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ORIGINAL ARTICLE

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Basal lamina molecules are concentrated in myogenic regions of the mouse limb bud

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Abstract Molecular components of basal lamina, such as laminin, stimulate the differentiation of skeletal muscle cells in culture, while interstitial matrix components such as fibronectin are inhibitory. However, the role of extracellular matrix (ECM) molecules in muscle cell differentiation in the embryo is less well understood. As a first step toward understanding the role of the ECM in embryonic myogenesis, the localization of basal lamina molecules in the mouse limb bud before and during muscle cell differentiation was determined by immunofluorescence. Laminin, collagen type IV and nidogen (entactin) were concentrated in myogenic regions of the limb bud both before and during differentiation of skeletal muscle cells. Punctate immunofluorescence for basal lamina molecules was concentrated in dorsal and ventral premuscle and muscle masses, when compared with other regions of limb mesenchyme. In contrast, immunofluorescence for fibronectin, an interstitial extracellular matrix molecule, was decreased in premuscle and muscle masses. These results suggest that basal lamina components play an important stimulatory role in early stages of skeletal muscle differentiation in the developing mouse limb bud.

Key words Extracellular matrix · Limb development · Laminin · Muscle differentiation · Myogenesis

Introduction

Little is known about the role of extracellular matrix (ECM) in the differentiation of skeletal muscle cells. However, ECM molecules have significant effects on myoblasts in culture, and major changes take place in the ECM of developing muscle during its differentiation in the embryo. When myoblasts are cultured on a substrate of laminin-1 – a major adhesive glycoprotein of basal laminae, cell adhesion, migration, division and differentiation are stimulated (Foster et al. 1987; Öcalan et al. 1988). However, interstitial ECM components such as fibronectin and hyaluronic acid inhibit myogenesis (Podleski et al. 1979; Kujawa et al. 1986). A gradual transition occurs in the ECM surrounding muscle cells early in myogenesis, from a matrix rich in interstitial molecules to one enriched in basal lamina components. For example, during the early phase of muscle differentiation in the chick embryo hindlimb, interstitial components such as hyaluronic acid and fibronectin decrease (Toole 1972; Chiquet et al. 1981; Tomasek et al. 1982), while the basal lamina components laminin and agrin increase (Godfrey et al. 1988).

To understand the role of basal lamina molecules in muscle cell differentiation in the embryo, it would be advantageous to be able to study the regulation of genes encoding them. The mouse embryo has unique advantages for studying the role of ECM molecules in myogenesis, including the availability of cloned cDNAs coding for ECM molecules and the possibility of altering their expression using transgenic technology. The transition in the composition of the ECM in early muscle masses has been demonstrated in chick but not mouse limb bud. The purpose of this study was to define by immunofluorescence the distribution of ECM components during the development of muscle in the limb bud of the mouse. This knowledge should provide a basis for future studies of the expression and regulation of genes encoding these molecules, and should aid in understanding the environmental cues that contribute to muscle cell differentiation in the embryo.

Materials and methods

Antibodies

We used the following antibodies: *laminin* 1:150 rabbit anti-mouse EHS laminin-1 (Dr. C. Little, Medical University of South Carolina; Little et al. 1989); *collagen type IV* 1:150 rabbit anti-mouse (Dr. C. Little; Little et al. 1989), *nidogen* 1:400 rabbit anti-mouse (Dr. R.

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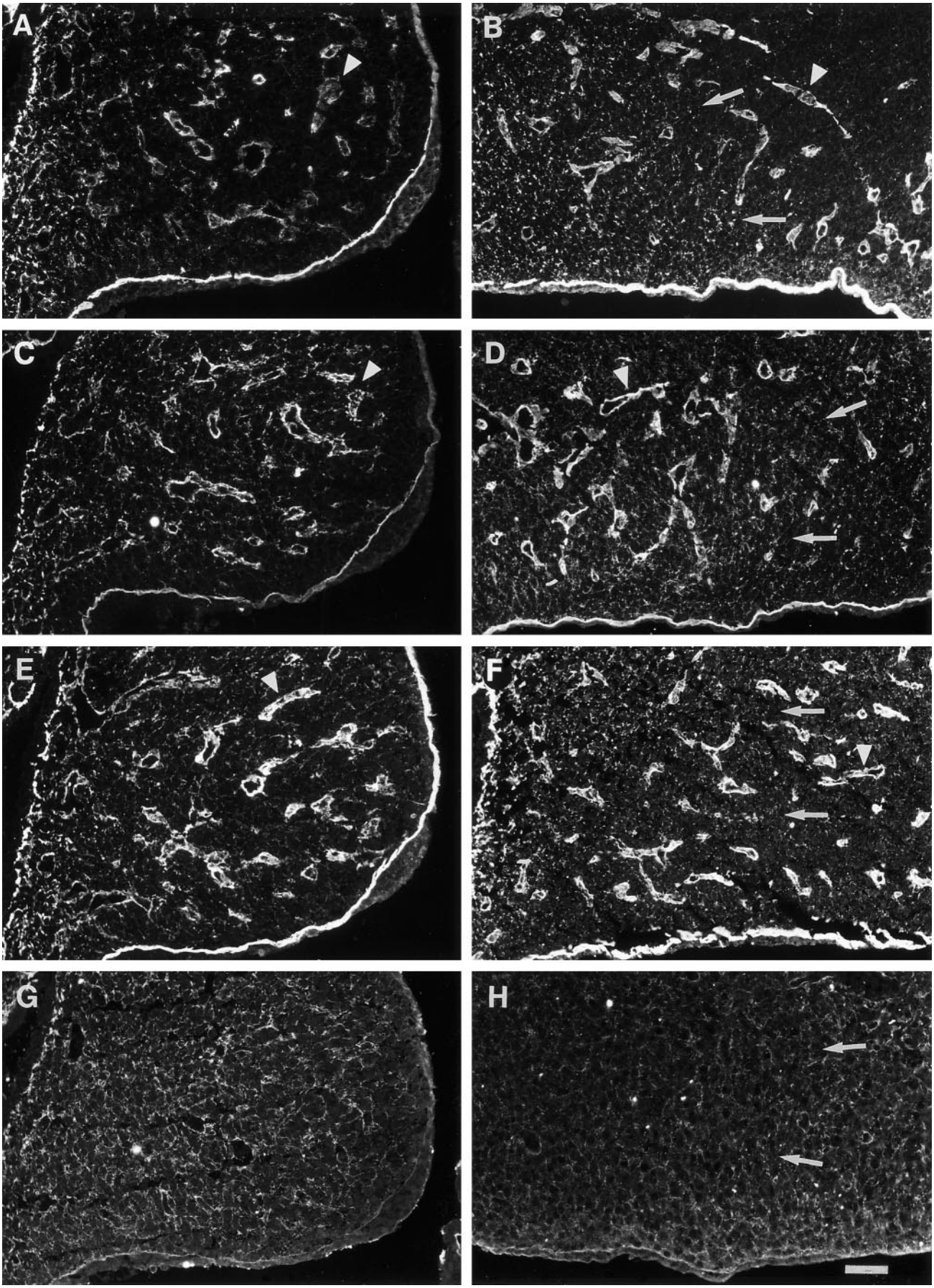
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Timpl, Max-Planck-Institut, Martinsreid, Germany; Paulsson et al. 1986); *fibronectin* 1:250 goat anti-human (Sigma); *myoD1* 1:400 rabbit anti-mouse (Dr. H. Weintraub, University of Washington); *myosin* mouse monoclonal anti-chicken antibody MF-20 (Bader et al. 1982; Developmental Studies Hybridoma Bank).

Immunofluorescence

We used our previous method (Godfrey et al. 1988) to visualize antibodies bound to cryostat sections of mouse embryo hindlimbs. Briefly, sections were fixed 5 min in 2% paraformaldehyde, blocked 1 h in PBS-BSA (5 mg/ml), indubated 1 h with primary antibody diluted in PBS-BSA, rinsed, incubated 1 h with secondary antibody labeled with FITC (rabbit anti-mouse, 1:100, rabbit anti-goat, 1:150, or rabbit anti-mouse, 1:100), rinsed, and mounted.

Results

Premuscle masses contain punctate deposits of basal lamina components

To determine the distribution of basal lamina components in premuscle masses, immunofluorescence staining with antibodies against ECM components was performed using sections of hindlimbs at embryonic day 10 (E10) and E11.5. Results are shown in Fig. 1. At E10 (panels on left), laminin (Fig. 1A), collagen type IV (Fig. 1B), and nidogen (Fig. 1C) were not obviously concentrated in dorsal and ventral areas of mesenchyme. However, these basal lamina components appeared to be somewhat more concentrated in ventral and dorsal (not shown) regions of mesenchyme at E11.5 (to the left of arrows in Fig. 1B, D and F). This concentration was less apparent for collagen type IV (Fig. 1D) than for the other basal lamina components, and took the form of punctate immunofluorescence in the regions of limb mesenchyme that give rise to muscle one day later in development. Fibronectin, however, appeared to be less concentrated in the premuscle regions than elsewhere in the limb at E11.5 (left of arrows in Fig. 1H), whereas it was uniformly distributed in mesenchyme at E10 (Fig. 1G). Thus, a higher concentration of basal lamina components was seen in premuscle regions of developing mouse embryo hindlimb, just as we had previously observed during chick embryo limb development.

Basal lamina components are concentrated in muscle masses

Muscle cells in the mouse embryo hindlimb begin to differentiate in dorsal and ventral muscle masses at E12. At this stage, laminin, a basal lamina glycoprotein, was found concentrated in a punctate distribution in muscle masses (Fig. 2A). The muscle-specific transcription factor myoD1 first appeared in cells of dorsal and ventral muscle masses at this stage (Fig. 2B), showing that molecules that direct myogenic differentiation were expressed. At a slightly later stage, E12.5, the concentration of basal lamina components in the muscle masses was more apparent. Basal lamina components concentrated in the muscle masses included collagen type IV (Fig. 3A, F), laminin (Fig. 3E), and nidogen (Fig. 3G). Myogenic differentiation was more advanced, as shown by the appearance of myoD1 (Fig. 3C), myosin (Fig. 3D) and desmin (not shown). In contrast to the basal lamina components, the interstitial ECM molecule fibronectin was less concentrated in muscle masses than in surrounding regions of mesenchyme (Fig. 3B, H). Thus, when myogenic differentiation begins in the muscle masses of the mouse embryo limb bud, these regions contain a higher concentration of basal lamina components and a lower concentration of interstitial ECM components than the surrounding mesenchyme.

Fig. 1A–H Premuscle masses (*arrows*, *right panels*) contain punctate immunofluorescence for basal lamina molecules. Mouse embryos were cut in cross-section at the level of the hindlimb bud. Panels on left are from E10 embryos; those at right are from E11.5. In all micrographs, ventral is down, proximal to the left, and distal to the right (*arrowheads* blood vessels). **A, B** Laminin. **C, D** Collagen type IV. **E, F** Nidogen (entactin). **G, H** Fibronectin. *Bar* 50 μm

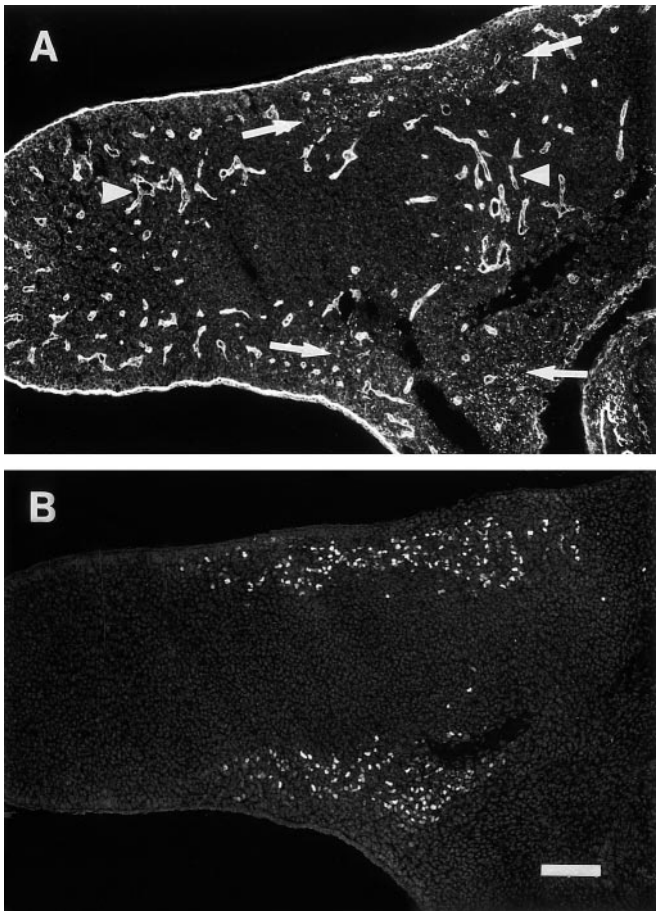
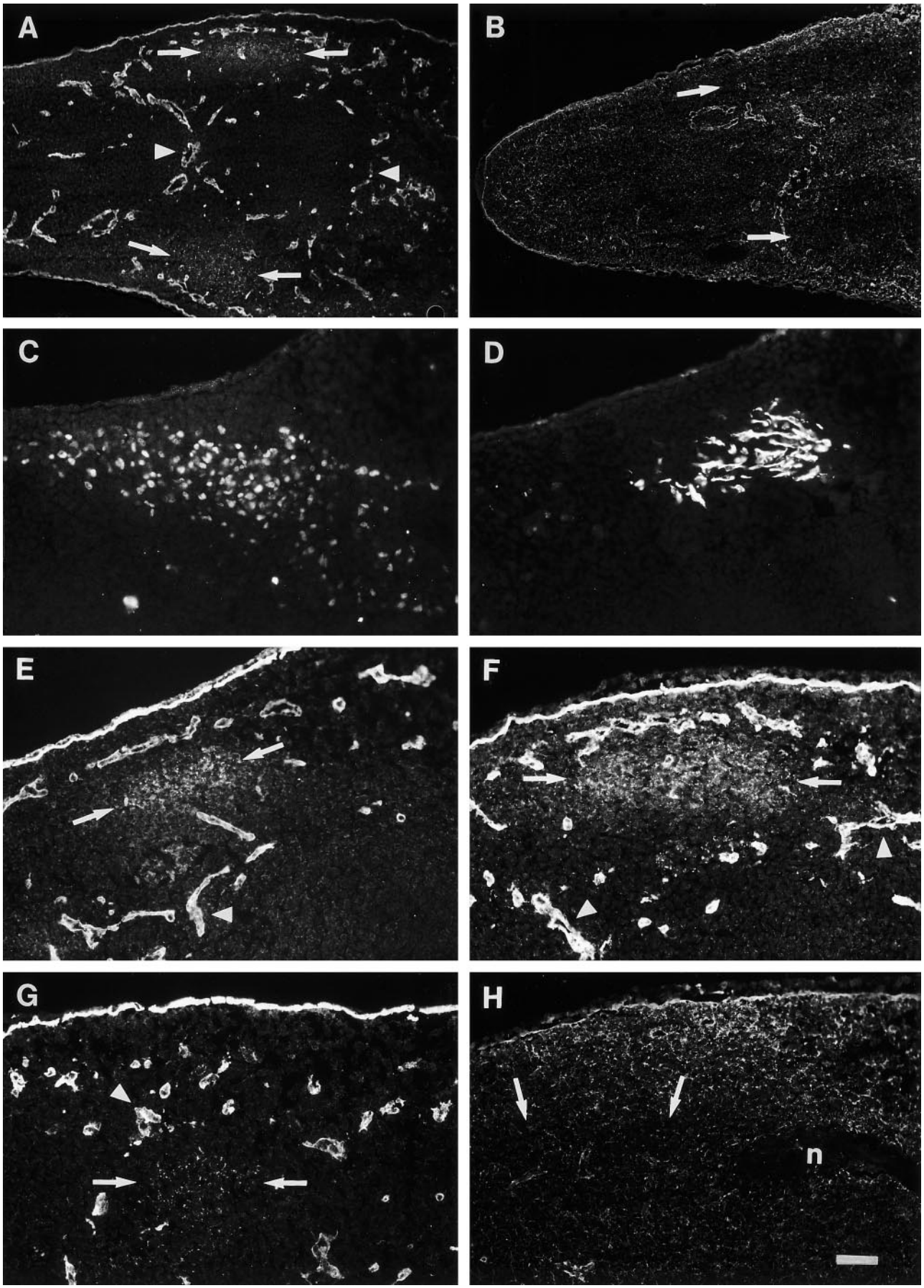


Fig. 2A, B Laminin is concentrated in limb premuscle masses early in myogenesis (E12). Cross-sections of embryo (longitudinal sections of hindlimb bud) were stained with antibodies against laminin (**A**) and myo D1 protein (**B**), which first appears in limb at this stage. Dorsal (at top) and ventral premuscle masses indicated by *arrows* in **A**. *Bar* 100 μm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Discussion

We found that basal lamina components were concentrated in both premuscle and muscle masses of the mouse embryo hindlimb bud, while the interstitial matrix component fibronectin was reduced in these regions compared to surrounding mesenchyme. These results parallel those seen in chick embryo limb development (Godfrey et al. 1988; Solursh and Jensen 1988), and suggest that basal lamina molecules play a stimulatory role in skeletal muscle cell differentiation in the limb bud, as they do in culture.

The mesenchyme of the limb bud has a dual origin. Cells which give rise to connective tissue and cartilage are derived from the somatopleure, while skeletal muscle is derived from cells which originate in the dermomyotome of the somites (Christ et al. 1974, 1977, 1983; Chevallier et al. 1977). These myogenic precursor cells migrate into the somatopleure just prior to limb bud formation (Jacob et al. 1978, 1979). In the epithelial somite, these cells are in contact with the somitic basal lamina (Christ and Ordahl 1995). During migration into the somatopleure and the limb bud, muscle precursor cells encounter a complex ECM, which includes punctate deposits of laminin and other basal lamina molecules (Godfrey et al. 1988). However, these cell-ECM interactions do not cause terminal differentiation into myoblasts. Perhaps the myogenic precursor cells are not competent to respond to differentiation signals from the matrix during these early stages. Alternatively, the somitic basal lamina and the ECM through which they migrate contain molecules such as fibronectin, which may inhibit myogenic differentiation of these cells (Podleski et al. 1979).

Interaction of muscle cell precursors with the ECM, and the composition of the ECM with which they interact, are important determinants of myogenic differentiation. The interstitial ECM components, hyaluronate and fibronectin, inhibit fusion and differentiation of myoblasts in culture (Toole 1972; Podleski et al. 1979). However, hyaluronate stimulates migration of myogenic precursor cells in the limb bud (Krenn et al. 1991). The lower concentration of fibronectin in muscle masses in the limb bud probably reflects less fibronectin synthesis by myogenic cells than by other limb mesenchyme cells (Tomasek et al. 1982). Basal lamina components, particularly laminin, stimulate myogenesis in culture. Laminin-1 stimulates the adhesion, spreading, motility, and proliferation of rat and mouse myoblasts (Foster et al. 1987; Öcalan et al. 1988). The predominant form of laminin in fetal and adult mouse skeletal muscle is merosin or laminin-2 (Leivo and Engvall 1988; Schuler and Sorokin 1995; Vachon et al. 1996). Both laminin-1 and merosin stimulate proliferation and differentiation of skeletal muscle cells, but merosin stabilizes myotubes while laminin-1 does not (Vachon et al. 1996). The antibodies used in this study were made against laminin-1, but since merosin (laminin-2) shares 2 of its 3 polypeptide chains with laminin-1, we cannot determine whether the laminin immunoreactivity we observed in premuscle and early muscle masses was due to one or both isoforms. However, we hypothesize that basal lamina molecules, including laminin isoforms, stimulate terminal differentiation of muscle cells in limb muscle masses in the embryo, as they do in culture.

The increase of basal lamina components in premuscle masses may be one of the first steps in the myogenic differentiation program. In fact, it precedes the appearance of the myoD1 protein. Expression of the *myoD1* gene, which encodes a DNA-binding protein that activates muscle-specific genes, is an early step in the myogenic program (Sassoon et al. 1989). In the mouse embryo limb, a related gene, *myf-5*, is expressed earlier, at E10.5 (Ott et al. 1991), about the time that increased expression of basal lamina molecules is first seen in premuscle regions. While it is unclear whether the change in the expression of basal lamina components precedes or follows the expression of *myf-5*, a causal connection is possible. In this regard, it would be interesting to examine the distribution of ECM components in the limbs of transgenic mice in which expression of *myoD1* and/or *myf-5* has been eliminated (Braun et al. 1992; Rudnicki et al. 1992). The change in extracellular matrix composition of differentiating muscle masses may be both a cause and an effect of activation of genes specifying myogenic differentiation.

Fig. 3A–H Basal lamina components are increased in early limb muscle masses (E12.5). **A** collagen type IV. **B** Fibronectin. **C** MyoD1 protein. **D** Myosin. **E** Laminin. **F** Collagen type IV (high power). **G** Nidogen (entactin). **H** Fibronectin (high power). Muscle masses are indicated by *arrows*. *Bar* **A, B** 100 µm; **C–H** 50 µm

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