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Abnormalities in neural crest cell migration in *laminin α5* mutant mice

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Abstract

Although numerous in vitro experiments suggest that extracellular matrix molecules like laminin can influence neural crest migration, little is known about their function in the embryo. Here, we show that *laminin α5*, a gene up-regulated during neural crest induction, is localized in regions of newly formed cranial and trunk neural folds and adjacent neural crest migratory pathways in a manner largely conserved between chick and mouse. In *laminin α5* mutant mice, neural crest migratory streams appear expanded in width compared to wild type. Conversely, neural folds exposed to laminin α5 in vitro show a reduction by half in the number of migratory neural crest cells. During gangliogenesis, *laminin α5* mutants exhibit defects in condensing cranial sensory and trunk sympathetic ganglia. However, ganglia apparently recover at later stages. These data suggest that the *laminin α5* subunit functions as a cue that restricts neural crest cells, focusing their migratory pathways and condensation into ganglia. Thus, it is required for proper migration and timely differentiation of some neural crest populations.
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Introduction

Presumptive neural crest cells form at the border between the neural plate and non-neural ectoderm by an inductive interaction between these two tissues (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Knecht and Bronner-Fraser, 2002). During the transformation of the neural plate into the brain and spinal cord, these precursors come to lie in the dorsal portion of the neural tube. Subsequently, neural crest cells delaminate from the neuroepithelium by undergoing an epithelial-to-mesenchymal transition. After emigration, these migratory cells move in a highly patterned fashion through neighboring tissues and arrive at specific sites, where they differentiate into a wide variety of derivatives including neurons and glia of the peripheral nervous system (Bronner-Fraser and Fraser, 1988; Stemple and Anderson, 1992; Christiansen et al., 2000; Basch et al., 2004).

Separation of neural crest cells from the dorsal neural epithelium and their locomotion though the embryo involves alterations in cell–cell adhesion, changes in cell–matrix interactions and reorganization of the extracellular matrix (ECM) (Le Douarin and Kalcheim, 1999; Perris and Perissinotto, 2000). A number of candidate permissive and inhibitory ECM molecules have been implicated in aspects of neural crest migration. Matrix glycoproteins thought to promote neural crest migration include fibronectin (Duband et al., 1986; Newgreen, 1989), collagen types I and IV (Perris et al., 1993a,b), tenascin (Tan et al., 1987; Bronner-Fraser, 1988) and laminin (Duband and Thiery, 1987). All of these components contribute to the highly organized ECM scaffolding contacted by neural crest cells as they migrate. In contrast, non-permissive cues are present in regions where crest cells fail to migrate and may act as barriers to migratory crest cells. Extracellular matrix molecules that prevent the attachment and migration of neural crest cells include aggrecan, versican (Landolt et al., 1995; Perris et al., 1991, 1996b) and collagen type IX (Ring et al., 1996).

The laminin family of glycoproteins represents a major component of the interstitial ECM as well as basement membranes (basal laminae) ECM. Laminins have been implicated in a broad array of biological processes including cell adhesion, migration, angiogenesis, differentiation, tumor growth and metastasis (Colognato and Yurchenco, 2000; Miner and Yurchenco, 2004). Laminins exist in the ECM as heterotrimers containing one α-, one β- and one γ-chain that assemble with each other via their coiled-coil domains to form

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a cross-like structure (Colognato and Yurchenco, 2000). To date, 5 distinct α-chains, 4 β-chains and 3 γ-chains have been shown to form at least 15 different laminin isoforms (Miner et al., 1997; Patton et al., 1997; Miner and Yurchenco, 2004). Laminin proteins have been suggested to play a role in neural crest cell emigration, specifically at cranial levels, as well as during gangliogenesis (Bronner-Fraser, 1985, 1986; Bronner-Fraser and Lallier, 1988; Lallier et al., 1994; Perris and Perissinotto, 2000). Moreover, different laminin heterotrimers have varying effects on neural crest cell migration in vitro; laminin-1 (α1 β1 γ1) and -8 (α4 β1 γ1) promote early neural crest cell migration, whereas laminin-2 (α2 β1 γ1), -4 (α2 β2 γ1) and -9 (α4 β2 γ1) do not (Lallier et al., 1994; Desban and Duband, 1997). More recently, the distribution of individual laminin chains has been reported in the chick; α1 chain transcripts are widely distributed in the early chick embryo, α4 chain is in the neural tube, and α5 chain is in the dorsal aspect of the neural tube (Gammill and Bronner-Fraser, 2002). Despite the evidence demonstrating the importance of laminins in neural crest development, it has been difficult to identify the precise function(s) of any one laminin subunit due to compensation by other chains and/or laminin heterotrimer(s) masking or buffering the phenotype that would otherwise result from the loss of one subunit alone. Little information is available regarding laminin loss-of-function phenotypes during stages of neural crest migration.

It is likely that different laminin isoforms individually and/or in combination will influence different aspects of neural crest development from maturation to emigration, migration or cessation. To address these issues, we have examined the function of the α5 subunit of laminin during neural crest cell development. This subunit was previously identified in a screen for genes up-regulated in response to neural crest induction (Gammill and Bronner-Fraser, 2002). In chick and mouse, the temporal and spatial expression pattern of *laminin α5* is consistent with a function in the emigration and/or migration of neural crest cells from the dorsal neural tube to their final location in the developing embryo. In *laminin α5* null mice, there is an expansion in neural crest migratory streams, suggesting that the normal function of this subunit may be to focus or restrict neural crest migration pathways. Conversely, culturing murine neural folds in the presence of laminin α5 subunit in vitro results in a reduction in the numbers of migratory neural crest cells by half. *Laminin α5*-deficient mice exhibit a delay in gangliogenesis of the peripheral ganglia compared to wild type. These data demonstrate that proper regulation of the α5 subunit of laminin is critical for normal neural crest emigration and timely differentiation.

Materials and methods

Analysis of mutant embryos

Mutant mice were generated as previously described (Miner et al., 1998). Heterozygous *laminin α5* mice were crossed to obtain homozygous progeny. Embryos were dissected between E8 and E11 days of development in Ringers solution and fixed in 4% paraformaldehyde prior to processing by in situ hybridization or immunohistochemistry. Since the developmental stage of a litter can vary, embryos were staged in accordance with described criteria (Kaufman and Bard, 1999). Embryos were genotyped by PCR using genomic DNA extracted from the allantois as previously described (Miner et al., 1998).

Whole-mount in situ hybridization

Briefly, to determine gene expression patterns for both chick and mouse embryos, linearized cDNAs were used to synthesize digoxigenin labeled antisense RNA probes and whole-mount in situ hybridizations performed using 'Protocol Four' as previously described (Xu and Wilkinson, 1998). Antisense probes against chick *laminin α5* (5kb), mouse *laminin α5* (392bp), mouse *Sox10* (2.2kb), mouse *Snail* (~500bp) and mouse *NeuroD* (1.6kb) were transcribed. The 2.2kb mouse Sox10 probe was hydrolyzed prior to use. Stained embryos were imaged in 70% glycerol using a camera mounted on a Zeiss Stemi SV11 microscope. Images were processed using Adobe Photoshop 7.0 (Adobe Systems).

Immunohistochemistry

Chick embryos processed by whole-mount in situ hybridization were subsequently stained with the migratory chick neural crest cell marker HNK-1. Embryos were cryostat sectioned at 14 μm and then incubated with mouse-anti-HNK-1 (American Type Culture, 1:200), which recognizes chick migratory neural crest cells (Sechrist et al., 1995). Mouse neural fold explants were incubated with rabbit-anti-p75 (gift from Lou Reichardt, 1:2000), which recognizes migratory mouse neural crest cells and nuclei were stained with DAPI. Bright-field and fluorescent images of sections were captured using a Zeiss Axiocam mounted on a Zeiss Axioskop 2 microscope and processed using Adobe Photoshop.

Mouse embryos were processed with the rabbit-anti-laminin α5 antibody (J. Miner, 1:600) with a Fluorochrome-conjugated Alexa-Fluor 488 donkey-anti-rabbit secondary antibody (Molecular Probes, 1:500) as previously described (Miner et al., 1997). Sections were stained with DAPI and mounted in PermaFluor Mountant Medium (Thermo) prior to imaging. The mouse anti-neuron-specific class III β-tubulin antibody, Tuj1 (IgG2a, BABCO, 1:500), was used as previously described (De Bellard et al., 2002), and a Alexa-Fluor 488 goat-anti-mouse IgG2a secondary antibody (Molecular Probes, 1:200). Embryos were z scanned using a Zeiss LSM410 confocal microscope (Beckman Imaging Center, Caltech) and projected (NIH-Image) into a single image.

Cell counts of Sox10 expressing cells

E8.5 wild-type and *laminin α5* null mouse embryos that had been hybridized with the *Sox10* probe were imaged, then embedded in gelatin and sectioned at 14 μm. These sections were stained with DAPI to enable the identification of individual nuclei and mounted for analysis. In cranial regions where neural crest cell migration had initiated, a minimum of 10 images of serial sections at the same cranial axial level were captured for 3 (8, 9 and 10 somites) wild-type and 3 (8, 9 and 10 somites) *laminin α5* mutant embryos. Every DAPI-positive nuclei surrounded by cytoplasmic *Sox10* staining was marked, counted and the numbers recorded. The number of *Sox10*-positive neural crest cells was determined for each embryo and averaged with like stage and genotyped embryo. To determine the variability of the data set, the standard deviation based on the entire population was calculated (Microsoft Excel).

In vitro migration assay

Mouse cranial neural folds from 8 dpc (5–9 somites) were isolated and cultured as previously described (De Bellard et al., 2003). Glass culture dishes were coated with 10 μg/ml of either Cultrex® mouse laminin-1 (R&D Systems) or the purest commercially available form of laminin-10, derived from human placenta (Sigma®). Neural crest cells cultured on laminin-1 in vitro have properties very similar to fibronectin (Desban and Duband, 1997). Explanted tissue was cultured in complete neural crest media without retinoic acid (Stemple and Anderson, 1992) for 24 h, the next day, migratory neural crest cells could be seen as a halo around the explants. Cells were fixed for 1 h with 4% PFA prior to immunological processing.

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