

Absence of Basement Membranes after Targeting the *LAMC1* Gene Results in Embryonic Lethality Due to Failure of Endoderm Differentiation

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Abstract. The *LAMC1* gene coding for the laminin $\gamma 1$ subunit was targeted by homologous recombination in mouse embryonic stem cells. Mice heterozygous for the mutation had a normal phenotype and were fertile, whereas homozygous mutant embryos did not survive beyond day 5.5 post coitum. These embryos lacked basement membranes and although the blastocysts had expanded, primitive endoderm cells remained in the inner cell mass, and the parietal yolk sac did not develop. Cultured embryonic stem cells appeared normal after targeting both *LAMC1* genes, but the embryoid bodies derived from them also lacked basement membranes, having disorganized extracellular deposits of the basement membrane proteins collagen IV and perlecan, and

the cells failed to differentiate into stable myotubes. Secretion of the linking protein nidogen and a truncated laminin $\alpha 1$ subunit did occur, but these were not deposited in the extracellular matrix. These results show that the laminin $\gamma 1$ subunit is necessary for laminin assembly and that laminin is in turn essential for the organization of other basement membrane components in vivo and in vitro. Surprisingly, basement membranes are not necessary for the formation of the first epithelium to develop during embryogenesis, but first become required for extra embryonic endoderm differentiation.

Key words: extracellular matrix • epithelium • embryogenesis • endoderm • laminin

ALTHOUGH basement membrane molecules have been shown to affect the differentiation and survival of cells (Streuli, 1996), the mechanisms regulating the assembly of basement membranes in vivo and the fundamental roles of basement membranes during embryogenesis are poorly defined. The best studied basement membrane proteins are the laminins which constitute the major noncollagenous basement membrane component (Timpl, 1996). Antibody inhibition of laminin binding to its cellular receptors or to other basement membrane components has been shown to perturb both basement membrane deposition and also epithelial morphogenesis in organ culture (Klein et al., 1988; Sorokin et al., 1990; Ekblom et al., 1994; Kadoya et al., 1995). However, it remains to be established if basement membranes are an absolute requirement for epithelial cell differentiation and at what

stages of development these fundamental extracellular matrix structures become essential.

All characterized laminin variants are heterotrimeric molecules formed by the covalent bonding of one polypeptide from the α , β , and γ laminin subunit families, each of which comprises multiple members encoded by individual genes (Maurer and Engel, 1996). Thus, many variant laminin trimers may potentially be formed, depending on differential subunit gene expression which occurs in a time- and cell-specific manner (Paulsson, 1996). Definitive evidence for the distinct roles of some laminin variants in vivo has been provided by the phenotypes of mutations in laminin subunit genes. For example, natural mutations in any of the genes coding for subunits of laminin type-5 (kalinin) can result in junctional epidermolysis bullosa (Burgeson, 1996). Similarly, mutations of the $\alpha 2$ subunit (merosin) can result in autosomal forms of muscular dystrophy (Helbling-Leclerc et al., 1995), and targeted disruption of the laminin $\beta 2$ chain (s-laminin) has been shown to result in disruption of neuromuscular junction development and of kidney function (Noakes et al., 1995). The characteristic postnatal phenotypes of all of these muta-

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tions reflect the restricted expression and specific functions of the minor laminin subunits concerned.

Laminin $\gamma 1$ is one of the earliest expressed laminin subunits which, together with the $\alpha 1$ and $\beta 1$ subunits of laminin type-1, is expressed in the preimplantation embryo (Shim et al., 1996) before the appearance of the first basement membrane of the trophoblastic epithelium (Dziadek and Timpl, 1985; Thorsteinsdottir, 1992). Furthermore, $\gamma 1$ is the most ubiquitously expressed laminin subunit, being present in 10 of the 11 known laminin isoforms (Burgeson et al., 1994; Miner et al., 1997). Indeed, the only isoform (type-5) shown to lack the $\gamma 1$ subunit has instead the other known member of this subunit family, $\gamma 2$ (Kallunki et al., 1992). However, the $\gamma 2$ subunit has a restricted distribution being associated with epithelial anchoring filaments rather than being a common component of basement membranes (Burgeson, 1996). This is most probably either because it has no nidogen binding domain (Mayer et al., 1993) or because it lacks the three self-interacting NH_2 -terminal globular domains necessary to link it to the other basement membrane components (Champlaud et al., 1996; Cheng et al., 1997). We therefore decided to use homologous recombination to target the mouse *LAMC1* gene because the resulting lack of the laminin $\gamma 1$ subunit would alter the formation of all known integral basement membrane laminin isoforms. This would therefore be expected to affect the structure and function of most if not all basement membranes. Analysis of the phenotype of this knockout thus defines the role of laminin in basement membrane formation in vivo, and in this in turn demonstrates the initial function of basement membranes in tissue development during embryogenesis.

Materials and Methods

Production of Targeting Constructs

A Lambda FIX[®]II genomic library (Stratagene) of the 129SVJ mouse line was screened using a PCR product comprising 372 bp of the 5' untranslated region and the first 128 bases of exon one of the *LAMC1* gene (Ogawa et al., 1988). Six different clones representing this area were isolated and mapped. Targeting construct 1 was formed by cloning the 6-kb HindIII fragment containing the first exon together with 2-kb upstream sequence and 3.5 kb of intron 1 into pUC 19. The IRES β -Geo cassette (Friedrich and Soriano, 1991; Mountford et al., 1994), which had a NotI linker added to its 3' end was inserted into the unique NotI site in the first exon (see Fig. 1). The use of the cap-independent translation initiation sequence removed the need to place a Neo resistance cassette in frame to obtain expression by the *LAMC1* promoter.

Construct 2 was an EcoRI/SacI fragment of *LAMC1* cloned into KSII Bluescript (Stratagene). The sequence was interrupted at the NotI site by the insertion of the phosphoglycerate kinase (*pgk*) promoter (Soriano et al., 1991) 5' to a hygromycin resistance cassette with poly A tail (*Hygro*). This divided the *LAMC1* fragment into two arms with 6-kb homology in the 5' arm and 2.5-kb homology in the 3' arm (see Fig. 2).

LAMC1 Gene Targeting in Embryonic Stem Cells

R1 mouse embryonic stem (ES)¹ cells were grown in standard ES conditions with DME supplemented with 15% (vol/vol) fetal bovine serum (FBS), 0.1 mM β mercaptoethanol, and 1,000 U/ml of LIF (ESGRO; GIBCO BRL). 5×10^6 cells were transfected by electroporation with 25

μg of linearized construct 1 and colonies selected for resistance to G418 at 380 $\mu\text{g}/\text{ml}$ in the culture medium. Surviving clones were picked, expanded, and then DNA extracted for Southern blotting. DNA from the cells digested with SacI was probed with an external 3' probe and an internal 5' probe (see Fig. 1). In cases of correct integration, the wild-type 11-kb fragment was reduced to 7 kb when probed with the external probe and a 7.5-kb band was seen with the internal probe (see Fig. 1).

Attempts using increased G418 concentrations up to 1.5 mg/ml failed to produce ES cells in which both the *LAMC1* alleles had been targeted (Mortensen et al., 1992). Therefore, the second targeting construct was used for disruption of the second *LAMC1* allele in ES cells previously targeted with construct 1 (see Fig. 2 A). After correct targeting, Southern blot hybridization of SacI genomic DNA digests with probe 1 resulted in the wild-type band being lost, whereas a 5.5-kb band appeared (see Fig. 2 B). Clones so targeted were checked for the absence of expression of the *LAMC1* gene by Northern hybridization of mRNA with a probe of *LAMC1* cDNA.

Production of Mice Lacking the *LAMC1* Gene

Two independent ES cell lines were used to generate germ line chimeras. Blastocysts were isolated from C57Bl/6 mice 3.5 d post coitum (pc) (plug date = 0.5 d pc) and were injected with five to seven +/- ES cells. Blastocysts were then transferred into the uteri of pseudopregnant CD1 foster mothers. Chimeric male progeny were mated to C57Bl/6 females and offspring were tested for germline transmission by Southern blots of DNA extracted from tail biopsies. Heterozygous animals were mated together to obtain homozygous embryos.

Immuno- and Fragmented DNA Staining

The rabbit polyclonal primary antibodies used were: anti-laminin $\alpha 1$ raised against recombinant domain IVa (Schulze et al., 1996); anti-laminin $\gamma 1$ raised against recombinant domain III LE3-5 (Mayer et al., 1993); anti-EHS laminin which recognizes all three subunits of laminin (Kücherer-Ehret et al., 1990); anti-nidogen raised against recombinant nidogen (Fox et al., 1991); and anti-perlecan raised against recombinant domain III3 (Schulze et al., 1995). Rabbit polyclonal antibodies against von Willebrand factor, the 200-kD neurofilament subunit, and skeletal myosin were obtained from Sigma.

Embryos were washed in phosphate buffered saline (PBS) before embedding and freezing in Tissue-Tek (Sakura Finetek Europe). Cryostat sections were fixed with 0.5% (wt/vol) paraformaldehyde in PBS for 10 min, washed with PBS, and then blocked with 5% (vol/vol) goat serum in PBS/0.1% (vol/vol) Tween 20. The primary antibodies (see below) and goat-anti rabbit Cy3 conjugate secondary antibodies (Jackson Immunodiagnostics) were used in the same solution before washing the sections in PBS and mounting in fluorescent mounting medium (Dako).

For visualization of fragmented nuclear DNA in situ, serial cryosections were fixed for 20 min in 4% (wt/vol) paraformaldehyde in PBS before staining by a modification of the terminal dUTP-biotin nick end labeling (TUNEL) method (Gavrieli et al., 1992). A TACS apoptosis detection kit[™] (Trevigen) was used according to the manufacturer's instructions, fragmented DNA being end labeled with biotinylated nucleotides using the Klenow fragment, followed by detection with streptavidin-horseradish peroxidase conjugates. The sections were then counterstained with eosin.

Embryo Culture

Embryos were isolated from heterozygous matings by flushing the uterus with M2 medium and the blastocysts were cultured in M16 medium at 37°C in 5% CO_2 until they had fully expanded or hatched. Where present, the *zona pellucida* was removed from the expanded blastocysts by a short incubation in acid tyrode solution and the blastocysts washed in PBS before fixation in 1% (wt/vol) paraformaldehyde for 10 min at room temperature. The blastocysts were permeabilized in PBS/0.02% (vol/vol) Triton X-100 containing 2% (wt/vol) bovine serum albumin for 30 min before incubation with antibodies and subsequent fluorescence microscopy.

Embryoid Bodies

Undifferentiated ES cells were trypsinized, triturated, and then resuspended in DME 10% FBS at a dilution of 1,000 cells/ μl . The cells were then placed in hanging drops of 20 μl on the lower surface of the lids of plastic Petri dishes containing PBS (Wobus et al., 1991). After 24 h of cul-

1. *Abbreviations used in this paper:* ES, embryonic stem; pc, post coitum; TUNEL, terminal dUTP-biotin nick end labeling.

ture as hanging drops, the cell aggregates were plated into plastic Petri dishes and the embryoid bodies were fixed with 4% (wt/vol) paraformaldehyde after varying culture periods before sectioning and immunostaining as described above. Frozen sections were also stained for lacZ expression as previously described (Beddington and Lawson, 1990).

To monitor cell phenotypes of differentiating ES cells after formation in hanging drops, the embryoid bodies were allowed to attach to tissue culture plastic and cultured in the above medium for 21 d. Preliminary experiments showed that under these conditions small numbers of myotubes differentiated (Kuang et al., 1998). The cultures were then fixed in 4% (wt/vol) paraformaldehyde and immunostained as above.

Northern Blots

Wild-type ES cells, and those heterozygous and homozygous for mutations in the *LAMC1* alleles, were preplated in tissue culture dishes for 10 min to deplete them of the embryonic fibroblast feeder cells. The nonadherent ES cells were isolated and cultured on a gelatin-coated plate in ES media with LIF for two or three days until almost confluent. The cells were lysed and RNA extracted with guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). 10 µg of total RNA was separated on a denaturing formaldehyde gel of 1% agarose and transferred by vacuum blotting to a nylon membrane (Hybond N; Amersham). After UV cross-linking of the RNA to the membrane, it was prehybridized with a 50% formamide containing buffer and hybridized against cDNA probes for laminin α1, β1, γ1, γ2, and glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNAs. After high stringency washing, the blots were exposed to autoradiographic film. The probe for the laminin γ1 mRNA was a BamHI-EcoRI fragment between bases 2,959 and 4,163 in the protein coding area (Sasaki and Yamada, 1987), whereas γ2 was a BamHI fragment spanning nucleotides 1,509–2,120 of the protein coding region (Sugiyama et al., 1995). The probe for the α1 chain was a PCR-generated fragment using GCG-CATCAGAACAACACTCAACG (sense) and CAAGGGTGGTCATCA-TAAGG (antisense) primers amplifying between bases 708 and 1,203 of the protein coding region (Sasaki et al., 1988). The probe for the β1 chain was a PCR product using primers GATAACTGTGTCAGCACAACACC (sense) and GTGAAGTAGTAACGGACTCC (antisense) giving a probe between bases 1,231 and 1,794 of the protein coding region (Sasaki et al., 1987).

Metabolic Labeling and Immunoprecipitation

Embryoid bodies were produced from 5×10^3 ES cells incubated in hanging drops for 2 d (Wobus et al., 1991). About 10 embryoid bodies were then cultured in 500 µl DME without methionine, supplemented with 1% FBS and 200 µCi/ml [³⁵S]methionine (specific activity >1,000 Ci/mmol; Amersham). Labeling was carried out overnight. The medium was then collected, centrifuged, and then supernatants were stored at -70°C. Cells were washed with complete DME supplemented FBS and lysed in 50 mM Tris HCl, pH 7.5, containing 1% (vol/vol) Triton X-100, 10 mM EDTA,

0.10 M NaCl, and protease inhibitors. After trituration, insoluble material was removed by centrifugation and the supernatants were stored at -70°C.

Immunoprecipitations were carried out in the extraction buffer using Pansorbin (Calbiochem-Novabiochem) to precipitate the antibody-antigen complexes. Proteins bound to the Pansorbin were removed using boiling SDS gel electrophoresis sample buffer. Proteins were fractionated by SDS-PAGE on 5% gels under reducing conditions or on 3 and 5% gels under nonreducing conditions before fluorography.

For immunoblot analysis, embryoid bodies were cultured as above but using complete DME supplemented with 10% FBS. Cell extracts and media were concentrated by immunoprecipitation with antilaminin antiserum before SDS-PAGE under reducing conditions. After electroblotting onto nitrocellulose, the membrane was incubated with the primary antibodies which were detected using goat anti-rabbit antibodies conjugated with horseradish peroxidase (Dako). The enzymatic activity was visualized using 4-chloro-1-naphthol.

Results

Targeted Disruption of *LAMC1* Genes in ES Cells

A diagram of the initial targeting of the *LAMC1* gene is shown in Fig. 1 A. Of the 50 G418-resistant ES cell clones expanded and analyzed on Southern blots by hybridization with probe 1, 17 had undergone recombination at the *LAMC1* gene, as shown by the appearance of a band of the expected size of 7 kb and of equal intensity to the remaining wild-type 11-kb band (Fig. 1 B). A single insertion was demonstrated by the internal probe 2 which hybridized to the expected 7.5-kb band (Fig. 1 C). Although expression of the neomycin resistance-lacZ fusion cassette was dependent on the laminin promoter (Ogawa et al., 1988) present in the targeting vector, the high frequency of homologous recombination (>30% of all G418-resistant clones) suggests that the regulatory elements needed for full expression of the *LAMC1* gene in ES cells were lacking in the construct. This agrees with the recent demonstration of a strong enhancer element in the first intron of *LAMC1*, which was absent from our targeting construct (Chang et al., 1996). As expected, the frequency of the second targeting event with the hygromycin resistance cassette (Fig. 2 A) was much lower than that of the first: out of 200 clones, 12 had undergone the second homologous

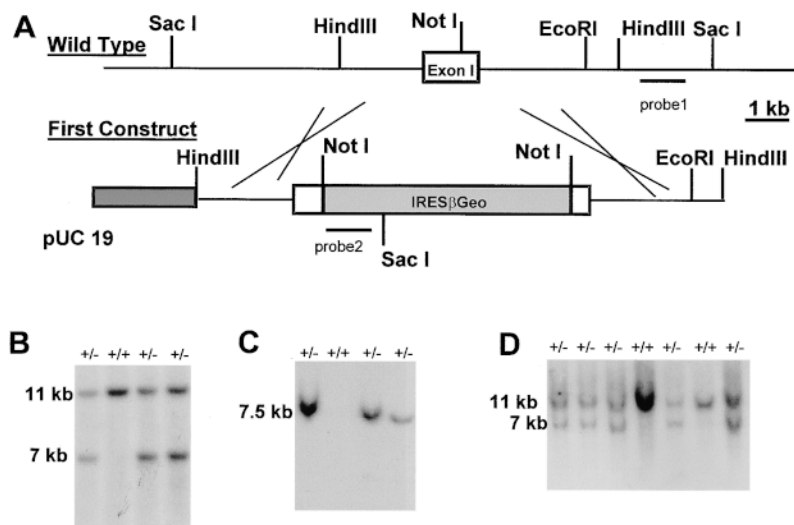


Figure 1. Homologous recombination in the *LAMC1* gene of ES cells and germline transmission in mice. (A) Restriction map including the first exon of the *LAMC1* gene and the targeting construct used to disrupt the gene. (B) SacI-digested DNA from ES clones analyzed with probe 1, showing appearance of a 7-kb band of equal intensity to the 11-kb wild-type band in clones having undergone homologous recombination. (C) A single integration event was confirmed using the internal probe 2, which produced a single 7.5-kb band when hybridized against SacI-digested DNA. (D) SacI-digested tail DNA of the offspring from *LAMC1* +/- mice matings hybridized with probe 1 to show the absence of animals homozygous for the mutation.

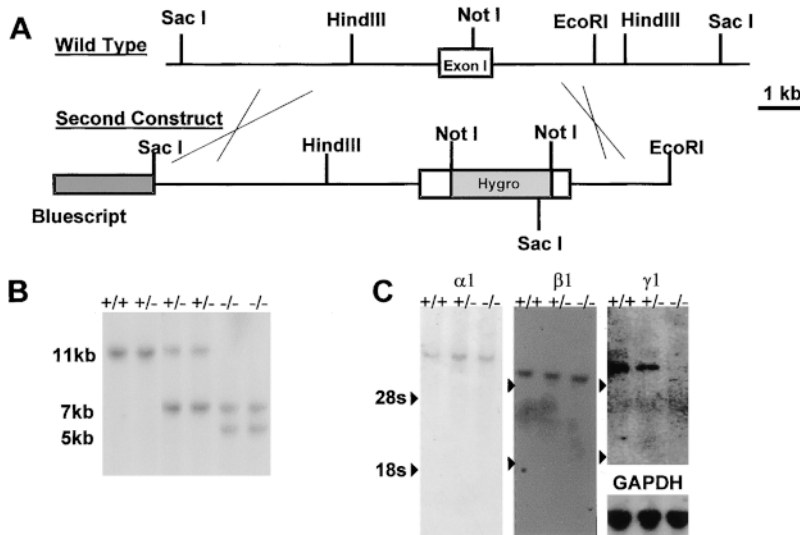


Figure 2. Analysis of ES cell lines generated by the homologous replacement of the second *LAMC1* allele. (A) Restriction map of the second targeting construct used to disrupt the remaining *LAMC1* allele by insertion of a hygromycin resistance cassette. (B) Southern blot analysis with probe 1 of *SacI*-digested DNA from ES clones. Loss of both *LAMC1* alleles results in the loss of the 11-kb wild-type band and the appearance of a 5-kb band in addition to the 7-kb band generated by the first targeting event. (C) Northern blot analysis of total RNA from ES cells *+/+*, *+/-*, and *-/-* for the *LAMC1*. The cDNA probes used were for the mRNAs of *LAMC1*($\alpha 1$), *LAMC1*($\beta 1$), and *LAMC1*($\gamma 1$). The GAPDH probe was used as a loading control. Arrowheads, positions of 28S and 18S rRNA bands on the three blots. The *LAMC1* message was reduced in the *+/-* ES cells and absent *-/-* cells, and there was no change observed in the levels of mRNAs coding for the other laminin subunits.

recombination shown by replacement of the wild-type *LAMC1* band by a 5-kb fragment (Fig. 2 B).

Northern blotting showed the absence of laminin $\gamma 1$ subunit mRNA in *-/-* ES cells, and the amount of *LAMC1* mRNA was reduced in *+/-* cells relative to the wild type (Fig. 2 C). However, levels of the mRNAs coding for laminin $\alpha 1$ and $\beta 1$ subunits were the same in undifferentiated *+/+*, *+/-*, and *-/-* cells (Fig. 2 C). The expression of *LAMC2* remained below the level of detection in all cases (data not shown).

Consequences of *LAMC1* Disruption In Vivo

LAMC1 *+/-* animals were phenotypically normal and have been intercrossed for at least seven generations and have also been bred into a C57Bl/6 background. More than 200 heterozygous matings produced no progeny homozygous for the mutation that were either born or found in utero after day 8.5 pc, indicating early embryonic lethality (Fig. 1 D). Day 3.5 pc preimplantation blastocysts from these matings were immunostained with polyclonal antibodies specific for the laminin $\gamma 1$ subunit (Fig. 3 A) and also with antibodies against laminin-1 which recognize the $\alpha 1$ and $\beta 1$ subunits as well as $\gamma 1$ (Kücherer-Ehret et al., 1990). The majority of blastocysts showed the reported pattern of immunostaining for laminin-1 (Thorsteinsdottir, 1992): the trophoctodermal basement membrane was stained together with apparently intracellular staining of cells throughout the blastocyst (Fig. 3 D). However, about one-quarter of the expanded and hatched embryos showed no immunostaining for the laminin $\gamma 1$ subunit (Fig. 3, B and C) and displayed only intracellular staining using the laminin-1 antibodies (Fig. 3 D).

The embryos found in decidua at day 4.5 pc appeared histologically normal (Fig. 4, A and F). However, eight out of the 40 embryos sectioned and stained did not display laminin $\gamma 1$ subunit immunoreactivity, although as expected there was immunoreactivity in the stroma and epithelial lining of maternal decidua (Fig. 4 J), which also

stained with the other antibodies used (Fig. 4, G–I). Embryos negative for $\gamma 1$ immunoreactivity demonstrated the absence of any extracellular laminin when stained with laminin-1 antibodies, although there was an accumulation of cells with intense laminin immunoreactivity in the inner cell mass (Fig. 4 G, inset). Very limited patchy deposits of nidogen and collagen type IV were seen under the tro-

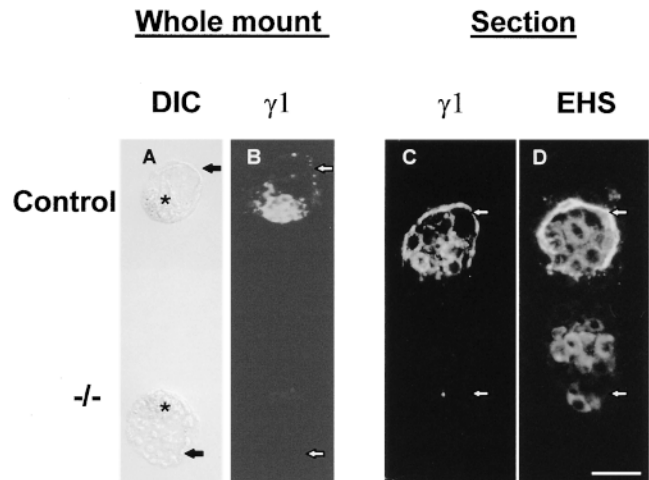


Figure 3. Appearance of 3.5 d pc preimplantation blastocysts from heterozygote matings. (A and B) Whole mount micrographs of the same pair of blastocysts. (C and D) Frozen 7- μ m serial sections. A shows the appearance of the blastocysts using Normarski optics. B and C are stained with $\gamma 1$ subunit antibodies and D is stained with antibodies against laminin-1. Arrows, location of the trophoctoderm; asterisks, inner cell mass. Embryos lacking laminin $\gamma 1$ immunoreactivity can expand to form blastocysts of normal appearance but lack the trophoctodermal basement membrane. Immunoreactivity of the other laminin subunits is intracellular, the cells showing cytoplasmic staining (D). Bars, 50 μ m.

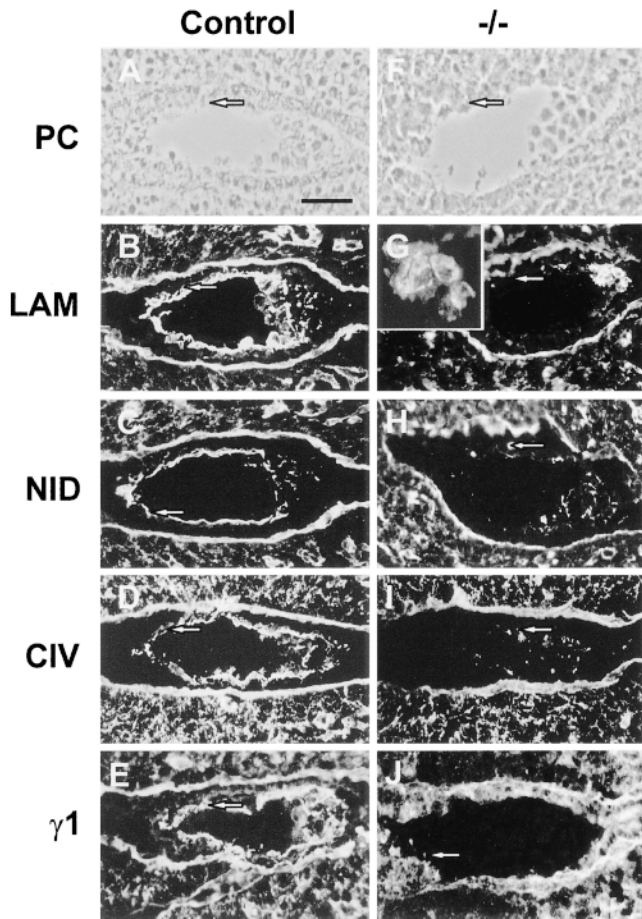


Figure 4. Immunofluorescence staining for basement membrane components in frozen sections of 4.5 d pc embryos in utero. (A–E) Wild-type or heterozygous embryos; (F–J) $-/-$ Embryos, as defined by lack of laminin γ 1 immunoreactivity (J). (A and F) Phase-contrast photomicrographs. Immunostaining was performed with antibodies directed against: laminin-1 (B and G); nidogen (C and H); collagen type IV (D and I); laminin γ 1 subunit (E and J). In wild-type or heterozygous embryos, all antibodies show staining under the trophoblast (arrows) and within the inner cell mass. Occasionally strongly staining parietal endoderm cells can be seen migrating over the trophoblast (B). In the $-/-$ embryos, discrete aggregates of nidogen and collagen IV immunoreactivity can be seen (H and I) whereas strong laminin 1 staining is mainly confined to the cells of the inner cell mass (G) and is apparently intracellular (G, inset). No basement membrane-like immunoreactivity can be seen associated with the trophoblast in these embryos (arrows), although the maternal basement membrane underlying the uterine epithelium is clearly visible in all cases. Bar, 50 μ m.

phoblast epithelium but there was no continuous sheet characteristic of the trophoblast basement membrane (Fig. 4, H and I).

Of the 80 decidua examined at 5.5 d pc from heterozygous matings, 28 contained laminin γ 1-negative accumulations of cells, none of which conformed to any recognizable embryonic structures (Fig. 5, B and C), whereas out of the 40 decidua examined from heterozygous/wild-type matings, only five contained no recognizable embryo. Detection of fragmented nuclear DNA by TUNEL staining

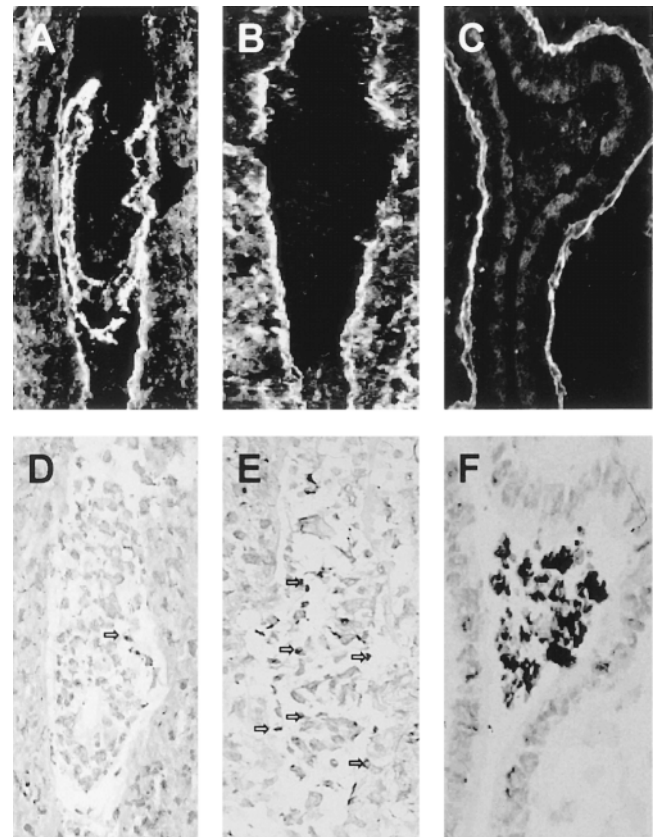


Figure 5. Appearance of 5.5 d pc embryos in utero. Frozen sections of embryos were immunostained with anti-laminin γ 1 antibodies to show: (A) a control embryo staining for γ 1 (either $+/+$ or $+/-$); (B and C) $-/-$ embryos lacking γ 1 staining. The γ 1-negative embryonic cells had lost recognizable structure, large aggregates of cells being present (E and F). TUNEL staining revealed low numbers of cells with fragmented DNA in serial sections from γ 1-positive embryos (D), whereas γ 1-negative embryos displayed either increased (E) or intense (F) staining.

of serial sections revealed very few stained cells in any embryos before 5.5 d pc (data not shown). However, at 5.5 d pc we detected low numbers of stained nuclei in the γ 1-positive embryos (Fig. 5 D), and the laminin γ 1-negative embryos displayed either increased numbers of TUNEL-positive cells (Fig. 5 E) or very intense labeling, indicative of extensive DNA fragmentation (Fig. 5 F).

Analysis of LAMC1 Disruption In Vitro

After 48 h of suspension culture, the differentiating $+/-$ and $-/-$ ES cells at the periphery of developing embryoid bodies began to display intense lacZ staining (Fig. 6), indicating the expression of the *LAMC1* gene. This reflects the differentiation of these cells into primitive endoderm-like cells with high levels of laminin expression (Doetschman et al., 1985). The deposition of a continuous basement membrane-like sheet of laminin, nidogen, perlecan, and collagen type IV immunoreactivity was observed towards the periphery of $+/-$ embryoid bodies after 7 d of culture (Fig. 7, A, C, E, and G). Outside this basement membrane,

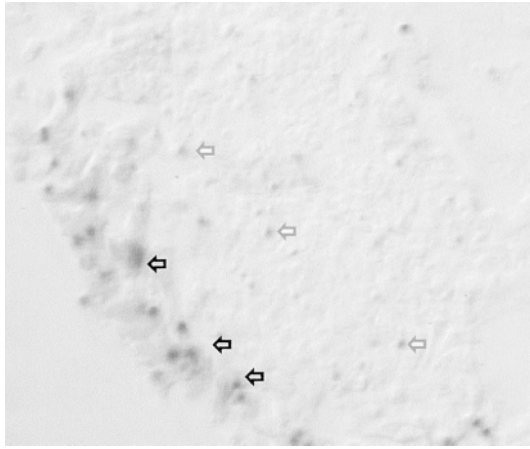


Figure 6. LacZ staining of +/- embryoid bodies after 48 h of culture. The lacZ reaction product is localized to cells in which the *LAMC1* promoter is active and are found mainly around the periphery of the embryoid body, although a few scattered weakly positive cells were also seen in the center of the embryoid body (arrows).

there was a sheet some one to three cells thick displaying laminin immunoreactivity (Fig. 7 A). No differences in these were observed between +/+ and +/- embryoid bodies (data not shown). In contrast, in addition to the expected lack of $\gamma 1$ immunoreactivity in the -/- embryoid bodies (data not shown), no deposition of basement membranes was detected when they were stained with any of the above antibodies: although there were patchy extracellular deposits of perlecan and collagen type IV, these molecules were not deposited in a continuous basement membrane-like sheet (Fig. 7, F and H). Although there was no obvious extracellular laminin staining, polyclonal antibodies to laminin-1 showed immunoreactivity to be accumulated mainly in the cells at the surface of the -/- embryoid bodies (Fig. 7 B). Staining with antibodies specific to $\alpha 1$ and $\beta 1$ showed the same patterns of distribution as that detected with the polyclonal antibodies to laminin 1 (data not shown). Little if any intracellular or extracellular nidogen immunoreactivity was detectable in the -/- embryoid bodies (Fig. 7 D) although it was present as expected in the basement membranes of +/+ and +/- embryoid bodies (Fig. 7 C).

To determine if the differentiation of ES cells was affected by the absence of the $\gamma 1$ subunit, embryoid bodies were allowed to attach to tissue culture plastic and cultured for up to 3 wk. At this time, the *LAMC1* +/- cells displayed basement membrane-like sheets of laminin immunoreactivity (Fig. 8 A), small numbers of isolated von Willebrand-positive cells were seen (Fig. 8 C) and neurofilament-positive cell bodies and neurites were identified (Fig. 8 E). Furthermore, the cultures contained low numbers of myotubes that stained with antibodies to skeletal myosin (Fig. 8 G). As expected from Fig. 7, the *LAMC1* -/- cells did not deposit basement membranes although individual permeabilized cells displayed intense laminin immunoreactivity (Fig. 8 B). Although no differences from controls were seen in the von Willebrand- and neurofilament-positive cells (Fig. 8, D and F, respectively),

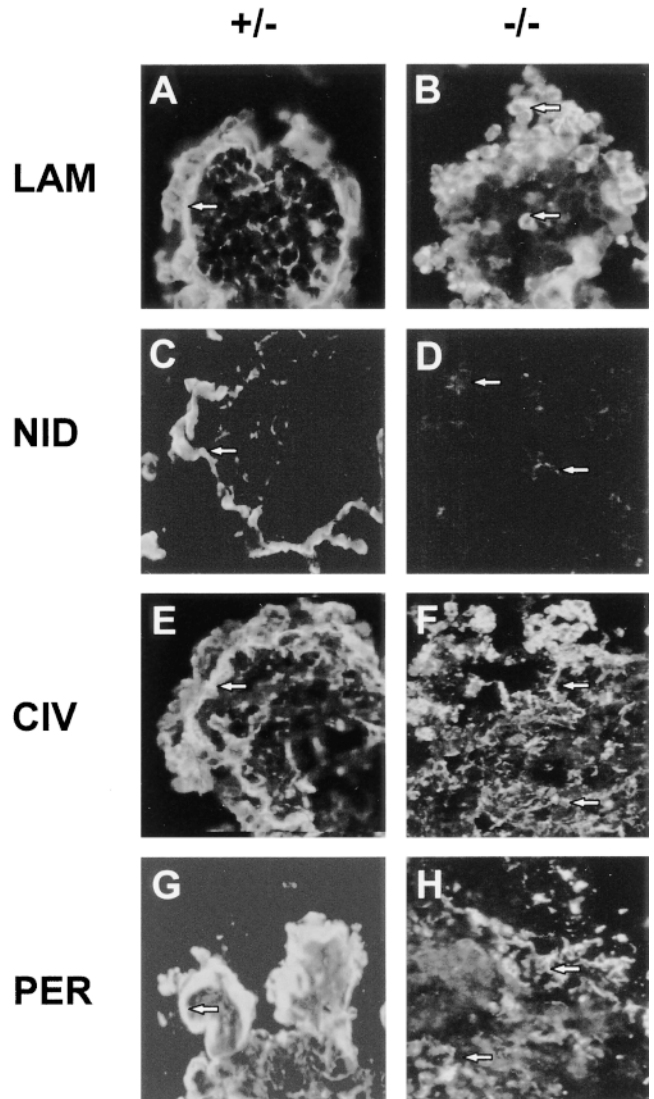


Figure 7. Immunofluorescence staining for basement membrane components in embryoid bodies after 7 d of culture. The primary polyclonal antibodies used were: anti-laminin-1 (A and B); anti-nidogen (C and D); anti-collagen type IV (E and F); and anti-perlecan (G and H). Genotypes of the ES cells are indicated. Arrows, location of immunofluorescence in the peripheral basement membrane of (+/-) embryoid bodies (A, C, E, and G). Arrows, intracellular laminin staining in (-/-) embryoid bodies (B) and disorganized extracellular staining of nidogen, collagen type IV, and perlecan in (-/-) embryoid bodies (D, F, and H, respectively). Note that there is very little nidogen immunoreactivity in the (-/-) embryoid body (D).

no normal myotubes were observed, myosin-positive cells being present in large aggregates which extended thin processes (Fig. 8 H).

After overnight [³⁵S]methionine metabolic labeling, a band of ~800 kD on nonreducing SDS-PAGE was immunoprecipitated with laminin-1 antibodies from both culture medium and extracts of the +/+ and +/- embryoid bodies (Fig. 9 A, lanes 1–4). This corresponds to the expected size of laminin type 1. In contrast, neither the cell extracts nor the culture medium from the -/- embryoid

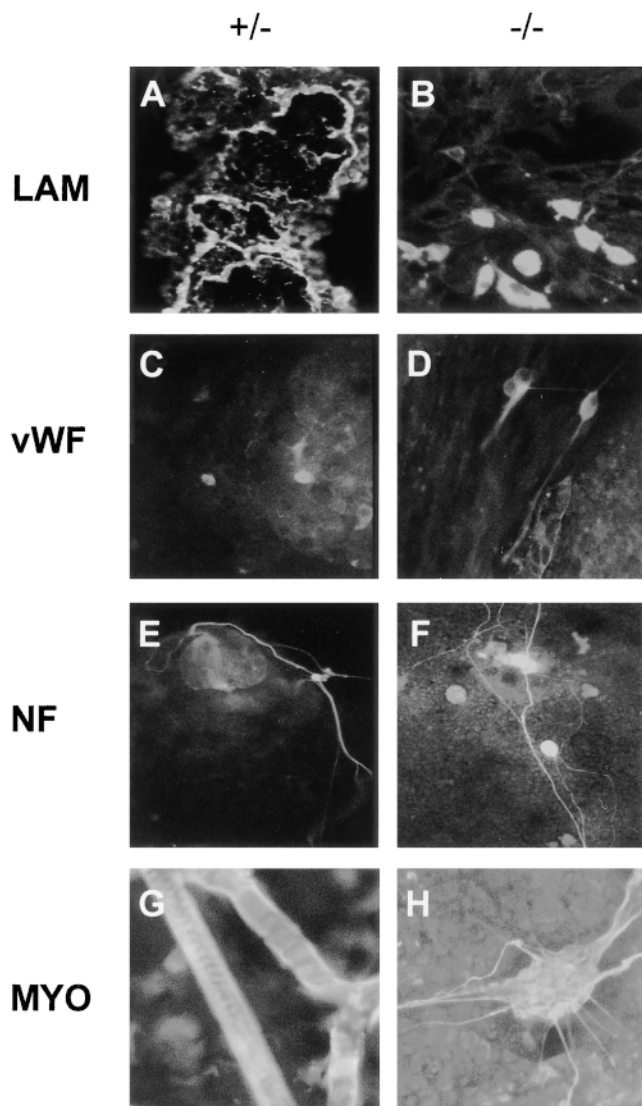


Figure 8. Immunofluorescence staining for laminin and cell-specific markers in +/- and -/- ES cells after 21 d of culture under differentiating conditions. The primary polyclonal antibodies used were: anti-laminin-1 (A and B); anti-von Willebrand factor (C and D); anti-neurofilament 200 subunit (E and F); and anti-skeletal myosin (G and H). Note the presence of myosin-positive accumulations of cells with processes rather than typical myotubes in the -/- ES cell cultures (H).

bodies contained detectable laminin of 800 kD, although lower molecular weight bands were immunoprecipitated (Fig. 9 A, lanes 5 and 6).

To better characterize these bands, electrophoresis was performed on higher percentage SDS-polyacrylamide gels under nonreducing and reducing conditions. The pattern of reduced protein bands immunoprecipitated from the media of the -/- embryoid bodies differed from that of the -/- embryoid body cell extracts (Fig. 9 B, lanes 7 and 8), whereas the patterns were the same from the +/- embryoid body cell extracts and media (Fig. 9 B, lanes 5 and 6). These differences are consistent with the observation that most of the laminin immunoreactivity of +/- and +/- embryoid bodies was seen in a basement membrane and

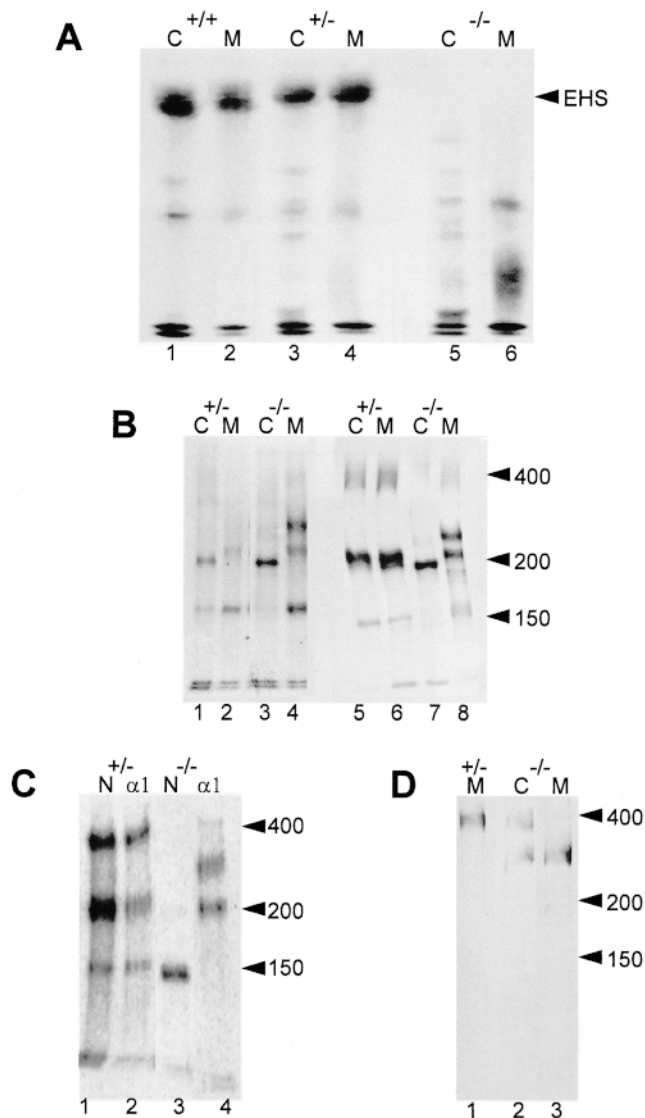


Figure 9. Embryoid bodies lacking the laminin $\gamma 1$ subunit fail to produce intact laminin but do secrete a truncated $\alpha 1$ subunit and free nidogen. (A) Immunoprecipitation with anti-laminin-1 antibodies and 3% SDS-PAGE under nonreducing conditions of ^{35}S -labeled laminin from cell extracts (1, 3, and 5) and media (2, 4, and 6) of embryoid bodies +/- (1 and 2), +/- (3 and 4) and -/- (5 and 6) for the $\gamma 1$ subunit. (B) 5% SDS-PAGE under nonreducing (1-4) and reducing conditions (5-8) of immunoprecipitated ^{35}S -labeled laminin from cell extracts (1, 3, 5, and 7) and media (2, 4, 6, and 8) obtained from +/- (1, 2, 5, and 6) and -/- (3, 4, 7, and 8) embryoid bodies. (C) Immunoprecipitation and electrophoresis under reducing conditions of ^{35}S -labeled protein from culture media of +/- (1 and 2) and -/- (3 and 4) embryoid bodies with antibodies specific for the laminin $\alpha 1$ subunit (2 and 4) and nidogen (1 and 3). (D) Anti-laminin $\alpha 1$ subunit immunoblots of proteins from +/- (1) and -/- (2 and 3) embryoid bodies after electrophoresis under reducing conditions. (1 and 3) Proteins present in the media; (2) proteins present in cell extracts.

hence extracellular (Fig. 6 A), whereas that seen in the -/- embryoid bodies appeared to be intracellular (Fig. 6 B). Although a nidogen band of 150 kD was present in the medium of these cells (Fig. 9 B, lanes 4 and 8; Fig. 9 C, lane

3), there was little or no nidogen detectable in the $-/-$ embryoid body extracts under both nonreducing (Fig. 9 B, lane 3) and reducing conditions (Fig. 9 B, lane 7). Thus, the lack of nidogen immunoreactivity in $-/-$ embryoid bodies (Fig. 9 D) is not due to lack of nidogen synthesis, but rather results from nidogen being lost from the embryoid bodies into the medium (Fig. 9 B, lanes 4 and 8).

The nonreduced extracts of $+/-$ and $-/-$ embryoid bodies were similar in that they contained a prominent band of ~ 200 kD (Fig. 9 B, lanes 1 and 3). However, a novel strong band of ~ 300 kD under both non-reducing and reducing conditions was immunoprecipitated from $-/-$ embryoid body medium (Fig. 9 B, lanes 4 and 8), indicating that this secreted protein was not disulfide-bonded to other laminin subunits. To identify the novel band, immunoprecipitation and immunoblotting experiments were performed with laminin $\alpha 1$ subunit-specific antibodies directed against the IVa domain. In addition to the 400-kD $\alpha 1$ chain, an equally strong band corresponding to the 300-kD protein was also found in $-/-$ cell extracts (Fig. 9 D, lane 2), and this band alone was detected in the $-/-$ medium (Fig. 9 D, lane 3) although it was absent from the $+/-$ medium (Fig. 9 D, lane 1). Immunoprecipitation of this band from the $-/-$ cell medium with antibodies to the laminin $\alpha 1$ subunit also coprecipitated a band of 200 kD but no nidogen was detected (Fig. 9 C, lane 4). Conversely, immunoprecipitation of nidogen from $-/-$ cell medium with antibodies against nidogen failed to precipitate any laminin subunits (Fig. 9 C, lane 3). Thus although the $-/-$ ES cells secrete a modified laminin $\alpha 1$ subunit and nidogen, they are not associated as normal (Fig. 9 C, lanes 1 and 2), consistent with the absence of the laminin $\gamma 1$ subunit in the $-/-$ ES cells.

Discussion

We have used homologous recombination to target one or both of the *LAMC1* alleles coding for the laminin $\gamma 1$ subunit in mouse embryonic stem cells. By so doing, we have disrupted the formation of all described laminin isoforms with the exception of laminin 5. Although the null mutation resulted in the absence of basement membranes and hence was an early embryonic lethal, surprisingly, preimplantation development appeared to be normal in that a pumping trophoblastic epithelium allowed expansion of the blastocysts. Basement membranes were first found to be necessary for differentiation of primitive endodermal cells, in their absence Reichert's membrane failing to form. In vitro analysis of the null mutation showed that the laminin $\gamma 1$ subunit was necessary for the differentiation of stable myotubes and for assembly of other covalently bonded laminin subunits. In turn, the lack of intact laminin deposition was necessary for the assimilation of other components into a continuous basement membrane in vitro, consistent with the disruption of formation of the first basement membrane to be formed during development in vivo.

The LAMC1-null Mutation Causes Early Embryonic Lethality

Although mice heterozygous for the *LAMC1* gene are

healthy and fertile, the analysis of different gestational ages showed that *LAMC1*-null mutant embryos did not survive later than day 5.5 pc. Immunostaining of pre- and postimplantation embryos up to 4.5 d pc showed laminin $\gamma 1$ -negative embryos to have an apparently normal morphology although they lacked basement membranes as defined by staining for other basement membrane components. Disruptions of cell-extracellular matrix interactions in the developing lung, kidney, and salivary gland have been shown to inhibit epithelial morphogenesis (Ekblom et al., 1994; Kadoya et al., 1997; Klein et al., 1988). However, the fact that the null mutant embryos described here could develop a functional pumping trophoblastic epithelium means that a basement membrane is not an absolute requirement for the differentiation of epithelia. Although this result points to the primary importance of cell-cell interactions in epithelial development (Watson et al., 1990), it does not rule out a role for basement membranes in the maintenance of specific epithelia or the differentiation of other epithelial cell properties. Indeed, although a decidual reaction occurred in the uterine wall adjacent to $-/-$ embryos, it may be that in the absence of the trophoblastic basement membrane that the trophoblast was unable to successfully implant into the uterus.

Although both Reichert's membrane and underlying parietal endoderm cells were absent in the *LAMC1*-null embryos, cells staining strongly for intracellular laminin $\alpha 1$ or $\beta 1$ subunits were seen in the inner cell mass, characteristic of primitive endodermal cells (Doetschman et al., 1985; Dziadek and Timpl, 1985). Thus, although these cells had evidently started to differentiate along the extra-embryonic endodermal pathway, the absence of a trophoblastic basement membrane had prevented further development involving the migration of parietal endodermal cells and/or the differentiation of primitive endodermal cells. The need for cellular interactions with laminin at this stage of development is consistent with the observation that embryos lacking the $\beta 1$ integrin subunit or α -dystroglycan also die rapidly after day 4.5 pc (Fässler and Meyer, 1995; Stephens et al., 1995). It has previously been shown that the formation of the proamniotic cavity in the epiblast is due to death of the cells unable to interact with the extracellular matrix via a $\beta 1$ -containing integrin receptor (Couchanis and Martin, 1995). At 5.5 d pc we were unable to find anything other than disrupted laminin $\gamma 1$ -negative embryos, the cells of which displayed increased DNA fragmentation. The fact that the increased extent of DNA fragmentation in these embryos varied widely is consistent with a rapid onset of apoptotic cell death subsequent to disruption of embryo structure.

The early embryonic lethality of the *LAMC1* knockout in vivo precludes an analysis of the roles of basement membranes in subsequent postimplantation development. However, by observing the differentiation of $-/-$ ES cells in culture we were able to see that developing myotubes were affected. It has recently been shown that the stability of myotubes derived from *LAM2A* $-/-$ ES cells is compromised (Kuang et al., 1998). Taken together with our observation of aggregates of myosin-positive *LAMC1* $-/-$ cells with processes characteristic of retraction, these results are consistent with the laminin $\gamma 1$ subunit being re-

quired for the formation of $\alpha 2/\gamma 1$ -containing laminin isoforms that are necessary for the maintenance of myotubes.

Covalent Laminin Trimers Fail to Form in the Absence of the $\gamma 1$ Subunit

In the absence of the $\gamma 1$ subunit, no covalently bonded laminin subunits were produced by differentiating ES cells. Previous studies have indicated that intracellular laminin transport and secretion is limited by the assembly of the $\alpha 1$ subunit to form a triple coiled-coil α -helix with preassembled $\beta \gamma$ dimers (Peters et al., 1985; Hunter et al., 1990; De Arcangelis et al., 1996; Yurchenco et al., 1997). However, despite the lack of extracellular laminin deposition in the embryoid bodies, our immunoprecipitation experiments clearly showed that some laminin subunits were secreted from the ES cells. However, the $\alpha 1$ chain had undergone cleavage to produce a fragment of ~ 300 kD that was released into the medium. The size of this fragment is consistent with cleavage occurring at or close to the terminal globular domain of the $\alpha 1$ subunit. A similar but incomplete cutting of the laminin $\alpha 1$ subunit upon secretion from transfected cells has recently been demonstrated, and it was suggested that when unable to assemble into a coiled-coil structure, the $\alpha 1$ subunit adopts a conformation laying it open to cleavage (Yurchenco et al., 1997). Although the truncated $\alpha 1$ subunit in the $-/-$ ES cell medium described here was noncovalently associated with a protein band of 200 kD, the fact that the $\alpha 1$ subunit was cleaved to a 300-kD fragment indicates that it is also unlikely to have been associated with any other laminin subunits via a coiled-coil interaction.

Basement Membrane Components Fail to Assemble in the Absence of Laminin

Experiments *in vitro* have shown that collagen type IV can self-assemble into a characteristic chicken wire network (Yurchenco and O'Rear, 1993). Furthermore, there are reports of basement membrane-like structures lacking either type IV collagen (Brauer and Keller, 1989) or laminin (Hahn et al., 1980). However, the present work demonstrates in both embryos and embryoid bodies that laminin is necessary for the incorporation of collagen type IV into a continuous basement membrane. Although we cannot rule out the hitherto undocumented existence of other laminin γ subunits, it is clear that the $\gamma 1$ isoform is a prerequisite for the formation of that laminin variant necessary for the assembly of the first basement membranes in the preimplantation embryo. Furthermore, the available data are consistent with that variant being laminin type-1, the $\alpha 1$, and $\beta 1$ subunits of which have also been shown to be expressed in the preimplantation embryo (Shim et al., 1996). In the absence of the laminin, collagen IV and perlecan were seen in disorganized deposits within the embryoid bodies. However, little if any nidogen was deposited with them, but instead it was released into the culture medium. Although nidogen has been shown to be able to bind to both collagen IV and perlecan in solid-phase binding assays *in vitro* (Battaglia et al., 1992; Dziadek et al., 1985), the present experiments indicate that this apparently does not occur in the absence of laminin in embryoid bodies. Clearly factors other than binding interactions be-

tween its individual components regulate basement membrane deposition *in vivo*. In this regard, it should be noted that basement membrane organization is disrupted by targeted deletions of the $\beta 1$ integrin subunit (Fässler and Meyer, 1995; Stephens et al., 1995) or α -dystroglycan (Williamson et al., 1997), pointing to the involvement of cellular receptors for laminin in basement membrane deposition.

Taken together, the present results show that other basement membrane components require laminin for their assembly into an organized basement membrane structure. Furthermore, although cell-cell contacts may be sufficient for epithelium formation during preimplantation development, these do not form a sufficient basis for postimplantation embryonic development when basement membranes are first required for endoderm differentiation.

We are greatly indebted to H. Thoenen (Max Planck Institute for Psychiatry, Munich, Germany) for providing encouragement, advice, and facilities for the initial stages of this work, to H. Thorun and B. Kunkel (both from Max Planck Institute for Psychiatry) who provided skilled technical assistance with tissue culture and microinjection, and to A. Fichard (Max Planck Institute for Psychiatry) who was involved in preliminary experiments leading to this project. We thank U. Mayer and R. Timpl (both from Max Planck Institute for Biochemistry) for generously making their antibodies available to us and also P. Soriano (Fred Hutchinson Cancer Center Research Center, Seattle, WA) and A. Nagy (Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, Canada) who provided the IRES construct and R1 ES cells, respectively.

This work was supported by a European Union SCIENCE Programme Grant (SCC-CT90-0021) and Wellcome Trust grant to D. Edgar. N. Smyth, C. Frie, and M. Paulsson were supported by a grant from the Bundesministerium fuer Bildung und Forschung in the framework of the Centre for Molecular Medicine Cologne. P. Murray was supported by a postgraduate research studentship (G610/47) of the Medical Research Council.

Received for publication 27 July 1998 and in revised form 19 November 1998.

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