The Role of Laminin in Embryonic Cell Polarization and Tissue Organization

Review

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Genetic analyses have revealed that members of the laminin glycoprotein family are required for basement membrane assembly and cell polarization, with subsequent effects on cell survival and tissue organization during metazoan embryogenesis. These functions depend upon the cooperation between laminin polymerization and cell anchorage mediated via interactions with β1-integrins, dystroglycan, and other cell surface receptors.

Introduction

Metazoan embryogenesis comprises a sequence of events that results in the generation of differentiated cells and in the organization of those cells within the organism. One of the fundamental processes involved in both differentiation and organization is the establishment of cell polarity. This cellular asymmetry provides the basis upon which higher orders of multicellular structure are ultimately based, being a prerequisite for the generation of epithelia, distinct tissues, and organogenesis. The initial generation of cell asymmetry may either be cell autonomous, resulting, for example, from the unequal distribution of cytoplasmic contents, or alternatively it may result from intercellular interactions. The spatial cues for the latter are provided by direct contacts between cells or by indirect intercellular interactions via extracellular matrices (ECMs), the components of which are synthesized by neighboring cells. In particular, basement membranes are specialized cell surface-associated ECMs of diverse animal tissues, found either as enveloping cell coats or as sheets underlying cell layers. Thus, they are ideally placed to mediate spatially specific signaling information.

Among the most ancient of animal ECMs, basement membranes first appear in the periimplantation period of development in the mouse and during gastrulation in the nematode and fly. Members of the laminin family, each an $\alpha_x\beta_y\gamma_z$ glycoprotein heterotrimer, are required for basement membrane assembly, a process thought to depend upon both the shared and distinct architecture-building and cell-anchoring properties of these heterotrimers. Correspondingly, laminin and its cellular receptors have been shown to regulate critical processes of

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differentiation (Table 1) and are phylogentically conserved (Hutter et al., 2000). Genetic analyses of the two laminins in the fly have shown changes of cell polarity, cell fate, and body axis, affecting morphogenesis (Fessler et al., 1987; Garcia-Alonso et al., 1996; Henchcliffe et al., 1993; Martin et al., 1999). Similarly, analysis of homologous laminins in the nematode *C. elegans* has revealed that loss of either of them results in embryonic lethality, characterized by defects of cell polarity, cell adhesion, and tissue cohesion (Huang et al., 2003). In mammals, there are at least 15 laminins derived from five α, three β, and three γ subunits, the α subunits of which are thought to have evolved from two ancestral chains corresponding to the two invertebrate α chains.

The mammalian laminins expressed earliest during embryogenesis are laminins-1 (α1β1γ1) and -10 (α5β1γ1). While these laminins, unlike their nematode homologs, partially overlap in developmental expression and functions, only laminin-1 appears to be essential in the periimplantation period (Smyth et al., 1999), with loss of laminin-10 not severely affecting embryogenesis until E14-17 (Miner et al., 1998). Mutations in other laminin subunits that likely evolved later, i.e., α2, α3, α4, β2, and β3, cause perinatal and postnatal defects detected in skeletal muscle and nerve, skin and mucosa, blood vessels, and neuromuscular junction and kidney, largely affecting cell adhesion and tissue maintenance (Table 1). Thus, mutations in the α3β3γ2 subunits of laminin-5 and the α2 subunit of laminins-2/4 are of clinical significance in that they result in Herlitz junctional epidermolysis bullosa and merosin-negative congenital muscular dystrophy, respectively. These disorders reflect the loss of epithelial adhesion strength in the former, and skeletal muscle/Schwann sheath integrity in the latter (Gullberg et al., 1999; Nakano et al., 2002; Tubridy et al., 2001).

This review focuses on the role that laminins play in the induction of cell polarity and consequently in the resulting organization of embryonic tissues. To do this, we will discuss the evidence demonstrating how the structure and corresponding functional properties of the laminins allow them to exert a specific and necessary role in metazoan development.

Embryonic Epithelial Polarization

Basement membrane laminins have long been implicated in epithelial morphogenesis (Klein et al., 1988; Streuli et al., 1995), and both the collagens and laminins have previously been shown to alter Madin-Darby canine kidney (MDCK) cell polarity within epithelial cysts (O'Brien et al., 2001; Wang et al., 1990a, 1990b). Several studies now provide evidence that laminin (Figure 1) forms the initial cell-anchored polymer required for basement membrane assembly and triggers processes required for epithelial cell polarization (Huang et al., 2003; Murray and Edgar, 2000). Supporting evidence now comes from an analysis of genetically engineered embryonic stem (ES) cells cultured to form embryoid bodies (EBs) that provide a molecular window into underlying mechanisms (Li et al., 2001a, 2002; Murray and

Table 1. Laminin and Receptor Mutations

Subunit	Organism	Expression	Null Phenotype	References
Laminin-αA (*lam-3*)	Nematode	Pharynx, nerves, and other tissues	Embryonic and larval lethals: pharyngeal cell polarization, and adhesion defects	Huang et al., 2003
Laminin-αB (*epi-1*)	Nematode	Body wall muscles, epidermis, gonad, intestine, and nerves	Embryonic, larval, and adult lethals: adhesion, muscle dense body, axonal, and other defects	Huang et al., 2003
Laminin-α1,2	Fly	Widespread	Multiple embryonic defects plus wing-blister in adult survivors	Martin et al., 1999
Laminin-α3,5	Fly	Widespread	Embryonic lethality: defects in mesodermally derived and other tissues	Fessler et al., 1987; Garcia-Alonso et al., 1996; Henchcliffe et al., 1993
Laminin-α1	Mouse	Blastocyst, neuroectodermally derived tissues, developing kidney, and other tissues	Not published	
Laminin-α2	Mouse	Skeletal and cardiac muscle, peripheral nerve, brain capillaries	Postnatal lethal: muscular dystrophy and peripheral neuropathy	Kuang et al., 1998; Miyagoe et al., 1997
Laminin-α3	Mouse	Epithelium	Neonatal lethal: epidermolysis bullosa	Ryan et al., 1999
Laminin-α4	Mouse	Microvasculature, smooth muscle	Viable: transient microvascular bleeding	Thyboll et al., 2002
Laminin-α5	Mouse	Multiple tissues during development; adult microvasculature and epithelia	E14-17 lethal: defective placental vasculature, neural tube closure, renal glomerular, and limb development	Miner et al., 1998
Laminin-β1	Mouse	Widespread	Not published	
Laminin-β2	Mouse	Neuromuscular junction, skeletal muscle, renal glomerulus	Neonatal lethal: defects in neuromuscular junction and renal glomerulus	Noakes et al., 1995b
Laminin-γ1	Mouse	Widespread	E5.5 lethal: failure of blastocyst development	Smyth et al., 1999
αina-1 integrin	Nematode	(Laminin-type α subunit associated with βpat-3)	Many defects include those of neuronal migration, axon fasciculation, and head morphogenesis	Baum and Garriga, 1997
Integrin-β1	Mouse	Widespread	E5.5 lethal: failure of blastocyst development	Fassler and Meyer, 1995; Stephens et al., 1995
Integrin-α6	Mouse	Widespread	Neonatal lethal: epidermolysis bullosa; neuronal ectopias, and retinal defects	Georges-Labouesse et al., 1996
Integrin-α3+α6	Mouse	Widespread	Embryonic defects of limb, neural tube closure, urogenital system, brain, and eye	De Arcangelis et al., 1999
Integrin-β4	Mouse	Epithelia/hemidesmosomes	Perinatal lethal: epidermolysis bullosa	Dowling et al., 1996
Dystroglycan	Mouse	Widespread	E6.5 lethal: failure of Reichert's membrane	Williamson et al., 1997

Edgar, 2000). While several conceptual links are still tentative and much remains to be understood at the molecular level, collectively the studies argue for a pivotal role of basement membrane laminin in early embryogenesis.

Depending on the type of embryo, epithelial differentiation occurs at different stages. During gastrulation in all bilateria, germline, endodermal, and mesodermal cells move into the interior, leaving only ectodermal cells on the surface of the embryo. In the nematode, gastrulation precedes epithelialization. Germline, endodermal, and mesodermal cells move to the interior through a furrow and the subsequent epithelializaton of the epidermis and intestine creates the pseudocoelomic cavity, which is homologous to the blastocoel of other organisms. Laminin gene expression is closely correlated with the events of gastrulation. Expression begins as the cells ingress through the furrow and after the cells have arranged in rows with laminin secreted between the layers (Huang et al., 2003). During fly embryogenesis, a cellular blastoderm forms, in which all the cells are arranged as an epithelium surrounding a large yolk core. During this
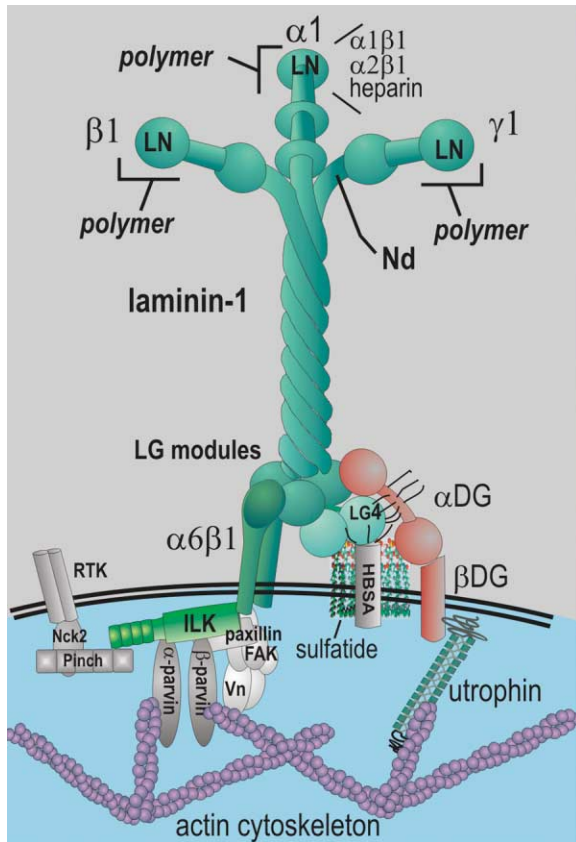
Figure 1. Laminin-1 Receptor Interactions

The N-terminal globular LN domains of the α1, β1, and γ1 allow laminin to polymerize. The LG modules allow laminin to interact with α6β1 integrin, α-dystroglycan (αDG), and other cell surface macromolecules (*h*eparin *b*inding-*s*ite *a*nchor, HBSA) such as the syndecans and sulfatides. Anchorage and signaling may minimally require two of three classes of receptor/receptor-like molecules to form a basement membrane and induce epiblast differentiation. β1-integrins can reversibly anchor the laminin matrix to the actin cytoskeleton through α- and β-parvins that bind to integrin-linked kinase (ILK) and other adaptor proteins (FAK, paxillin, vinculin shown). ILK is, in turn, bound to Pinch, which is linked to Gsk3 and receptor tyrosine kinases (RTK). In EBs, ILK downregulates the amount of F-actin adjacent to basement membrane. The β subunit of dystroglycan similarly has the potential to anchor laminin to F-actin through C-terminal interactions with utrophin. Laminin-10, another embryonic laminin, shares polymerization, α6β1 integrin, heparin, and α-dystroglycan binding, but also interactions with α3β1 integrin and the Lutheran antigen receptor, the latter mediated through LG3.

cellularization, transient basal adherens junctions form. Gastrulation occurs as endoderm and mesoderm cells invaginate as intact epithelial sheets, which subsequently undergo an epithelial-mesenchymal transition. Ectodermal and the mesodermal cells converge and migrate to form the germband, a collection of cells that will form the trunk of the embryo. By the extended germband stage, the epidermis has matured into a typical invertebrate epithelium. Laminin transcripts are detected before and during germband extension and laminin protein is detected between the ectoderm and mesoderm, when the mesenchymal-epithelial transition of the principal midgut epithelial cells occurs (Kusche-Gullberg et al., 1992; Montell and Goodman, 1989).

In mammals, epithelialization precedes gastrulation. Following compaction of mouse blastomeres at about the eight-cell stage to form the morula, the first bifurcation of cell lineages occurs with the development of trophectodermal cells that surround the blastocoelic cavity and ICM. Laminin-1 intracellular assembly and secretion is initiated at about the eight-cell stage by the expression of laminin-α subunits present in limiting the amount that follows expression of β1 and γ1 subunits (Cooper and MacQueen, 1983; Leivo et al., 1980; Yurchenco et al., 1997). It is important to note that the formation of the trophectodermal epithelium that results from compaction does not need a basement membrane (Smyth et al., 1999). Furthermore, it is not until the blastocyst stage that two continuous basement membranes form, one deposited between the newly differentiated primitive endoderm cells and the remaining interior ICM cells and the other accumulating on trophectoderm (Leivo et al., 1980; Salamat et al., 1995; Smyth et al., 1999). Following blastocyst implantation at embryonic (E) day 4.5, the ICM cells adjacent to this basement membrane develop into the epiblast (primitive ectoderm) epithelium and a central proamniotic cavity forms by E5.5–E6.0.

The processes of endodermal and ectodermal differentiation can be recapitulated in cultured embryoid bodies (EBs) derived from suspended aggregates of mouse embryonic stem (ES) cells (Figures 2 and 3). After 2 days of culturing, the outer cells of the EB become flat in shape and begin to resemble the primitive endoderm of the blastocyst, which like the trophectodermal epithelium does not require a basement membrane for its formation. Instead, the spatial cues for both of these two initial mammalian epithelia appear to result from direct cell-cell contacts and/or their position at the surface of cell aggregates. The primitive endodermal cells upregulate expression of the laminin chains and secrete laminin-1, laminin-10, and type IV collagen. A basement membrane containing laminin-1, type IV collagen, nidogen, and perlecan then assembles (3–4 days) between endoderm and remaining nonpolarized cells of the EB (Li et al., 2001a, 2002; Smyth et al., 1999). Integrin α6β1 (a major integrin heterodimer at this stage) and α/β-dystroglycan redistribute from a diffuse pericellular location to a predominantly subbasement membrane location (Li et al., 2002). Following basement membrane assembly, β-catenin, E-cadherin, and F-actin, initially subpericellular and cytoplasmic within the undifferentiated EB, accumulate circumferentially in a zone midway between the endoderm and EB center. Apoptosis initially occurs in cells located in a midzonal circumferential distribution within the ICM, and slit-like clefts with peripheral F-actin replace the apoptotic zone. Adherens junctions form in the cells adjacent to the clefts. Since these junctional complexes are located not only on the apical face of cells between basement membrane and cleft, but also on the apical face of cells between cleft and central core, the cavity, rather than basement membrane, may stimulate induction of these structures. Apoptosis and cavitation then extend into the central portion of surviving EB. The partially polarized cells that form a rim around this cavity become pseudostratified and the nuclei elongate. Following appearance of the epiblast,
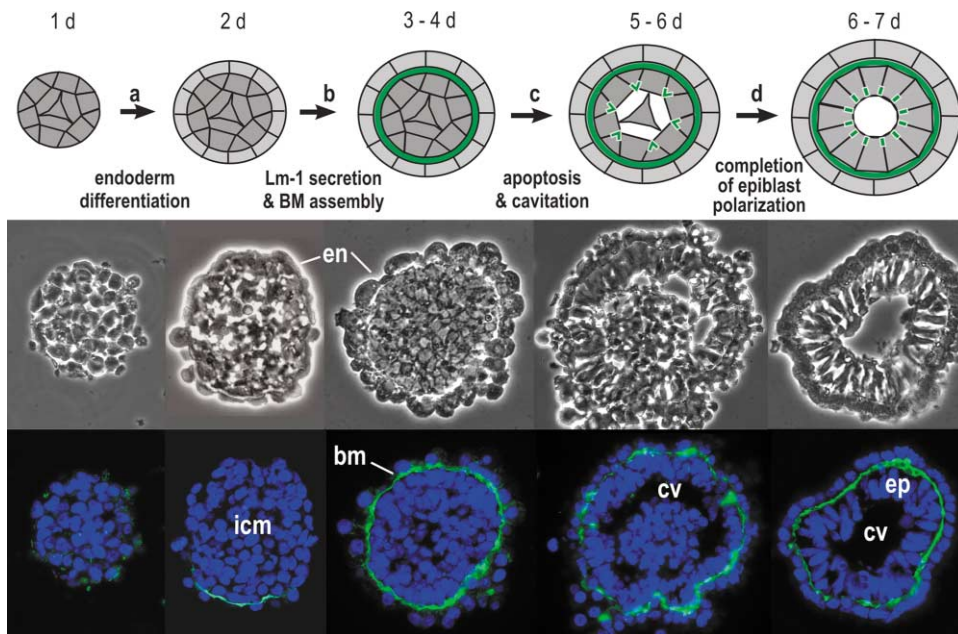
Figure 2. Embryoid Body Morphology at Different Stages

The progression of EB differentiation shown (upper panel, from left to right) is characteristic of (A) the undifferentiated state, (B) endoderm and well-formed basement membrane (indicated here in green) but with nonpolarized ectoderm, (C) formation of slit cavities, and (D) well-developed EB with endoderm, basement membrane, epiblast, and central cavity. Junctional complexes (green bars, lower panels) form at the time of initial cavitation. Phase-contrast images (middle panels) and DAPI (blue) and laminin-γ1 (green) immunostained sections of EBs (lower panels) are shown at corresponding stages of differentiation. Labels: en, endoderm; cv, cavity; ep, epiblast.

molecular markers for definitive endoderm, ectoderm, and mesoderm are detected (Li et al., 2002).

Cell polarity defects are prevalent in laminin-γ1 null EBs (Figure 4), affecting primarily the ICM cells that remain polygonal in shape, that do not develop basement membrane, and that fail to elongate and orient along a radial axis or form adherens junctions (Li et al., 2002). The corresponding laminin-γ1 null embryos die by about E5.5 with involution of the implanted blastocyst (Smyth et al., 1999). In the nematode (Figure 4), the lack of either of the laminins most often results in embryonic lethality, primarily caused by improper separation of tissues and/or detachment of cells (Huang et al., 2003). However, in rare animals that continue development and in the case of mutants with partial loss-of-function alleles, cell polarity defects are observed. For example, at body wall muscles the integrin-containing dense bodies, which link muscles to the basement membrane and overlying epidermis, are missing and ectopically assemble at other muscle surfaces. Moreover, the myofilament lattices are disoriented. In the pharynx, myofilaments and intermediate filaments, which are normally oriented between apical and basal membranes, are disordered, with some running to the lateral membranes. In general, the pharyngeal cells have greatly increased basal cell membrane with very little lateral membrane and lateral pharyngeal cell-cell contact. These results suggest that in laminin mutants the apical-basal polarity of cells is compromised as well as the ability to maintain or establish lateral identity.

Integrin receptors are thought to play critical roles in cell adhesion to, and migration on, basement membranes and other extracellular matrices, providing critical linkages between the extracellular structure and the cytoskeleton (reviewed in Bokel and Brown, 2002; Brakebusch et al., 2002). Many of the studies have focused on the dynamic links that are established in focal adhesion sites (FAs) of adherent cells. Cytosolic adaptor proteins and kinases known to be recruited into FAs represent a class of downstream mediators of cell polarization (Figure 5). Integrin linked kinase (ILK) is such an adaptor protein that binds to the cytoplasmic domain of β1 integrin, forming a link with the actin cytoskeleton in focal adhesions through the parvin family. Other adaptor proteins such as PINCH and Nck2 are also recruited into FAs. They modulate actin dynamics through Rho GTPase-dependent as well as -independent manners (Wu and Dedhar, 2001). In EBs, the activity of ILK assures that F-actin is localized to the apical zone of the polarizing epiblast. In ILK null EBs, F-actin accumulates to unusually high levels at the basement membrane zone of the epiblast (Sakai et al., 2003). The ILK-associated defect reorganizing the F-actin may be causally related to failure to develop full apoptosis and well-formed cavities and the impaired polarization of the ectoderm.

Apoptosis and Cavitation

Epithelial polarization often occurs between a cavity and a basement membrane. The cavity can form as a result of cell migrations and cell-joining to define a tube (e.g., nematode pharynx and dorsal closure in fly), the displacement of cells resulting from the pumping activity of polarized epithelial cells to produce lumen or cysts (e.g., mammalian trophectodermal blastocoel cavity), or apoptosis and cavitation (e.g., mammalian proamniotic cavity and mammary gland acini) (Lubarsky and Krasnow, 2003). The cavity that forms in embryoid body is
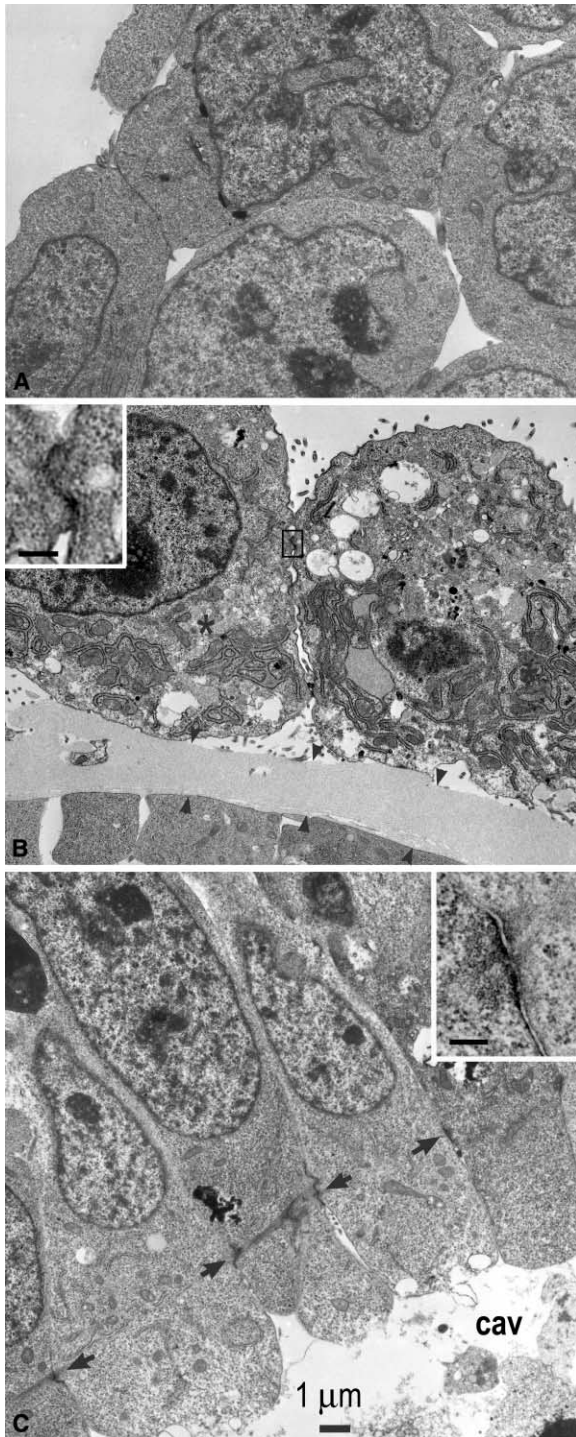
Figure 3. Ultrastructural Changes in Embryoid Body Differentiation

The electron micrographs shown are of undifferentiated (A) and differentiated EBs (B and C) cultured from laminin-γ1 null ES cells. EBs shown in (B) and (C) were treated with laminin-1 (25 μg/ml) and allowed to differentiate for 7 days.
(A) EB prior to development of an endodermal layer.
(B) EB with secretory endoderm containing basal endoplasmic reticulum (*) and tight junctions (box, enlarged in inset) and overlying a thick basement membrane (between arrowheads).
(C) Polarized ectoderm (epiblast), pseudostratified from region below cells in panel (B). These cells possess a symmetrical distribution
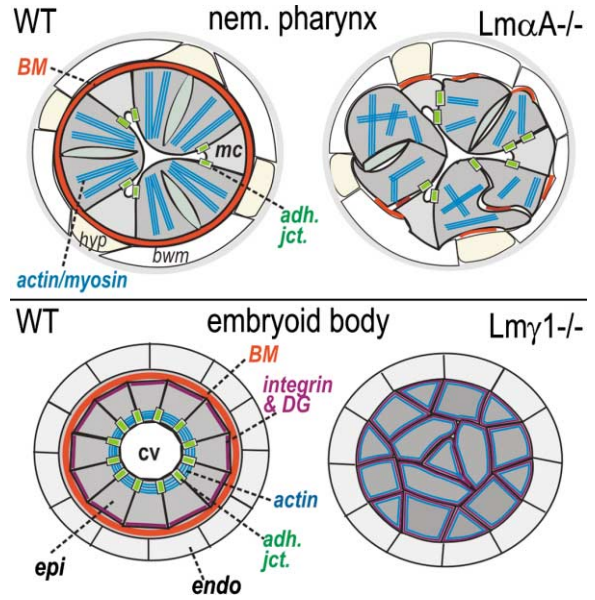

Figure 4. Polarity Defects Resulting from Laminin⁻ Mutations
Nematode pharynx, upper panel: Wild-type (WT) radially oriented muscle cells and intervening secretory cells (marginal cells [mc]) face a lumen and are bounded by a basement membrane (BM) that contains αA and αB laminins. The basement membrane is adjacent to body wall muscle (bwm) and hypodermis (hyp) on its outer aspect. αA mutations result in basement membrane disruption, loss of radial actin-myosin bundles, basal-lateral cell protrusions, disruptions of cell-cell contacts, with preserved and ectopic adherens junctions (adh. jct.).
Embryoid body, lower panel: The outer endoderm (endo) cell layer secretes laminins and type IV collagen. The wild-type epiblast (ep) is radially polarized between basement membrane and central cavity (cv). E-cadherin and β-catenin, found in adherens junctions and associated with an F-actin belt, are located at the apex, while β1-integrins and dystroglycan are found in the basement membrane zone. The γ1-laminin null mutation is associated with a near total failure of epiblast polarization and cavitation. The persistent internal cells, essentially uncommitted ICM cells that are precursor to epiblast, are polygonal in shape and possess a nonpolarized and largely overlapping peripheral distribution of actin, E-cadherin, β-catenin, β1-integrin, and dystroglycan.

an in vitro equivalent of the proamniotic cavity and, importantly, requires apoptosis to form (Coucouvanis and Martin, 1995). In studies conducted on MCF-10A cell-derived mammary acini cultured under the inductive influence of Matrigel, a laminin-1 polymer-rich environment, interactions between cell proliferation and apoptosis occurred, and full inhibition of cavitation required both suppression of apoptosis and stimulation of cell cycle progression (Debnath et al., 2002). Laminin expression, indirectly or directly, appears to play a role in the induction of EB cavitation (Murray and Edgar, 2000). The apoptosis following laminin expression first occurs in the circumferential pattern within the ICM, with a sharp transition between apoptotic and living cells and initial sparing of a core island, and is followed by cavitation.

of adherens junctions (arrows, and enlarged inset) with associated lateral actin filaments near apical surfaces that face the central cavity (cav). Inset magnification bars, 0.2 μm.
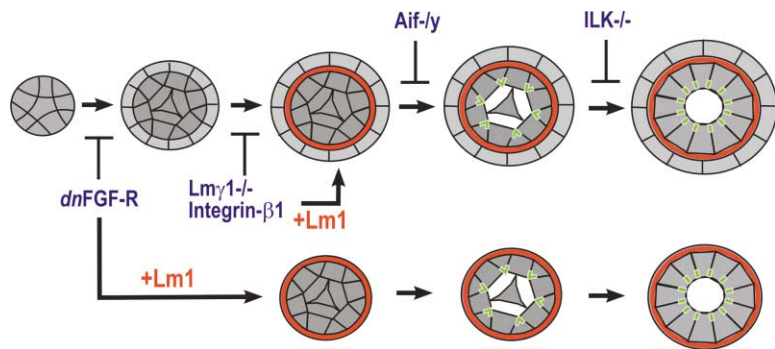
Figure 5. Mutations that Affect Embryoid Body Differentiation

Diagram illustrating the stages at which EB differentiation arrests in response to null mutations in the genes for laminin-γ1, β1-integrin, AIF, and ILK, or dominant-negative expression of *Fgfr2*. EBs with defects that resulted in a failure of heterotrimeric laminin synthesis, i.e., laminin-γ1$^{-/-}$, β1-integrin$^{-/-}$, and dn-Fgfr2, could be partially rescued with exogenous laminin-1, even in the absence of endoderm. Rescue of laminin-γ1$^{-/-}$ EBs was blocked with laminin-1 fragments that inhibit polymerization and anchorage through terminal LG modules.

While apoptosis can occur in the laminin-γ1 null and β1-integrin null EBs, the characteristics are different, i.e., scattered cells that undergo apoptosis in a central and diffuse pattern that usually does not result in cavitation. This latter type of apoptosis, particularly prominent in integrin β1 null EBs, may be responsible for the demise of the embryo by E.5.5 in vivo (Fässler and Meyer, 1995; Stephens et al., 1995).

One gene product affecting cavitation in EBs is apoptosis inducing factor (AIF), a mitochondrial flavoprotein that can induce a caspase-independent form of apoptosis (Joza et al., 2001). In particular, genetically modified EBs lacking expression of AIF (*Aif* −/y) were found to fail to undergo morphological signs of apoptosis or to cavitate (Joza et al., 2001). The study raises the interesting possibility that AIF apoptosis is triggered by laminin and that the apoptosis, which develops in the absence of laminin and does not lead to cavitation, is mediated by a different mechanism.

Embryonic Basement Membrane Assembly
Endodermal Regulation of Basement Membrane Assembly

Analysis of wild-type and mutant EBs has provided a model system to analyze the role that basement membrane components, especially laminins, exert during embryonic development. The endodermal cells of these tissues are the main source of laminin and type IV collagen secretion in the developing EBs and hence can regulate basement membrane assembly (Murray and Edgar, 2001b; Smyth et al., 1999). Expression of laminin subunits depends upon COUP-TF I/II and GATA transcription factors (Fujikura et al., 2002; Murray and Edgar, 2001b) and requires FGF-receptor activity, the latter mediated in part through the activation of Akt/PKB (Chen et al., 2000; Li et al., 2001b). Recombinant expression of a truncated *Fgfr2* lacking the cytoplasmic domain in ES cells results in a dominant-negative receptor (*dn*FGF-receptor) that causes a developmental block in endoderm and a failure of the EBs to form basement membrane, cavitate, or develop epiblast. β1-integrin has been found to selectively enable laminin-α subunit expression without greatly affecting laminin-β1 and -γ1 expression (Aumailley et al., 2000; Li et al., 2002). In the absence of an α subunit, β1 and γ1 are confined to an intracellular endodermal distribution and cannot be secreted (Yurchenco et al., 1997). Since the phenotype of the β1-integrin mouse gene knockout in mice was found to be embryonic lethal at E5.5 characterized by degeneration of the blastocyst ICM (Fässler and Meyer, 1995; Stephens et al., 1995), the earliest defect may result from a loss of laminin expression. At present, neither the ligand for the integrins (if any) nor the α chain integrin partners that activate laminin-α subunit expression are currently known. The loss of basement membranes in laminin-γ1 null, β1-integrin null, and *dn*FGF-receptor EBs can be restored simply by the addition of purified laminin-1 to the culture medium (Li et al., 2001a, 2002; Murray and Edgar, 2001b), underscoring the central role of endodermal laminin for basement membrane assembly. Genetic analysis in the nematode also supports a central role for laminin in the organization of basement membrane architecture. In the nematode, laminin is secreted and becomes localized to cell surfaces before the expression of type IV collagen, nidogen or perlecan, and in laminin mutants basement membranes are missing or are severely disrupted (Huang et al., 2003).

While laminin may be the principal secretory product of endoderm that is both necessary and sufficient to form basement membrane and promote epithelial differentiation, the effects of laminin alone probably are not sufficient for the characteristic ectodermal gene expression profile of the epithelial cells. Instead, other factors produced by the endodermal cells appear to be necessary for full ectodermal differentiation (Coucouvanis and Martin, 1995; Murray and Edgar, 2000; Murray and Edgar, 2001a). Thus, while endodermal cells are responsible for all of these events, they direct epithelialization via basement membrane assembly and induce the ectodermal expression profile via some unknown factor such as bone morphogenetic protein or hedgehog proteins (Byrd et al., 2002; Coucouvanis and Martin, 1995; Maye et al., 2000).

Laminin Polymerization and Cell Anchorage

If laminin acts as an embryonic induction factor, then it is an unusual one in that laminin cell binding is alone insufficient for imparting a differentiating signal. The laminin must also self-assemble to form a nascent basement membrane. This self-assembly into a polymer occurs spontaneously through a reversible, cooperative (i.e., nucleation propagation), calcium-dependent mechanism (laminin-1 polymer critical concentration of 0.1 μM) in which an LN domain from each short arm participates in the tessellating bond (Cheng et al., 1997; Garbe et al., 2002; Yurchenco and Cheng, 1993; Yurchenco et al.,

1992). In a study to evaluate the role of laminin polymerization in EB differentiation, laminin-γ1 null EBs were treated with laminin-1 with molar excess of specific fragment to evaluate their potential to inhibit laminin rescue of the phenotype (Li et al., 2002). While the EBs treated with laminin-1 assembled basement membrane and formed epiblast, those treated with polymer-inhibiting fragments did not. These results provided evidence that laminin polymerization is essential for basement membrane and its downstream developmental consequences.

Laminin has been found to assemble only on "competent" cell surfaces, i.e., surfaces capable of anchoring laminin (Tsiper and Yurchenco, 2002). A domain implicated in such anchorage of laminin is G domain, composed of five LG modules, each a ~20 kDa β sandwich (Tisi et al., 2000). These LG modules have been found to bind to α6β1 in LG1-3, α-dystroglycan in LG4, and heparin/sulfatide in LG4 (Andac et al., 1999; Sung et al., 1993; Talts et al., 1999). There is a delay of several days before laminin accumulation on the outer surface of the nonpolarized ectoderm is detected, suggesting that laminin must first induce competency. When laminin-γ1 null EBs were treated with laminin-1 with potentially inhibiting laminin fragments, only an LG4-containing fragment blocked phenotypic rescue of epithelialization and cavitation (Li et al., 2002), suggesting that either dystroglycan and/or the heparin binding activities of G domain contributed the most critical anchorage-type activities. The specificity of the interaction was further localized with recombinant LG4-5 with a mutation of the heparin/sulfatide binding residues 2792-4 within LG4.

A hypothesis to explain the preferential cell surface location of laminin is that of "anchorage-facilitated self-assembly" (Colognato and Yurchenco, 2000) in which the affinity of laminin for receptors and/or other surface binding molecules drives an increase of the local cell surface concentration of laminin, facilitating its polymerization (Figure 6). Of particular interest have been those receptors that, through binding of their cytoplasmic tails to adaptor and other proteins, can link the extracellular matrix to the cytoskeleton, i.e., β1 integrins (especially α6β1) and α-dystroglycan (Brown, 2000; Colognato et al., 1999; Rybakova et al., 2000; Wu and Dedhar, 2001), both present in the basal pole of epiblast epithelia. Indeed, several investigators have proposed that these integrins and dystroglycan are not only important for subsequent cell differentiation (Brakebusch et al., 2000; Deng et al., 2003), but for basement membrane assembly itself (DiPersio et al., 1997; Henry and Campbell, 1998; Raghavan et al., 2000).

As the laminin polymer accumulates on the target cell surface through a process of polymerization and anchorage, type IV collagen, nidogen, and perlecan are integrated into the structure. Laminin binds to nidogen-1, produced by the ectoderm, through a noncovalent high-affinity bond between the rod domain LE modules (domain III) of the laminin-γ1 subunit and the nidogen C-terminal globular domain (Timpl and Brown, 1996). Nidogen, in turn, can bind to type IV collagen and other basement membrane components, forming bridging links. Type IV collagen forms a three-dimensional polymer that provides a second stabilizing basement membrane network (Yurchenco, 1994). Its assembly is due to mass action-driven interactions in which monomers form linear antiparallel dimers through C-terminal globular (NC1) domain interactions, four-armed tetramers by the end overlapping of the N-terminal segments ("7S" domain), and laterally associated branching complexes. Perlecan, a heparan sulfate proteoglycan, binds to nidogen-1 through its core protein and may bind to the G domain of laminin (Timpl and Brown, 1996). However, genetic inactivation of the nidogen binding in laminin in mice, of nidogen in the nematode, and of perlecan in mice have provided evidence that these components are not required for early embryonic basement membrane assembly (Costell et al., 1999; Kim and Wadsworth, 2000; Willem et al., 2002). When laminin-1 is not secreted, the other basement membrane components accumulate in nonlinear discontinuous deposits within the interior of EBs at low levels and in the culture medium and ultrastructurally recognizable basement membranes do not form (Smyth et al., 1999; Li et al., 2002). By analogy, multiple layers, large whorls, and clumps of extracellular material accumulate in the pseudocoelomic cavity of nematodes lacking either laminin (Huang et al., 2003). Nonlaminin components likely provide structural stability, increasingly required as development progresses, and additional ligands on the basement membrane scaffold for growth factor storage and presentation to cell surface receptors (Costell et al., 1999; Halfter et al., 2002).

Receptors Implicated in Basement Membrane Assembly

β1-integrin and dystroglycan are candidate receptors for mediating basement membrane assembly on cell surfaces. Laminin-1 is found to interact with only those integrins with β1 (α1β1, α2β1, α6β1, α7β1) or β4 (α6β4) subunits (reviewed in Colognato and Yurchenco, 2000). β1-integrin null embryos die by E5.5 and β1-integrin null EBs, because they are unable to express the laminin-α subunit, arrest at the same stage as the laminin-γ1 null EBs (Aumailley et al., 2000; Fässler and Meyer, 1995; Li et al., 2002; Stephens et al., 1995). When treated with laminin-1, partial phenotypic rescue was observed with about half the efficiency seen with wild-type EBs (Li et al., 2002). The absence of detectable α6 integrin (or β4 integrin) in the laminin-rescued EBs provided evidence that β4 integrin compensation had not occurred. Thus, while β1-integrin is essential for laminin expression, it does not appear to be essential for basement membrane assembly or initial cell anchorage. The rescued EBs, however, had defects in cell adhesion to the basement membrane accompanied by accelerated apoptosis and delay or loss of endodermal differentiation. Thus β1-integrins, while not necessary for initiation of epithelialization, appear to be critical for robust epithelial/basement membrane adhesion and survival and may be important for subsequent cell migrations required during gastrulation.

Dystroglycan, which binds to laminins, agrin, and perlecan through its α subunit, has been another candidate for mediation of basement membrane assembly (Henry and Campbell, 1998). Its presence is required for at least some basement membranes, but not necessarily at the level of assembly. The mouse *Dag1* (dystroglycan) knockout was found to be an E6.5 lethal due to a failure
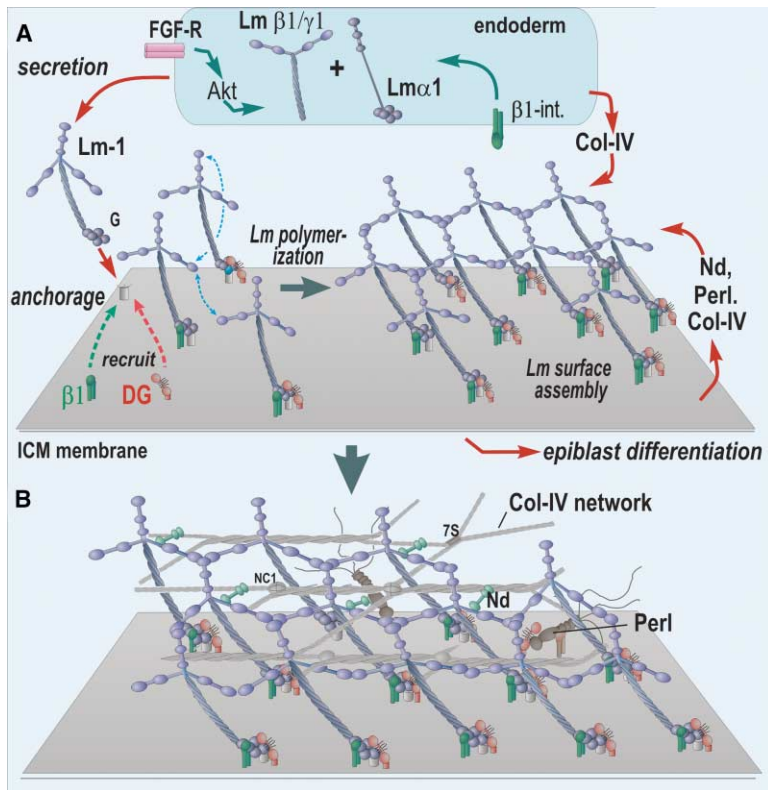
Figure 6. A Model of Embryonic Basement Membrane Assembly

(A) β1-integrin initiates laminin-α subunit expression in endoderm, enabling heterotrimer formation with existent laminin β1 and γ1 subunits. The secreted laminin-1 becomes anchored to the outer ICM cell surface through its G domain, substantially mediated by LG module 4, and with participation of β1-integrins and α/β-dystroglycan (DG). Laminin, concentrated on the cell surface, polymerizes through its short arms creating a multivalent network. The undifferentiated ectoderm, requiring this network, but not separately requiring integrin or dystroglycan at this stage, becomes polarized and converted to epiblast. Inset shows laminin fragments used to probe domain function.

(B) Type IV collagen, largely secreted by endoderm, forms a second network that becomes linked to laminin by ICM/epiblast-derived nidogen (Nd), and perlecan binds to nidogen and laminin, stabilizing the basement membrane. The collagen can interact with the cell surface through several β1 integrins (α1β1, α2β1, α3β1), while perlecan can interact through integrins (α3β1, αvβ3) and dystroglycan.

of Reichert's membrane but with formation of the embryonic basement membrane adjacent to epiblast (Henry and Campbell, 1998; Henry and Campbell, 1999; Williamson et al., 1997). When EBs null for dystroglycan were allowed to develop in culture, basement membrane, cavitation, and epiblast differentiation all occurred spontaneously, findings in agreement with the mouse data (Li et al., 2002). However, the dystroglycan null epiblasts were less elongated and underwent apoptotic degeneration at an accelerated rate, suggesting that dystroglycan plays a role in subsequent epithelial differentiation and, like β1-integrins, in cell survival. In agreement with the former point, dystroglycan has also been implicated in mediating polarization of epithelial cells in *Drosophila* (Deng et al., 2003). The *Large^myd* mouse lacks a glycosyl transferase required to complete the mannosyl O-linked oligosaccharide chain required for α-dystroglycan binding to laminin-1, agrin, and perlecan (Michele et al., 2002; Moore et al., 2002). There is no reported defect in the periimplantation period and the mice reach gestation. The defects are those of ruptures in the basement membrane of the *glia limitans* with associated cell defects of the developing brain cortex and a muscular dystrophy. The difference in phenotype between the *Dag1^−/−* embryo, and the *Large^myd* mouse suggests that the role of dystroglycan in development of Reichert's membrane and its associated cell layers may actually be independent of a receptor-ligand interaction involving basement membrane components.

Cell Adhesion and Tissue Organization
A critical function of basement membranes is to mediate cell adhesions to different cell types, a process related to cell polarization that contributes to cell organization and stability within developing tissues. In fact, the inability of cells to polarize and correctly associate with their neighbors in the nematode embryo likely accounts for the embryonic lethality in laminin-null animals (Huang et al., 2003). Moreover, in nematode and fly laminin mutants, tissues adhere to each other when they should not or fail to adhere when they should (Martin et al., 1999; Huang et al., 2003). Related to these polarity and adhesion defects is the ability of some cells to invade neighboring tissues, disrupting tissue organization and integrity.

The integrin family of receptors features prominently in the mediation of cell adhesion to basement membrane components, in particular laminins (reviewed in Bokel and Brown, 2002). Mutations in these integrins have resulted in defects of epithelial and muscle adhesion and integrity in the worm, fly, and mouse. Other nonintegrin receptors implicated in laminin adhesion are not only dystroglycan, but also syndecans, HNK-bearing proteins, and the Lutheran antigen (Hall et al., 1997; Matsumura et al., 1997; Parsons et al., 2001; Salmivirta et al., 1994; Shimizu et al., 1999; Woods and Couchman, 1994). These receptors, with the exception of mammalian α1 and α2 integrins, bind to the G domain of laminin. The laminin-α5 subunit, like its α1 paralog, possesses a polymerization LN domain at the end of a short arm with a similar, but nonidentical, arrangement of globules, LE rod domain modules, and terminal globular LG modules (Garbe et al., 2002; Miner et al., 1995). α6β1 and α3β1 integrin binding maps to LG1-3 while α-dystroglycan, heparin, and sulfatide binding map to LG4 (Yu and Talts, 2003). One property of the C-terminal region specific to

α5 laminins is that the Lutheran blood group antigen receptor, found in different epithelia, binds to LG3 (Kikkawa et al., 2002). The mouse laminin-α5 knockout results in late embryonic lethality with neural tube, limb, placental vasculature, and kidney defects (Miner et al., 1998). Further analysis of the kidney phenotype has revealed that during the capillary loop stage when a switch occurs from α1 to α5 expression in the glomerular basement membrane (GBM), the endothelial cells and mesangium are extruded and the glomerulus fails to develop (Miner and Li, 2000). Transgenic expression of laminin-α5 corrected the defect, while expression of a chimeric laminin-α5 containing the α1-LG4-5 subdomains only partially rescued this defect with failure of mesangial adhesion to GBM (Kikkawa et al., 2003). In vitro analysis of cell adhesion suggests that the defect is a loss of the Lutheran antigen receptor binding site, providing evidence for a direct link between cell-selective adhesion and cell organization to form a functional tissue.

Another mammalian example of a laminin tissue organization role during development is seen with the laminin-β2 subunit which is joined with either α2 or α5 along the neuromuscular axis and which is concentrated at the synaptic cleft (Patton et al., 1997). The laminin-β2 mouse knockout is characterized by a late developing defect of the neuromuscular junction in which the Schwann cell inappropriately covers the synaptic cleft (Noakes et al., 1995a; Patton et al., 1998). The defect may be due to the loss of inhibition of Schwann cell migration and/or adhesion mediated by laminin-11.

Conclusions

Recent work analyzing the biological role of laminins has demonstrated that these multifunctional extracellular matrix molecules have a profound influence on the generation of complex tissues. This ability is dependent upon the properties of distinct functional domains of the laminin molecule that coordinate self-association with cell anchorage and receptor-mediated signal transduction. Interestingly, the recent insights into the fundamental roles of laminins in tissue development raise several important questions related to the molecular mechanisms involved that remain to be tackled.

One question concerns the requirement for laminin anchorage in basement membrane assembly. The nature of this anchorage may be particularly important with respect to epithelial development, since recruitment of anchors may begin the process of cell polarization leading to epithelialization. Clearly β1-integrins and dystroglycan are important for cell interactions with laminin, including cell adhesion, survival, and migration. However, the initial process of cell polarization does not appear to be primarily dependent upon the activity of either receptor. This may be due to receptor redundancy, or alternatively, due to the participation of other receptors whose identities remain to be established. The generation of mutant ES cell clones lacking both the β1-integrin gene and the dystroglycan gene, as well as the generation of laminins that are deficient in the receptor binding sites, will permit addressing this issue. Another question relates to the nature of the tethers that exist between laminin and the actin cytoskeleton. While the establishment and strengthening of such connections has been axiomatic in the development of focal adhesions (Hynes, 2002), it is not clear if this is the case in cell contacts mediated through basement membranes. Indeed, the analysis of ILK null EBs suggests that a reverse transition resulting in a weakening of intracellular associations occurs as the basement membrane is assembled. The establishment of ES and their progeny cells lacking proteins localized to focal adhesions should aid in the determination of how the cytoskeleton is anchored at basement membrane adhesion sites. A third question concerns the role of polymerization in cell signaling. We propose that the mechanical properties of the laminin polymer, modulated by the fully formed type IV collagen polymer, may be as important as receptor specificity and inherent signal transduction properties in determining the behavior of cells and cell layers on basement membrane. Evidence that mechanical effects play important roles has been seen in focal adhesion integrin interactions (Jalali et al., 2001; Li and Xu, 2000; Schwartz, 2001; Wang et al., 1993), and these studies may provide useful conceptual clues in an analysis that may be approached by introducing mutations that affect the mechanical properties of the laminin polymer. A final question concerns the functions of different members of the laminin family. While α1- and α5-laminins are found to contribute to the generation of polarity in different epithelia, it is unclear if other laminins (e.g., laminin-2) serves a parallel role in the differentiation of mesodermally derived cells or whether, as is suggested by the null phenotypes, the functions are primarily ones of adhesion and maintenance. These roles will likely be further delineated as laminin and receptor mutations continued to be studied in both invertebrates and vertebrates.

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