Published in final edited form as:

Matrix Biol. 2011 May ; 30(4): 301–309. doi:10.1016/j.matbio.2011.04.001.

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

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Abstract

Many significant human birth defects originate around the time of neural tube closure or early during post-closure nervous system development. For example, failure of the neural tube to close generates anencephaly and spina bifida, faulty cell cycle progression is implicated in primary microcephaly, while defective migration of neuroblasts can lead to neuronal migration disorders such as lissencephaly. At the stage of neural tube closure, basement membranes are becoming organised around the neuroepithelium, and beneath the adjacent non-neural surface ectoderm. While there is circumstantial evidence to implicate basement membrane dynamics in neural tube and surface ectodermal development, we have an incomplete understanding of the molecular composition of basement membranes at this stage. In the present study, we examined the developing basement membranes of the mouse embryo at mid-gestation (embryonic day 9.5), with particular reference to laminin composition. We performed *in situ* hybridization to detect the mRNAs of all eleven individual laminin chains, and immunohistochemistry to identify which laminin chains are present in the basement membranes. From this information, we inferred the likely laminin variants and their tissues of origin: that is, whether a given basement membrane laminin is contributed by epithelium, mesenchyme, or both. Our findings reveal major differences in basement composition along the body axis, with the rostral neural tube (at mandibular arch and heart levels) exhibiting many distinct laminin variants, while the lumbar level where the neural tube is just closing shows a much simpler laminin profile. Moreover, there appears to be a marked difference in the extent to which the mesenchyme contributes laminin variants to the basement membrane, with potential contribution of several laminins rostrally, but no contribution caudally. This information paves the way towards a mechanistic analysis of basement membrane laminin function during early neural tube development in mammals.

Keywords

Neural tube closure; laminin; mouse embryo; neural tube defects

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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 post-mitotic 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 perlecan (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; Martins-Green and Erickson, 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 $\alpha 5$ (Miner et al., 1998) and in 5% of laminin $\gamma 1$ 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 epithelia 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 $\beta 1$ transcript exhibits strong expression in the caudal region, with much less intense expression more rostrally (Fig. 1F). In contrast, $\beta 2$ and $\beta 3$ mRNAs have an almost complementary pattern, with expression confined largely to the rostral-most levels of the embryonic axis (Fig. 1G). The α mRNAs, do not show a similar regional specificity, although they exhibit markedly different intensities of expression with strong expression of $\alpha 3$ and $\alpha 4$ (Fig. 1C, D), less intense expression of $\alpha 1$ and $\alpha 5$ (Fig. 1A, E), and almost no expression of $\alpha 2$ (Fig. 1B). Similarly, $\gamma 1$ is more strongly expressed than $\gamma 3$, while $\gamma 2$ 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 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.

2.1.- Laminin chain expression at mandibular arch level

2.1.1- Neural tube expression—Within the closing or recently closed neural tube (Fig. 2A), we detected expression of $\alpha 1$, $\alpha 3$, $\beta 2$ and $\gamma 1$ mRNAs. $\alpha 1$ transcripts are present in the whole neural tube, although expression appears more intense ventrally and close to the luminal surface (Fig. 2B). Immunofluorescence reveals $\alpha 1$ protein in the basement membrane around the entire neural tube. $\alpha 3$ mRNA expression is also enhanced in the

ventral part of the neural tube (Fig. 2D) but, in contrast to $\alpha 1$, the $\alpha 3$ protein was detected only in the portion of the neural tube basement membrane immediately beneath the floor plate (arrowhead in Fig. 2O). $\beta 2$ 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 $\beta 2$ expression in the floor plate correlates exactly with a localised region of low $\alpha 3$ mRNA abundance (arrowhead in Fig. 2D). $\beta 2$ protein also exhibits a stronger signal in the basement membrane around the dorsal part of the neural tube, together with a small area of $\beta 2$ immunofluorescence adjacent to the floor plate (data not shown, but corroborated in other sections, see subsection 2.2.1 and Fig. 3S). $\gamma 1$ mRNA exhibits low intensity, uniform expression throughout the neural folds (Fig. 2J) that correlates with uniform deposition of $\gamma 1$ 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 $\alpha 1$, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 2$, $\gamma 1$, and $\gamma 2$ expression reproducibly. Low intensity $\alpha 1$ mRNA expression (Fig. 2B) is associated with strong, uniform distribution of $\alpha 1$ protein throughout the basement membrane underlying the surface ectoderm (Fig. 2M). $\alpha 3$ mRNA is expressed at low intensity by surface ectoderm, and $\alpha 3$ protein was strongly detected in the underlying basement membrane. $\alpha 5$ 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 $\alpha 5$ protein throughout the underlying basement membrane (Fig. 2Q). $\beta 1$ shows robust mRNA expression in the surface ectoderm and is immunolocalised in the basement membrane underneath (Fig. 2G, R). $\beta 2$ 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). $\beta 2$ protein is strongly present throughout the basement membrane underlying the surface ectoderm (Fig. 2S). $\gamma 1$ transcripts are present throughout the surface ectoderm, with intense expression on the ventral surface of the mandibular arches (Fig. 2J). As with $\beta 2$ expression, $\gamma 1$ mRNA is particularly abundant in the surface ectoderm contacting the dorsal tips of the neural folds (asterisks in Fig. 2H and 2J). Despite these variations in mRNA abundance, $\gamma 1$ protein appears uniform throughout the surface ectoderm basement membrane (Fig. 2U). $\gamma 2$ mRNA is expressed uniformly throughout the surface ectoderm (Fig. 2K), with abundant $\gamma 2$ protein in the underlying basement membrane (Fig. 2V).

2.1.3- Mesenchyme expression—mRNAs for $\alpha 1$, $\alpha 3$, $\alpha 4$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, and $\gamma 3$ are all expressed by the cranial mesenchyme. $\alpha 1$ 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 $\alpha 1$ 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. $\alpha 3$ 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 (arrow in Fig. 2D). Despite this expression, immunofluorescence failed to detect $\alpha 3$ chains in the mesenchyme. $\alpha 4$ exhibits an mRNA pattern that is largely complementary to that of $\alpha 3$, with transcripts detected in the dorso-lateral mesenchyme, and in the $\alpha 3$ -negative area adjacent to the ventral neural tube (Fig. 2E). $\alpha 4$ 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). $\alpha 4$ chains are strongly deposited in vascular tissues and the surface ectoderm basement membrane (Fig. 2P), and in view of the absence of $\alpha 4$ transcripts from the surface ectoderm (Fig. 2E), it seems possible that this laminin is contributed by the mesenchyme. $\beta 1$ exhibits uniform

mRNA expression in the mesenchyme (Fig. 2G), in association with widespread clumps of $\beta 1$ protein-positive cells both adjacent to the neural tube and within the mandibular arches (Fig. 2M). Mesenchymal $\beta 2$ expression is of similar low intensity to $\beta 1$ (Fig. 2H) and is matched by $\beta 2$ protein which is of low abundance in the mesenchyme. However, the intense $\beta 2$ expression in the surface ectoderm basement membrane may derive in part from mesenchymal synthesis. $\gamma 1$ mRNA expression is distributed evenly throughout the mesenchyme (Fig. 2J), and while $\gamma 1$ immunofluorescence is detected in groups of cells within the mandibular arches (Fig. 2U), there is little $\gamma 1$ protein in mesenchyme adjacent to the neural tube. $\gamma 2$ and $\gamma 3$ exhibit very similar, specific mRNA patterns, with expression mainly detected in the mesenchyme that is sandwiched between the dorsal neural tube and surface ectoderm (arrows in Fig. 2K, L). In contrast, $\gamma 2$ and $\gamma 3$ proteins are not seen within the cranial mesenchyme, although both are abundant in the basement membranes around the neural tube and beneath the surface ectoderm (Fig. 2V, W). Whereas $\gamma 2$ protein entirely encircles the neural tube, $\gamma 3$ 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 $\alpha 5$ protein in the neural tube basement membrane (Fig. 2Q). While $\alpha 5$ transcripts are present in surface ectoderm, expression is not detectable in neural tube or cranial mesenchyme (Fig. 2F). It is possible that $\alpha 5$ 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 $\alpha 1$, $\alpha 3$, $\alpha 4$, $\beta 2$, $\beta 3$ and $\gamma 1$ 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. $\alpha 1$ mRNA expression is detected towards the luminal surface of the entire neural tube (Fig. 3B) and, consistent with this, $\alpha 1$ protein is localised throughout the basement membrane surrounding the neural tube (Fig. 3M). $\alpha 3$ and $\alpha 4$ mRNAs are confined to the ventral part of the neural tube, although transcripts are absent from the floor plate (Fig. 3D, E). Neither $\alpha 3$ nor $\alpha 4$ protein is detectable in the basement membrane surrounding the neural tube (Fig. 3O, P). $\beta 2$ transcripts are present throughout the neural tube, with most intense expression at the ventral and dorsal poles (asterisks in Fig. 3H). $\beta 2$ protein is present throughout the neural tube basement membrane (Fig. 3S) together with a small area of $\beta 2$ immunofluorescence within to the floor plate (arrowhead in Fig. 3S). $\beta 3$ transcripts are weakly detected in the neural tube (Fig. 3I), corresponding to weak expression of $\beta 3$ protein around the neural tube (Fig. 3T). $\gamma 1$ mRNA is expressed throughout the neural tube, but with apparently greater intensity either side of the roof plate (asterisk in Fig. 3J). $\gamma 1$ protein appears evenly deposited throughout the neural tube basement membrane (Fig. 3U).

2.2.2- Surface ectoderm expression—mRNAs for $\alpha 1$, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$ and $\gamma 2$ are all detectable in the surface ectoderm at the level of the heart. $\alpha 1$, $\alpha 3$ and $\alpha 5$ are all expressed throughout the surface ectoderm with $\alpha 5$ exhibiting most intense expression and $\alpha 3$ the least intense (Fig. 3B,D,F). All three laminin chains are detected by immunofluorescence throughout the basement membrane underlying the surface ectoderm, although $\alpha 3$ exhibits rather diffuse signal (Fig. 3O) compared with the very specific staining for $\alpha 1$ and $\alpha 5$ (Fig. 3M,Q). $\beta 1$ and $\beta 2$ 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). $\gamma 1$, $\gamma 2$ and $\gamma 3$ mRNAs are all expressed by the surface ectoderm with more intense expression of $\gamma 1$ than $\gamma 2$, and only weak expression of $\gamma 3$ (Fig. 3J, K, L). All three laminin chains: $\gamma 1$, $\gamma 2$ and $\gamma 3$

are present, uniformly and at similar intensity, in the surface ectoderm basement membrane (Fig. 3U,V,W).

2.2.3- Mesenchyme expression—Transcripts for *α1*, *α3*, *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. 3O,P), 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. 4Q). *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 $\alpha 1$ and $\beta 1$ mRNAs are extremely strongly expressed in the paraxial mesenchyme (Fig. 4B,G), there is little or no detectable $\alpha 1$ or $\beta 1$ protein within the mesoderm itself (Fig. 4M,R). The $\alpha 1$ and $\beta 1$ laminin chains may be exported into the adjacent neural tube and surface ectoderm basement membranes which are both strongly positive. Both $\alpha 4$ mRNA and protein are detectable in blood vessel walls (arrowheads in Fig. 4E,P). The notochord expresses $\alpha 5$, $\beta 1$ and $\gamma 1$ mRNAs, corresponding to $\alpha 5$, $\beta 1$ and $\gamma 1$ laminins in the basement membrane surrounding the notochord (Fig. 4Q,R,U). The source of the $\alpha 1$ 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 $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$ and $\gamma 2$ (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. 4M,N,O,P,Q,R,T,U,V).

2.3.4- Discordancy between mRNA and protein expression— $\gamma 2$ 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, $\gamma 3$ laminin is clearly expressed in the surface ectoderm and hindgut basement membranes (Fig. 4W), whereas $\gamma 3$ 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 underlying the surface ectoderm is assumed to be synthesised by surface ectoderm or adjacent mesodermal cells (Fig. 5).

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

Table 2 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 3 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, the neural tube seems to contribute only a single ‘unique’ laminin (521), and even here there is uncertainty as mRNA for laminin $\alpha 5$ was not detected in neural tube cells (see below). In contrast to the cranial level, in the upper spine (heart level) only laminin 121 is contributed by the mesoderm (shared with neural tube) and it appears to supply no ‘unique’ laminins. Most strikingly, at lumbar spine level, we could detect no laminin

variants at all contributed by mesoderm. At this caudal-most level, the basement membrane surrounding the neural tube appears to contain only two laminin variants (111, 511), both synthesized by the neural tube itself.

A similar rostro-caudal trend is seen in relation to the basement membrane underlying the surface ectoderm (Table 3). At mandibular arch and heart levels, mesoderm could supply four different laminin variants (111, 121, 311, 321) although all are shared with the surface ectoderm cells. 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 had closed 24 hours earlier, we also detected laminins 121 and 521, but we could not identify laminin 321. The $\alpha 3$ 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, $\alpha 3$ 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 $\beta 1$ chain, which appears to be part of laminins 111 and 511, and which is absent from the neural tube basement membrane at more rostral levels. While laminin 511 is unique to the caudal-most neural tube basement membrane, laminin 111 is also present more rostrally in the surface ectoderm basement membrane, secreted both by the mesenchyme and surface ectoderm cells. The caudal-most end of the E9.5 embryo (i.e. the tail bud) represents a very early stage of development in several tissue lineages: the neural plate is differentiating from adjacent surface ectoderm, the notochord and hindgut are originating from the remnant of the node, and the lateral mesenchyme is differentiating into presomitic mesoderm (Tam, 1986; Catala et al., 1996; Wilson and Beddington, 1996; Le Douarin et al., 1998). It is striking, therefore, that the predominance of laminins 111 and 511 in the neural tube basement membrane is identical to the laminins previously shown to characterise much earlier developmental stages, including the implanting blastocyst and gastrula (Smyth et al., 1999; Miner and Yurchenco, 2004; Tzu and Marinkovich, 2008). Mouse embryos genetically null for either laminin $\beta 1$ or $\gamma 1$ die soon after implantation, indicating the essential nature of these early-expressed laminins. There is more flexibility about whether $\alpha 1$ or $\alpha 5$ is present as $\alpha 1$ null embryos die later, around E7.5, and over-expression of $\alpha 5$ is able to partially rescue the embryonic defects in $\alpha 1$ nulls, although extraembryonic tissue viability cannot be rescued (Miner et al., 2004). It remains to be determined whether $\beta 1$ - and $\gamma 1$ -containing laminins are also essential for early neural tube development, including the closure process. This would require a conditional gene targeting approach, in view of the earlier lethality of the null embryos.

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 $\alpha 5$ -containing variants, several of which are

expressed by surface ectodermal cells, and are present in the basement membrane at all three axial levels. $\alpha 5$ 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, $\alpha 5$ 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 $\alpha 5$, $\beta 2$ and $\gamma 1$ mRNA expression, suggesting that the critical $\alpha 5$ expression for cranial neurulation and neural crest migration might 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 epithelio-mesenchymal interfaces (Simon-Assmann et al., 1988; Marinkovich et al., 1993). However, in a few cases we detected robust laminin chain expression in the absence of mRNA in adjacent cells. For example, laminin $\alpha 5$ is clearly expressed in the neural tube basement membrane at all levels and yet we could only detect $\alpha 5$ mRNA in sections of the lumbar spinal cord, but not at more rostral levels. Similarly, the $\gamma 2$ chain is expressed in the caudal-most neural tube basement membrane and yet neither neural tube nor adjacent mesenchyme express the mRNA. These findings could be explained by low intensity gene expression that went undetected by our *in situ* hybridization technique. However, an alternative possibility is that some extracellular matrix components may be transported over many cell diameters before being deposited in basement membranes. Hence, the $\alpha 5$ and $\gamma 2$ chains detected in neural tube basement membrane could have originated from surface ectoderm synthesis (Fig. 5). In support of this idea, soluble laminin 111 was able to restore the myotomal basement membrane in mouse embryos null for both *Shh* and *Gli3* (Anderson et al., 2009) and extracellular matrix materials were shown to be transferred between germ layers in quail-chick chimeras (Harrisson et al., 1985). It remains to be determined whether movement of ECM components is a widespread phenomenon and, moreover, what might be the cellular mechanisms of such transport.

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 through 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 $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$, $\gamma 2$ and $\gamma 3$. 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 1, cloned into 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 $7\text{ }\mu\text{m}$, 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. The sections were then processed for immunohistochemistry using primary antibodies specific for laminin chains $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 1$, $\beta 2$ (Sorokin et al., 1992; Schuler and Sorokin, 1995; Sorokin et al., 1997; Sixt et al., 2001; Agrawal et al., 2006), $\alpha 3$, $\alpha 4$, $\beta 3$, $\gamma 2$ and $\gamma 3$ (Talts et al., 2000; Sasaki et al., 2001; Gersdorff et al., 2005a) and $\gamma 1$ (Chemicon) followed by incubation with the appropriate fluorescently tagged secondary antibody. Slides were mounted with Vectashield containing DAPI (Vector Laboratories, Peterborough, UK). Selected sections were photographed with an Olympus BX-61 microscope. Illustrated immunofluorescence patterns are based on serial sections through a minimum of 3 embryos.

Acknowledgments

The authors gratefully acknowledge financial support for this work by the de Instituto de Salud Carlos III project CP08/00111 and PS09/00050 (to PY-G), and by Wellcome Trust grant 06883 (to AJC and NDG). The authors are grateful to Dr Iain Patten for helpful discussions during writing of the manuscript

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Figure 1. Expression of laminin chain mRNAs, as indicated on each panel, by whole mount *in situ* hybridisation 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 μ m.

Figure 2. Expression of laminin chain mRNAs and proteins at the mandibular arch/hindbrain level of E9.5 embryos

(A) Diagram of a representative section at this axial 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: MA, mandibular arch; 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. Scale bar in M = 20 μ m and applies to all other figure parts.

Figure 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 autofluorescence from blood cells. Scale bar in C = 20 μ m and applies to all other figure parts.

Figure 4. Expression of laminin chain mRNAs and proteins at the lumbar 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: HG, hindgut; 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. Scale bar in M = 10 μ m and applies to all other figure parts.

Figure 5.

Diagram showing possible ways in which basement membrane components such as laminins may be generated. Black arrows indicate the conventional view that matrix components are deposited in basement membranes from adjacent epithelial and mesenchymal tissues. The neural tube (blue) and mesoderm (hatched) contribute to the neural tube basement membrane (blue dashed line) while the surface ectoderm (pink) and mesoderm contribute to the surface ectodermal basement membrane (red dashed line). The green arrow indicates a different view, that some molecular components may be transported from non-adjacent tissues. In the present study, $\alpha 5$ and $\gamma 2$ chains may be synthesised in the surface ectoderm and deposited in the neural tube basement membrane.

Table 1
PCR primers used to generate cDNA probes for specific laminin isoforms

Laminin isoform	Forward primer	Reverse primer	GenBank accession no.
$\alpha 1$	5' aggtgaccaatatgtacggg3'	5' agaatccagcctatagagcg3'	J04064
$\alpha 2$	5' caacctgactactccatcc3'	5' cagtgcctgcagttacaaacc3'	NM_008481
$\alpha 3$ *	5' aagcagcatgttgcaagtc3'	5' ttctggactgaggtcattgg3'	XM_981828
$\alpha 4$	5' caattcccatgagtgcttg3'	5' cacttatcacagccttagg3'	NM_010681, XM_125563
$\alpha 5$	5' caatgacacgagagaacagg3'	5' ctacattcctgacaagtggc3'	U37501
$\beta 1$	5' aaggcagtcattcaggacc3'	5' tctgcagacatctgtcatgg3'	NM_008482
$\beta 2$	5' ctgaagctgaaactgatcg3'	5' gggatcaatgtttccactgc3'	BC026051
$\beta 3$	5' gtcacagaatgatgtgagcc3'	5' tagaacctccaggattagg3'	BC008516
$\gamma 1$	5' ttggccattcttctgtgtgc3'	5' accacggatcttgatagagg3'	J02930
$\gamma 2$	5' ttccaagtgtgactgtgacc3'	5' aggtccgcagtaaccttcg3'	NM_008485
$\gamma 3$	5' atactgacctccaagtacc3'	5' tctctctgtatcccagagc3'	BC096366

* Two different $\alpha 3$ 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 $\alpha 3A$ from $\alpha 3B$. Hence, both chains are described as laminin $\alpha 3$.

Table 2
Distribution of laminin chain mRNAs and proteins

	Mandibular arch level						Heart level						Lumbar spine level					
	NT			M			NT			M			NT			M**		
	mR	P	mR	mR	P	mR	mR	P	mR	mR	P	mR	mR	P	mR	mR	P	mR
LC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α5	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β1	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
β2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β3	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
γ1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
γ2	-	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
γ3	-	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-

Abbreviations: LC : Laminin chain; NT, neural tube; M, mesoderm; SE, surface ectoderm. mR: mRNA; P: Protein deposited in the basement membrane.

* Expression in mesoderm adjacent to neural tube only

** Paraxial mesoderm only

Table 3
Possible laminin variants in basement membranes and their tissue of origin

Mandibular arch level			Heart level			Lumbar spine level		
N. tube BM		S. ect BM	N. tube BM		S. ect BM	N. tube BM		S. ect BM
NT	Mes	Mes	S. ect	NT	Mes	S. ect	NT	Mes
121	121	111	111	121	111	111	111	111
321	122	121	121	131	121	121	511	121
521 *	123	311	112	521 *	311	112		112
	321	321	122	531 *	321	113		122
	322		311			122		311
	323		321			123		321
			312			311		312
			322			321		322
			511			312		511
			521			322		521
			512			313		512
			522			323		522
						511		
						521		
						512		
						522		
						513		
						523		

Abbreviations: BM, basement membrane ; NT, neural tube; Mes, mesoderm; S. ect, surface ectoderm.

* These laminin variants are included because $\alpha 5$ 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 $\alpha 5$ chains (see Discussion).