Regional differences in the expression of laminin isoforms during mouse neural tube development

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1. Introduction

The neural tube is the developmental precursor of the central nervous system and much of the peripheral nervous system, (Schoenwolf, 1984; Schoenwolf and Nichols, 1984; Copp et al., 2003). The process of primary neurulation, which is responsible for formation of the brain and most of the spinal cord, is characterised by elevation, adhesion and fusion of the neural folds along the body axis. However, the mechanisms involved in the closure of the neural folds appear to vary at different axial levels as evidenced by the heterogeneity of neural tube defect (NTD) phenotypes in mutant and gene knockout mice (Greene and Copp, 2009). Eighty percent of mutants develop a single type of NTD: either exencephaly, typically in the midbrain (70%), open spina bifida in the lumbo-sacral region (5%), or craniorachischisis, with almost total failure of closure along the body axis (5%). Only 20% of mutants exhibit NTDs at more than one site (Harris and Juriloff, 2007; Harris and Juriloff, 2010). This spectrum of neural tube closure defects reflects the range of NTDs typically observed also in humans (Harding and Copp, 2008).

Simultaneous with the morphogenetic events of neural tube closure, other critical events of early nervous system development are underway including rapid cell proliferation within the ventricular zone and the onset of asymmetric division to generate the first postmitotic neuronal progenitors. These cells migrate along the fibres of radial glia to take up more peripheral positions in the neural tube,
either as proliferative sub-ventricular zone cells in the brain or as early differentiating neuroblasts (e.g. motor neurons) in the spinal cord (Campbell and Gotz, 2002). Associated disorders in humans include primary microcephaly, in which a series of gene mutations affect proteins required for the centrosome function during the cell cycle, in order to maintain renewing cell divisions in the early neural tube (Thornton and Woods, 2009). Neuronal migration disorders such as lissencephaly, whose genetic causation relates largely to cytoskeletal function within migrating neuroblasts (Wynshaw-Boris et al., 2010), also have their developmental origin at this stage.

Basement membranes are specialized extracellular matrices found closely apposed to the basal surface of epithelia at the interface with mesenchyme or, in a few cases, other epithelia. They are important for providing mechanical stability to the epithelium and adhesion to neighbouring tissues. In addition, basement membranes initiate intracellular signalling, particularly via integrin receptors (Kruegel and Miosge, 2010) that can affect cellular survival, polarity, cytoskeletal dynamics, and other epithelial functions (Rozario and DeSimone, 2010). Laminins, a family of trimeric glycoproteins, constitute one of the main components of basement membranes. Laminins exist in the extracellular matrix as heterotrimers containing one α-, one β- and one γ-chain that assemble with each other via their coiled-coil domains to form a cross-like structure (Colognato and Yurchenco, 2000). To date, five distinct α-chains, three β-chains and three γ-chains have been shown to form at least 15 different laminin isoforms (Miner and Yurchenco, 2004). Laminin trimers assemble and undergo glycosylation intracellularly, prior to secretion into the extracellular matrix (Yurchenco et al., 1997; Schneider et al., 2007; Tzu and Marinkovich, 2008). Once secreted, higher order laminin structures are formed, enabling interaction with other basement membrane components including collagen IV, nidogen and perlecans (Miner, 2008).

It is known that rapid switches occur in the expression of laminins during development (Wan et al., 1984; Miner and Sanes, 1994; Gersdorff et al., 2005b), potentially leading to dramatic differences in the biological properties of the embryonic basement membranes. Around the time of neural tube closure, and during early post-closure development, the basement membranes around the neural tube and underlying the non-neural surface ectoderm become organised and undergo remodeling, with particularly dynamic alterations in the dorsal neural folds, where the two epithelia are contiguous (Martins-Green and Erickson, 1986, 1987). In addition, matrix disturbances have been identified in avian spontaneous NTDs (Newgreen et al., 1997), while exencephaly has been observed in 60% of mouse embryos lacking laminin α5 (Miner et al., 1998) and in 5% of laminin γ1 mutants (Halfter et al., 2002).

There is relatively little information on the trimeric laminin variants that make up the basement membranes of the early neural tube and surface ectoderm. In the present study, therefore, we determined both the mRNA and protein expression patterns of all the individual laminin chains in the neural tube and surface ectodermal basement membranes along the body axis in the embryonic day (E) 9.5 embryo. From this information, we inferred the likely laminin variants and their tissue sources. This analysis reveals marked differences in basement membrane composition, and relative contribution from epithelium and mesoderm, along the rostro-caudal body axis that may relate to developmental events occurring in nervous system development at this stage.

2. Results

The aim of the study was to determine which laminin chains are synthesised and exported into the developing basement membranes along the E9.5 mouse embryonic body axis. This information is necessary to identify the tissue sources of the laminin isoforms, and to understand the likely chain composition of the extracellular laminin variants present in association with the neural tube at this stage of development.

Whole mount in situ hybridization on E9.5 embryos demonstrated a wide variation in the abundance and distribution of laminin mRNAs along the body axis (Fig. 1). For example, the β1 transcript exhibits strong expression in the caudal region, with much less intense expression more rostrally (Fig. 1F). In contrast, β2 and β3 mRNAs have an almost complementary pattern, with expression confined largely to the rostral-most levels of the embryonic axis (Fig. 1G). The α1 mRNAs do not show a similar regional specificity, although they exhibit markedly different intensities of expression with strong expression of α3 and α4 (Fig. 1C, D), less intense expression of α1 and α5 (Fig. 1A,E), and almost no expression of α2 (Fig. 1B). Similarly, γ1 is more strongly expressed than γ3, while γ2 exhibits lowest transcript abundance (Fig. 1I–K). To further investigate these apparent differences in expression of the laminin mRNAs, and to compare with the distribution of extracellular matrix laminin proteins, sections from the whole mount in situ hybridized embryos were compared with embryo sections subjected to immunohistochemistry.

Three different levels along the body axes of E9.5 embryos were chosen for analysis: (i) mandibular arch/hindbrain level, where the neural tube is either closing or has recently closed (Fig. 2); (ii) heart/cervical neural tube level, where the neural tube has closed 24 h prior to E9.5 (Fig. 3); (iii) lumbar level, which at E9.5 represents the caudal-most region of closing or recently closed spinal neural tube (Fig. 4). This analysis of sections at different levels of the body axis in embryos

![Fig. 1. Expression of laminin chain mRNAs, as indicated on each panel, by whole mount in situ hybridization of E9.5 mouse embryos. Dashed lines in (E) indicate level of sections through the mandibular arch/hindbrain (2: see Fig. 2), heart/cervical spine (3: see Fig. 3) and lumbar spine (4: see Fig. 4). Scale bar = 50 μm.](image-url)
of a single gestational stage allowed comparison of laminin distribution in cranial versus spinal tissues, and also in the vicinity of closed versus closing neural tube.

2. Laminin chain expression at mandibular arch level

2.1. Neural tube expression

Within the closing or recently closed neural tube (Fig. 2A), we detected expression of α1, α3, β2 and γ1 mRNAs. α1 transcripts are present in the whole neural tube, although expression appears more intense ventrally and close to the luminal surface (Fig. 2B). Immunofluorescence reveals α1 protein in the basement membrane around the entire neural tube.

α3 mRNA expression is also enhanced in the ventral part of the neural tube (Fig. 2D) but, in contrast to α1, the α3 protein was detected only in the portion of the neural tube basement membrane immediately beneath the floor plate (arrowhead in Fig. 2O). β2 transcript is distributed on the inner surface of the neural tube along its entire dorso-ventral extent, with particularly intense signal in the dorsal tips of the neural folds and in the floor plate (asterisks in Fig. 2H). It is worth noting that the zone of high β2 expression in the floor plate correlates exactly with a localised region of low α3 mRNA abundance (arrowhead in Fig. 2D). β2 protein also exhibits a stronger signal in the basement membrane around the dorsal part of the neural tube, together with a small area of β2 immunofluorescence adjacent to the floor plate (data not shown, but corroborated in other sections, see Subsection 2.2.1 and Fig. 3S). γ1 mRNA exhibits low intensity, uniform expression throughout the neural folds (Fig. 2J) that correlates with uniform deposition of γ1 protein throughout the neural tube basement membrane (Fig. 2U).

2.1.2. Surface ectoderm expression

While detecting in situ hybridization signal in the thin surface ectoderm layer can be challenging, we identified α1, α3, α5, β1, β2, γ1, and γ2 expression reproducibly. Low intensity α1 mRNA expression (Fig. 2B) is associated with strong, uniform distribution of α1 protein throughout the basement membrane underlying the surface ectoderm (Fig. 2M). α3 mRNA is expressed at low intensity by surface ectoderm, and α3 protein was strongly detected in the underlying basement membrane. α5 is the most intensely expressed α chain mRNA in the surface ectoderm with especially strong signal at the tips of the neural folds (asterisk in Fig. 2F). There is strong, uniform distribution of α5 protein throughout the underlying basement membrane (Fig. 2Q). β1 shows robust mRNA expression in the surface ectoderm and is immunolocalised in the basement membrane underneath (Fig. 2G, R). β2 has very low intensity expression in the surface ectoderm, except at its junction with the dorsal neural folds, where expression is intense (top asterisk in Fig. 2H). β2 protein is strongly present throughout the basement membrane underlying the surface ectoderm (Fig. 2S). γ1 mRNA is particularly abundant in the surface ectoderm contacting the dorsal tips of the neural folds (asterisks in Fig. 2H and J). Despite these variations in mRNA abundance, γ1 protein appears uniform throughout the surface ectoderm basement membrane (Fig. 2U). γ2 mRNA is
expressed uniformly throughout the surface ectoderm (Fig. 2K), with abundant γ2 protein in the underlying basement membrane (Fig. 2V).

2.1.2. Mesenchyme expression

mRNAs for α1, α3, α4, β1, β2, γ1, γ2, and γ3 are all expressed by the cranial mesenchyme. α1 shows low intensity mRNA expression throughout the mesenchyme, with marginally stronger signal dorsally beneath the surface ectoderm and adjacent to the ventrolateral neural tube (arrowheads in Fig. 2B). Laminin α1 immunofluorescence is intense in association with clumps of cells in the dorsal mesenchyme and within the mandibular arches (Fig. 2M). This protein accumulation may relate to blood cells. α3 mRNA is strongly expressed in ventro-medial mesenchyme, except for a narrow zone of cells immediately adjacent to the ventral neural tube that appears negative.

Fig. 3. Expression of laminin chain mRNAs and proteins at the heart/cervical spine level of E9.5 embryos. (A) Diagram of a representative section at this level where the main tissues are marked. Dorsal is to the top of each section. In red is marked the narrow zone of mesenchyme adjacent to the neural tube where some laminin mRNA synthesis is observed. Abbreviations: MS, mesenchyme; NT, neural tube; SE, surface ectoderm. (B–L) Laminin mRNA distribution in vibratome sections following whole mount in situ hybridization. (M–W). Immunofluorescence showing localisation of laminin chain proteins. Intense staining of single cells or cell groups in M, Q, R and U likely represents FITC auto-fluorescence from blood cells. Scale bar in C = 20 μm and applies to all other figure parts.
 Despite this expression, immunofluorescence failed to detect α3 chains in the mesenchyme. α4 exhibits an mRNA pattern that is largely complementary to that of α3, with transcripts detected in the dorso-lateral mesenchyme, and in the α3-negative area adjacent to the ventral neural tube (Fig. 2E). α4 protein is present at low intensity in the mesenchyme in a similar pattern to the mRNA, with stronger immunofluorescence visible in the narrow zone of mesenchyme adjacent to the neural tube (arrow in Fig. 2P). α4 chains are strongly deposited in vascular tissues and the surface ectoderm basement membrane (Fig. 2P), and in view of the absence of α4 transcripts from the surface ectoderm (Fig. 2E), it seems possible that this laminin is contributed by the mesenchyme. β1 exhibits uniform mRNA expression in the mesenchyme (Fig. 2G), in association with widespread clumps of β1 protein-positive cells both adjacent to the neural tube and within the mandibular arches (Fig. 2M). Mesenchymal β2 expression is of similar low intensity to β1 (Fig. 2H) and is matched by β2 protein which is of low abundance in the mesenchyme. However, the intense β2 expression in the surface ectoderm basement membrane may derive in part from mesenchymal synthesis. γ1 mRNA expression is of similar low intensity to β1 (Fig. 2H) and is matched by γ1 protein which is of low abundance in the mesenchyme. whereas γ2 protein entirely encircles the neural tube, γ3 protein is less intense in the ventral neural tube basement membrane (arrow in Fig. 2W).

2.1.4. Discordancy between mRNA and protein expression

It is not immediately obvious what might be the tissue of origin of the abundant α5 protein in the neural tube basement membrane (Fig. 2Q). While α5 transcripts are present in surface ectoderm, expression is not detectable in neural tube or cranial mesenchyme (Fig. 2F). It is possible that α5 chains secreted by the surface ectoderm are deposited in the neural tube basement membrane, but the mechanism by which this might be achieved is unclear.

2.2. Laminin chain expression at heart level

2.2.1. Neural tube expression

Transcripts of α1, α3, α4, β2, β3 and γ1 are detectable in the E9.5 neural tube which, at the level of the heart, has been closed for approximately 24 hours prior to the stage of analysis. α1 mRNA expression is detected towards the luminal surface of the entire neural tube (Fig. 3B) and, consistent with this, α1 protein is localised throughout the basement membrane surrounding the neural tube (Fig. 3M). α3 and α4 mRNAs are confined to the ventral part of the neural tube, although transcripts are absent from the floor plate (Fig. 3D, E). Neither α3 nor α4 protein is detectable in the basement membrane surrounding the neural tube (Fig. 3O, P). β2 transcripts are present throughout the neural tube, with most intense expression at the ventral and dorsal poles (asterisks in Fig. 3H). β2 protein is present throughout the neural tube basement membrane (Fig. 3S).
together with a small area of β2 immunofluorescence within to the floor plate (arrowhead in Fig. 3S), β3 transcripts are weakly detected in the neural tube (Fig. 3I), corresponding to weak expression of β3 protein around the neural tube (Fig. 3T). γ1 mRNA is expressed throughout the neural tube, but with apparently greater intensity either side of the roof plate (asterisk in Fig. 3J). γ1 protein appears evenly deposited throughout the neural tube basement membrane (Fig. 3U).

2.2. Surface ectoderm expression

mRNAs for α1, α3, α5, β1, β2, β3, γ1 and γ2 are all detectable in the surface ectoderm at the level of the heart. α1, α3 and α5 are all expressed throughout the surface ectoderm with α5 exhibiting most intense expression and α3 the least intense (Fig. 3B, D, F). All three laminin chains are detected by immunofluorescence throughout the basement membrane underlying the surface ectoderm, although α3 exhibits rather diffuse signal (Fig. 3O) compared with the very specific staining for α1 and α5 (Fig. 3M, Q). β1 and β2 both have well defined mRNA expression in surface ectoderm (Fig. 3G, H), with the corresponding laminin chains detectable by immunofluorescence in the underlying basement membrane (Fig. 3R, S). γ1, γ2 and γ3 mRNAs are all expressed by the surface ectoderm with more intense expression of γ1 than γ2, and only weak expression of γ3 (Fig. 3K, L). All three laminin chains: γ1, γ2 and γ3 are present, uniformly and at similar intensity, in the surface ectodermal basement membrane (Fig. 3U, V, W).

2.2.3. Mesenchyme expression

Transcripts for α1, α2, α4, β1, β2, γ1, γ2 and γ3 were detected in the mesenchyme. α1 expression is most intense in cells close to the neural tube (arrow in Fig. 3B), perhaps indicating a contribution to the neural tube basement membrane which is strongly positive for α1 protein (Fig. 3M). α3 and α4 are both expressed by ventro-medial mesenchyme (arrows in Fig. 3D, E), although α3 mRNA is absent from a narrow zone of cells adjacent to the ventral neural tube (arrowhead in Fig. 3D). Neither laminin chain is strongly expressed in the mesenchyme (Fig. 3P), although α4 is present faintly in cells close to the ventral neural tube (arrow in Fig. 3P) and in vascular cells. β1 is strongly expressed by mesenchyme (Fig. 3G) although, remarkably, no β1 protein can be detected in either mesenchyme or neural tube basement membrane at this level (Fig. 3R). β2 expression is weak in mesenchyme and no β2 protein is detected (Fig. 3H, S). γ1 mRNA expression is intense and uniform throughout the mesenchyme (Fig. 3J), and there is clear, albeit low intensity γ1 immunofluorescence throughout the tissue (Fig. 3U). Additionally, both neural tube and surface ectoderm basement membranes are strongly positive for γ1, raising the possibility of a mesenchymal contribution. Both γ2 and γ3 show highly localised mRNA expression in mesenchymal cells immediately adjacent to the neural tube (arrows in Fig. 3K, L) whereas there is no particular localisation of either γ2 or γ3 proteins in mesenchyme at this location, nor to neural tube basement membrane (Fig. 3V, W).

2.2.4. Discordancy between mRNA and protein expression

As at mandibular arch level, α5 laminin is strongly detected within the basement membrane surrounding the neural tube (Fig. 3Q), despite the apparent absence of mRNA from both neural tube and mesoderm (Fig. 3F).

2.3. Laminin chain expression at lumbar spinal level

2.3.1. Neural tube/neural plate expression

α1, α5, β1, β2, γ1 mRNAs were detected in the closing or recently closed neural plate, at the future lumbar level of the E9.5 embryo. α1 is expressed uniformly throughout the neuroepithelium, with a corresponding protein distribution throughout the surrounding basement membrane (Fig. 4B, M). α5 mRNA is also strongly expressed by neural tube cells, but shows a graded distribution from intense expression dorsally to near absence in the floor plate (Fig. 4F). This is in striking contrast with the complete absence of α5 transcript from neural tube at the mandibular arch and heart levels (compare with Figs. 2F and 3F). α5 chain is deposited in an even distribution throughout the neural tube basement membrane (Fig. 4O). β1 transcripts are present uniformly throughout the neural tube, with β1 protein strongly expressed in the surrounding basement membrane (Fig. 4G, R). β2 mRNA is detected only at the tips of the neural folds (Fig. 4H) and, correspondingly, β2 protein is also confined to the dorsal part of the neural tube; it is absent from the neural tube basement membrane (Fig. 4N). γ1 mRNA is weakly expressed in the neural tube at E9.5 (Fig. 4J), whereas more intense signal was detected in earlier stage embryos (data not shown). γ1 protein is present within the neural tube basement membrane, with most intense expression dorsally between surface ectoderm and neural tube roof plate (Fig. 4U).

2.3.2. Surface ectoderm expression

mRNAs for α1, α3, α5, β1, β2, γ1 and γ2 are all present in the surface ectoderm overlying the neural tube or fusing neural folds at the lumbar level of E9.5 embryos (Fig. 4B, D, F, G, H, J, K). Each of their laminin chain products can be detected in the basement membrane beneath the surface ectodermal layer (Fig. 4M, O, Q, R, S, U, V).

2.3.3. Paraxial mesoderm, notochord and hindgut expression

While α1 and β1 mRNAs are extremely strongly expressed in the paraxial mesenchyme (Fig. 4B, G), there is little or no detectable α1 or β1 protein within the mesoderm itself (Fig. 4M, R). The α1 and β1 laminin chains may be exported into the adjacent neural tube and surface ectoderm basement membranes which are both strongly positive. Both α4 mRNA and protein are detectable in blood vessel walls (arrowheads in Fig. 4E, P). The notochord expresses α5, β1 and γ1 mRNAs, corresponding to α5, β1 and γ1 laminins in the basement membrane surrounding the notochord (Fig. 4O, R, U). The source of the α1 in the notochordal basement membrane (Fig. 4M) may be low level synthesis in the notochord itself or deposition from adjacent paraxial mesoderm (Fig. 4B). The hindgut endoderm appears to be a prolific producer of laminins, containing mRNAs for α1, α2, α3, α4, α5, β1, β2, β3, γ1 and γ2 (Fig. 4B, C, D, E, F, G, H, I, J, K) which closely correlates with the corresponding laminin chains in the hindgut basement membrane (Fig. 4N, O, P, Q, R, T, U, V).

2.3.4. Discordancy between mRNA and protein expression

γ2 laminin is distinctly expressed in the neural tube basement membrane (Fig. 4V), but in the apparent absence of mRNA in either neural tube or paraxial mesoderm (Fig. 4K). Moreover, γ3 laminin is clearly expressed in the surface ectoderm and hindgut basement membranes (Fig. 4W), whereas γ3 mRNA is not detectable in either surface ectoderm, hindgut endoderm or adjacent paraxial mesoderm (Fig. 4L).

3. Discussion

Detection of each individual laminin chain by immunohistochemistry does not identify which trimeric laminin variants are present since, in the same basement membrane, several different laminin variants may co-exist. However, combining immunohistochemistry with in situ hybridization provides more information on the possible variants. This is because laminin trimers are assembled before secretion (Yurchenco et al., 1997; Schneider et al., 2007; Tzu and Marinkovich, 2008) so that the mRNAs encoding all three chains of any given trimer should be present in the same cell type. With this in mind, we analyzed our in situ hybridization and immunohistochemistry findings to infer which laminin variants are likely to be present in the vicinity of the E9.5 neural tube, along the body axis. We assumed
that laminins are deposited in embryonic basement membranes by the epithelium or mesenchyme (or both) that immediately contacts the membrane (Simon-Assmann et al., 1988; Marinkovich et al., 1993). That is, laminins in the basement membrane surrounding the neural tube were assumed to be synthesised by neural tube or adjacent mesodermal cells, while the basement membrane underlining the surface ectoderm is assumed to be synthesised in the surface ectoderm and deposited in the neural tube basement membrane.

### 3.1. Rostro-caudal differences in tissue contribution of laminins to basement membranes

Table 1 summarises the distribution of laminin chains in each of the two basement membranes, together with the distribution of laminin mRNAs in the adjacent tissues. Based on this information, Table 2 then lists the sources of the possible laminin variants in each basement membrane. A striking finding from this analysis is the varying extent along the body axis to which the mesoderm contributes to the neural tube basement membrane. At mandibular arch level, the mesoderm may contribute up to six laminin variants: four unique to the mesoderm (122, 123, 322, 323) and two shared with the neural tube cells (121, 321). In contrast, at lumbar spine level we could detect no laminin variants contributed by the mesoderm. The surface ectoderm basement membrane appears much better endowed than the neural tube basement membrane in terms of diversity of laminin variants, with 12 distinct laminins at mandibular arch and lumbar spine levels, and 18 at heart level. Strikingly, all of these laminins appear to be contributed by the surface ectodermal layer itself.

### 3.2. Variations in laminin composition in the neural tube basement membrane

At mandibular arch level, we detected laminins 121, 321 and 521 in the basement membrane surrounding the newly formed hindbrain neural tube. Similarly, at heart level, where the cervical neural tube

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**Table 1**

Distribution of laminin chain mRNAs and proteins.

<table>
<thead>
<tr>
<th></th>
<th>Mandibular arch level</th>
<th>Heart level</th>
<th>Lumbar spine level</th>
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**Abbreviations:** LC: laminin chain; NT: neural tube; M: mesoderm; SE: surface ectoderm. mR: mRNA; P: protein deposited in the basement membrane.

* Paraxial mesoderm only.

* Expression in mesoderm adjacent to neural tube only.

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**Table 2**

Possible laminin variants in basement membranes and their tissue of origin.

<table>
<thead>
<tr>
<th></th>
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<th>Lumbar spine level</th>
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<td>S. ect BM</td>
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**Abbreviations:** BM: basement membrane; NT: neural tube; Mes: mesoderm; S. ect: surface ectoderm.

* These laminin variants are included because α5 can be clearly detected by immunohistochemistry in the neural tube basement membrane at both mandibular arch and heart levels. However, there is no corresponding mRNA in either neural tube or adjacent mesoderm cells to account for its synthesis, calling into question the origin of the α5 chains (see Discussion).
had closed 24 hours earlier, we also detected laminins 121 and 521, but we could not identify laminin 321. The α3 laminin chain was observed at mandibular arch level only in the basement membrane beneath the floor plate, suggesting that it could be an early feature of floor plate induction or establishment. However, α3 null mutant mice develop throughout gestation without obvious neural tube abnormalities (Ryan et al., 1999), calling into question a specific role for laminin 321 in early neural tube development.

In the caudal-most (lumbar spine) region, we found a completely different set of laminins in the neural tube basement membrane. This is principally a result of the predominance of the different set of laminins in the neural tube basement membrane. This malformations (Ryan et al., 1999), calling into question a specific role for laminin 321 in early neural tube development.

Interestingly, this is a very similar location to our surface ectoderm close to the tips of the cranial neural folds. This is consistent with the view that basement membrane components are deposited locally at epithelial-mesenchymal interfaces.

### 3.3. Abundance of laminin variants in the surface ectoderm basement membrane

The basement membrane beneath the surface ectoderm in the E9.5 embryo contains a wide variety of laminin chains, with potential for up to 18 different laminin variants in the trunk at the level of the heart. This includes laminin α5-containing variants, several of which are expressed by surface ectodermal cells, and are present in the basement membrane at all three axial levels. α5 has previously been implicated in both cranial neural tube closure (Miner et al., 1998) and migration and differentiation of neural crest cells (Coles et al., 2006). Indeed, α5 deficient embryos developing exencephaly were found to have an abnormally thin, patchy basement membrane beneath the surface ectoderm close to the tips of the cranial neural folds. Interestingly, this is a very similar location to our finding of intense α5, β2 and γ1 mRNA expression, suggesting that the critical α5 expression for cranial neurulation and neural crest migration correspond to laminin 521.

### 3.4. Are laminins deposited in basement membranes after synthesis in non-adjacent tissues?

The great majority of laminin chains that we detected by immunohistochemistry in basement membranes were adjacent to epithelial or mesenchymal cells expressing the same laminin mRNA. This is consistent with the view that basement membrane components are deposited locally at epithelial-mesenchymal interfaces.

### 4. Materials and methods

#### 4.1. Mouse embryo preparation

Random-bred CD1 mice were mated overnight and the day of finding a copulation plug was designated embryonic day (E) 0.5. Embryos at E9.5 were dissected from the uterus in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (FCS), rinsed in phosphate buffered saline (PBS) and fixed overnight in either 4% paraformaldehyde in PBS at 4 °C, for in situ hybridization, or in zinc fixative (3 mM calcium acetate, 0.023 M zinc acetate, 0.036 M zinc chloride in 0.1 M Tris buffer pH 7.4), for immunohistochemistry.

#### 4.2. Whole mount in situ hybridization

After fixation, embryos were dehydrated though a graded series of methanols to 100% methanol, in which they were stored at −20 °C prior to further processing. Production of CRNA probes was undertaken for laminin subunits α1, α2, α3, α4, β5, β1, β2, β3, γ1, γ2 and γ3. Total RNA was obtained from E9.5 CD1 mouse embryos using TRIzol reagent (Invitrogen), and reverse transcriptase was used to generate first-strand cDNA. Sequences specific for each laminin subunit were amplified using PCR primers in Table 3, cloned into

<table>
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**Table 3**

PCR primers used to generate cDNA probes for specific laminin isoforms.

Two different α3 chains, A and B, are encoded by a single LAMA3 gene as a result of alternative promoter usage (Miner, 2008). In our study, we could not differentiate α3A from α3B. Hence, both chains are described as laminin α3.
pGEMT, and sequenced to confirm identity. cRNA probe synthesis, whole mount in situ hybridization, and preparation of vibratome sections through hybridized embryos were performed as described previously (Ybot-Gonzalez et al., 2005). Illustrated expression patterns are based on observation of a minimum of 3 hybridized embryos.

4.3. Immunohistochemistry

Zinc fixed embryos were embedded in gelatine-sucrose and stored frozen at 80 °C. Transverse cryo-sections, thickness 7 μm, were incubated for 30 min in PBS to remove the cryoprotectant, digested with 0.05% hyaluronidase in PBS for 2 h at 37 °C, and blocked in PBS containing 10% heat inactivated sheep serum and 0.1% Triton X-100. The sections were then processed for immunohistochemistry using primary antibodies specific for laminin chains α1, α2, α5, β1, β2 (Sorokin et al., 1992; Schuler and Sorokin, 1995; Sorokin et al., 1997; Sixt et al., 2001; Agrawal et al., 2006), α3, α4, β2 and γ3 (Taïts et al., 2000; Sasaki et al., 2001; Gersdorff et al., 2005a) and γ1 (Chemicon) followed by incubation with the appropriate fluoroescently-tagged secondary antibody. Slides were mounted with Vectashield containing DAPI (Vector Laboratories, Peterborough, UK). Selected sections were photographed on an Olympus BX-61 microscope. Illustrated immunofluorescence patterns are based on serial sections through a minimum of 3 embryos.

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References


2011) 301–309