundant, second product to be generated from 5’ splice site readthrough of exon 11 (into exon 11e); therefore, consisting of exons 1–11 plus 11e (Fig. 1A). This additional exon encoded a termination codon 11-bp downstream leading to the generation of a protein of 496 amino acids in size. Both transcripts contained non-consensus polyadenylation signal sequences (38).

RT-PCR Analyses Indicate Tissue-specific Expression Patterns for These Novel Laminin Isoforms—The expression profiles of the newly identified laminin transcripts identified above were studied using multiple tissue cDNA (MTC) panels, which include cDNA from leukocytes and which were supplemented with cDNA from cultured keratinocytes (25–27). Semi-quantitative RT-PCR was performed for each transcript with samples removed at regular intervals, and we scored expression based on signal intensity for each tissue relative to housekeeping genes (Fig. 2A and Table 1).

For LAMASLN1, relatively strong expression was detected in heart, placenta, prostate, testis, small intestine, leukocyte, and keratinocyte (Fig. 2A). Lower levels were detected in pancreas, brain, ovary, and fibroblasts (Fig. 2A). No products were present in the colon, liver, skeletal muscle, kidney, thymus, or spleen.

FIGURE 3. LaNt α31 protein expression. In A, HaCaT total protein and ECM extracts were prepared for Western blotting with a rabbit polyclonal antibody against LaNt α31. B shows immunofluorescence images of human foreskin sections stained for LaNt α31 (LaNt31, upper panel) or LM332 (lower panel). e, epidermis; d, dermis. Arrowheads indicate discontinuous staining basal staining. Bar, 20 μm. In C, HaCaT cells were plated onto glass coverslips, fixed, and then processed using antibodies LaNt α31. Cells were imaged by confocal immunofluorescence microscopy. Note punctate distribution of LaNt α31 staining around peripheral regions of both individual (C, upper panels) and groups of cells (lower panels). In D, HaCaT cells were prepared for double labeling using antibodies against LaNt α31 (green) and LM332 (red). Note the area of overlap of stain toward the edge of the cell. Bars, 50 μm.
LA3LN1 Is Translated and Secreted by Keratinocytes.—Of all the transcripts identified here, only the LA3LN1 transcript encodes a unique sequence of sufficient length (amino acids 423–487) that would allow generation of an antibody probe specific for the putative product of the transcript. This antibody probe would not recognize full length laminin α3a or α3b protein. A rabbit polyclonal antibody raised against this unique region recognizes a protein at the predicted molecular mass of ~64 kDa in total cell lysate extracts derived from the cultured human keratinocytes prepared for immunoblotting (Fig. 3A). These data indicate that there is a bona fide product translated from the LA3LN1 transcript. Based on the structure of the protein, we suggest that it be termed LaNt (Laminin N terminus) α31.

Although LaNt α31 migrated around 64 kDa in extracts of keratinocytes, it exhibits a difference in mobility in ECM extracts of cultured keratinocytes. The higher mobility suggests the possibility that LaNt α31 undergoes proteolytic processing upon secretion (Fig. 3A).

LaNt α31 antibody generated bright staining along the basement membrane underlying the keratinocytes in the basal layer of the epidermis (Fig. 3B). This staining was not continuous and was distinct from the localization of LM332 along the basement membrane (Fig. 3B). We also evaluated the localization of LaNt α31 by confocal immunofluorescence microscopy in HaCaT cells. The LaNt α31 antibody generated a punctate stain toward the periphery of both individual cells and around the outer edges of groups of cells (Fig. 3C). These puncta were distributed along the substratum-attached surface of the cells and showed partial overlap with staining generated by antibody GB3, which recognizes assembled LM332 trimers (41, 42) (Fig. 3D).

RNAi-mediated Knockdown of LA3LN1—LN domains have been implicated in cell adhesion, neurite outgrowth, bind-
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Compared with controls, LAMA3LN1 siRNA-treated cells initially show a significant delay in adhesion (Fig. 5A; 54% of mock-transfected cell adhesion, \( p = 0.001 \) at 30 min). By 60 min, differences between controls and knockdown cells were not significant suggesting that the adhesion defect primarily affected the initial stages of attachment. For cell spreading, cells were imaged and scored by phenotype 2 h after re-seeding onto uncoated tissue culture plates. In comparison to controls, LAMA3LN1 knockdown cells showed a slight reduction in spreading at 2 h after replating that is not seen at later time points (Fig. 5B). However, differences were below significance (\( p = 0.09 \)).

During routine handling of the siRNA-transfected cells, we became aware that the LAMA3LN1 knockdown cells responded more rapidly to trypsin exposure than control-treated cells. To quantify this, we exposed knockdown and control siRNA-treated keratinocytes to a diluted trypsin (final concentration 0.005% trypsin, 0.0004% EDTA) and scored cell rounding over time. LaNt α31 knockdown cells responded significantly faster than control siRNA-transfected cells (Fig. 5C).

LaNt α31 is up-regulated during wound healing and knockdown impacts wound closure in vitro—To determine if LaNt α31 expression levels change during wound healing of monolayers of keratinocytes in vitro, ECM extracts were derived from keratinocytes before and after 30 min, 2 h, 6 h, and 24 h following introduction of scratch wounds. LaNt α31 protein expression levels increased following wounding, showing maximal expression around 6 h postwounding, suggesting a potential role in the wound-healing process (Fig. 6A). Moreover, conditioned medium of either confluent or scratch-wounded keratinocytes grown for 24 h in serum-free medium then concentrated with ammonium sulfate revealed a dramatic increase in the level of LaNt α31 secreted into the scratch-wounded culture medium (Fig. 6B).

To determine if LaNt α31 is required for the closure of wounds, a scratch was introduced into a confluent monolayer of LAMA3LN1 siRNA or control-treated keratinocytes, and images at representative points along the wound margins were taken at 20-min intervals over 36 h (Fig. 6C and supplemental materials). All control keratinocytes had completely closed the introduced wound by 24 h after wounding. In contrast, there were still gaps between the migrating fronts of cells in which LaNt α31 was knocked down (Fig. 6C). Repetition of this experiment in the presence of mitomycin C gave identical results, indicating that the differences in wound closure time was not due to any difference in proliferation rate (not shown).

Denser analyses of the acquired images revealed that the wound closure to be distinctly biphasic (Fig. 6D). During the first phase, cells migrated rapidly over the first ~6 h to form a distinct line along the edge of the scratch site. Both control and LaNt α31 knockdown cells migrated at similar rates over this period (~20 μm/h). In the second phase, control cells continued in a slightly slower manner (~14 μm/h from hours 6–12) during which time they presented numerous forward cellular projections. In contrast, the LaNt α31 knockdown cells projected fewer lamellipodia along their leading edge as they advanced and showed a markedly reduced migration rate relative to controls (8 μm/hr from 6–12 h postscratching). Differ-

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FIGURE 5. LaNt α31 knockdown cells display impaired adhesion relative to controls. In A, HaCaT cells, 72-h post-transfection with siRNAs directed against either LAMA3LN1 or lamin a/c, were trypsinized and plated into 96-well plates, allowed to attach for 30, 60, and 360 min, and the percentage of cells attached determined relative to the 360-min time point by staining. Results are plotted as mean ± S.E. from three repetitions, 9 wells per repetition (*, \( p < 0.001 \)). In B, HaCaT cells, 72-h post-transfection, were plated into 6-well plates, allowed to adhere for 2 h, and their spreading profile scored. Results are plotted as mean percentage cells spread ≥ S.E. as determined from counting 6 randomly selected fields per treatment, three repetitions (\( p = 0.09 \) LAMA3LN1 siRNA relative to control siRNA). In C, HaCaT cells, 72-h post-transfection with LAMA3LN1 siRNA or control siRNA, were exposed to a dilute trypsin (0.005% trypsin, 0.0004% EDTA), and images were collected every 5 min. Cells were scored based on phenotypic appearance, with round cells scored as “detached.” Values represent the mean of three repetitions, 6 fields per repetition (\( p < 0.01 \) from 15 to 30 min). \( p \) values determined using paired Student’s t-test.
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Because of the short regions of unique sequence in several of the transcripts we have been unable to generate appropriate tools to confirm their existence at the protein level and to further investigate their functionality. This is not the case with transcript LAMA3LN1. The longer extension to exon 9 has allowed us to generate specific probes, including siRNAs to the LAMA3LN1 mRNA and antibodies to the putative protein product encoded by this transcript. Our antibody confirmed the existence of a secreted protein, which we have termed LaNt α31. The LaNt α31 antibody stains the basement membrane of intact human epithelia although the pattern is distinct from that generated by a LM332 antibody. Likewise, the LaNt α31 antibody localizes along the substra-
tum-associated region of both individual and groups of cultured keratinocytes. Although it fails to perfectly co-localize with LM332 matrix secreted by keratinocytes, its localization partially overlaps with that of LM332. Most important, functional analyses, utilizing RNAi-mediated knockdown, demonstrate a role for this novel protein in cell attachment and cell migration.

One interesting aspect of our study is the identification of tissue-specific expression profiles of the different LaNt encoding transcripts. Of particular note is the differences observed between LAMA3B and LAMA3LN1, which share a common promoter. For example, LAMA3B mRNA is highly expressed in placenta, lung, pancreas, and prostate whereas the LAMA3LN1 message is expressed at a higher level in heart, testis, small intestine, and by leukocytes. These data suggest that transcription of LAMA3LN1 is not purely a consequence of expression of LAMA3B. Rather, this implies that LAMA3LN1 production can be regulated independently of the “full length” transcript. Interestingly, in this regard, our finding that the LAMA3LN1 transcript is conserved between human and mouse implies there has been evolutionary pressure to maintain its expression (1).

DISCUSSION

The laminins are an important family of ECM proteins whose functions and interactions have been extensively studied because of their involvement in development, wound healing, cancer, and genetic diseases (14, 47). Here, through in silico analyses and RT-PCR, we have identified multiple novel iso-
forms derived from the 5′-end of laminin α3 and α5-encoding genes and have mapped their tissue expression profiles. Each of these identified transcripts predicts expression of short polypeptides comprising a laminin N-terminal domain followed by a short stretch of laminin-type epidermal growth fac-
tor-like repeats, which therefore structurally resemble the netrin family of axonal guidance cues (11, 48–50). However, all of the predicted proteins of this novel family lack the character-
istic C-terminal basic domain found in netrins (48, 50).
α3β laminin N terminus inhibits LM111 polymerization *in vitro* (45). We are currently investigating such possibilities.

Alternative splicing is clearly an important mechanism via which the diversity of expressed proteins can be expanded. We speculate that LaNt family members may play important roles in regulating laminin matrix functions; this would be consistent with our finding that LaNt protein expression is up-regulated during wound healing and its knockdown modulates keratinocyte adhesion and migration, even though LM332 matrix appears unperturbed, and its secretion is apparently unaffected. How, precisely, LaNt protein members do so should be an interesting avenue of investigation in the future.

REFERENCES
1. Kim, E., Goren, A., and Ast, G. (2008) *Bioessays* **30**, 38–47
2. Modrek, B., and Lee, C. J. (2003) *Nat. Genet.* **34**, 177–180
3. Sakabe, N. J., Vibranovski, M. D., and de Souza, S. J. (2004) *Genet. Mol. Res.* **3**, 532–544
4. Boyd, C. D., Pierce, R. A., Schwarzbauer, J. E., Doege, K., and Sandell, L. J. (1996) *Cell. Mol. Neurosci.* **19**, 344–358
5. O'Toole, E. A. (2001) *Clin. Exp. Dermatol.* **26**, 525–530
6. Schor, S. L., Ellis, I. R., Jones, S. J., Baillie, R., Seneviratne, K., Clausen, J., Deak, S. B., and Boyd, C. D. (1995) *Matrix Biol.* **14**, 635–641
7. Sandell, L. J. (1996) *Connect Tissue Res.* **35**, 1–6
8. Kosmehl, H., Berndt, A., Katenkamp, D., Mandel, U., Bohle, R., Gabler, U., and Celada, D. (1995) *Pathol. Res. Pract.* **191**, 1105–1113
9. Timpl, R. (1993) *Experientia* **49**, 417–428
10. White, E. S., Baralle, F. E., and Muro, A. F. (2008) *J. Biol. Chem.* **283**, 1–14
11. Yim, Y., Miner, J. H., and Sanes, J. R. (2002) *Mol. Cell. Neurosci.* **19**, 344–358
12. Schor, S. L., Ellis, I. R., Jones, S. J., Baillie, R., Seneviratne, K., Clausen, J., Deak, S. B., and Boyd, C. D. (1995) *Matrix Biol.* **14**, 635–641
13. Sandell, L. J. (1996) *Connect Tissue Res.* **35**, 1–6
14. Kosmehl, H., Berndt, A., Katenkamp, D., Mandel, U., Bohle, R., Gabler, U., and Celada, D. (1995) *Pathol. Res. Pract.* **191**, 1105–1113
15. Timpl, R. (1993) *Experientia* **49**, 417–428
16. White, E. S., Baralle, F. E., and Muro, A. F. (2008) *J. Biol. Chem.* **283**, 1–14
17. Yim, Y., Miner, J. H., and Sanes, J. R. (2002) *Mol. Cell. Neurosci.* **19**, 344–358
18. Schor, S. L., Ellis, I. R., Jones, S. J., Baillie, R., Seneviratne, K., Clausen, J., Deak, S. B., and Boyd, C. D. (1995) *Matrix Biol.* **14**, 635–641
19. Sandell, L. J. (1996) *Connect Tissue Res.* **35**, 1–6
20. Kosmehl, H., Berndt, A., Katenkamp, D., Mandel, U., Bohle, R., Gabler, U., and Celada, D. (1995) *Pathol. Res. Pract.* **191**, 1105–1113
21. Timpl, R. (1993) *Experientia* **49**, 417–428
22. White, E. S., Baralle, F. E., and Muro, A. F. (2008) *J. Biol. Chem.* **283**, 1–14
23. Yim, Y., Miner, J. H., and Sanes, J. R. (2002) *Mol. Cell. Neurosci.* **19**, 344–358
24. Boyd, C. D., Pierce, R. A., Schwarzbauer, J. E., Doege, K., and Sandell, L. J. (1996) *Cell. Mol. Neurosci.* **19**, 344–358
25. O'Toole, E. A. (2001) *Clin. Exp. Dermatol.* **26**, 525–530
26. Schor, S. L., Ellis, I. R., Jones, S. J., Baillie, R., Seneviratne, K., Clausen, J., Deak, S. B., and Boyd, C. D. (1995) *Matrix Biol.* **14**, 635–641
27. Sandell, L. J. (1996) *Connect Tissue Res.* **35**, 1–6
28. Kosmehl, H., Berndt, A., Katenkamp, D., Mandel, U., Bohle, R., Gabler, U., and Celada, D. (1995) *Pathol. Res. Pract.* **191**, 1105–1113
29. Timpl, R. (1993) *Experientia* **49**, 417–428
30. White, E. S., Baralle, F. E., and Muro, A. F. (2008) *J. Biol. Chem.* **283**, 1–14
31. Yim, Y., Miner, J. H., and Sanes, J. R. (2002) *Mol. Cell. Neurosci.* **19**, 344–358
32. Schor, S. L., Ellis, I. R., Jones, S. J., Baillie, R., Seneviratne, K., Clausen, J., Deak, S. B., and Boyd, C. D. (1995) *Matrix Biol.* **14**, 635–641
33. Sandell, L. J. (1996) *Connect Tissue Res.* **35**, 1–6
34. Kosmehl, H., Berndt, A., Katenkamp, D., Mandel, U., Bohle, R., Gabler, U., and Celada, D. (1995) *Pathol. Res. Pract.* **191**, 1105–1113
35. Timpl, R. (1993) *Experientia* **49**, 417–428
36. White, E. S., Baralle, F. E., and Muro, A. F. (2008) *J. Biol. Chem.* **283**, 1–14
37. Yim, Y., Miner, J. H., and Sanes, J. R. (2002) *Mol. Cell. Neurosci.* **19**, 344–358
38. Schor, S. L., Ellis, I. R., Jones, S. J., Baillie, R., Seneviratne, K., Clausen, J., Deak, S. B., and Boyd, C. D. (1995) *Matrix Biol.* **14**, 635–641
39. Sandell, L. J. (1996) *Connect Tissue Res.* **35**, 1–6
40. Kosmehl, H., Berndt, A., Katenkamp, D., Mandel, U., Bohle, R., Gabler, U., and Celada, D. (1995) *Pathol. Res. Pract.* **191**, 1105–1113
41. Timpl, R. (1993) *Experientia* **49**, 417–428