Integrin α6β1-laminin interactions regulate early myotome formation in the mouse embryo

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We addressed the potential role of cell-laminin interactions during epaxial myotome formation in the mouse embryo. Assembly of the myotomal laminin matrix occurs as epaxial myogenic precursor cells enter the myotome. Most Myf5-positive and myogenin-negative myogenic precursor cells localise near assembled laminin, while myogenin-expressing cells are located either away from this matrix or in areas where it is being assembled. In Myf5nlacZ/nlacZ (Myf5-null) embryos, laminin, collagen type IV and perlecan are present extracellularly near myogenic precursor cells, but do not form a basement membrane and cells are not contained in the myotomal compartment. Unlike wild-type myogenic precursor cells, Myf5-null cells do not express the α6β1 integrin, a laminin receptor, suggesting that integrin α6β1-laminin interactions are required for myotomal laminin matrix assembly. Blocking α6β1-laminin binding in cultured wild-type mouse embryo explants resulted in dispersion of Myf5-positive cells, a phenotype also seen in Myf5nlacZ/nlacZ embryos. Furthermore, inhibition of α6β1 resulted in an increase in Myf5 protein and ectopic myogenin expression in dermomyotomal cells, suggesting that αβ1-laminin interactions normally repress myogenesis in the dermomyotome. We conclude that Myf5 is required for maintaining α6β1 expression on myogenic precursor cells, and that α6β1 is necessary for myotomal laminin matrix assembly and cell guidance into the myotome. Engagement of laminin by α6β1 also plays a role in maintaining the undifferentiated state of cells in the dermomyotome prior to their entry into the myotome.

KEY WORDS: Integrin, Laminin, Mouse embryo, Myotome, Dermomyotome, Extracellular matrix, Myf5

INTRODUCTION

Trunk muscles such as the deep back and intercostal muscles are derived from the myotomes. These are transiently segmented structures that form when cells in the dermomyotome, in response to epaxial and hypaxial signals, translocate into the underlying myotomal space. Recent models for myotome formation propose that the first myogenic precursor cells (MPCs) delaminate from the epaxial lip of the dermomyotome, migrate to the space underneath it and elongate bi-directionally to form myocytes. Later, MPCs from all lips of the dermomyotome give rise to sequential waves of myocytes populating the myotome (Denetclaw et al., 1997; Kahane et al., 1998; Venters et al., 1999; Gros et al., 2004). Epaxial myotome formation is dependent on the myogenic regulatory factor (MRF) Myf5. In Myf5nlacZ/nlacZ (Myf5-null) mouse embryos, the epaxial MPCs delaminate from the dermomyotome but fail to form the myotome (Tajbakhsh et al., 1996). Rather, MPCs accumulate around the epaxial edge of the dermomyotome or migrate aberrantly. The myotome is normally delimited by a laminin-rich basement membrane that separates it from the underlying sclerotome (Tosney et al., 1994; Bajanca et al., 2004). The role of this laminin-rich matrix during myotome formation is unknown, but, strikingly, it does not form in Myf5nlacZ/nlacZ embryos (Tajbakhsh et al., 1996).

Laminins are a family of extracellular matrix (ECM) glycoproteins, composed of α, β and γ chains, which associate to form a trimeric cross-like structure. To date, five α, three β and three γ chains have been described that associate to form 12 different laminin isoforms (reviewed by Tunggal et al., 2000; Ekblom et al., 2003). Laminins, together with collagen type IV, perlecan and nidogen, are the major components of basement membranes. Studies using C2C12 myoblasts and embryonic stem cells suggest that laminin matrix formation is dependent on specific interactions with cellular receptors (Sasaki et al., 1998; Fleischmajer et al., 1998; Cachaço et al., 2005), dystroglycan, for an initial recruitment of laminin molecules, and β1 integrins, supporting their assembly (Cochaço et al., 1999; Henry et al., 2001). Furthermore, assembly of the laminin matrix is essential for the organisation of the remaining basement membrane components into a continuous basement membrane (Yurchenco et al., 2004; Yurchenco and Wadhsworth, 2004).

Cells sense their ECM environment through cell-surface receptors, the most common of which belong to the integrin family (Miranti and Brugge, 2002). Integrins are heterodimers composed of an α and a β subunit, which together determine the ligand specificity of each integrin (Hynes, 1992) (reviewed by van der Flier and Sonnenberg, 2001). The extracellular region of an integrin binds to specific ECM ligands, while the intracellular region interacts with the cytoskeleton and associated proteins, and can modulate numerous intracellular signalling pathways, regulating processes such as survival, proliferation, migration and differentiation (reviewed by Miranti and Brugge, 2002; Danen and Sonnenberg, 2003). Integrin expression patterns change during the course of skeletal muscle development (Gullberg et al., 1998; Bajanca and Thorsteinsdóttir, 2002; Bajanca et al., 2004), suggesting constant modulation of cell-ECM interactions during the different stages of myogenesis. Although the exact roles of these
cell-ECM interactions in vivo are not well understood, it is clear that \(\beta_1\) integrins play a role in skeletal muscle development. When the ubiquitously expressed \(\beta_1\)A subunit is substituted by the striated muscle specific splice variant \(\beta_1D\) (van der Flier et al., 1997), primary myogenesis is impaired, leading to a reduction in muscle mass (Cachazo et al., 2003). Furthermore, when the integrin \(\beta_1\) gene (Igb1) is inactivated specifically in myogenic cells, mutant animals die at birth with highly underdeveloped muscles (Schwander et al., 2003).

Recently, we described the expression patterns of several \(\beta_1\) integrins during myotome formation in the mouse. The laminin receptor, \(\alpha_6\beta_1\), is expressed in epithelial somites and in the dermomyotome, and remains strongly expressed on MPCs that colonise the epaxial myotome (Bajanca et al., 2004). In the present study, we analysed the normal pattern of laminin matrix assembly in the mouse myotome. We then addressed the issue of why myotomal laminin assembly fails in \(\text{M}yf_{5}\text{nlacZ}^{\text{nlacZ}}\) embryos. We show that the major basement membrane components are present around \(\text{M}yf_{5}\)-null cells, but \(\alpha_6\beta_1\) integrin is absent from MPCs when they delaminate from the dermomyotome. Blocking \(\alpha_6\beta_1\) binding to laminin in cultured explants of wild-type mouse embryos resulted in dispersion of \(\text{M}yf_{5}\)-expressing MPCs, a phenotype resembling the one observed in \(\text{M}yf_{5}\text{nlacZ}^{\text{nlacZ}}\) embryos. Surprisingly, when binding of \(\alpha_6\beta_1\) to laminin was blocked, we observed an increase in \(\text{M}yf_{5}\)-expressing cells and ectopic myogenin expression in the dermomyotome. Together, our results suggest that \(\alpha_6\beta_1\)-laminin interactions are involved not only in myotome formation, but that they also prevent precocious myogenesis in the dermomyotome.

### MATERIALS AND METHODS

#### Mice, embryo collection and genotyping

Gene targeting of the \(\text{M}yf_{5}\) locus with the \(\text{nlacZ}\) reporter gene has been described previously (Tajbaksh et al., 1996). Owing to its proximity to \(\text{M}yf_{5}\), within the same locus, the \(\text{M}yf_{5}\) gene is also affected in these mutants. These \(\text{M}yf_{5}\text{nlacZ}^{\text{nlacZ}}\) embryos are thus functional knockouts for \(\text{M}yf_{5}\) and \(\text{M}yf_{4}\) in the early embryo (Kassar-Duchossoy et al., 2004). In normal embryos, \(\text{M}yf_{4}\) mRNA transcripts only start being detected at E9.5, so \(\text{M}yf_{5}\) protein is the only MRK driving epaxial myotome formation before that time. Furthermore, in \(\text{M}yf_{5}\) mutant mice where the \(\text{M}yf_{4}\) locus is not affected, \(\text{M}yf_{4}\) expression is not sufficient to rescue normal epaxial myotome development (Kassar-Duchossoy et al., 2004). Thus, it is unlikely that defects in epaxial myotome formation observed in \(\text{M}yf_{5}\text{nlacZ}^{\text{nlacZ}}\) embryos are due to the absence of \(\text{M}yf_{4}\). Rather, they should be due to the absence of \(\text{M}yf_{5}\) protein.

Embryos were collected from crossings between \(\text{M}yf_{5}^{\text{nlacZ}^{\text{nlacZ}}}\) animals. The day of the vaginal plug was designated as embryonic day (E) 0.5. Embryos were staged as described by Houzelstein et al. (Houzelstein et al., 1999). DNA was isolated from yolk sacs and genotyping was carried out as described (Tajbaksh et al., 1997). Wild-type embryos were obtained from crossings of outbred Hsd:ICR(CD-1) mice (Harlan Interfauna Iberica).

#### Immunohistochemistry and \(\beta\)-galactosidase staining

Immunohistochemistry on cryostat (Bright Clinicut) sections (10 or 30 \(\mu\)m) and whole mounts was performed as described by Bajanca et al. (Bajanca et al., 2004). Primary antibodies were: anti-\(\beta\)-galactosidase (Promega), anti-EHS-laminin (Sigma), anti-collagen type IV (Chemicon), anti-\(\alpha_1\beta_1\) integrin (L-16; Santa Cruz Biotechnology), anti-myogenin (F5D; Santa Cruz Biotechnology) and anti-laminin \(\alpha_5\) chain (AL-4; Chemicon) diluted 1:20; and anti-Myf5 (C-20; Santa Cruz Biotechnology) diluted 1:2000; anti-myosin (F-59; a gift from F. Stockdale) diluted 1:20; and anti-desmin (D3; Developmental Studies Hybridoma Bank) and anti-laminin \(\alpha_1\) chain (7B83) (Sonnenberg et al., 1986) as undiluted supernatants. The polyclonal anti-EHS-laminin antibody raised against laminin 1 chain composition \(\alpha_1\beta_1\gamma_1\) recognises the \(\alpha_1\), \(\beta_1\) and \(\gamma_1\) chains, but may also crossreact with other laminins (Paulsson, 1994).

Secondary antibodies were Alexa Fluor 568-conjugated anti-mouse IgG, Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 546-conjugated anti-rabbit IgG, all F(ab’2) fragments (Molecular Probes), diluted 1:1000; FITC-conjugated anti-rat IgG and anti-goat IgG (Sigma), diluted 1:100. Some slides were stained with 4,6-Diamidino-2-phenylindole (DAPI, Sigma). \(\beta\)-Galactosidase staining with X-gal substrate was performed as described by Hogan et al. (Hogan et al., 1986).

#### Experimental interfering with \(\alpha_6\beta_1\) integrin-laminin interactions

Wild-type E8.0-E9.5 embryos were collected in cold culture medium with 10 mM HEPES and immediately processed. The head region above the otic vesicles and the visceral were removed, but all structures surrounding the somites were left intact. Explants were prepared from two littermates with the same number of somites, one being the control explant and the other the experimental one. All explants were placed on a Millipore filter (pore size 0.8 \(\mu\)m) in DMEM/Ham’s F12 medium (Gibco) supplemented with 4 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin and 8 mg/ml streptomycin, and cultured at 37 °C in a 5% CO2 atmosphere (Kil et al., 1998; Webb et al., 2002). The GoH3 rat monoclonal antibody against the \(\alpha_6\) integrin subunit (Sonnenberg et al., 1987; Sonnenberg et al., 1990) specifically blocks the binding of \(\alpha_6\beta_1\) to laminin, without causing activation of the integrin (e.g. Sonnenberg et al., 1988; Sonnenberg et al., 1990; Burrows et al., 1995; Falk et al., 1996; Jiang et al., 2001). Pilot experiments were performed with several different concentrations of GoH3. A 1:10 dilution of the supernatant and 20 \(\mu\)g/ml of purified antibody provided the most reproducible results and were used in all experiments. Control explants were cultured in: (1) medium only, (2) medium with a 1:10 dilution of the supernatant containing the rat monoclonal antibody CA5 against integrin \(\alpha_7\) (absent at this stage) (Bajanca et al., 2004) or (3) medium with 20 \(\mu\)g/ml of J5E3 (rat monoclonal antibody against an irrelevant 50 kDa cell surface antigen) (Sonnenberg et al., 1986). Incubation periods ranged from 14 to 24 hours. Penetration of the GoH3 antibody was verified by exposing some GoH3-treated explants to a FITC-conjugated anti-rat IgG antibody. Immunoreactivity was observed in the interior of the explants, on cells of the dermomyotome and myotome among others (data not shown).

Control experiments were performed to determine the quality of the culture conditions: immediately fixed (i.e. not cultured) explants, control explants and experimental explants were compared in terms of apoptosis levels (TUNEL assay, Roche), proliferation (phospho-H3 immunohistochemistry; Upstate) and cell and tissue morphology (phalloidin and DAPI staining). Although apoptosis levels were slightly increased in cultured explants compared with immediately fixed ones, no difference was observed between control explants and explants cultured with GoH3. There was no difference between the proliferation levels and morphology of \(\alpha_6\)-negative embryonic structures between explant types, indicating that GoH3 did not have non-specific effects.

#### Imaging

Sections processed for immunohistochemistry were photographed using an Olympus DP50 digital camera coupled to an Olympus BX60 microscope equipped with Normaski optics and epifluorescence. Optical \(z\)-series of whole mount embryos and thick cryostat sections were obtained in a Leica SP2 confocal microscope. Embryos stained with X-gal were photographed using an Olympus Camedia C-4040 digital camera coupled to a Wild M8 stereo microscope. Images were edited in Adobe Photoshop 7.0.

Quantification of \(\text{Myf}_{5}\) and myogenin immunoreactivity in dermomyotomes was performed using the ImageJ software on unprocessed images of embryo sections. The mean grey-level intensity of dermomyotomes (including or excluding lips) and neural tube was calculated for each section. The neural tube was selected to measure nonspecific fluorescence as it has a similar cell density, but has no specific immunoreactivity for \(\text{Myf}_{5}\) and myogenin. To compensate for differences in fluorescence intensity among embryos and sections, we computed a ratio of fluorescence intensity among sections of 'mean fluorescence intensity for dermomyotome/neural tube' for each section. These ratios represent the \(n\)-fold increase of \(\text{Myf}_{5}\) or myogenin in the dermomyotome compared with the neural tube. Fluorescence intensity was measured in 7-26 sections from each control (\(n=3\) for \(\text{Myf}_{5}\); \(n=4\) for...
RESULTS
Myogenic cells interact with laminin at several stages of myotome formation
We used co-immunohistochemistry with antibodies against laminins and against markers for different phases of myogenesis, to analyse how laminin distribution correlates with myotome formation in normal embryos. Before myotome formation, laminin is restricted to the basal, i.e. dorsal, surface of the dermomyotome (Tosney et al., 1994) (data not shown). As the first MPCs delaminate from the epaxial dermomyotome to form the myotome, a laminin matrix starts being laid down (Fig. 1A,B). As more cells enter the myotome, this matrix becomes sheet like, separating the epaxial myotome from the sclerotome (Fig. 1C,D). The myotomal laminin matrix, detected with antibodies against laminin α5-, α1- and β1-chains, is then found along the full extent of the epaxial myotome (Fig. 1E-G).

Immunoreactivity for the laminin α2-chain was not detected in the myotome at E9.5 (data not shown). Therefore the basement membranes delimiting the dermomyotome dorsally and separating the myotome from the sclerotome at E9.5 contain laminin 1 (α1β1γ1), and laminin 10 (α5β1γ1), but not α2-chain laminins (laminin 2, laminin 4 and laminin 12).

Co-immunohistochemistry for laminins and Myf5 shows that Myf5-positive cells are often in close contact with the laminin-containing myotomal basement membrane (arrows in Fig. 1F,G). Confocal sectioning through a sagittal plane confirms that Myf5-positive cells are rare in the area of the myotome closest to the dermomyotome (Fig. 1H) and that most Myf5-positive cells within the myotome are located near the myotome-sclerotome interface (Fig. 11-K) (Venters et al., 1999), the area where the myotomal laminin matrix is localised (Fig. 1E-G,L-M). Most myogenin-positive cells are located away from the myotome-sclerotome interface (compare progression from Fig. 1H-J) (Venters et al., 1999) and, thus, far from the laminin matrix. However, the central band of myogenin-positive nuclei reaches this interface in the middle of the myotome.

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Fig. 1. Characterisation of laminin matrix assembly in the myotome and its interaction with myotomal cells. (A-D) A Myf5nlacZ/+ embryo at E9.5 processed for whole-mount immunostaining for β-gal (A,C) and laminin (A-D; polyclonal antibody). Confocal imaging showing a sagittal plane of the myotome in caudal (A,B) and interlimb (C,D) somites. z-series projection of: (A) 10×1 μm, (B) 6×1 μm (starting 1 μm ventral to last section in A) and (C,D) 24×1 μm optical sections. Shortly after the myotome (white cells in A) begins to form, a laminin matrix appears under these cells (B). In an interlimb somite of the same embryo, more cells have entered the myotome (C) and the laminin matrix accompanies this growth (C,D). (E-M) Transverse (E-G), sagittal (H-J) and longitudinal (K-M) cryostat sections of interlimb myotomes showing a sagittal plane of the myotome in: (A) 24×1 μm optical sections.

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myotome (arrowheads in Fig. 1I–K). It is notable that most of the Myf5-positive cells localised at this interface are positioned rostral and caudal to the central area containing myogenin-positive nuclei (Fig. 1H–K). Cells co-expressing Myf5 and myogenin are preferentially found centrally, but close to the sclerotome (arrowheads in Fig. 1I,J), suggesting that cells are beginning myogenic differentiation at this location. Interestingly, in this area, the laminin matrix is discontinuous (Fig. 1L,M), suggesting that it is an area where new laminin matrix is assembled to accommodate for the medial growth of the myotome. Myogenin-positive cells located in this area are not full length (arrowheads in Fig. 1L), but are elongating, as shown by desmin labelling at the cell extremities (arrows in Fig. 1M), which are in contact with the assembled laminin matrix at the myotome-sclerotome interface (arrows in Fig. 1M).

**Basement membrane components are produced, but fail to assemble correctly in Myf5nlacZ/nlacZ embryos**

MPCs of Myf5nlacZ/nlacZ embryos delaminate from the dermomyotome, but disperse and fail to form a myotome; no laminin-rich myotomal basement membrane is detected at E11.5 (Tajbakhsh et al., 1996). Thus, the absence of laminin could contribute to the phenotype of Myf5nlacZ/nlacZ embryos.

We first determined whether laminin was present near Myf5-null cells at early stages of myotome development and confirmed that assembled laminin is absent both near the β-galactosidase (β-gal)-positive cells and at the sclerotome interface in E9.5 Myf5nlacZ/nlacZ embryos (compare Fig. 2B,E with 2A,D).

The absence of a laminin-rich basement membrane adjacent to Myf5-null cells could be due to one of several causes (reviewed by Ghosh and Stack, 2000; Tunggal et al., 2000). First, production of laminin chains might not occur, suggesting that Myf5, directly or indirectly, regulates transcription of laminin (Lam) genes. Second, intracellular assembly or secretion of the laminin trimer might be affected. Finally, extracellular assembly of laminin molecules into a basement membrane might be impaired. A high magnification of cryosections immunolabelled for β-gal and laminin shows the presence of laminin near β-gal-positive MPCs in Myf5nlacZ/nlacZ embryos (Fig. 2C). This indicates that laminin molecules are produced and secreted but are not assembled.

In normal embryos, the myotomal basement membrane is not only rich in laminin (Fig. 2A,D), but also in collagen type IV (Fig. 2F) and perlecan (Fig. 2H). Although a continuous basement membrane is not formed in Myf5nlacZ/nlacZ embryos, there is evident immunoreactivity for laminin, perlecan and collagen type IV (Fig. 2E,E’,G,G’,I,I’). In fact, collagen type IV (Fig. 2G,G’) and perlecan...
(Fig. 2I,I') form patches among Myf5-null MPCs (Fig. 2G,I). However, β-gal-positive Myf5-null cells disperse through this matrix (Fig. 2G,I), which, unlike the normal basement membrane, does not appear to restrict cells successfully within the myotomal space. Our results thus suggest that the major basement membrane components are present in the myotomal area of Myf5nlacZ/nlacZ embryos but a functional basement membrane fails to assemble.

**Myf5-null MPCs fail to maintain α6β1 integrin expression when they detach from the dermomyotome**

The laminin receptor integrin α6β1 is strongly expressed by early epaxial MPCs (Fig. 3A,C) (Bajanca et al., 2004). This integrin is normally present on dermomyotomal and myotomal cells (Fig. 3A,C) but, although present on dermomyotomal cells in Myf5nlacZ/nlacZ embryos, it is undetectable on most MPCs (β-gal-positive cells) that have delaminated from the epaxial lip of the dermomyotome (arrows in Fig. 3B,D). These results suggest that, while (or soon after) delaminating from the dermomyotome, Myf5-null MPCs are not able to maintain α6β1 on their cell surface, and thus lose the ability to interact with extracellular laminin through this integrin. This may result in: (1) the impairment of laminin assembly; and, in the absence of a basement membrane barrier, (2) cell dispersal.

**Blocking α6β1-laminin binding perturbs myotome formation**

We hypothesised that the defect in myotome colonisation and laminin matrix formation in Myf5nlacZ/nlacZ embryos could be due to a failure of MPCs to bind laminin. To test this hypothesis, we used a well characterised integrin α6-blocking antibody (GoH3) to inhibit specifically α6β1 binding to laminin in wild type (i.e. Myf5-expressing) embryo explants cultured in vitro.

The expression of Myf5, laminin and desmin, shows that myotome development is normal in control explants in culture (Fig. 4A,C,G-I), whereas the presence of GoH3 perturbed myotome formation (Fig. 4B,D,J-L).

When E8.0 embryo explants are cultured with GoH3 for 14 hours, the Myf5-positive MPCs that delaminated from the dermomyotome are found near the neural tube instead of being positioned in the myotomal area (arrows in Fig. 4B). When slightly older (E9.5) embryo explants were cultured with GoH3 for 24 hours, a dramatic scattering of Myf5-positive cells was observed (compare Fig. 4G with 4J) and very few Myf5-expressing cells are found in the central area of the myotome (bracket in Fig. 4J). Interestingly, this distribution pattern of Myf5-positive cells is very similar to the distribution of MPCs in Myf5nlacZ/nlacZ embryos (compare Fig. 4J with 4F).

The pattern of desmin immunoreactivity is also disorganised in GoH3-treated explants compared with controls. Some dispersed cells upregulate desmin but elongated cells were not detected among them (Fig. 4K,L). The same pattern is detected in the central myotomes, suggesting that the differentiation pattern of cells that were already there at the time of the addition of the antibody is perturbed. Myosin immunoreactivity is unperturbed in GoH3-treated explants (data not shown), suggesting that fully elongated myocytes are unaffected by GoH3.

We hypothesised that the myotomal laminin matrix might constitute a barrier restraining myogenic cells inside the myotomal space. The immunoreactivity for laminin is discontinuous and diffuse in GoH3-treated embryos both in the myotomal basement membrane (compare Fig. 4D with 4C) and at the epaxial lip (arrowheads in Fig. 4C,D).

Our results thus suggest that α6β1-laminin interactions are necessary for the normal translocation of MPCs from the dermomyotome into the myotome, for the normal differentiation of cells in the myotome and for laminin assembly at the myotome-sclerotome interface.

**Blocking α6β1-laminin binding leads to a precocious activation of the myogenic programme in dermomyotomal cells**

We noticed that in embryo explants cultured with GoH3, the expression of Myf5 was often unusually widespread in the dermomyotome (compare Fig. 4A with 4B), raising the possibility that dermomyotomal cells might be differentiating precociously. To address this, we cultured explants of E9.0 embryos with GoH3 for 14 hours and tested for the expression of Myf5 and myogenin. Control explants showed a normal expression pattern for these proteins (Fig. 5A,C). Myf5 is expressed in the myotome and in dermomyotomal lips just prior to MPC delamination and entry into the myotomal space (Ott et al., 1991; Venters et al., 1999) and myogenin is upregulated in myotomal cells (Fig. 5A,C). Embryos incubated with GoH3, showed a striking increase in Myf5 expression and, surprisingly, an ectopic expression of myogenin in the dermomyotome (Fig. 5B,D). This effect is not restricted to the epaxial lip in this experiment, but extends towards the hypaxial dermomyotome. Quantification of the fluorescence intensity in the whole dermomyotome showed an increase in both Myf5 and myogenin fluorescence in the presence of GoH3 (Fig. 5E). The increase in myogenin fluorescence was significant (P=0.009), but the increase in Myf5 fluorescence, although considerable, did not.
Fig. 4. Incubation of embryo explants with GoH3 perturbs myotome formation. (A-D) Transverse cryostat sections of E8.0 (A,B) and E9.5 (C,D) wild-type embryo explants cultured for 14 hours under control conditions (A,C) or with GoH3 (B,D). In E8.0 (+14 hours) control explants, a few Myf5-positive cells are localised under the dermomyotome in a typical myotomal position (arrow in A). In E8.0 (+14 hours) explants cultured with GoH3, Myf5-positive cells are found near the neural tube (arrows in B). Myf5 expression is present in the dermomyotome (asterisks in B). Laminin immunoreactivity at the myotome-sclerotome interface is discontinuous in GoH3-treated embryos (arrows in D; also see amplification in inserts), and laminin staining is interrupted at the epaxial lip (arrowheads). (E,F) X-gal staining of Myf5\textsuperscript{nlacZ/\textsuperscript{+}} embryos (E) shows normal distribution of reporter-expressing cells in the myotome, while in Myf5\textsuperscript{nlacZ/nlacZ} embryos (F), these cells disperse. (G-L) Whole mount co-immunohistochemistry of E9.5 embryo explants cultured under control conditions for 24 hours show a normal expression pattern of Myf5 (G,I) and desmin (H,I), while the presence of GoH3 resulted in abnormal dispersion of Myf5-positive cells (J,L), particularly in interlimb-level somites. Furthermore, Myf5-positive cells fail to invade the myotome (absence in the area indicated by brackets). These effects are very similar to the defect observed in Myf5\textsuperscript{nlacZ/nlacZ} embryos (compare J with F). A disorganised pattern of desmin immunoreactivity (K,L) is also observed (area indicated by the brackets and patches among dispersed cells). Anterior is towards the right in E-L. ep, epaxial; hyp, hypaxial; nt, neural tube; fl, forelimb. Scale bars: 100 μm in A-D; 80 μm in G-L.

Fig. 5. Incubation of embryo explants with GoH3 leads to precocious myogenic differentiation in the dermomyotome. (A-D) Co-immunohistochemistry for Myf5 and myogenin on cryostat sections of E9.5 wild-type embryo explants cultured for 14 hours (A,B, transverse; C,D, longitudinal). Control explants (A,C) show normal expression of Myf5 and myogenin, including a few Myf5-expressing cells in the dermomyotomal lips (arrowheads in C). In the presence of GoH3 (B,D), myogenin is ectopically expressed in the dermomyotome (arrows) and Myf5-expression has extended (arrowheads). This effect is particularly evident in interlimb-level somites. (E,F) Graphical representation of the increase in fluorescence intensity for Myf5 and myogenin in the dermomyotome compared with the neural tube in control versus GoH3-treated explants. This value was obtained for whole dermomyotomes (E) and for dermomyotomes excluding lips (F). ep, epaxial; nt, neural tube; dm, dermomyotome; myog, myogenin. *P<0.05; **P<0.01; bars represent s.d. of means. Scale bars: 100 μm in A-D.
score statistically significant ($P=0.09$). As Myf5 is normally expressed in the dermomyotome lips, we reasoned that excluding lips in our measurements would better identify whether a difference exists in the remaining areas of the dermomyotome. Measurements were made for both Myf5 and myogenin (Fig. 5F), and there was a statistically significant difference between control and GoH3-treated explants ($P=0.03$ for Myf5; $P=0.01$ for myogenin). We therefore conclude that α6β1-laminin interactions normally repress myogenesis while cells are in the dermomyotome.

**DISCUSSION**

Our results show that the expression of Myf5 is necessary for the maintenance of α6β1 by MPCs colonising the myotome, which strongly suggests that the cause of the failure of laminin matrix assembly and the consequent cell dispersal in $Myf5^{nlacZ/nlacZ}$ embryos is the absence of this integrin on myotomal cells. Blocking α6β1-laminin binding in mouse embryo explants not only confirmed these conclusions, but in addition revealed an unexpected role for α6β1-laminin interactions in the repression of myogenesis in the dermomyotome.

**The relationship between myogenic cells and laminin changes with their progressive maturation**

Myogenic cells interact with laminin at several phases of their differentiation programme (schematised in Fig. 6). MPCs in the epaxial dermomyotomal lip detach from their basement membrane when undergoing an epithelium to mesenchyme transition, but soon after entering the myotomal space, they accumulate electron dense basement membrane material on the side facing the sclerotome (Tosney et al., 1994). This agrees with our detection of patchy laminin immunoreactivity between early epaxial MPCs and the sclerotome (Fig. 6A). Thus, we suggest that these first MPCs facilitate the assembly of laminin very soon after they enter the myotomal space and the observation that laminin fails to assemble in $Myf5^{nlacZ/nlacZ}$ embryos reinforces this conclusion. After the initial deposition of the laminin matrix, the myotome grows in thickness and, consequently, new extracellular material has to be added to enable an increase in the surface area of the basement membrane. The identification of areas of unassembled laminin at the myotome-sclerotome interface, midway between rostral and caudal edges of the myotome (Fig. 6B), suggests that the basement membrane is growing at this point.

As the myotome grows and myotomal cells advance in their differentiation programme, their interaction with the laminin matrix changes. The great majority of young (Myf5-positive, myogenin-negative) cells that enter the myotome are found near the myotome-sclerotome interface, i.e. closely apposed to the pre-existing, assembled laminin matrix (see Fig. 6B), suggesting that they use this matrix as a migration substrate. More differentiated (Myf5- and myogenin-positive) cells are generally found in a central position near the myotome-sclerotome interface (i.e. midway between rostral and caudal dermomyotomal lips) (Venters et al., 1999), an area where the laminin matrix is discontinuous (see Fig. 6B). We speculate that the loss of contact with assembled laminin might play a role in myogenin activation and initiation of myocyte elongation observed in this area (step 4; Fig. 6B). Alternatively, this may be an indirect effect resulting from a unique growth factor environment at this site.

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**Fig. 6. Potential roles of α6β1-laminin interactions during early myotome formation in the mouse.** (A) (1) Our results strongly suggest that α6β1-laminin interactions repress myogenesis in the dermomyotome (yellow cells). This repression is lifted when α6β1-laminin binding is broken, an event that normally occurs at the epaxial lip (green cells). (2) α6β1 expression is maintained on early Myf5-expressing MPCs and our results demonstrate that α6β1 on early MPCs is required for the assembly of the first myotomal laminin matrix. (B) (3) At slightly later stages, Myf5-positive MPCs continue to colonise the myotomal space and their guidance into this space is also dependent on α6β1-laminin interactions. The majority of these cells stay in close contact with laminin and do not proceed further in their differentiation programme until they reach the central area of the myotome. (4) This central area near the myotome-sclerotome interface is the area where the laminin matrix is discontinuous and cells expressing Myf5 and myogenin are found. (5) With the addition of younger cells at the myotome-sclerotome interface, elongating myocytes lose contact with laminin except at their caudal and cranial extremities. This laminin matrix may play a role in supporting their elongation.
As the myotomal cells differentiate even further, they elongate in a cranial and caudal direction and start synthesising muscle structural proteins, such as desmin and myosins (Buckingham et al., 1992; Venter et al., 1999). Myosin is present in fully elongated cells, which are localised far from the laminin matrix, while the expression pattern of desmin shows that the tips of elongating cells contact this matrix. We thus propose that, as new myogenic cells are being added, intercalating between the myotome and its laminin matrix, the elongating cells are displaced, most probably only retaining contact with the laminin matrix through their tips (Fig. 6B).

Assembly of the myotomal basement membrane depends on Myf5 and α6β1-laminin interactions

The formation of the myotomal laminin matrix is severely affected in Myf5/H9251/H9252 embryos (Tajbakhsh et al., 1996) (this study). However, dotted immunoreactivity for laminin and patchy immunoreactivity for collagen IV and perlecan was present around Myf5-null cells. A scaffold of assembled laminin is a prerequisite for the subsequent integration of the other basement membrane components into a mature basement membrane (Henry et al., 2001; Yurchenco et al., 2004; Yurchenco and Wadsworth, 2004). Thus, our results suggest that although the production of basement membrane components is unaffected in Myf5/H9251/H9252 embryos, the assembly of a laminin matrix is greatly impaired, resulting in the absence of the myotomal basement membrane.

Laminin polymeric networks can be induced to self-assemble in vitro (Yurchenco et al., 1985; Cheng et al., 1997; Yurchenco and Cheng, 1993), but there is growing evidence that β1 integrins are involved in the assembly of laminin-based basement membranes in vivo (reviewed by Tunggal et al., 2000; Yurchenco et al., 2004). Embryos null for the integrin β1 subunit gene (Itgb1) die early (at E5.5) and basement membranes are not detected (Stephens et al., 1995; Li et al., 2002). Furthermore, conditional inactivation of Itgb1 in skeletal muscle cells (cre/skeletal α-actin promoter) leads to discontinuities in muscle basement membranes (Schwander et al., 2003). Inactivation of α integrin subunits tends to produce more subtle phenotypes, probably owing to compensation by other related α-subunits (Hynes, 1996). However, in embryos null for the α6 subunit gene (Itga6), laminin deposition in neural tissues is abnormal (Georges-Labouesse et al., 1998) and newborn Itga3-null mice display severe defects in several epithelial basement membranes (Kreidberg et al., 1996; DiPersio et al., 1997). In addition, embryos null for both Itga6 and Itga3 present basement membrane discontinuities and cell detachment (De Arcangelis et al., 1999).

The most likely β1 integrin to mediate ES cell basement membrane assembly is α6β1 (Henry et al., 2001). Here, we show that Myf5/H9251/H9252 embryos display a dotted pattern of laminin immunoreactivity around MPCs and that MPCs fail to maintain the α6β1 integrin after they delaminate from the dermomyotome. This strongly suggests that the absence of α6β1 on Myf5-null MPCs is the cause of their failure to assemble laminin.

Colonisation of the myotome is dependent on α6β1-laminin binding

We demonstrate that α6β1-laminin binding is also important for the colonisation of the myotome by MPCs. In explants placed in culture with GoH3 at E9.5, myotome formation had already been initiated and thus some laminin matrix was present in these myotomes before the beginning of the culture period. In spite of the presence of this laminin matrix, MPCs scattered and failed to colonise the myotomal space. This shows that an interaction between α6β1 and laminin is not only important for laminin matrix assembly during early myotome formation, but also serves to correctly position MPCs in the myotomal space.

The abnormal staining pattern for desmin suggests that the inhibition by GoH3 leads to a disturbance of elongating myocytes. Our experiments do not allow us to determine whether this effect is due to a detachment of the tips of elongating myocytes or whether it is due to the inability of differentiating cells to initiate their elongation (see Fig. 6B; longitudinal view). By contrast, fully elongated myocytes do not appear to be affected by incubation with GoH3. In fact, these myocytes also express α6β1, which colocalises with its ligand fibronectin, at their tips (Bajanca et al., 2004), so they may not exclusively depend on α6β1 for the maintenance of their elongated phenotype. Interestingly, α6β1-laminin interactions have been implicated in embryonic retinal neurite outgrowth (de Curtis and Reichardt, 1993) but whether a similar mechanism occurs in elongating myocytes remains an unanswered question.

Inhibition of α6β1-laminin binding leads to activation of myogenesis in the dermomyotome

Our results show that inhibiting the binding of dermomyotomal α6β1 to laminin leads to an activation of the myogenic programme in that both Myf5 and myogenin are upregulated. Furthermore, the most significant upregulation of Myf5 occurs in the central myotome. We propose that the antibody perturbation experiments mimic the disengagement between α6β1 and laminin that normally only occurs epaxially (at the epaxial lip). It is commonly believed that the environment at the epaxial lip is such that inducing signals (e.g. Wnts, Shh) override repressive signals (e.g. BMPs) leading to the induction of myogenesis (reviewed by Currie and Ingham, 1998; Cosson and Borello, 1999; Tajbakhsh and Buckingham, 2000; Pownall et al., 2002). By contrast, in other regions of the dermomyotome where myogenic progenitor cells are also present (see Relaix et al., 2005; Kassar-Duchossoy et al., 2005; Gros et al., 2005; Ben-Yair et al., 2005) repressive signals override potential inducing signals at the stages under study. Our results suggest that the experimental disengagement between α6β1 and laminin in those regions of the dermomyotome pushes the equilibrium such that repressive signals no longer prevail. This strongly implicates α6β1 disengagement from laminin as one of the factors that drive myogenesis epaxially.

The observation that chick epiblast cells activate MyoD after dissociation in culture, has led to the suggestion that myogenesis is normally repressed in the embryo (George-Weinstein et al., 1996) (see Cosson et al., 1996; Currie and Ingham, 1998). Interestingly, perturbation of β1 integrin-laminin interaction has been shown to lead to precocious differentiation in other systems. Precursors of cerebellar granule cells are attached to laminin in the meningeal basement membrane by α6β1 and α7β1, and under those conditions they proliferate and remain undifferentiated (Pons et al., 2001; Blass et al., 2004). Differentiation normally occurs when these cells detach from the basement membrane and migrate into the deeper layers of the cerebellum. Conditional inactivation of Itgb1 in the central nervous system resulted in impaired laminin matrix formation by cerebellar granule cell precursors, reduced cell proliferation and precocious expression of differentiation markers (Blass et al., 2004). These data suggest that α6β1 and/or α7β1 binding to laminin normally prevents the differentiation of these cells and that their detachment from laminin causes differentiation. During pancreas development, the proliferation of pancreatic insulin-producing β cell precursors is dependent on their binding to laminin 1 via α6β1, and differentiation occurs when this binding is
blocked by the addition of GoH3 to organ cultures (Jiang et al., 2001). Interestingly, no alterations in β-cell differentiation were detected in Igalb-null embryos (Jiang et al., 2001). Thus, at least in this system, blocking αβ1 function has a more severe effect than inactivation of the Igalb gene. It is possible that other laminin receptors (e.g. the closely related α3β1 or α7β1 integrins) are upregulated in the absence of Igalb expression (see Hynes, 1996). α6β1-laminin interactions have been shown to promote differentiation in some cell types, including skeletal muscle (Sastry et al., 1996) and lens (Walker and Menko, 1999; Walker et al., 2002). However, in these two situations, the differentiation-promoting effect was attributed to the presence of the α6A variant. The α6 subunit exists in two splice variant forms, α6A and α6B (Hogervorst et al., 1991; Tamura et al., 1991), but only the α6B variant is present during early (E8.5-E9.5) myotome formation (Thorsteinsdóttir et al., 1995). There is evidence that the two α6 variants modulate α6β1 signalling in different ways. For example α6Aβ1, but not α6Bβ1, activates ERK (Wei et al., 1998; Ferletta et al., 2003). We suggest that the binding of laminin by the α6Bβ1 integrin contributes to a signalling pathway that represses myogenesis in the dermomyotome and that the detachment of MPCs from the dermomyotomal laminin matrix permits the activation of the myogenic programme during the early stages of myotome development. Whether this signalling pathway is dependent only on engagement of α6β1 or whether synergism with a pathway activated by growth factors occurs (Colognato et al., 2002; Blaess et al., 2004) (reviewed by Danen and Sonnenberg, 2003), remains to be determined.

We are grateful to Jeff Miner and Frank Stockdale for the laminin α5 and myosin antibodies respectively. Anti-desmin (D3; D. A. Fischman) was from DSHB developed under the auspices of NICHD and maintained by the University of Iowa. We thank Luis Marques and Ingrid Kuikman for help with some experiments, Jorge Palmeirim for statistical advice, Leonor Sauder for reading the manuscript and Prof. E. G. Crespo for constant support. This study was financed by Fundação para a Ciência e a Tecnologia (FCT/FEDER) via POCI/BCI/47681/2002 (to S. Thorsteinsdóttir and F.B.) and by FP6/EU Network POCTI/BCI/47681/2002 (to S. Thorsteinsdóttir and F.B.) and by FP6/EU Network of Excellence: Developmental Cell Signalling and the Developing Nervous System. Cell programs in CNS and Head, EMBO J. 31525-31532.

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