Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation

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Summary

Laminins are components of all basement membranes and have well demonstrated roles in diverse developmental processes, from the peri-implantation period onwards. Laminin 1 (α1β1γ1) is a major laminin found at early stages of embryogenesis in both embryonic and extraembryonic basement membranes. The laminin γ1 chain has been shown by targeted mutation to be required for endodermal differentiation and formation of basement membranes; *Lamc1*$^{-/-}$ embryos die within a day of implantation. We report the generation of mice lacking laminin α1 and laminin β1, the remaining two laminin 1 chains. Mutagenic insertions in both *Lama1* and *Lamb1* were obtained in a secretory gene trap screen. *Lamb1*$^{-/-}$ embryos are similar to *Lamc1*$^{-/-}$ embryos in that they lack basement membranes and do not survive beyond embryonic day (E) 5.5. However, in *Lama1*$^{-/-}$ embryos, the embryonic basement membrane forms, the embryonic ectoderm cavitates and the parietal endoderm differentiates, apparently because laminin 10 (α5β1γ1) partially compensates for the absent laminin 1. However, such compensation did not occur for Reichert's membrane, which was absent, and the embryos died by E7. Overexpression of laminin α5 from a transgene improved the phenotype of *Lama1*$^{-/-}$ embryos to the point that they initiated gastrulation, but this overexpression did not rescue Reichert's membrane, and trophoblast cells did not form blood sinuses. These data suggest that both the molecular composition and the integrity of basement membranes are crucial for early developmental events.

Key words: Laminin, Basement membrane, Embryogenesis, Mouse

Introduction

Basement membranes are thin sheets of specialized extracellular matrix that surround epithelia, endothelia, muscle cells, fat cells, Schwann cells and peripheral nerves, as well as the entire central nervous system. They play important roles in maintaining tissue integrity and compartmentalization, in filtration and in diverse developmental processes. All basement membranes contain type IV collagen, nidogen, sulfated proteoglycans and laminin. Laminins are large heterotrimeric glycoproteins composed of one α, one β and one γ chain (Colognato and Yurchenco, 2000). There are currently five α, four β and three γ chain genes that have been described in vertebrates, and the chains can assemble into at least 15 different heterotrimers (Burgeson et al., 1994; Koch et al., 1999; Libby et al., 2000; Miner et al., 1997; Parsons et al., 2002). Some laminins have been shown to self-associate to form polymeric networks that interact with cellular receptors, such as dystroglycan and integrins (Cheng et al., 1997; Colognato et al., 1999). These networks are thought to recruit other matrix proteins that interact both with laminin and with each other to form the insoluble, highly crosslinked basement membrane.

Both naturally occurring and targeted mutations in laminin genes have demonstrated the importance of laminin and its isoform composition to basement membrane structure and function. For example, mutations in *Lama2* (α2) cause congenital muscular dystrophy (Helbling-Leclerc et al., 1995; Xu et al., 1994); mutations in laminin 5 (α3β3γ2) component genes cause severe skin blistering (reviewed by Pulkkinen and Uitto, 1999); mutation of *Lama4* causes a mild muscular dystrophy and a mild bleeding disorder (Patton et al., 2001; Thyboll et al., 2002); mutation of *Lama5* causes defects in neural tube closure, digit septation, placentation, kidney formation and vascularization, lung lobe septation, hair morphogenesis, and intestinal smooth muscle differentiation (Bolcato-Bellemin et al., 2003; Li et al., 2003a; Miner et al., 1998; Miner and Li, 2000; Nguyen et al., 2002); and mutation in *Lamb2* causes severe defects in neuromuscular junction differentiation and kidney filtration (Noakes et al., 1995a; Noakes et al., 1995b). Finally, null mutation in *Lamc1* results in very early embryonic lethality (embryonic day [E] 5) because of the absence of basement membranes, resulting in failure of endoderm differentiation (Smyth et al., 1999). Similarly, in vitro laminin γ1 is required for proper organization and differentiation of embryoid bodies (reviewed

by Li et al., 2003b; Li et al., 2002; Murray and Edgar, 2000; Murray and Edgar, 2001).

That mutation in *Lamc1* causes such early lethality probably stems from the fact that laminin γ1 is found in 10 out of the 15 known laminin trimers. Thus, removing γ1 effectively prevents assembly of almost all basement membrane-associated laminins, though only two laminins, laminin 1 (α1β1γ1) and laminin 10 (α5β1γ1), are detectable at significant levels in peri-implantation embryos (Klaffky et al., 2001). We now report the generation and characterization of mice with mutations in *Lama1* and *Lamb1*, which encode the remaining two laminin 1 subunits, α1 and β1. These mice were derived from embryonic stem (ES) cell clones isolated in a gene trap screen designed to capture genes encoding secreted and cell surface proteins (Leighton et al., 2001; Mitchell et al., 2001; Skarnes et al., 1995). The gene trap vector contained a splice acceptor, a transmembrane (TM) domain, and *βgeo*, a reporter (*lacZ*)/selectable marker (*neo*) fusion, but it lacked a promoter to drive expression. Upon electroporation into ES cells, this vector integrated into introns of many genes (Mitchell et al., 2001), resulting in production of membrane-tethered fusion proteins that contained the N terminus of the protein encoded by the trapped gene, a TM domain, and a cytoplasmic βgeo. By forcing production of such fusion proteins, the gene trap vector not only prevents expression of the full-length endogenous proteins (i.e. it is mutagenic), but it also results in expression of the *lacZ* reporter under the control of the endogenous regulatory elements of the trapped genes. Thus, the gene trap insertions in *Lama1* and *Lamb1* have allowed us to: (1) generate knockout mice that lack laminin α1 or β1, and (2) characterize the expression pattern of the two genes using X-gal staining. In addition, we have used a widely expressed laminin α5 transgene to show that α5 can compensate somewhat for the absent α1 in the embryonic basement membrane but not in Reichert's membrane (RM). This compensation is highly significant in that it allows for initiation of gastrulation even in the absence of RM.

Materials and methods

Generation of knockout and transgenic mice

ES cell lines with insertions in *Lama1* (PST008) and *Lamb1* (PST084) were provided by William Skarnes, University of California at Berkeley (presently at The Wellcome Trust Sanger Institute, Cambridge, UK). Their isolation and identification and production of heterozygous mice have been reported (Leighton et al., 2001; Mitchell et al., 2001; Yin et al., 2003). Generation of *Lama5*$^{-/-}$ mice has been previously described (Miner et al., 1998).

Mice carrying a laminin α5 transgene (Mr5) have been previously described (Kikkawa et al., 2002; Kikkawa et al., 2003; Moulson et al., 2001). The full-length mouse laminin α5 cDNA-coding region was placed under the control of the widely active regulatory element miw, which contains the Rous Sarcoma long terminal repeat inserted into the chicken β-actin promoter (Suemori et al., 1990). Of five transgenic founders, two produced offspring that expressed the transgene widely throughout embryogenesis. One of these lines was used in the experiments described here.

X-gal staining

For wholemount staining, embryos or dissected tissues were fixed in 2% paraformaldehyde in PBS for 1 to 6 hours at room temperature. They were then incubated at 37°C for 2-12 hours in staining solution containing 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.1% NP-40, 0.2% sodium deoxycholate and 0.8 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in PBS.

For staining of sections, tissues were first immersed in OCT and frozen in dry ice/ethanol-cooled 2-methylbutane. Sections were cut in a cryostat (10 μm), air-dried at room temperature, and then fixed and stained as described above. After staining, sections were rinsed in PBS, counterstained with 0.1% nuclear Fast Red (Fluka/Sigma-Aldrich, St. Louis, MO) in 5% aluminum sulfate, and mounted in 90% glycerol.

Antibodies and immunofluorescence

The antibodies to mouse laminin chains used were: rat anti-α1 clone 8B3 and rat anti-β1 clone 5A2 (Abrahamson et al., 1989; St John et al., 2001); rabbit anti-α5 serum 8948 (Miner et al., 1997); rabbit anti-β1 and anti-β2 (Sasaki et al., 2002); rat anti-γ1, MAB1914 (Chemicon, Temecula, CA); and rabbit anti-laminin-1 (α1β1γ1) serum R23 (Sanes et al., 1990). Other antibodies used were: goat anti-collagen α1,α2(IV) (Southern Biotechnology Associates, Birmingham, AL); rat anti-nidogen/entactin, MAB1946 (Chemicon); and rat anti-cytokeratin 8 clone TROMA-1 (Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA).

For cryosectioning of peri- and post-implantation embryos (E5 to E7.5), uterine horns were removed from timed mated females, immersed in OCT, and frozen in dry ice-ethanol cooled 2-methylbutane. In some cases, decidua were first removed from the uterus and then frozen in OCT. Sections (7-10 μm) were cut in a cryostat and either fixed in 2% paraformaldehyde in PBS for 10 minutes or stained unfixed. After rinsing in PBS, antibodies diluted in 1% BSA in PBS were applied for 1 hour. Sections were then rinsed in PBS, and FITC- or Cy3-conjugated secondary antibodies (Chemicon), along with 1 μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR) to label nuclei, were applied for 1 hour. After rinsing, sections were mounted in 1 mg/ml *p*-phenylenediamine/90% glycerol/0.1× PBS and viewed under epifluorescence with a Nikon Eclipse 800 microscope. Images were captured with a Spot2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI).

In situ hybridization

E7.5 embryos were frozen and sectioned as described above. In situ hybridizations were carried out essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993) using a *T* (brachyury) riboprobe labeled with a digoxigenin-UTP labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). The probe was made from an 800 bp *Eco*RI fragment of a brachyury expressed sequence tag (GenBank accession number AA163572) subcloned into Bluescript II SK+ (Stratagene, La Jolla, CA).

Results

Production and characterization of *Lama1* and *Lamb1* gene trap mice

Skarnes and colleagues performed a gene trap screen that identified secreted and transmembrane proteins expressed in ES cells (Mitchell et al., 2001; Skarnes et al., 1995). Because the gene trap vector used did not carry a promoter, expression of the βgeo (*lacZ*/neo) reporter/selectable marker was driven by regulatory elements of the trapped genes. Four out of the 12 known laminin chain genes were trapped in ES cells (Leighton et al., 2001; Mitchell et al., 2001), and they encode the four chains found in laminin 1 (α1β1γ1) and laminin 10 (α5β1γ1). Although the screen was not saturating, we can nevertheless conclude that laminin 1 and 10 are expressed by

ES cells. Nidogen 2, perlecan and agrin were also trapped (Leighton et al., 2001; Mitchell et al., 2001). Thus, expression of basement membrane proteins is a general feature of the ES cells used, perhaps reflecting their in vivo counterpart, the epiblast/embryonic ectoderm. The epiblast probably contributes to the basement membrane that normally lies between the ectoderm and the visceral endoderm in vivo.

We obtained the *Lama1* and *Lamb1* gene trap ES cell lines and produced mice carrying the insertions (Yin et al., 2003). Based on 5′ RACE analysis and comparisons with the Celera Genomics Mouse Genome Assembly, we determined that in the *Lama1* trap line the gene trap vector (pGT0TMp) inserted in the 20.4 kb third intron of *Lama1* to produce a fusion protein containing the first 120 amino acids of laminin α1 (part of domain VI), the vector-encoded TM domain and βgeo (Fig. 1A) (Yin et al., 2003). In the *Lamb1* trap line, the vector (pGT1TMpfs) inserted into the 10.2 kb 23rd intron to produce a fusion protein containing the first 1178 amino acids of laminin β1 (domains III through VI), the TM domain and βgeo (Fig. 1B) (Yin et al., 2003).

Analysis of gene expression with X-gal

Because the βgeo fusion proteins are expressed under the control of the *Lama1* and *Lamb1* endogenous regulatory elements, we used X-gal staining to investigate the expression patterns of the two genes during embryogenesis. A number of in situ hybridization and antibody studies have been performed previously to examine expression of these genes, so we compared our results with published studies.

Lama1

In general, *Lama1* is considered to have a restricted pattern of expression (Virtanen et al., 2000), and our results are consistent with this notion. The major site of expression from E9 to E13 was the central nervous system (CNS). Intense X-gal staining was observed in several regions of the brain, as well as in the ependymal layer of the spinal cord, which lines the central canal, and in meningeal cells (Fig. 2 and J.H.M., unpublished). This is consistent with in situ hybridization analyses (Lentz et al., 1997; Thomas and Dziadek, 1993). The major basement membrane associated with the CNS that contains laminin α1 is the pial or meningeal basement membrane, which covers the entire CNS outer surface. This basement membrane is synthesized by meningeal cells, which explains their staining with X-gal. However, it is not clear why ependymal cells express *Lama1* so robustly. Staining could represent the radial glia, which extend from the ependyma to the pia and may deposit laminin 1 (α1β1γ1) in the pial basement membrane. Expression was also observed in the lens of the eye (Fig. 2E), as we showed previously by in situ hybridization (Lentz et al., 1997). Embryos lacking the *Lama1* gene trap insertion did not stain significantly with X-gal (Fig. 2B).

Another major site of expression was the urogenital system. Staining was prominent in newly formed epithelia in the developing kidney and in the vas deferens (Fig. 2F). Similar expression in kidney was found previously by other methods (Ekblom et al., 1990). One major site of *Lama1* expression not

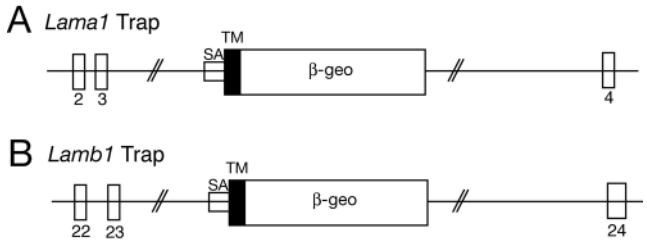

Fig. 1. Insertion of secretory gene trap vectors into *Lama1* and *Lamb1*. (A) The vector pGT0TMp inserted into the third and largest intron of *Lama1*, which is 20.4 kb. The remaining 59 downstream exons and introns are not shown. (B) The vector pGT1TMpfs inserted into the 23rd and largest intron of *Lamb1*, which is 10.2 kb. The remaining exons (of the 33 total) and introns are not shown.

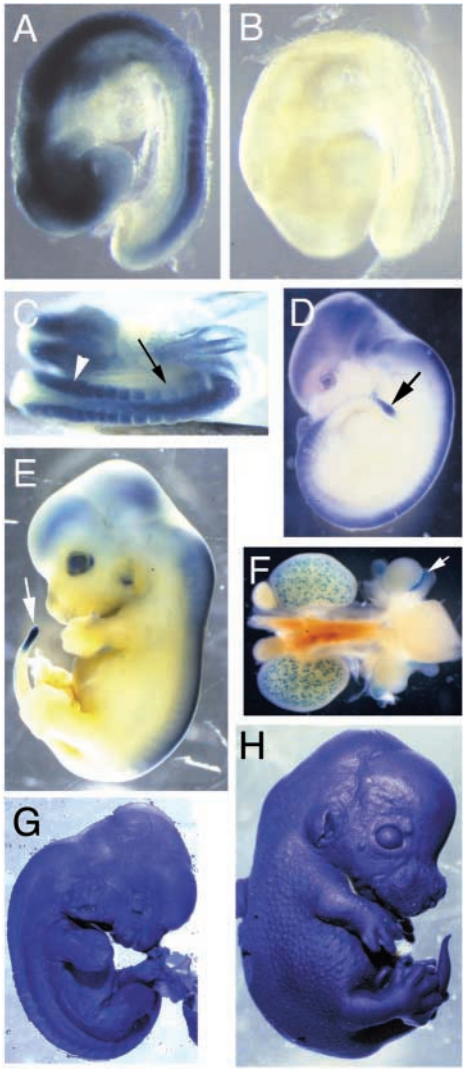

Fig. 2. X-gal staining of *Lama1* Trap and *Lamb1* Trap embryos. Whole *Lama1* Trap embryos are shown at E9.5 (A,C), E11.5 (D) and E13.5 (E). A control E9.5 littermate stained in X-gal is also shown (B). There is intense X-gal staining in the central nervous system and in presomitic mesoderm (arrowhead in C, black arrow in D, white arrow in E). Once formed, somites soon lose X-gal reactivity (black arrow in C). (F) The urogenital region was dissected out of an E16.5 male *Lama1* Trap embryo and then stained with X-gal. Prominent staining of the vas deferens (white arrow) and punctate staining in kidneys was observed. Whole *Lamb1* Trap embryos are shown at E11.5 (G) and E13.5 (H).

reported before was the pre-somitic mesoderm, which was stained intensely by X-gal, and expression was downregulated after somites had formed (Fig. 2C,D). Somitogenesis involves conversion of pre-somitic mesoderm into an epithelial sphere surrounded by a basement membrane. Laminin α1 is present in this basement membrane (J. H. Miner, unpublished) and may be required for proper cell polarization.

Lamb1

Lamb1 is considered to be deposited almost ubiquitously in basement membranes. Consistent with this, X-gal stained whole *Lamb1*$^{+/-}$ gene trap embryos completely blue (Fig. 2G,H). Tissues dissected out of older embryos were also stained completely blue; however, X-gal staining of frozen sections, albeit a less sensitive assay, revealed that not all cells were stained (J.H.M., unpublished). Interestingly, many cells not adjacent to basement membranes were stained blue. This indicates that there may be promiscuous expression of *Lamb1* in cells that do not normally secrete laminin. Indeed, we found significant expression of *Lamb1* by a large population of CNS neurons, but very little laminin β1 protein associated with most of them (Yin et al., 2003).

Both laminin 1 (α1β1γ1) and laminin 10 (α5β1γ1) are present in early postimplantation embryos

Owing to the peri-implantation lethality caused by targeted mutation of *Lamc1*, we predicted that *Lama1*$^{-/-}$ and *Lamb1*$^{-/-}$ embryos would show phenotypes at similarly early gestational ages. We therefore examined expression of laminin α and β chains in early postimplantation embryos to gain a better understanding of where α1 and β1 were expressed and whether compensation by other chains might be expected.

From E5.5 to E7.5, we could only detect laminins α1, α5, β1 and γ1 (Fig. 3 and J.H.M., unpublished), which indicates the presence of laminin 1 and laminin 10. But these isoforms did not accumulate equally in basement membranes. Laminin 1 was the prominent laminin in RM at all stages (Fig. 3, arrows), though laminin 10 was also present at E6.5 in the region of RM nearest the ectoplacental cone (Fig. 3B, asterisks), as previously shown (Klaffky et al., 2001). The other

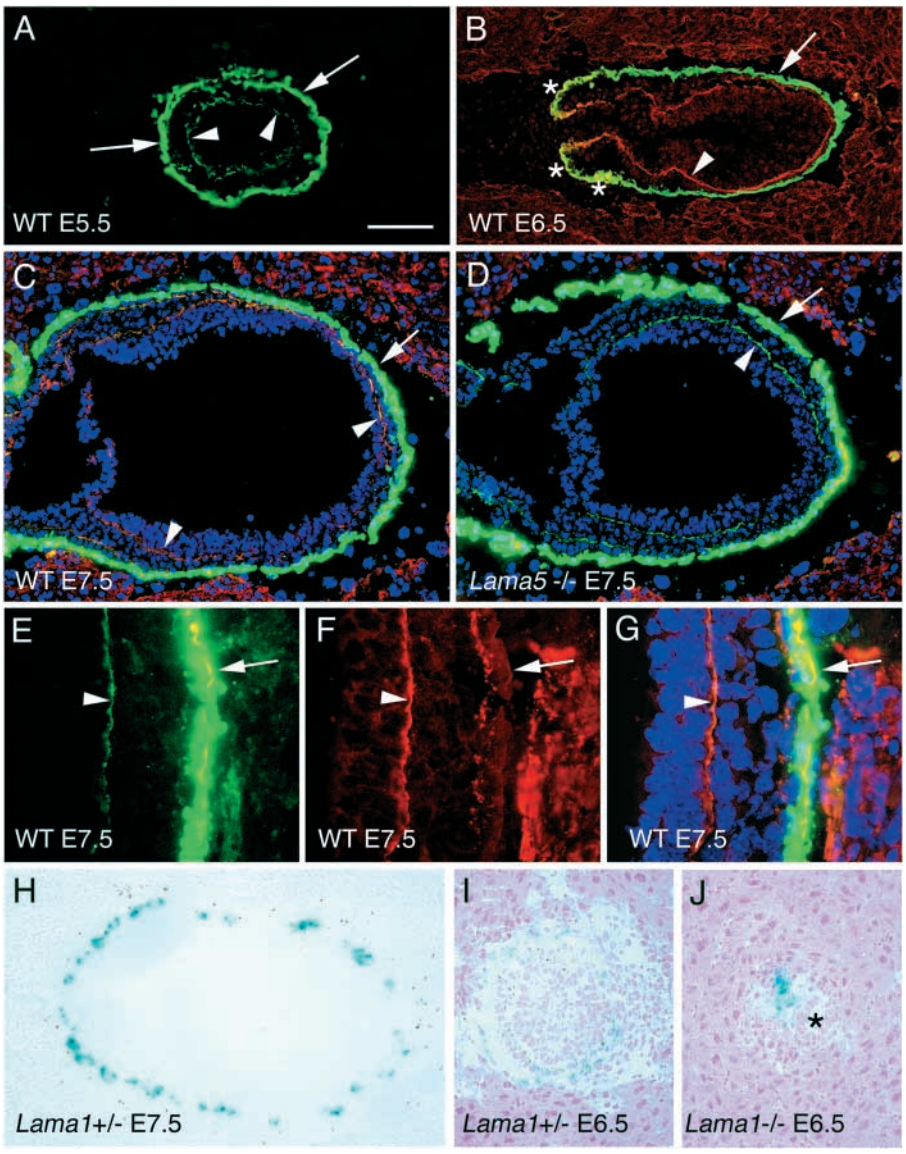

Fig. 3. Expression of laminin chains in normal and mutant early postimplantation embryos. (A-G) Arrowheads indicate the embryonic basement membrane and the arrow indicates RM. (A) At E5.5, laminin α1 was detected in the embryonic basement membrane and in RM. (B) At E6.5, laminin α5 (red) is detected primarily in the embryonic basement membrane, though some is apparent in RM at the ectoplacental pole (asterisks). Laminin α1 (green) is abundant in RM, but its presence in the embryonic basement membrane is obscured by the α5 staining. (C,E-G) At E7.5 in wild type, laminin α1 (green) is present in both the embryonic basement membrane and in RM, but laminin α5 (red) appears confined primarily to the embryonic basement membrane. (D) This embryo is *Lama5*$^{-/-}$, demonstrating the specificity of the α5 antibody. Note staining for α5 in the surrounding maternal tissue. (F) Note the absence of α5 in RM. (G) Nuclei (blue) are visible in the merged images. (H-J) X-gal staining of sections of *Lama1*$^{+/-}$ and *Lama1*$^{-/-}$ embryos, as indicated. Cells of the PE at the periphery of the normal embryos stain blue (H,I). In the mutant, the cells stain blue but are found in a cluster next to the small embryo (asterisk in J) owing to failure to migrate. Scale bar in A: 100 μm for A-D,H-J; 33.3 μm for E-G.

major basement membrane at these early stages is the embryonic basement membrane, which lies between the embryonic ectoderm and the visceral endoderm (Fig. 3, arrowheads). Laminin 10 appeared most prominent in the embryonic basement membrane (Fig. 3B,C,F,G), though laminin 1 was also clearly detectable (Fig. 3A,E). Given the nature of immunofluorescence, it is not possible to make a quantitative assessment of the level of laminin 1 versus that of laminin 10 in this basement membrane.

To confirm the results for laminin α5, we sectioned and stained a limited number of E7.5 *Lama5*$^{-/-}$ embryos. The anti-α5 antibody stained neither the embryonic basement membrane nor RM in a subset of embryos derived from *Lama5*$^{+/-}$ intercrosses (Fig. 3D), and we conclude that these represent *Lama5*$^{-/-}$ embryos. Furthermore, these results confirm the specificity of our α5 antibody. Staining for laminin α1 in the *Lama5*$^{-/-}$ embryos was similar to controls, and no other α chains could be detected (Fig. 3D and J.H.M., unpublished). Thus, the laminin 1 in the embryonic basement membrane is sufficient to maintain its structure and function and compensates for the absent laminin 10 in *Lama5*$^{-/-}$ embryos, which can develop to very late fetal stages (Miner et al., 1998). Similarly, the normal pattern of deposition of laminin 10 at E5.5 to E7.5 suggests that it could compensate for laminin 1 in the embryonic basement membrane, but probably not in RM, in *Lama1*$^{-/-}$ embryos.

Identification of *Lama1*$^{-/-}$ and *Lamb1*$^{-/-}$ embryos

Lama1$^{+/-}$ and *Lamb1*$^{+/-}$ mice were intercrossed to generate potential *Lama1*$^{-/-}$ and *Lamb1*$^{-/-}$ embryos at an expected Mendelian frequency of 25%. The uterine horns of timed pregnant females were removed, frozen in OCT and sectioned on a cryostat. Embryo implantation sites (E5.5 to E7.5) were localized using the trophoblast marker antibody TROMA 1 (anti-cytokeratin 8), which labeled the invading trophoblast cells as well as endoderm cells in the embryo. We then used monoclonal antibodies to laminin α1 (8B3) and β1 (5A2) to identify control *Lama1*$^{+/-}$ and *Lamb1*$^{+/-}$ embryos, respectively. Embryos that were not stained with these antibodies in a basement membrane-like pattern were judged to be *Lama1*$^{-/-}$ and *Lamb1*$^{-/-}$, respectively (see below).

In many cases, several sections were stained with X-gal to take advantage of the expression of βgeo to determine which embryos contained at least one mutated (gene trapped) allele. In *Lama1*$^{+/-}$ and *Lama1*$^{-/-}$ embryos, only cells of the parietal endoderm (PE), which secrete the components of RM, stained blue (Fig. 3H-J); the lack of staining in the visceral endoderm, which contributes to the embryonic basement membrane, indicates that *Lama1* expression is weak in those cells. However, while the blue parietal endodermal cells were localized at the periphery of laminin α1 antibody-positive embryos (Fig. 3H,I), blue cells were clustered in those embryos that were laminin α1-negative (Fig. 3J), all of which were small, suggesting that the PE was unable to migrate on the inner surface of the blastocoele, a phenotype reported for *Lamc1*$^{-/-}$ embryos (Smyth et al., 1999). Absence of X-gal staining was used to identify wild-type *Lama1*$^{+/+}$ embryos. Of 55 scored embryos from *Lama1*$^{+/-}$ intercrosses, 25% were +/+, 51% were +/–, 20% were –/– and 4% could not be unambiguously genotyped. Of 77 scored embryos from *Lamb1*$^{+/-}$ intercrosses, 13% were +/+, 57% were +/–, 22% were –/– and 8% could not be genotyped.

Phenotypes of *Lama1*$^{-/-}$ and *Lamb1*$^{-/-}$ embryos

Lama1

At E7.5, after gastrulation has normally ensued and embryos should be relatively large (Fig. 3C), *Lama1*$^{-/-}$ embryos had already died, as little embryonic tissue remained at the center of the deciduum (J.H.M., unpublished). However, at E6.5, *Lama1*$^{-/-}$ embryos still exhibited some degree of organization and had cavitated, though they were significantly smaller than wild-type and heterozygous littermates (Fig. 4C-F). RM was not present in the *Lama1*$^{-/-}$ embryos, as judged by staining with antibodies to type IV collagen, nidogen and laminin γ1 (Fig. 4F and J.H.M., unpublished). At E5.5, *Lama1*$^{-/-}$ embryos were already smaller than control embryos (Fig. 4A,B). RM was not present at this earlier stage either, indicating that it did not form and then break down because of the absence of

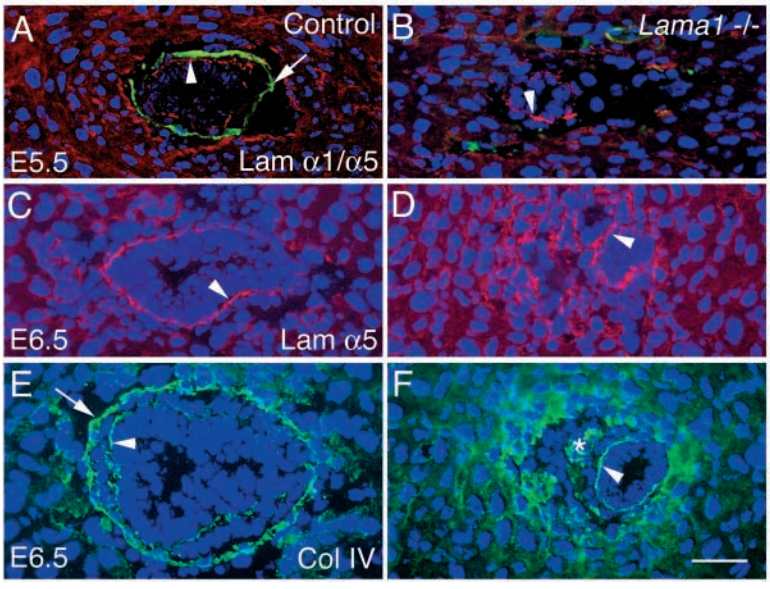

Fig. 4. Analysis of basement membranes in *Lama1*$^{-/-}$ embryos. (A,C,E) Wild type; (B,D,F) *Lama1*$^{-/-}$. Arrowheads indicate the embryonic basement membrane, and arrows indicate RM, when present. (A,B) Triple staining for laminin α1 (green), laminin α5 (red), and nuclei (blue) at E5.5. Both control and mutant embryos have a laminin α5-positive embryonic basement membrane, but the mutant lacks immunoreactivity of α1 and RM. (C,D) Double staining for laminin α5 (red) and nuclei (blue) at E6.5. Laminin α5 is present in the embryonic basement membrane in both embryos. (E,F) Double staining for collagen α1, α2(IV) (green) and nuclei (blue). Collagen IV is present in both the embryonic basement membrane and RM in the control, but only the embryonic basement membrane is present in the *Lama1* mutant. Asterisk in F probably represents non-basement membrane associated collagen IV made by PE, clustered as in Fig. 3J. Note that the arrangement of nuclei in B and F suggest that cavitation occurred in *Lama1* mutant embryos. Scale bar in F: 100 μm.

laminin 1. Staining with antibodies to laminin α5, β1 and γ1 revealed that there were significant levels of laminin 10 (α5β1γ1) in both control and *Lama1*$^{-/-}$ embryonic basement membranes (Fig. 4A-D and J.H.M., unpublished). It is therefore likely that compensation by laminin 10 for the missing laminin 1 allows for maintenance of the embryonic basement membrane and for successful polarization and cavitation of the embryo proper.

Lamb1

By comparison with the *Lama1* mutant phenotype, the *Lamb1* mutant phenotype was much more severe. At E6.5, *Lamb1*$^{-/-}$ embryos were not detected. At E5.5, however, there were some normal features, although the mutants lacked RM and there was no detectable laminin in the region where the embryonic basement membrane should have been (Fig. 5D-F and J.H.M., unpublished). This striking difference between the *Lama1* and *Lamb1* mutants could explain the more severe phenotype of the latter. As discussed above, laminin α5 is normally detected in the embryonic basement membrane and may compensate somewhat for the missing α1 in *Lama1*$^{-/-}$ embryos. However, laminin β2 is not normally found in the embryonic basement membrane and was not expressed in the absence of β1 (J.H.M., unpublished). With no laminin β chain present, no laminin can accumulate and no basement membranes can form, thus leading to the more severe phenotype.

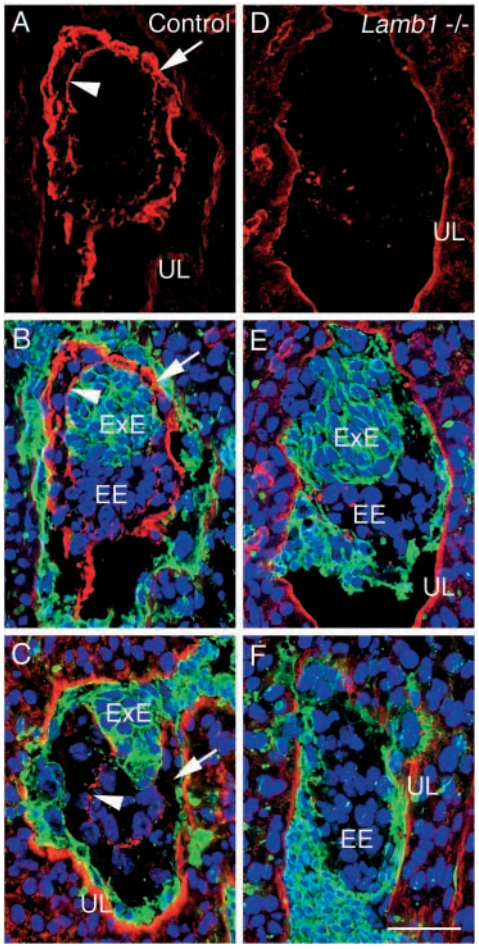

Fig. 5. Analysis of *Lamb1*$^{-/-}$ embryos. Arrowheads indicate the embryonic basement membrane, and arrows indicate RM, when present. E5.5 control (A-C) and *Lamb1*$^{-/-}$ (D-F) embryos were stained for laminin β1 (A,D); for laminin β1 (red), cytokeratin 8 (green) and nuclei (blue) (B,E); and for laminin α5 (red), ctyokeratin 8 (green) and nuclei (blue) (C,F). Note the absence of basement membranes in the *Lamb1*$^{-/-}$ embryos, other than those associated with the uterine lining (UL). Anti-cytokeratin 8 stains the extra-embryonic ectoderm (ExE), the PE and trophoblasts. More trophoblast invasion is apparent in the control than in the *Lamb1*$^{-/-}$ embryos. EE, embryonic ectoderm. Scale bar in F: 50 μm.

Although *Lamb1*$^{-/-}$ embryos had distinct embryonic and extra-embryonic regions, the embryonic region exhibited no signs of cavitation, and the mural trophectoderm and PE were disorganized and bunched together (Fig. 5E,F), suggesting that the blastocoele wall had collapsed after the onset of implantation. Trophoblast invasion was evident in some embryos, as assessed by degeneration of the uterine epithelial basement membrane, but the extent of invasion was greatly reduced compared with control embryos (Fig. 5B,E). This in vivo phenotype may be analogous to those observed in vitro for embryoid bodies that either do not express laminin or that cannot organize a basement membrane (Li et al., 2002; Li et al., 2001; Murray and Edgar, 2000; Murray and Edgar, 2001).

Overexpression of laminin α5 promotes increased compensation in *Lama1* mutants

If our hypothesis that endogenous laminin α5 compensates for the missing α1 to promote formation and maintenance of the embryonic basement membrane is correct, then increasing the level of α5 expression in the embryo should improve embryonic development in the mutant and perhaps even rescue RM formation. To test this possibility, we crossed a widely expressed laminin α5 transgene (Mr5) onto the *Lama1*$^{+/-}$ background and intercrossed the resulting offspring to generate *Lama1*$^{+/-}$; Mr5/Mr5 mice (homozygous for the Mr5 transgene). Then, in order to generate *Lama1*$^{-/-}$ embryos that carry the Mr5 transgene, *Lama1*$^{+/-}$; Mr5/Mr5 males were mated to *Lama1*$^{+/-}$ females to produce *Lama1*$^{-/-}$; Mr5 embryos at an expected frequency of 25%. The resulting anti-laminin α1-negative, obligate hemizygous Mr5 embryos always appeared larger and more developed than *Lama1*$^{-/-}$ embryos at E6.5 (compare Fig. 4 with Fig. 6A,B). At E7.5, when *Lama1*$^{-/-}$ embryos were already dead, *Lama1*$^{-/-}$; Mr5 embryos were alive, although they were significantly smaller than controls (Fig. 6C-F). The embryonic region was particularly small, while the extra-embryonic ectoderm and ectoplacental cone regions were relatively larger. The embryonic ectoderm had cavitated and exhibited strong staining for laminin in the embryonic basement membrane. Significantly, there were very few trophoblast giant cells at the periphery of the embryo, and the blood sinuses of the yolk sac placenta, which were quite evident in controls, were absent in *Lama1*$^{-/-}$; Mr5 embryos (Fig. 6E,F).

In no cases did *Lama1*$^{-/-}$; Mr5 embryos have a properly formed RM. In some embryos, laminin β1 staining could be detected in a pattern that was consistent with a RM localization, with fairly strong staining proximally and weak and inconsistent staining distally (Fig. 6F). These observations suggested that Mr5 either is not expressed well by the PE that secretes RM, or that laminin 10 is simply not capable of substituting for laminin 1 to promote formation of RM. To distinguish between these possibilities, we determined the

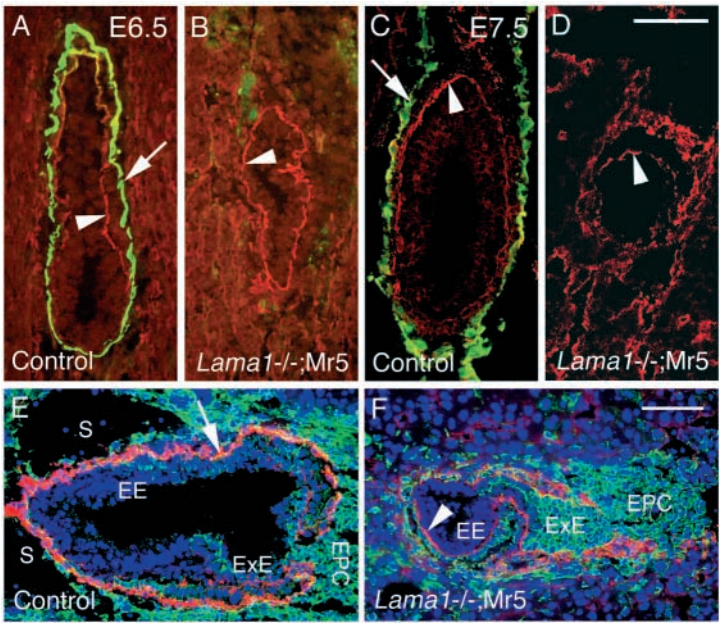

Fig. 6. Analysis of basement membranes in *Lama1*$^{-/-}$; Mr5 embryos. (A-D) Embryos were double stained for laminin α1 (green) and laminin α5 (red). Arrowheads indicate the embryonic basement membrane, and arrows indicate RM, when present. (A,B) At E6.5, the *Lama1*$^{-/-}$; Mr5 embryo (B) has laminin α5 in the embryonic basement membrane but lacks laminin α1 and RM, when compared with the control (A). (C,D) At E7.5, *Lama1*$^{-/-}$; Mr5 embryos appear to still be alive and to have an embryonic basement membrane containing laminin α5. (E,F) Embryos were triple stained for laminin β1 (red), cytokeratin 8 (green) and nuclei (blue). The *Lama1*$^{-/-}$; Mr5 embryo is much smaller than the control, but both contain embryonic ectoderm (EE), extra-embryonic ectoderm (ExE) and ectoplacental cone (EPC). Trophoblast blood sinuses (S) are present in the control but are lacking in the *Lama1*$^{-/-}$; Mr5 embryo. Scale bars: in D, 100 μm for A-D; in F, 100 μm for E,F.

pattern of expression of the Mr5 transgene in early embryos in the context of the *Lama5*$^{-/-}$ background. In *Lama5*$^{-/-}$; Mr5 embryos, all laminin α5 must originate from the Mr5 transgene, so immunolocalization of laminin α5 reveals specifically where transgene-derived protein is deposited. Because of the difficulty of genotyping such early embryos by DNA analysis, we intercrossed *Lama5*$^{-/-}$; Mr5$^{+/-}$ adult mice, which are viable and fertile (J.H.M., unpublished). All resulting offspring will be *Lama5*$^{-/-}$, and 75% (on average) will have at least one copy of the Mr5 transgene. As shown in Fig. 7A,B, transgene-derived laminin α5 was deposited in both the embryonic basement membrane and in RM, while no deposition was observed in a *Lama5*$^{-/-}$ littermate that apparently lacked the transgene (Fig. 7C,D).

These results demonstrate that the Mr5 transgene can indeed be expressed by the PE, and that the transgene-derived α5 protein can be incorporated into RM, where it could theoretically substitute for any lack of laminin α1. However, compensation was incomplete at best in *Lama1*$^{-/-}$; Mr5 embryos (Fig. 6B,D,F), suggesting that laminin 10 on its own is incapable of organizing RM. There was also decreased PE migration, suggesting that these cells either require specific interactions with laminin 1 to facilitate migration, or that in the absence of a properly organized RM, migration is inhibited.

Because gastrulation begins on the seventh day of gestation, we asked whether the *Lama1*$^{-/-}$; Mr5 embryos were capable of initiating gastrulation, despite their abnormalities. To investigate this, we performed in situ hybridization using a probe for brachyury (*T*), a mesodermal marker that is expressed only in embryos that have begun to gastrulate. E7.5 *Lama1*$^{-/-}$; Mr5 embryos did express brachyury (Fig. 8A-D), suggesting that they initiated gastrulation even in the absence of laminin 1 and RM. *Lama1*$^{-/-}$ embryos without the transgene were never found alive at E7.5. Conversely, *Lama5*$^{-/-}$ embryos initiate gastrulation (Fig. 8E,F) and carry out a somewhat normal developmental program (Miner et al., 1998). Taken together, these data suggest that either laminin 1 or laminin 10 alone in the embryonic basement membrane can support initiation of gastrulation, but supernormal levels of laminin-10 are required in the absence of laminin 1.

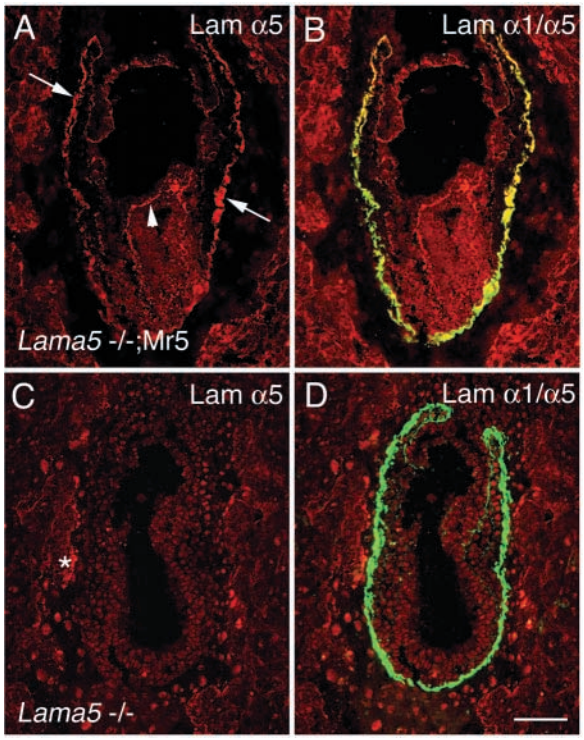

Fig. 7. Analysis of Mr5 transgene expression on the *Lama5*$^{-/-}$ genetic background. Sections were double stained for laminin α1 (green) and α5 (red). The red channel is shown in A and C, and both red and green channels are shown in B and D. (A,B) At E7.5, transgene-derived laminin α5 was detected in the embryonic basement membrane and in RM (A,B), but no α5 was detected in a *Lama5*$^{-/-}$ embryo that lacked the transgene (C,D). Scale bar: 100 μm.

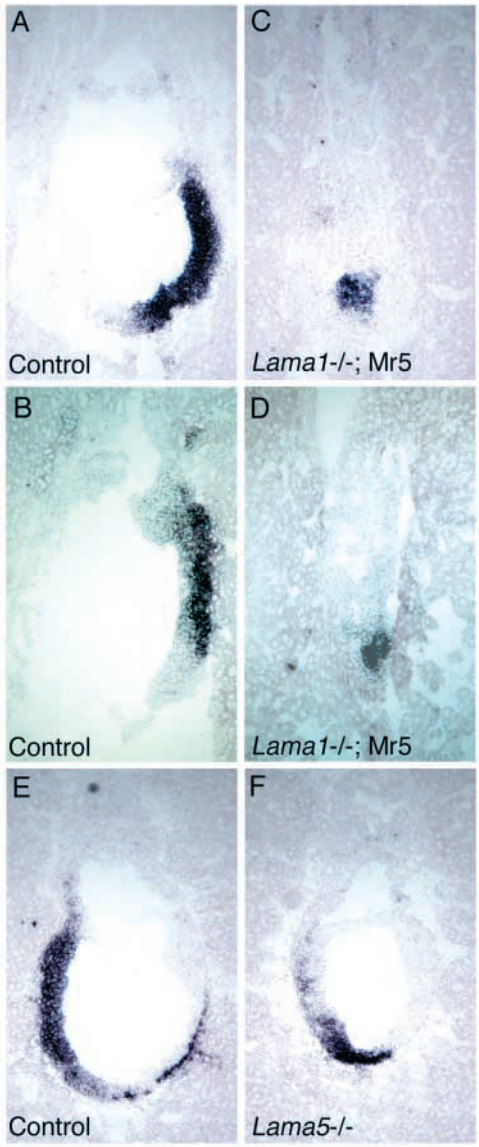

Fig. 8. Gastrulation initiates in *Lama1*$^{-/-}$; Mr5 embryos, as demonstrated by in situ hybridization of a digoxigenin-labeled brachyury (*T*) cRNA probe to frozen sections of E7.5 embryos. (A-D) Two control/*Lama1*$^{-/-}$; Mr5 embryo pairs from two different litters. Despite the aberrant shape of the *Lama1*$^{-/-}$; Mr5 embryos, robust expression of brachyury is evident. (E,F) Brachyury expression in a *Lama5*$^{-/-}$ embryo is similar to the control.

Discussion

We have used two fortuitous gene trap ES cell lines to generate mouse embryos lacking either laminin α1 or β1. These knockouts have provided new insights into the roles of laminin and basement membranes in early postimplantation development. Our results show that both laminin α1 and β1 are required for normal embryogenesis. *Lama1*$^{-/-}$ and *Lamb1*$^{-/-}$ embryos died in the early postimplantation period, but *Lamb1*$^{-/-}$ embryos became disrupted sooner than did *Lama1* mutants (Figs 4, 5). Based on comparisons with the phenotypes of *Lamc1* and *Dag1* (dystroglycan)-null embryos, this can be explained by considering that there are dual requirements for laminin and basement membranes during early postimplantation development.

The first requirement involves the embryonic basement membrane that separates the epiblast/embryonic ectoderm from the visceral endoderm. This basement membrane has been shown to be necessary in vitro for survival, differentiation, polarization, and cavitation of the epiblast (Coucouvanis and Martin, 1995; Li et al., 2002; Li et al., 2001; Murray and Edgar, 2000; Murray and Edgar, 2001). The embryonic basement membrane normally contains both laminin 1 (α1β1γ1) and laminin 10 (α5β1γ1) (Fig. 3) (Klaffky et al., 2001), neither of which is able to form in the *Lamb1* and *Lamc1* nulls because β1 and γ1 are components of both trimers. However, laminin 10 is present in the *Lama1* null and appears capable – at least to some extent – of promoting embryonic basement membrane formation and cavitation of the embryonic ectoderm (Fig. 4). Thus, *Lamb1* and *Lamc1* mutants have no detectable laminin trimers or basement membranes, but *Lama1* mutants have laminin 10 and an embryonic basement membrane; this makes them somewhat healthier, but they are still small and abnormal at E6.5. *Dag1* mutants also have an embryonic basement membrane, suggesting that dystroglycan is not required for its formation. Interestingly, *Dag1* mutants are found as late as E10, though they are severely disrupted and do not gastrulate (Williamson et al., 1997). In comparison with the *Lama1*, *Lamb1* and *Lamc1* nulls, the integrity of the embryonic basement membrane is greater in the *Dag1*$^{-/-}$ and *Lama1*$^{-/-}$; Mr5 embryos. Why the latter but not the former embryos are able to initiate gastrulation is unknown, but it probably indicates a requirement for dystroglycan in this crucial developmental process.

The inability of laminin 10 to fully compensate for a lack of laminin 1 in the embryonic basement membrane is an important question to be answered with future studies. It is unlikely that the structure of the basement membrane is obligatorily disrupted by the absence of laminin 1, for there are many basement membranes at various stages of development that contain only laminin 10. One possibility is that laminin 1 interacts with an important molecule – perhaps another basement membrane component; perhaps a cellular receptor, such as dystroglycan; or perhaps a growth factor that needs to be concentrated in the basement membrane – for which laminin 10 has less affinity. Presumably, by increasing the amount of laminin 10 in the embryonic basement membrane through expression of the Mr5 transgene, we have increased the binding of such a molecule to the basement membrane and enhanced its function.

The second requirement for laminin involves RM. The exact function of RM is not known, but it appears to serve as a barrier between the maternal blood in the yolk sac placenta and the developing embryo, and as a facilitator of materno-embryonic exchange of nutrients and gases (Williamson et al., 1997). Mutation in any of the laminin 1 subunit genes, or in *Dag1*, results in the absence of RM. Consideration of the different phenotypes in the *Dag1*$^{-/-}$ mice and the *Lama1*$^{-/-}$ mice illustrate two major functions of laminin 1 in RM. The first is structural: laminin polymerization into a basement membrane provides for the barrier function of RM. The importance of the structural attributes is evident in the *Dag1*$^{-/-}$ mutants, where the PE cells do migrate, but are unable to assemble the secreted

laminin 1 into an organized RM (Williamson et al., 1997). In these embryos, the resulting RM has its normal cellular components (PE and trophoblast giant cells) and architecture, but loses its barrier capacity. The second function of laminin 1 in RM is informational: interaction of both PE and trophoblast with laminin 1 influences their differentiation and behavior. When there is a complete lack of laminin, as demonstrated in the *Lama1*, *Lamb1* and *Lamc1* mutants, both the cellular and molecular components of RM are lacking. The PE cells fail to differentiate and fail to migrate properly to the periphery of the embryo (Smyth et al., 1999), while the trophoblast cells do not differentiate to giant cells and do not form the blood sinuses. Dystroglycan is, thus, the crucial receptor for assembly of RM, but not for transmitting specific signals from laminin 1 to the overlying trophoblast cells, or for promoting PE migration. It will be interesting to determine the mechanism of RM assembly in *Myd* (*Large* – Mouse Genome Informatics) mutant mice, which have defects in glycosylation and produce a dystroglycan molecule unable to interact with laminin in adults (Michele et al., 2002).

It has been suggested that the absence of RM contributes to death of the embryo due to exposure to maternal blood (Williamson et al., 1997). This may be true for the *Dag1*$^{-/-}$ phenotype, but in *Lama1*$^{-/-}$ embryos the lack of RM does not lead to exposure to maternal blood. Rather, in the absence of laminin 1 the trophoblast cells do not undergo their normal morphogenesis to form the blood sinuses of the yolk sac placenta. The lack of these spaces leads to a severe deficit in nutrients and oxygen for the embryonic region, and this is likely the fatal deficit in these embryos.

The compositional requirement for laminin 1 in RM is somewhat surprising, because laminin 10 is present in part of RM in normal embryos (Fig. 3). However, laminin 10 is neither necessary (Fig. 6D) (Miner et al., 1998) nor sufficient (Figs 4, 6) for formation of RM. In addition, despite the fact that the Mr5 transgene can direct deposition of laminin 10 into RM (Fig. 7A), the presence of the transgene in *Lama1*$^{-/-}$; Mr5 embryos does not restore RM, and does not rescue the formation of the yolk sac placenta (Fig. 6F). These observations suggest that there is a unique and crucial function of laminin 1 in RM for initiating morphogenesis of the yolk sac placenta. Based on the phenotype of the *Lama1*$^{-/-}$; Mr5 embryos, we hypothesize that the crucial step may be between the onset of invasive behavior and the final differentiation of the trophoblast cells to the giant cell phenotype (Sutherland, 2003). The trophoblast cells of the *Lama1*$^{-/-}$; Mr5 mutant embryos are able to initiate invasion and displace the uterine epithelium, but they do not have significant numbers of giant cells and do not undergo appropriate morphogenesis.

Finally, the βgeo insertion in *Lama1* and *Lamb1* allowed us to determine their expression patterns. Of note, we identified a previously unreported site of robust *Lama1* expression, the presomitic mesoderm (Fig. 2). Currently, we are unable to determine the importance of this expression, but it could be required for the mesenchyme to epithelium transition that these cells undergo. If true, this would be analogous to the situation in developing kidney. There, loose mesenchyme condenses to form the epithelial renal vesicle in the first step of nephrogenesis (Saxen, 1987), and this is accompanied by intense laminin α1 expression (Ekblom et al., 1990) (Fig. 2). Inhibition of laminin polymerization or its recognition by cellular receptors with monoclonal antibodies to α1 prevents polarization of these cells in organ culture, demonstrating the importance of laminin α1 in this process (Falk et al., 1996; Klein et al., 1988; Sorokin et al., 1992).

The βgeo insertion in *Lamb1* revealed very widespread expression, even in cells that are not adjacent to basement membrane and would not be expected to secrete laminin. We recently reported similar findings in the brains of these mice, which exhibit widespread neuronal expression of *Lamb1* but little accumulation of immunoreactive protein (Yin et al., 2003). A widespread pattern of gene expression was not found in studies aimed at defining the *Lamb1* regulatory region, suggesting that the transgenic constructs used in those studies contained some but not all regulatory elements that normally regulate *Lamb1* expression (Sharif et al., 2001).

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