Title

Laminin N-terminus α 31 protein distribution in adult human tissues

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Abstract

Laminin N terminus $\alpha 31$ (LaNt $\alpha 31$) is a netrin-like protein derived from alternative splicing of the laminin $\alpha 3$ gene. Although LaNt $\alpha 31$ has been demonstrated to influence corneal and skin epithelial cell function, its expression has not been investigated beyond these tissues. In this study, we used immunohistochemistry to characterise the distribution of this protein in a wide-array of human tissue sections in comparison to laminin $\alpha 3$. These data revealed widespread LaNt $\alpha 31$ expression. In epithelial tissue, LaNt $\alpha 31$ was present in the basal layer of the epidermis, throughout the epithelium of the digestive tract, and much of the epithelium of the reproductive system. LaNt $\alpha 31$ was also found throughout the vasculature of most tissues, with enrichment in reticular-like fibres in the extracellular matrix surrounding large vessels. A similar matrix pattern was observed around the terminal ducts in the breast and around the alveolar epithelium in the lung, where basement membrane staining was also evident. Specific enrichment of LaNt $\alpha 31$ was identified in sub-populations of cells of the kidney, liver, pancreas, and spleen, with variations in intensity between different cell types in the collecting ducts and glomeruli of the kidney being of particular note. Intriguingly, LaNt $\alpha 31$ immunoreactivity was also evident in neurons of the central nervous system, in the cerebellum, cerebral cortex, and spinal cord. Together these findings suggest that LaNt $\alpha 31$ may be functionally relevant in a wider range of tissue contexts than previously thought, and provides a valuable basis for investigation into this interesting protein.

Abbreviations

LaNt (Laminin N-terminal),
 LM (laminin),
 ECM (extracellular matrix),
 BM (basement membrane),
 LCC (laminin-coiled coil),

- 39 LE-repeats (laminin-type epidermal growth factors-like domains),
- 40 LN-domain (laminin N-terminal domain),
- 41 Carboxyl-terminus (C-terminus),
- 42 Amino terminus (N-terminus),

43 Introduction

44 Laminins (LMs) are essential extracellular matrix (ECM) structural proteins required
 45 for the assembly and function of basement membranes (BMs)[1, 2]. BMs provide cell-ECM
 46 interaction sites for epithelial, endothelial, nerve, and muscle cells, as well as providing a
 47 substrate for cell migration during tissue remodelling, and signals to define lineage
 48 specification and differentiation patterning, as reviewed in[1-4]. LMs have been studied
 49 extensively over the past 40+ years and 12 LM encoding genes have been identified in
 50 higher organisms[5]; however, in 2009 the complexity of the family grew with the
 51 identification of a series of transcripts encoding non-laminin proteins generated by
 52 alternative splicing from the 5' end of the LM α 3 (LAMA3) and LM α 5 (LAMA5) genes[6]. At
 53 least one of these transcripts is translated into a functional protein, termed Laminin N-
 54 terminus (LaNt) α 31 (LaNt α 31)[6-8].

55 LaNt α 31 is produced from the LAMA3 gene via a process of intron retention and
 56 polyadenylation. LAMA3 is unique within the LM family in that it has two promoters giving
 57 rise to structurally distinct isoforms; a short LM α 3a form and a longer LM α 3b. These proteins
 58 share common carboxyl-terminal (C-terminal) regions but differ in the length of their amino
 59 terminus (N-terminus)[9, 10]. LM α 3a has been reported as largely restricted in its expression
 60 to squamous epithelia. In contrast, LM α 3b, which shares its promoter with LaNt α 31, has a
 61 much wider distribution profile, though often at lower levels than LM α 3a[6, 9-12]. LaNt α 31
 62 mRNA (*LAMA3LN1*) is also expressed in a wide array of tissues including; the heart, brain,
 63 placenta, lung, pancreas, spleen, thymus, prostate, testis, ovaries, small intestine, and in
 64 leukocytes as determined by semi-quantitative reverse transcription polymerase chain

reaction from whole tissue extracts[6]. At the protein level, LaNt α 31 studies have focused on epithelial tissues[6-8]. LaNt α 31 protein was identified in the basal layers of the skin and corneal epithelia with localised enrichment in limbal epithelial sub-populations, and in stromal structures including blood vessels. Upregulation of the protein was observed during ex vivo corneal wound repair and in stem cell activation assays, and functional studies using knockdown and overexpression indicate a role for this comparatively unstudied protein in the regulation of keratinocyte migration and adhesion[6-8].

Although derived from the LAMA3 gene, LaNt α 31 is much smaller and does not contain the some of the characteristic LMs features. Specifically, whereas LMs form heterotrimers via a laminin coiled-coil-domain (LCC-domain) located toward the C-terminus of each subunit[13-16], LaNt α 31 lacks this critical LCC domain. Indeed, as their name suggests, the most striking structural feature of LaNt α 31 is a laminin N-terminal domain (LN-domain). This LN domain is followed by stretch of laminin-type epidermal growth factor-like domains (LE-repeats) and a unique C-terminus without any characteristic structural architecture and which is not conserved with LMs[6]. LN-domains play an essential role in BM assembly by providing the points through which LM to LM interactions occur; a process which involves formation of a ternary nodes between LN-domains between an α , β and γ LM subunits[1, 2, 17-20].

LaNt α 31 protein architecture closely resembles other members of the LM superfamily; the netrins, with a notable exception that the netrins have distinct C-terminal domains not present in LaNt α 31[21-24]. Netrins can be broadly classified into two groups; γ -netrins and β -netrins. Netrins-1, 3 and 5 share evolutionary ancestors with the γ laminins[23, 25] and are considered primarily as signalling molecules via binding of their LN domains (domain VI) and LE-repeats (LE2-LE3) to the cell receptors neogenin, deleted in colorectal cancer (DCC), members of the uncoordinated 5 family, and Down-syndrome cell adhesion molecule, as reviewed in[24, 26, 27]. Netrin-4, in contrast, which arose independently from β laminin ancestors[23, 25], appears to be functionally distinct in that it can disrupt LM

networks via competition of LM ternary nodes[28], with implications for directed cell migration during angiogenesis and neurogenesis[29-35]. LMs and netrins are known to play context-specific roles across a range of tissues types, with local expression patterns and abundance providing valuable clues to functionality, as reviewed in[2-4, 13, 22, 24, 26, 36-41]. However, the equivalent expression and distribution of LaNt α 31 protein have not been determined. The LaNt α 31 expression data are of particular interest, as LaNt α 31 is likely to play distinct roles in different tissues dependent on local cell-surface receptor and BM make-up.

Here we used LaNt α 31 specific monoclonal antibodies to determine the protein distribution across a wide range of human adult tissues for the first time, while comparing to total LM α 3 localisation.

Methods

Ethical approval

Ethical approval for working with human tissue was conferred by the University of Liverpool Research Ethics Committees (approval number:7488). Microarray sections of 10% neutral buffered formalin-fixed and paraffin-embedded human tissue sections were acquired from Reveal Biosciences (NT02, Reveal Biosciences, San Diego, California, US) and US Biomax (MBN481, US Biomax, Rockville, Maryland, US). Human tongue tissue was acquired from the Liverpool Head and Neck Bioresource (research ethical committee approval number: EC 47.0).

Antibodies

Mouse monoclonal antibodies (clone 3E11) against residues 437 to 451 of the unique region of LaNt α 31 were described previously[7], these were used for immunohistochemistry (IHC) at 0.225 μ g mL⁻¹, immunoblotting (IB) at 1.8 μ g mL⁻¹, and enzyme-linked immunosorbent assay (ELISA) at 18 μ g mL⁻¹. Rabbit polyclonal antibodies raised against a glutathione S transferase fusion protein containing the 54 amino acid unique region of LaNt α 31 were used as described previously for IHC[6, 7], mouse anti- LM α 3a and LM α 3b antibodies (mapped to EINSLQSDFT, corresponding to residues within the LCC domain) were used at 50 μ g mL⁻¹ (clone CL3112, AMAB91123, Sigma-Aldrich, St. Louis, Missouri, US) and mouse IgG for IHC (31903, ThermoFisher Scientific, Waltham, Massachusetts, US).

Immunohistochemistry

Sections were processed using a Leica Bond autostainer and the Bond™ Polymer Refine Detection system (Leica Biosystems, Wetzlar, Germany). Briefly, sections were dewaxed and rehydrated through a series of xylene and decreasing ethanol concentrations. Antigen retrieval was performed by incubating in Tris/EDTA buffer pH 9 for 20 mins at 60°C for anti-LaNt α 31 antibodies and mouse IgG, or in Tris/citrate buffer pH 6 for 30 mins at 60°C

for anti-LM α 3 antibodies. Endogenous peroxidases were blocked with Bond hydrogen peroxide solution for 5 mins at room temperature. Sections were then incubated with primary antibodies at room temperature for 30 mins LaNt α 31 and mouse IgG or for 60 mins LM α 3) in Bond primary Ab solution (tris-buffered saline [TBS], containing surfactant and protein stabilizer, complete composition not provided). Secondary rabbit anti-mouse IgG Abs with 10% v/v animal serum in TBS were added for 15 mins (LaNt α 31 and mouse IgG) or 30 mins (LM α 3) at room temperature. Polymer anti-rabbit poly-HRP-IgG Abs (<25 μ g mL⁻¹) in 10% v/v animal serum in TBS were added at room temperature for 20 mins for LaNt α 31 and mouse IgG, or 30 mins for LM α 3. 66 mM DAB chromogen substrate was added for 10 mins (LaNt α 31 and mouse IgG) or 30 mins (LM α 3) at room temperature, then slides were counterstained with 0.1% w/v haematoxylin for 5 mins. At each stage, washes were performed with Bond wash solution (TBS containing surfactant, complete composition not provided), and with deionized water after counterstaining. Sections were dehydrated through a series of ascending ethanol concentrations and xylene, then mounted in Pertex® (Pioneer Research Chemicals Limited, Essex, UK).

Slides were scanned at 20x using an Zeis Axio-slidescanner Z1 equipped with an Axiocam colour CCD camera and processed using ZEN Blue software (all from Carl Zeiss AG, Oberkochen, Germany) and Fiji (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, US). Figures were prepared using CorelDRAW 2017 (Corel, Ottawa, Canada).

Short hairpin RNA (shRNA) and LaNt α 31 expression construct generation

shRNA sequences targeting the unique portion of the *LAMA3LN1* transcript were designed using the BROAD institute design algorithm (<http://www.broadinstitute.org/rnai/public/seq/search>). A gblock containing the shRNA sequence (CCCTCTCTCTTCAGAGTATT) or non-silencing sequence (TCTCGCTTGGGCGAGAGTAAG), as well as stem loop sequence with *Bam*HI and *Xho*I restriction enzyme compatible overhangs (synthesized by Integrated DNA Technologies,

Coralville, Iowa, US), was cloned into the miRNA adapted pGIPz plasmid (Open Biosystems, Huntsville, Alabama, US). Lentiviral particles were generated as previously described[42] using 293T packaging cells (Gene Hunter Corporation, Nashville, Tennessee, US) with help from the DNA/RNA Delivery Core at Northwestern University (DNA/RNA Delivery Core at Northwestern University, Chicago, Illinois, US).

To generate lentiviral particles for LaNt α 31-PAmCherry expression, a gBlock containing the coding sequence for LaNt α 31-PAmCherry with *EcoR1* and *NheI* restriction enzyme compatible overhangs (synthesised by Integrated DNA Technologies) was inserted into the pLenti-puromycin vector and packaged in lentiviral particles (produced by Origene). (PS100109, OriGene, Rockville, Maryland, US).

hTCEpi cells[43] cultured in Gibco keratinocyte serum-free medium supplemented 5 ng mL⁻¹ EGF, 0.05 mg mL⁻¹ BPE, and 0.15 mM CaCl₂ (all ThermoFisher Scientific), were seeded at 200,000 cells/well of 6 well plates and transduced with lentiviral particles at final MOI of 0.5 with 8 µg/ml polybrene (Sigma-Aldrich). Transduced cells were selected in 10 µg/ml puromycin (Gibco, ThermoFisher Scientific) for 7 days. Selected cells were seeded at 2.5x 10⁵ in 6-well plates for 24 hours then scraped into 90 µL urea/sodium dodecyl sulphate (SDS) buffer (10 mM Tris-HCl pH 6.8, 6.7 M Urea, 1% w/v SDS, 10% v/v Glycerol and 7.4 µM bromophenol blue, containing 50 µM phenylmethanesulfonyl fluoride (PMSF) and 50 µM N-methylmaleimide (all Sigma-Aldrich), then sonicated and 10% v/v β-mercaptoethanol added (Sigma-Aldrich). Proteins were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis using 10% polyacrylamide gels (1.5 M Tris, 0.4 % w/v SDS, 10 % acrylamide/ bis-acrylamide, [Sigma-Aldrich]), in electrophoresis buffer (25 mM Tris, 190 mM glycine [Sigma-Aldrich], 0.1 % w/v SDS, pH 8.5). Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) using the TurboBlot™ system (Bio-Rad Laboratories) and blocked at room temperature in Odyssey® TBS-Blocking Buffer (Li-COR Biosciences, Lincoln, Nebraska, US) for one hour, then probed overnight at 4°C with mouse monoclonal antibodies diluted in blocking buffer, washed 3 x 5 mins in phosphate-buffered

saline (PBS) with 0.1% Tween-20 (both Sigma-Aldrich) and probed for 1 hour at room temperature in the dark with IRDye® 800CW conjugated goat anti-mouse secondary antibodies (LiCOR Biosciences), diluted in Odyssey® TBS-Blocking Buffer buffer at 0.05 µg mL⁻¹. Membranes were then washed for 3 x 5 mins in PBS with 0.1% Tween-20, rinsed with distilled H₂O and imaged using the Odyssey® CLX 9120 infrared imaging system (LiCOR Biosciences). Image Studio Light v.5.2 was used to process scanned membranes (https://www.licor.com/bio/products/software/image_studio_lite/).

Recombinant LaNt α31 production

To guarantee LaNt α31 protein expression and expression tag cleavage, we cloned the maltose-binding protein (MBP) expression enhancer tag[44] followed by a spacer sequence into a modified sleeping beauty vector[45] containing an N-terminal BM40 signal peptide sequence followed by a double Strep II tag. The LaNt α31 gene sequence encoding for aa: 36-488 was generated by polymerase chain reaction and inserted into the multiple cloning site downstream of the MBP sequence. HEK293 cells (ThermoFisher Scientific) were cultured in DMEM/F-12, GlutaMAX™ (ThermoFisher Scientific) containing 10% foetal bovine serum (Labtech, East Sussex, UK) and seeded at 90% confluency in a 6-well plate overnight, then transfected with the MBP-LaNt construct using FuGENE® HD (Promega, Madison, Wisconsin, US). In brief, FuGENE® HD was added to DMEM/F-12, GlutaMAX™ at room temperature for 10 mins, and then incubated with the plasmid DNA mix (transposase vector and LaNt α31 expression vector, ratio 1:9) for additional 20 min. Transfection reagent to plasmid DNA mix ratio was 1:3 (0.5 µg plasmid per well). The transfection DNA mixture was added to the wells and incubated for 24 hrs. Cells were then selected with 4 µg mL⁻¹ puromycin (Sigma-Aldrich) for 3 days and expanded prior to seeding into triple flasks (Nunc® Sigma-aldrich). Expression was induced with 1 µg mL⁻¹ doxycyclin (Sigma-Aldrich) in 2% serum-containing media when 90% confluency was reached. Media was harvested every 48 hrs for 6 days, filtered, pre-cleared with gelatin sepharose 4B (GE Healthcare, Chicago, Illinois, US) and the MBP-LaNt α31 purified using Strep-Tactin® sepharose 50%

suspension (IBA Lifesciences, Göttingen, Germany), eluted with 1.875 mg ml⁻¹ desthiobiotin in TBS. The purified protein was dialysed against 1 x PBS and concentration measured on a Nanodrop 2000 (ThermoFisher Scientific) using the molecular weight and the extinction coefficient of the produced protein.

Enzyme-linked immunosorbent assay

1 µg of MBP-LaNt α31 or a 0.125 to 1 µg dilution series of LM332 or LM511 in PBS (BioLamina AB, Sundbyberg Municipality, Sweden) was added to wells of a 96-well plate (Greiner Bio-One, Kremsmünster, Austria) in duplicate in a final volume of 100 µL and adsorbed overnight at 4°C. Wells were blocked 1% BSA in PBS (Sigma-Aldrich) for 2 hours at room temperature, washed, and mouse anti-LaNt α31 antibodies added in PBS for 1 hour at room temperature with rocking, washed, then probed for 1 hr at room temperature with HRP-conjugated goat anti-mouse IgG secondary antibodies 1:500 in PBS (Dako, Agilent Technologies, Santa Clara, California, US). Final washes were performed and the plate developed with 100 µL tetramethylbenzidine substrate for 10 mins (KPL, SeraCare Life Sciences Inc., Milford Massachusetts, US), and stopped with 20 µL 0.5 M sulphuric acid (Sigma-Aldrich). Absorbance was read at 450 nm on a FLUOstar Optima plate reader (B M G Labtech Ltd, Aylesbury, UK). All wash steps were performed 3 x 5 mins with PBS with 0.1% Tween-20.

Results

Antibodies validation

The C-terminus of LaNt α31 is unique to this protein and not conserved with any human LMs or netrins (Figure 1A and B). Previous publications have validated two separate antibodies that specifically recognise human LaNt α31; rabbit polyclonal antibodies raised against the entire unique region of human LaNt α31[6], and mouse monoclonal antibodies (clone 3E11) raised against a peptide within the LaNt α31-specific region[7] (Figure 1A, B). Consistent with previous findings, near identical distribution of immunoreactivity was

observed in human tongue sections processed with these antibodies (Figure 1C). We extended the validation of 3E11 by immunoblotting total protein extracts from immortalised limbal-derived corneal epithelial cells (hTCEPi cells[43]) stably transduced with either shRNAs targeting LaNt α 31, scrambled shRNA controls, or full-length LaNt α 31-with a PAmCherry tag cDNA. This confirmed that the 3E11 antibodies display affinity for appropriately sized products, whose expression were reduced upon knockdown (Figure 1D). The 3E11 antibodies also recognised recombinant MBP-tagged LaNt α 31 but not netrin-4 (Figure 1E). We further confirmed that 3E11 does not display cross-reactivity to LM332 or LM511 in their native forms using ELISA with recombinant LMs and recombinant LaNt α 31 protein as positive control (Figure 1E and F).

Figure 1- 3E11 mouse monoclonal antibodies against the unique C-terminus of LaNt α 31 validation.

(A) Diagram of the laminin and laminin-related protein architecture. (B) Multiple sequence alignment of LaNt α 31 C-terminus with equivalent regions of laminin N-terminal sequences. Peptide unique to LaNt α 31 used to generate antibodies highlighted by red box. (C) Paraffin-embedded human tongue tissue sections processed for immunohistochemistry with rabbit polyclonal antibodies against LaNt α 31 or mouse monoclonal antibodies against LaNt α 31 (3E11). Scale bars represent 200 μ m or 100 μ m in magnified images. (D) Immunoblot of total protein from hTCEPi expressing either: shRNAs specific to LaNt α 31, scrambled shRNA controls, or full-length LaNt α 31-PAmCherry cDNA. Ponceau S stained membrane below. (E) Immunoblot of dilution series of recombinant MBP-LaNt α 31, and MBP-netrin-4 probed with 3E11 antibodies. Diagram below shows the structure of the recombinant MBP-LaNt α 31 and Netrin-4. (F) ELISA using recombinant MBP-LaNt α 31, LM511, or LM332. Wells were coated overnight, blocked with BSA, and then probed with 3E11 antibodies. Bound 3E11 antibodies were detected with HRP-conjugated secondary antibodies, HRP-substrate added and absorbance read at 450 nm.

The LaNt α 31 protein has been shown to be present in the BM of skin and enriched in basal epithelial cells of the corneal limbus[7], while in situ hybridisation demonstrated that the mRNA (*LAMA3LN1*) is enriched in the basal layer of the epidermis and hair follicles[6]. Using the 3E11 monoclonal antibodies, we observed a similar expression profile. Specifically, LaNt α 31 expression was predominantly restricted to the basal layer of the epidermis, with localised enrichment within a sub-population of cells (Figure 2A, arrowheads). As expected, monoclonal antibodies raised against the LCC domain that is shared between both LM α 3a and LM α 3b displayed immunoreactivity along BM at the dermal-epidermal junction (Figure 2B, arrowheads). In addition to the epithelial reactivity, both the anti-LaNt α 31 and anti-LM α 3 antibodies bound to sub-populations of stromal cells (Figure 2A-B).

Figure 2: LaNt α 31 is enriched in a sub-population of basal epidermal keratinocytes. Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11, A), laminin α 3 (B), or mouse IgG (C). Scale bars represent 200 μ m or 100 μ m in magnified images.

LaNt α 31 is expressed in the blood vasculature

LaNt α 31 displayed immunoreactivity in and around blood vessels in all tissues tested (Figure 3A-D). Specifically, LaNt α 31 immunoreactivity was evident in larger vessels, arterioles and venules, and in the larger capillaries likely in vascular endothelial cells and pericytes. In many cases, particularly for arterioles, the strongest signal was observed in individual cells around the vessel and in vessel walls (Figure 3, arrowheads). The pattern was not uniform to all vasculature; around large venules a distinct ECM / BM-like staining was obtained in the stroma surrounding vessels, with a reticular fibre-like ECM pattern

(Figure 3, chevrons). There was no apparent LaNt α 31 expression in the lymphatic vessels of these sections (Fig 3, asterisks).

Figure 3- LaNt α 31 is expressed throughout the blood vasculature.

Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11). Representative images taken from: (A) breast, (B) colon, (C) small intestine, (D) oesophagus. Scale bars represent 60 μ m.

LaNt α 31 in the breast and lung is assembled into basement membrane structures.

In breast tissue, both the inner luminal epithelial and outer myoepithelial cells displayed weak affinity for LaNt α 31 antibodies (Figure 4A). Remarkably, the ECM surrounding large collecting ducts of the terminal duct lobular units displayed particularly intense immunoreactivity (Figure 4A, arrowheads), although the smaller lobular acini were negative. These distributions are similar to the reticular fibre-like distribution surrounding larger vessels and ducts (compare Figure 3A and 4A), and resembles that reported for fibulin-2[46-48]. Although sections from precisely matched tissue were not available for LM α 3, in the closest comparison possible, a similar ECM pattern of immunoreactivity was also observed (Figure 4B, arrowheads); however, in contrast to LaNt α 31, immunoreactivity for LM α 3 was present around the smaller lobular acini (Figure 4B, chevrons). In the lung tissue, LaNt α 31 immunoreactivity was observed in the BM underlying the alveolar epithelium that encompass the air sacks (Figure 5A arrowheads), and in similar reticular fibre-like structures (Figure 5A chevrons). In contrast, LM α 3 displayed weak immunoreactivity which was restricted to the alveolar BMs (Figure 5B arrowheads).

Figure 4- LaNt α 31 is enriched in the ECM surround terminal ducts in breast tissue.

Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11, A), laminin α 3 (B), or mouse IgG (C). Scale bars represent 200 μ m or 100 μ m in magnified images.

Figure 5- LaNt α 31 localises to the alveolar basement membranes in lung tissue.

Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11, A), laminin α 3 (B), or mouse IgG (C). Scale bars represent 200 μ m or 100 μ m in magnified images.

LaNt α 31 is expressed throughout the epithelium of the digestive tract

LaNt α 31 immunoreactivity was detected throughout the oesophagus (Figure 6A), stomach (Figure 6D), small intestine (Figure 6G), colon (Figure 6J), and rectum (Figure 6M), where it displayed a diffuse distribution throughout all layers of the epithelium. LM α 3 largely matched the LaNt α 31 in all except oesophageal and rectal tissue (Figure 6B and N, respectively), where LM α 3 was restricted to the epithelial BM of the oesophagus (Figure 6B, arrowhead), or extremely weak in the rectal epithelium. In addition to the vasculature, the LaNt α 31 and LM α 3 antibodies both also labelled a sub-population of stromal cells in all tissue throughout the digestive tract,.

Figure 6: LaNt α 31 is expressed throughout the epithelium of the digestive tract.

Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11), laminin α 3, or mouse IgG. (A-C) oesophagus, (D-F) stomach, (G-I) small intestine, colon (J-L), rectum (M-O). Scale bars represent 200 μ m or 100 μ m in magnified images.

LaNt α 31 is enriched in specific cell sub-populations in kidney, pancreas, liver and spleen

In the kidney, LaNt α 31 immunoreactivity was observed in the cuboidal epithelium of proximal and distal tubules and collecting ducts, interestingly the intensity was markedly different between structures; with some ducts showing particularly high reactivity (Figure 7As arrowheads). LaNt α 31 immunoreactivity was also observed throughout the parietal epithelium of the Bowman's capsule (Fig 7A, chevron), and in a sub-population of cells in the glomeruli and glomeruli-surrounding stroma. LM α 3 largely matched LaNt α 31 in the tubules, collecting ducts, Bowman's capsule and glomeruli, but only displayed weak immunoreactivity in some stromal cells, and its intensity did not vary as markedly between ductal structures (Figure 7B).

LaNt α 31 immunoreactivity in the liver was observed throughout the cords of hepatocytes (Figure 7D), with a higher intensity in the branches of the bile ducts (Figure 7D, arrowheads). No LaNt α 31 was observed in the vascular sinusoid endothelium between hepatocyte cords, nor in stromal tissue, including the accompanying Kupffer cells and fibroblasts. Sections from precisely matched tissue location were not available for LM α 3; however, in the closest comparative section, a similar distribution of weak immunoreactivity for LM α 3 was observed in the hepatocyte cords and negative the stromal cells (Figure 7E).

In the pancreas, LaNt α 31 was found in the acini epithelium, enriched around the basal layer towards the outer edge (Figure 7G). LM α 3 immunoreactivity was also present in the same locations, with basal enrichment (Figure 7H).

In the spleen, LaNt α 31 was found throughout the tissue. In the red pulp, the most notable immunoreactivity was observed in the splenic cords of Billroth (Figure 7J, arrowheads), while venous sinuses were comparatively weak (Figure 7J). In the white pulp, comprised of lymphatic tissue, reactivity was much more intense in a sub-population of immune cells, while negative in others. This distribution pattern was largely paralleled by LM α 3 (Figure 7K).

Figure 7: LaNt α 31 expression in kidney, pancreas, liver, and spleen, is largely similar to that of LM α 3. Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11), laminin α 3, or mouse IgG. (A-C) kidney, (D-F) liver, (G-I) pancreas. Scale bars represent 200 μ m or 100 μ m in magnified images.

LaNt α 31 is widely expressed in reproductive organs

LaNt α 31 immunoreactivity was found throughout the female and male reproductive systems. In the ovaries, patchy distribution was observed in the follicular cells of the primordial follicles (figure 8A, arrowheads). Weak reactivity was also observed in the primary follicles (figure 8A, chevrons), and intense reactivity in the vasculature (figure 8A, double chevrons). LM α 3 immunoreactivity was also observed in the same structures (Figure 8B: primary follicles, chevrons; vasculature, double chevrons), but was more uniform in distribution in the follicular cells of the primordial follicles (Figure 8B, arrowheads). In the fallopian tube tissue, both LaNt α 31 (Figure 8D) and LM α 3 (Figure 8E) were found throughout uterine epithelium; however, the intensity of the LaNt α 31 reactivity was notably higher in peg cells compared to ciliated cells (Figure 8D arrowheads and chevrons, respectively). Very similar distribution for both LaNt α 31 and LM α 3 immunoreactivity was observed in the uterine gland of the endometrium (Figure 8G and H, respectively, arrowheads). LaNt α 31 in the cervix was relatively weak compared to the other reproductive tissues. However, LaNt α 31 again localised to reticular fibre-like structures in the stromal tissue (Figure 8J, arrowhead), with a similar distribution as in lung tissue (compare with Figure 5A). This reticular fibre-like distribution of LaNt α 31 was in contrast to the stratified squamous epithelium BM structure recognised by the LM α 3 antibodies (Figure 8K).

Figure 8: LaNt α 31 is expressed throughout the female reproductive system. Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11), laminin α 3, or mouse IgG. (A-C) ovary, (D-F) fallopian tube, (G-I) endometrium, (J-L) cervix. Scale bars represent 200 μ m or 100 μ m in magnified images.

In the testis, LaNt α 31 was present throughout the seminiferous epithelium. Weak reactivity was observed in the Sertoli cells (Figure 9A, chevrons), with more intense signal from a sub-population of the spermatogonia (Figure 9A, arrowheads). A BM-like distribution was observed under the smooth muscles cells that encompass the seminiferous epithelium (Figure 9A, double chevrons), while the smooth muscle layers themselves were negative. Although sections from precisely matched tissue were not available for LM α 3, similar BM-like immunoreactivity was evident, with some weak reactivity present within the seminiferous epithelium in the closest comparison possible, (Figure 9B).

In the prostate, LaNt α 31 immunoreactivity was observed throughout the acini (Figure 9D), however, intensity was stronger in the basal cells (Figure 9D, arrowheads) compared to the acinar cells (Figure 9D, chevrons). LM α 3 was also observed throughout the acini (Figure 9E), although the intensity was more uniform.

Figure 9: LaNt α 31 is expressed in the testis and prostate. Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11), laminin α 3, or mouse IgG. (A-C) testis, (D-F) prostate. Scale bars represent 200 μ m or 100 μ m in magnified images.

LaNt α 31 is expressed in neurons of the central nervous system

In the cerebellum, LaNt α 31 immunoreactivity was observed in Purkinje neurons in the ganglionic layer (Figure 10A, arrowheads) and axons throughout granular layer (Figure

10A, chevrons), whereas the granule cells in the granular layer, with the exception of a few cells, were negative (Figure 10A, red box). In the cerebral cortex, LaNt α 31 immunoreactivity was observed in the cell bodies towards the axon hillock of pyramidal cells (Figure 10D, arrowheads) and in some glial cells (Figure 10D, chevrons). This distribution was striking similar to that observed in the spinal cord tissue taken from a new-born, where LaNt α 31 was evident in the motor neurons, but appeared throughout the entire cell body and not just toward the axon hillock (Figure 10G, arrowheads). LM α 3 was not detected in either the cerebellum (Figure 10B), = cerebral cortex (Figure 10E), nor spinal cord (Figure 10H).

Figure 10: LaNt α 31 is expressed by neurons in the central nervous system. Paraffin-embedded human tissue microarray sections processed for immunohistochemistry with mouse monoclonal antibodies against LaNt α 31 (3E11), laminin α 3, or mouse IgG. (A-C) cerebellum, (D-F) cerebral cortex, (G-I) spinal cord. Scale bars represent 200 μ m or 100 μ m in magnified images.

Discussion

The findings reported here have revealed that LaNt α 31 is widely distributed across multiple tissues including the epithelia, vascular and stromal cells throughout most tissues, and in neurons in the central nervous system. Moreover, we have identified that LaNt α 31 is present in distinct structures and generally more widespread than LM α 3 across the tissues tested, despite their shared genetic origins. Surprisingly, in most situations the strongest LaNt α 31 signal was obtained from cells rather than BM structures; however, ECM enrichment was observed in reticular fibre-like structures were frequently observed, particularly surrounding large vessels, terminal ducts in the breast, and alveolar sacks in the lungs. These findings naturally lead to questions regarding the role of LaNt α 31 in different structures and contexts.

As LaNt α 31 and LM α 3b share a promoter, we predicted a broadly similar distribution pattern between the two proteins. Consistent with this, LM α 3b has been described in the BM at the dermal-epidermal junction of skin and underlying the epithelium of the oesophagus, breast, lungs, and the endothelium of vessels, and the LaNt α 31 immunoreactivity was also strong in these regions[12, 49, 50]. For the other tissues examined, there are no published data available at the protein level. However, at the transcript level *LAMA3LN1* and *LAMA3B* have been described as having widespread expression, although were not detected in liver, kidney, or spleen tissue-derived cDNAs[6, 11]. *LAMA3B* was reported as being highly enriched in the developing mouse brain[51] but expressed at extremely low levels in adult human brain[6]. The latter are reflected here by the observed absence of LM α 3 but positive LaNt α 31 immunoreactivity in the neurons throughout the CNS. Together, these collective data suggest differences between mRNA and protein expression, stability, and turnover, and also imply that changes in splicing behaviour occurs through development and ageing. To address these questions, it could be valuable in the future to investigate mRNA production and splicing regulation using approaches such as RNA in situ-hybridisation alongside protein distribution data.

The distinct tissue expression and localisation profiles identified here for LaNt α 31 suggests that it could play context-specific roles. When considering the LaNt α 31 findings, the LMs present in each BM may be of particularly relevance as LaNt α 31 is essentially a biologically-active α -type LM LN-domain, which potentially could bind to β and γ LN-domain complexes[49]. LaNt α 31 may behave differently in tissues with BMs that contain predominantly a single LN-domain LMs, such as the epidermis and squamous epithelium of the digestive tract which contain predominantly LM3a32, compared with two LN-domain LMs, such as the endothelium in small vessels where LM411 is abundant, and compared again to those tissues where three LN-domain LMs predominate, such as the kidney glomeruli, as reviewed in[3, 4, 52-54]. In this interpretation, clues as to LaNt α 31 function are suggested by the structural similarities with netrin-4, which binds with high affinity to the

LM α 1 subunit, and can disrupt LM networks[28, 55]. However, the affinity of the LaNt α 31 LN-domain for LN domains is much lower than netrin-4[28, 49], and therefore LaNt α 31 could only achieve the same disruptive functionality if present at very high concentrations. However, one could envisage situations where local partial network disruption could be beneficial, for example, in amoeboid-style migration through BMs such as immune cell infiltration through the blood vessel BMs[56-58].

Context-specific signalling roles for LaNt α 31 could also be possible. LN-domain containing LM fragments have repeatedly been implicated in integrin-mediated signalling, with the proteolytically released LN-domain containing fragment from LM α 3b being shown to support keratinocyte adhesion and proliferation via α 3 β 1 or α 6 β 1 integrins[59], while the α 1 LN-domain containing short-arm of LM111 can bind integrins α 1 β 1 and α 2 β 1, albeit at low affinity[60]. Signalling from a LN-domain containing fragment from LM β 1 has been shown to induce a shift from epithelial to mesenchymal-related gene expression via α 3 β 1 integrin[61]. While integrin-mediated signalling is most likely, netrin-like signalling via classical netrin receptors cannot be ruled out. Netrins are well established as being central mediators of numerous biological processes including; neurogenesis, lymphangiogenesis, and haemangiogenesis[29-34, 62]. The expression of LaNt α 31 in neurons, throughout the blood but not lymphatic vasculature puts it in appropriate locations to influence similar processes either directly as a signalling molecule in its own right, or indirectly by modulating netrin-mediated signalling.

Conclusions

LaNt α 31 is an interesting protein that is much more widely expressed than previously thought, with the differences in localisation and distribution suggesting a context specificity of function. These findings provide a platform for future functional studies were ECM remodelling is a hallmark, such as during development and ageing, and in pathological situations such as chronic wound repair and cancer.

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LDT designed and conducted the experiments, analysed the data, and made the figures. RR generated the plasmids and recombinant LaNt α 31/ netrin-4 proteins. CJS assisted in making figures. KJH designed the study and assisted in making figures. All authors contributed to the interpretation of the data, and writing/ editing of the manuscript.

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Figure 1

A

anti-LaNt $\alpha 31$

B

anti-laminin $\alpha 3$

C

Isotype control

Figure 2

Figure 3

A

B

C

Figure 4

A

B

C

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10