# Early Mouse Development

A series of manuscripts submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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# Preface

The five manuscripts presented here concern events that occur during the early stages of mouse development, beginning with differentiation of primitive endoderm and ending with formation of the proamniotic cavity. The main focus of the work has been the role of the basement membrane in early development, with particular reference to its role in the regulation of epiblast and extraembryonic endodermal cell differentiation, parietal endodermal cell migration and programmed cell death (manuscripts 1, 3 and 4). However, the critical role played by the basement membrane in these processes prompted an investigation into the mechanisms regulating the deposition of this specialised extracellular matrix (manuscript 2).

Central to all four manuscripts has been the use of an extremely good in vitro model system, the embryoid body. Embryoid bodies are derived from embryonic stem cells of the mouse blastocyst, and their development closely resembles that of the peri-implantation embryo. Of particular use, has been the availability of *LAMC1-/-* ES cells that are unable to express the laminin γ1 chain (manuscript 5). This defect renders the cells incapable of assembling a functional laminin type-1 trimer, which is necessary for basement membrane deposition. Hence, embryoid bodies derived from *LAMC1-/-* ES cells lack basement membranes, allowing the role of the basement membrane in early development to be analysed.

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## Summary



### Appendices

1. Murray, P. and Edgar, D. (2000) Regulation of programmed cell death by basement membranes in embryonic development. *J.Cell Biol.* 150: 1215-1221.

2. Murray, P. and Edgar, D. (2001) Regulation of laminin and COUP-TF expression in extraembryonic endodermal cells. *Mech. Dev.* 101: 213-215.

3. Murray, P. and Edgar, D. (2001) Regulation of the differentiation and behaviour of extra-embryonic endodermal cells by basement membranes. *J. Cell Sci.* 114: 931-939.

4. Murray, P. and Edgar, D. (2001) The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF). *Differentiation: In Press.*

5. Smyth, N., Vatansever, S., Murray, P., Meyer, M., Frie, C., Paulsson, M. and Edgar, D. (1999) Absence of basement membranes after targeting the *LAMC1* gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol.* 144: 151-160.

## Abbreviations

| | |
|---|---|
| BM | basement membrane |
| CEE | columnar epiblast epithelium |
| COUP-TF | chick ovalbumin upstream promoter transcription factor |
| E | embryonic day |
| EB | embryoid body |
| EC | embryonal carcinoma |
| ECM | extra-cellular matrix |
| ES | embryonic stem |
| ICM | inner cell mass |
| LAMB1 | laminin β1 chain |
| LAMC1 | laminin γ1 chain |
| LIF | leukaemia inhibitory factor |
| PAC | proamniotic cavity |
| PTH | parathyroid hormone |
| PTHrP | parathyroid hormone-related peptide |
| PTH/PTHrPR | parathyroid hormone/parathyroid hormone-related peptide receptor |

## The peri-implantation stage of mouse development

In this work, the peri-implantation stage is defined as beginning with the differentiation of primitive endoderm cells from undifferentiated inner cell mass cells at embryonic day 3.5 (E3.5), and ending with the formation of the proamniotic cavity at E5.5-E6.

The first differentiation event in mammalian development occurs at compaction, where loosely adherent blastomeres are seen to maximise their contact with each other via tight junctions to form a compact ball of cells known as the morula. Upon morula formation, a subset of blastomeres undergo horizontal cleavage, and the daughter cells that form basally generate the pluripotent embryonic stem (ES) cells of the inner cell mass (ICM) that gives rise to the embryo proper. The cells positioned on the outside of the morula are the progenitors of an epithelium called the trophectoderm. The trophectoderm cells pump fluid into the morula, creating a fluid-filled cavity surrounded by a ring of trophectoderm with the ICM aggregated at one side (Fleming, 1992). This structure is called the blastocyst. The trophectoderm cells also deposit the first basement membrane (BM) of the embryo, which separates these cells from the blastocoel (Salamat et al., 1995) (see fig. 1). In the mouse, compaction occurs on the second embryonic day at the 8 to 16 cell-stage, and the blastocyst is formed by E3-E3.5.

# Formation of the blastocyst

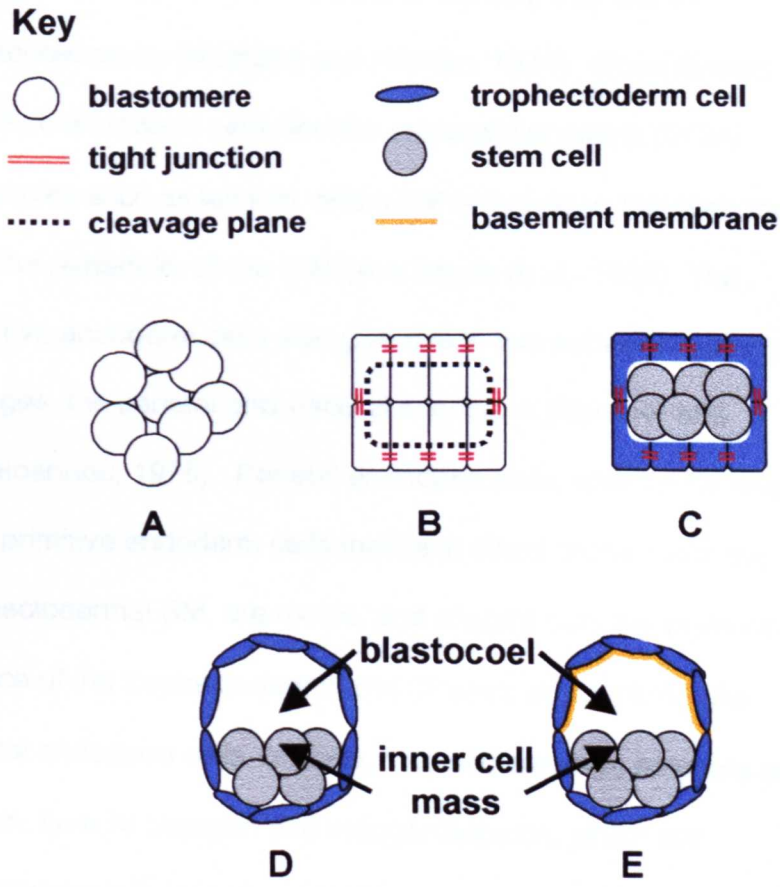


**Figure 1.** Schematic representation of blastocyst formation. (A) At the early 8-cell stage the embryo consists of loosely adherent blastomeres. (B) Compaction: blastomeres maximise their contact with each other via tight junctions to form a compact ball of cells known as the morula. (C) Upon morula formation, a subset of blastomeres undergo horizontal cleavage, and the daughter cells that form basally generate the pluripotent stem cells of the inner cell mass that gives rise to the embryo proper. All of the cells positioned on the outside of the morula are the progenitors of the trophectoderm. (D) The trophectoderm cells pump fluid into the morula, creating a fluid-filled cavity called the blastocoel that is surrounded by a ring of trophectoderm with the inner cell mass aggregated at one side. This structure is called the blastocyst. (E) The trophectoderm cells also deposit the first basement membrane (BM) of the embryo, which separates these cells from the blastocoel.

The second differentiation event takes place almost immediately after blastocyst formation, and involves the formation of a layer of primitive endoderm from the cells of the ICM that line the blastocoel cavity (Nadijcka and Hillman, 1974). Once formed, the primitive endoderm cells secrete extracellular matrix (ECM) molecules such as laminin, and a BM is laid down between them and the remainder of the ICM (see Smyth et al., 1999). The primitive endoderm cells also give rise to two extraembryonic cell lineages, the parietal and visceral endoderm (Gardner and Papaioannou, 1975). Parietal endoderm cells, which differentiate from primitive endoderm cells that lie in direct contact with the trophectodermal BM, are motile, and migrate over the blastocoelic surface of the trophectodermal BM (Enders et al., 1978). As parietal endoderm cells migrate, they secrete large amounts of laminin, type IV collagen and nidogen/entactin, which are incorporated into an extremely thick BM called Reichert's membrane that is laid down on the BM of the trophectoderm (Salamat et al., 1995). Following implantation, which occurs during E5, visceral endoderm cells differentiate from the primitive endoderm cells that remain attached to their underlying BM (Gardner, 1982) (see fig. 2). In addition to their critical role in nutrient exchange (Jollie, 1990), these cells have been shown to regulate the development of the embryo proper because the elimination of genes necessary for VE differentiation results in

# Formation of extraembryonic endoderm

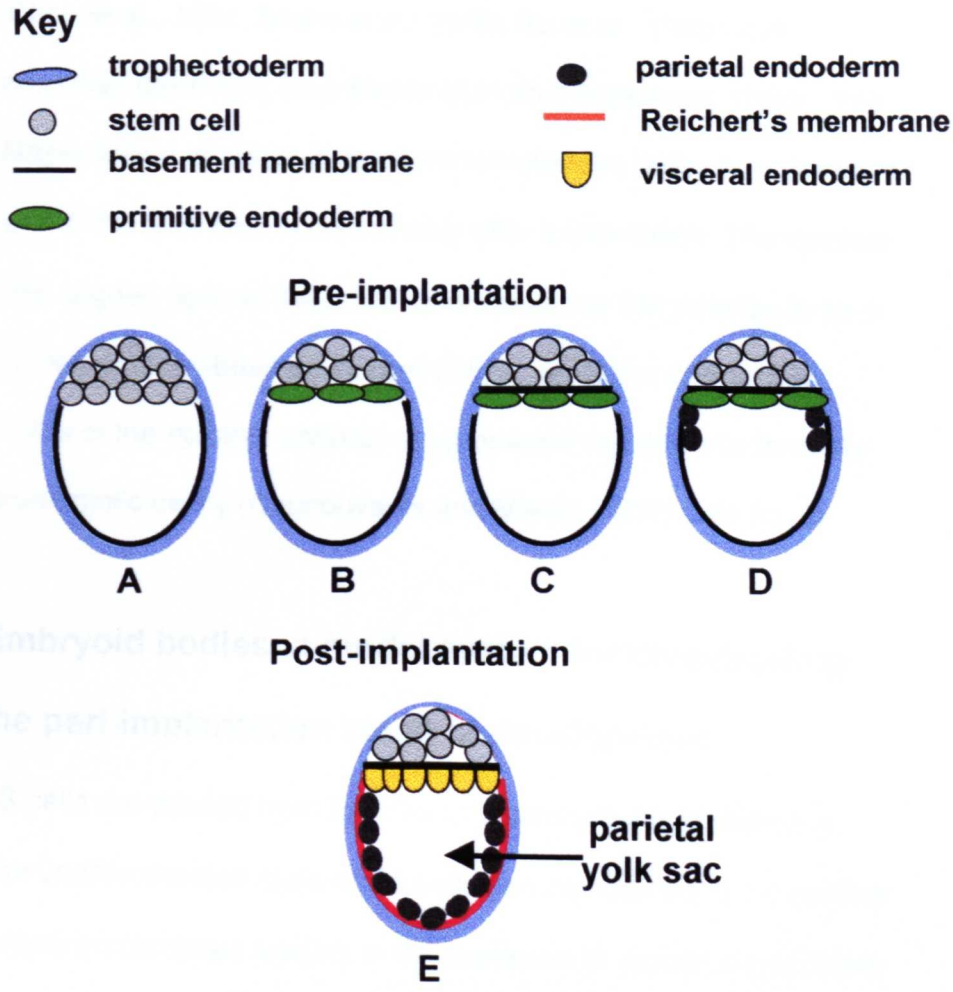


**Figure 2.** Schematic representation of extraembryonic endoderm formation. (A) Upon blastocyst formation the inner cell mass consists of equivalent stem cells. (B) Shortly afterwards, the stem cells on the surface of the inner cell mass differentiate to become primitive endoderm cells. (C) The primitive endoderm cells deposit a basement membrane between themselves and the remaining undifferentiated stem cells of the inner cell mass. (D) Subsequently, the primitive endoderm cells that lie adjacent to the trophectodermal basement membrane differentiate to become parietal endoderm cells and begin to migrate and deposit Reichert's membrane. (E) Following implantation, parietal endoderm cells complete their migration and deposition of Reichert's membrane, and the parietal yolk sac is formed. The primitive endoderm cells overlying the inner cell mass that remain attached to their basement membrane, differentiate to become visceral endoderm cells.

defects in growth (Spyropoulos and Capecchi, 1994), gastrulation (Chen et al., 1994; Sirard et al., 1999; Gu et al., 1999) and molecular patterning (see Beddington and Robertson, 1998). The differentiation of epiblast, or primitive ectoderm cells, from the stem cells of the ICM also occurs shortly after implantation. The epiblast cells aligned against the primitive endodermal BM polarise to form the columnar epiblast epithelium (CEE), while the cells at the centre of the epiblast undergo programmed cell death to form the proamniotic cavity (Coucouvanis and Martin, 1995) (see fig. 3).

## Embryoid bodies: a model system for investigating the peri-implantation stage of development

ES cells are derived from the ICM of blastocyst-stage embryos. The undifferentiated state of ES cells can be retained by monolayer culture on fibroblast feeders in the presence of leukaemia inhibitory factor (LIF). Alternatively, cells can be allowed to form aggregates by culturing in suspension in the absence of LIF; under which conditions, ES cells spontaneously differentiate to form embryoid bodies (EBs) (Robertson, 1987). The initial stage of EB development occurs on the first day following growth in suspension culture, and involves ES cells clumping together to form cellular aggregates. By day 2, the cells on the periphery of the EBs have begun to differentiate to become primitive endoderm cells (Murray and Edgar, 2000). These cells start to express ECM molecules

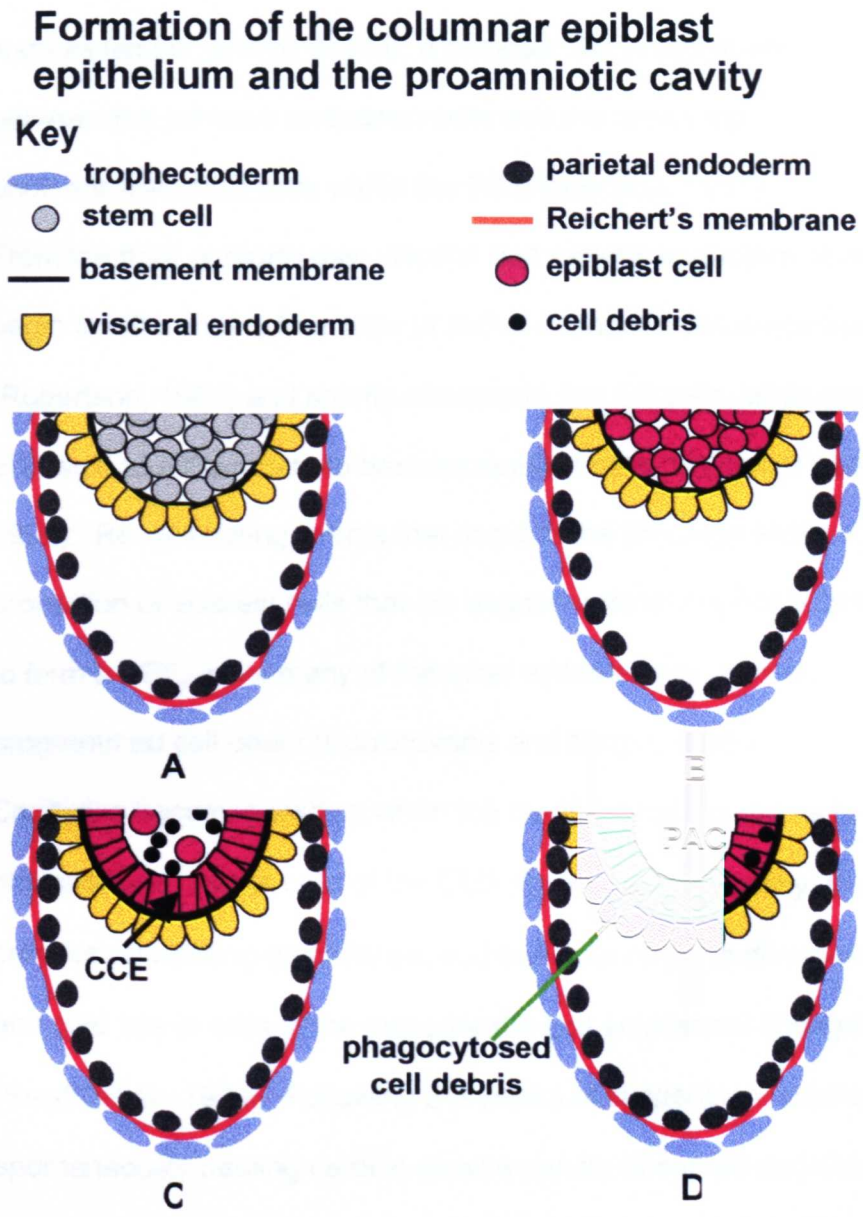


**Figure 3.** Schematic representation of formation of the columnar epiblast epithelium and proamniotic cavity. The proximal part of the embryo, which at this stage consists of extraembryonic ectoderm covered by a layer of visceral endoderm, has been omitted for clarity. (A) Upon implantation, the primitive endoderm cells that directly overlie the inner cell mass differentiate to become visceral endoderm cells. (B) Shortly afterwards the stem cells of the inner cell mass differentiate to become epiblast cells. (C) The epiblast cells in contact with the basement membrane that was initially laid down by the primitive endoderm cells become polarized to form the columnar epiblast epithelium (CEE). The epiblast cells at the apical surface of the CEE that do not contact the basement membrane undergo programmed cell death. (D) The cell debris is then phagocytosed by the cells of the CEE and the proamniotic cavity (PAC) is revealed.

such as laminin and by day4 6 a compete BM is laid down between the primitive endoderm cells and the remaining undifferentiated ES cells within the EB (Robertson 1987) From the third or fourth day visceral and parietal endoderm cells begin to differentiate from their primitive endoderm cell precursors (Robertson 1987) and shortly afterwards the ES cells within the EB begin to differentiate to become epiblast cells (Shen and Leder 1992) Recapitulating events that occur in the E5-stage embryo a proportion of epiblast cells that are aligned against the BM polarise to form a CEE while many of the inner epiblast cells undergo programmed cell death (Coucouvanis and Martin 1995) Cavitation becomes evident when the resultant cellular debris is phagocytosed by the cells of the CEE (fig 4) Subsequently in a process resembling gastrulation epiblast cells begin to differentiate and give rise to cells of the mesodermal and ectodermal lineages (Smyth et al 1999) Following 2-3 weeks of suspension culture spontaneously beating cardiac muscle can be observed and some EBs undergo vesiculation whereby they develop large fluid-filled cysts that are thought to resemble the visceral yolk sac (Doetschman et al 1985) The process of vesiculation in EBs should be distinguished from that of cavitation The former is analogous to the process of exocoelom formation occurring shortly after the commencement of gastrulation in the embryo and appears to involve the infiltration of fluid across the visceral

endoderm cell layer (Bonnevie,1950). Cavitation, on the other hand, is analogous to the process of proamniotic cavity formation and involves epithelialisation and programmed cell death (Coucouvanis and Martin, 1995).

It is easy to see, therefore, that EBs provide an excellent model system for investigating the differentiation events that occur during the peri-implantation stage of mouse development. It should be noted, however, that there are two major differences between EB development and early mouse embryogenesis. Firstly, trophectoderm cells do not differentiate in EBs (Doetschman et al., 1985). In vivo, it has been claimed that trophectoderm is necessary for the differentiation and migration of parietal endoderm cells, for in microdissected fragments of E6.5 embryos, trophoblast giant cell precursors were found to induce the transdifferentiation of visceral to parietal endoderm (Hogan and Tilly, 1981). Additionally, it has been shown that parietal endoderm is generated at the trophectoderm-visceral endoderm contact zone (Hogan et al., 1984). Thus, it is interesting that in EBs, which lack trophectodermal tissue, parietal endoderm cells are formed (Robertson, 1987). Secondly, in the E4 mouse blastocyst, the BM that forms between primitive endoderm cells and the ICM is thin, while in EBs the BM that is laid down between the outer layer of endoderm and the undifferentiated core cells is extremely thick,

# Embryoid body development

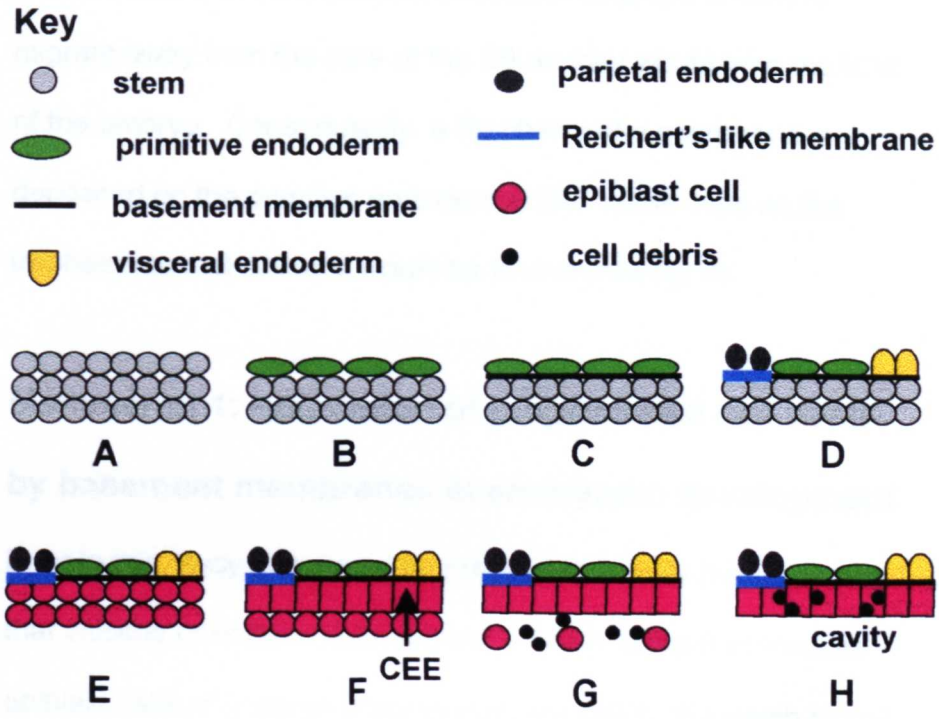


**Figure 4.** Schematic representation of EB development. (A) The first stage involves the clumping together of undifferentiated ES cells to form a cellular aggregate. (B) The cells on the surface of the aggregate then differentiate to become primitive endoderm cells. (C) The primitive endoderm cells deposit a BM between themselves and the undifferentiated core cells of the EB. (D) Some of the primitive endoderm cells differentiate to become visceral end parietal endoderm cells. The parietal endoderm cells deposit a very thick Reichert's-like BM. (E) The ES cells within the core of the EB differentiate to become epiblast cells. (F) The epiblast cells in contact with the BM polarize to form the CEE. (G) Epiblast cells positioned at the apical surface of the CEE detach and undergo programmed cell death. (H) The cell debris is phagocytosed by the cells of the CEE to reveal the cavity.

resembling Reichert's membrane (Martin et al., 1977; Inoue et al., 1983). The reason for this is that because of the absence of trophectoderm in EBs, parietal endoderm cells are unable to migrate away from the core of the EB as they would from the ICM of the embryo. Consequently, a Reichert's-like membrane is deposited on the primitive endodermal BM, rather than on the trophectodermal BM as it would be in vivo (see fig. 4).

## Manuscript 1: Regulation of programmed cell death by basement membranes in embryonic development

Prior to this study, the model for proamniotic cavity formation was that visceral endoderm cells secrete a death signal that induces epiblast cells to undergo programmed cell death: the death signal being counteracted by a life signal mediated by attachment to the BM (Coucouvanis and Martin, 1995). Consequently, the only epiblast cells to survive are those aligned against the BM, which become polarised to form the CCE (Coucouvanis and Martin, 1995). This model, however, was based on experiments performed using EC cell lines, which are derived from the stem cells of teratocarcinomas, and often have restricted developmental potential; whereas ES cells are derived directly from the pluripotent cells of pre-implantation embryos. Thus, to investigate the role of the BM in cavitation, use has been made of BM-deficient EBs derived from *LAMC1-/-* ES cells.

Here it is shown that in the absence of the BM, although visceral and epiblast cells differentiate, the epiblast cells in contact with the BM fail to polarise to form the CEE. Additionally, the epiblast cells within the EB do not undergo programmed cell death, and hence, there is a failure of cavitation.

## Manuscript 2: Regulation of laminin and COUP-TF expression in extraembryonic endodermal cells

The primitive endodermal BM is necessary for CEE and proamniotic cavity formation, and thus is required for embryogenesis to proceed beyond its earliest stages (Murray and Edgar, 2000). Furthermore, laminin-1 which is secreted by the primitive endoderm cells, is the key orchestrator in the deposition of this BM (Smyth et al., 1999; Murray and Edgar, 2000). Prior to this study, however, the mechanisms controlling the up-regulation of laminin genes by primitive endoderm cells had not been investigated. In order to begin to elucidate the molecular mechanisms controlling laminin gene expression in primitive endoderm cells, it was deemed necessary to identify the transcription factor(s) responsible for the induction of the laminin genes *LAMB1* and *LAMC1*, encoding the laminin β1 and γ1 subunits, respectively.

In this manuscript it is shown that *COUP-TFs* are induced in the primitive endoderm cells of the pre-implantation embryo and ES cell-derived EBs. Consistent with a role for COUP-TFs in the induction of laminin genes, the appearance of *COUP-TFs* precedes that of *LAMB1* and *LAMC1* in the differentiating primitive endoderm cells of EBs. In addition, transfection studies show that expression of either *COUP-TFI* or *COUP-TFII* can up-regulate expression of *LAMB1* and *LAMC1* genes in cells that normally express only minimal amounts of *COUP-TFs* and laminin. The results suggest, therefore, that the increased expression of *COUP-TFs* in differentiating primitive endoderm cells mediates laminin expression and hence, subsequent embryonic development dependent upon BM formation.

## Manuscript 3: Regulation of the differentiation of extraembryonic endoderm cells by basement membrane and parathyroid hormone-related polypeptide (PTHrP)

Shortly after the primitive endoderm cells deposit a BM, some of them differentiate to become visceral endoderm, while others become parietal endoderm. In order to investigate the role of the BM in extraembryonic endoderm differentiation, use has been made of EBs derived from *LAMC1-/-* ES cells that do not express

the laminin γ1 subunit and so cannot synthesise BMs (Smyth et al., 1999). Here it is shown that in the absence of the BM, primitive, visceral and parietal endoderm cells differentiate, but fail to form organized epithelia. Furthermore, the numbers of visceral and parietal endoderm cells differentiating from their primitive endoderm cell precursors are increased in *LAMC1-/-* EBs. It is concluded, therefore, that the BM is not only required for the proper organization of visceral and parietal endoderm cells but also restricts their differentiation to maintain the population of primitive endodermal stem cells.

Previous analysis of *LAMC1-/-* embryos has shown that although a functional trophectodermal epithelium can form in the absence of a BM, the embryos nevertheless die very shortly after implantation (Smyth et al., 1999). *LAMC1-/-* embryos appear to undergo some aspects of extra-embryonic endoderm differentiation; yet despite this, there is no evidence of parietal endoderm cell migration and consequently, the parietal yolk sac and Reichert's membrane fail to form. It is possible that the failure of parietal endoderm cell migration results from a defect in the differentiation of these cells. Alternatively, the lack of parietal endoderm cell migration may be due to the absence of the trophectodermal BM that is the normal substrate for migrating parietal endoderm cells (Enders et al., 1978).

It has been shown in vitro that PTHrP stimulates parietal endoderm cell migration on a variety of ECM substrates (Behrendtsen et al., 1995). In vivo, PTHrP is expressed by the trophectoderm cells and the PTH/ PTHrP receptor (PTH/PTHrPR) is expressed by extra-embryonic endoderm cells. Highest levels of PTH/PTHrPR are expressed by the extra-embryonic endoderm cells that lie adjacent to the trophectoderm (Verheijen et al., 1999). Until now, it has not been clear whether PTHrP functions to stimulate migration, or instead stimulates delamination from the primitive endodermal BM, which as is shown here, is a necessary prerequisite for parietal endoderm cells to become migratory.

By utilising EBs derived from *LAMC1-/-* ES cells it has been possible to analyse the effect of BM deficiency and PTHrP on parietal endoderm cell migration. In this manuscript it is shown that parietal endoderm cells migrate more readily from *LAMC1-/-* EB outgrowths than from controls with BM. Furthermore, cells will only migrate away from control EBs in the presence of PTHrP, whereas migration away from *LAMC1-/-* EBs does not require PTHrP and is unaffected by its presence. It is concluded that failure of parietal endoderm cell migration in vivo is caused by absence of the trophectodermal BM, the basal surfaces of the trophectodermal cells presumably being a non-permissive substrate for parietal endoderm cell migration. Moreover, the attachment to the primitive

endodermal BM normally inhibits parietal endoderm cell migration, as these cells must first delaminate from the BM in order for them to migrate. Thus, the major role of PTHrP in stimulating parietal endoderm cell migration is to induce delamination from the primitive endodermal BM.

## Manuscript 4: The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF)

It has been shown previously that LIF blocks the differentiation of epiblast cells in ES cell-derived EBs, but has no effect on the differentiation of primitive endoderm cells (Shen and Leder, 1992). Thus, to ascertain if epiblast cell differentiation is obligatory for the formation of a columnar epithelium and cavitation, we cultured EBs in the presence of LIF.

In agreement with previous work (Shen and Leder, 1992) it is shown here that while LIF blocks epiblast cell differentiation, it has no effect on the differentiation of primitive endoderm cells. Furthermore, although LIF does not affect deposition of the primitive endodermal BM, it does inhibit visceral and parietal endoderm cell differentiation. Interestingly, in the absence of epiblast cell differentiation, it was found that the stem cells aligned against the primitive endodermal BM were able to form a columnar epithelium that was morphologically identical to the CEE.

Furthermore, the stem cells at the apical surface of this epithelium underwent programmed cell death to form a cavity. From the results of this work and those of manuscript 1, it is concluded that while visceral endoderm is necessary for ES cells to differentiate into epiblast cells, it is not required for the polarisation of epiblast cells that leads to CEE formation; nor for the programmed cell death that leads to cavity formation. Conversely, although the BM is necessary for columnar epithelial formation and cavitation, it is not required for ES to epiblast cell differentiation.

## Manuscript 5: Absence of basement membranes after targeting the *LAMC1* gene results in embryonic lethality due to failure of endoderm differentiation

To elucidate the role of the BM in development, the *LAMC1* gene coding for the laminin γ1 subunit was targeted by homologous recombination in mouse embryonic stem cells. It was reasoned that the absence of laminin γ1 would prevent laminin trimer formation, and consequently, block BM formation. It is shown here that laminin γ1 is indeed necessary for BM formation in both early stage embryos in vivo and EBs in vitro. Moreover, the BM was found to be essential for embryonic development to proceed beyond E5.5. Analysis of *LAMC1* disruption in vitro revealed that despite the absence of the BM, epiblast cells were nonetheless able to differentiate.

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# Appendix 1

# *Report*

# Regulation of Programmed Cell Death by Basement Membranes in Embryonic Development

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*Abstract.* The formation of the proamniotic cavity in the mammalian embryo is the earliest of many instances throughout development in which programmed cell death and the formation of epithelia play fundamental roles (Coucouvanis, E., and G.R. Martin. 1995. *Cell.* 83:279–287). To determine the role of the basement membrane (BM) in cavitation, we use embryoid bodies derived from mouse embryonic stem cells in which the *LAMC1* genes have been inactivated to prevent BM deposition (Smyth, N., H.S. Vatansever, P. Murray, M. Meyer, C. Frie, M. Paulsson, and D. Edgar. 1999. *J. Cell Biol.* 144:151–610). We demonstrate here that *LAMC1−/−* embryoid bodies are unable to cavitate, and do not form an epiblast epithelium in the absence of a BM, although both embryonic ectodermal cells and extraembryonic endodermal cells do differentiate, as evidenced by the expression of cell-specific markers. Acceleration or rescue of BM deposition by exogenous laminin in wild-type or *LAMC1−/−* embryoid bodies, respectively, results in cavitation that is temporally and spatially associated with restoration of epiblast epithelial development. We conclude that the BM not only directly regulates development of epiblast epithelial cells, but also indirectly regulates the programmed cell death necessary for cavity formation.

Key words: organogenesis • extracellular matrix • laminin • apoptosis • stem cells

## *Introduction*

The formation of cavities in solid blocks of cells is a widespread event in organogenesis throughout embryonic development. Over the last decade, it has become apparent that programmed cell death (PCD)[1] plays a fundamental role in cavity formation in many tissues (Coles et al., 1993; Coucouvanis and Martin, 1995; Humphreys et al., 1996; Jacobson et al., 1997). Although it is known that basement membranes (BM) are necessary for the survival and differentiation of epithelial cells surrounding the cavities (Ekblom et al., 1980; Coucouvanis and Martin, 1995; Streuli, 1996), any involvement of BMs in the regulation of PCD and the mechanisms coordinating epithelialization with PCD during cavitation remain unknown.

Formation of the proamniotic cavity is the first instance of cavitation during mammalian development. Shortly before implantation, the inner cell mass (ICM) of the mouse embryo consists of a small group of cells separated from an outer layer of primitive endoderm by a BM (Salamat et al., 1995). Subsequently, the primitive endoderm cells remaining in contact with this BM differentiate to become visceral endoderm (VE), while the remaining ICM cells differentiate to become the epiblast, or primitive ectoderm (see Fig. 1). Initially, the differentiation of epiblast cells is reflected by an alteration in the profile of expressed genes, and is not accompanied by any obvious morphological differentiation (Kaufman, 1992; Rathjen et al., 1999). However, a few hours later, the epiblast cells in contact with the BM become polarized to form the columnar epiblast epithelium (CEE), while cells at the center of the ICM undergo PCD, thereby giving rise to the proamniotic cavity (Coucouvanis and Martin, 1995).

Embryoid bodies (EBs), which are derived from differ-

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[1]*Abbreviations used in this paper:* AFP, α-fetoprotein; BM, basement membrane; CEE, columnar epiblast epithelium; EB, embryoid body; EC, embryonal carcinoma; ES, embryonic stem; ICM, inner cell mass; PCD, programmed cell death; RT-PCR, reverse transcription–PCR; TUNEL, terminal transferase–mediated biotinylated-dNTP end labeling; VE, visceral endoderm.

entiating mouse embryonic stem (ES) or embryonal carcinoma (EC) cells, are widely used model systems suitable for analysis of such events, cell differentiation in EBs closely reflecting that of the ICM during the peri-implantation period (Robertson, 1987). Thus, cavitating and noncavitating EBs, which are derived from PSA1 and S2 EC cell lines, respectively, have been used to show that VE cells secrete a diffusible factor that induces PCD of the majority of epiblast cells (Coucouvanis and Martin, 1995). However, the BM that lies between the VE and the epiblast supports the survival of the CEE cells in contact with it (Coucouvanis and Martin, 1995). More recent studies using these EC cells have implicated bone morphogenetic proteins 2 and/or 4 in the apoptosis of epiblast cells by showing BMP4 only to be expressed by cavitating PSA1 EBs and being able to induce cell death in normally noncavitating S2 EBs (Coucouvanis and Martin, 1999).

To investigate the role of BMs during embryonic development in vivo and EB development in vitro, we previously used homologous recombination in mouse ES cells to knockout one or both copies of the *LAMC1* gene encoding the laminin γ1 subunit (Smyth et al., 1999). This defect renders the cells incapable of assembling a laminin type-1 trimer, which is necessary for BM deposition; hence, both *LAMC1−/−* preimplantation embryos and EBs lack BMs (Smyth et al., 1999). In vivo, the consequence of the lack of BMs is that the embryo dies during the peri-implantation period around the time when cavitation occurs, although the cause of this remains to be established (Smyth et al., 1999). However, the *LAMC1−/−* ES cells offer a unique system to help delineate the reasons for this lethality by establishing the role of the BM in proamniotic cavitation (Coucouvanis and Martin, 1995). By being able to experimentally manipulate BM deposition in *LAMC1−/−* EBs by addition of exogenous laminin, we are able to demonstrate that the BM is not only necessary for formation of the columnar epiblast epithelium, but is also necessary for the cell death leading to cavitation. Involvement of the BM in both these processes indicates that this extracellular matrix structure plays a key role in the coordination of events necessary for cavitation in developing tissues.

## Materials and Methods

### ES Cell and EB Culture

The production of R1 mouse *LAMC1+/−* and *LAMC1−/−* ES cells has been described in detail previously (Smyth et al., 1999). The *LAMC1+/−* ES cells, used here as controls, were from the clone previously used to produce healthy heterozygous germline animals (Smyth et al., 1999). The absence of clonal artefacts in the *LAMC1−/−* cells used here was confirmed by rescue of the phenotype by adding laminin type-1 (Sigma Chemical Co.) to developing *LAMC1−/−* EBs (see Fig. 4). ES cells were cultured on mitomycin-treated STO feeder cells in gelatinized 3.5-cm tissue culture dishes. The culture medium was DME (GIBCO BRL) supplemented with 15% (vol/vol) ES grade FBS (GIBCO BRL), 0.1 mM β mercaptoethanol, 1 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 1,000 U/ml of LIF (ESGRO; GIBCO BRL). ES cells were subcultured every 2 d. Before EB formation, ES cells were passaged once on gelatinized tissue culture dishes and incubated in the above medium for 2 d to eliminate STO cells. To make EBs, ES cells were trypsinized, triturated, and split 1:10 by replating into bacterial petri dishes, under which conditions the ES cells remained in suspension and formed aggregates. The EB culture medium was as above, except that LIF was omitted and the FBS content was reduced to 10% (vol/vol). After 2 d of suspension culture, the EB population of each 10-cm petri dish was divided into two and supplemented with fresh EB culture medium, after which the medium was changed on every second day. For the rescue experiment, 20 μg/ml of laminin type-1 was added to the culture medium immediately after replating of ES cells into petri dishes. For toluidine blue staining, immunostaining and terminal transferase–mediated biotinylated-dNTP end labeling (TUNEL) analysis, EBs were fixed for 1 h with 4% (wt/vol) paraformaldehyde and gelatin-embedded for preparation of frozen cryostat sections. For transmission EM, EBs were fixed for 1 h in 2:4% glutaraldehyde/paraformaldehyde (wt/vol) and processed as previously described (Fleming et al., 1984).

### Immuno- and Fragmented DNA Staining

The primary antibodies used were rabbit anti-EHS laminin that recognizes all three subunits of laminin type-1 (Kücherer-Ehret et al., 1990), and so could be used to localize α1 and β1 subunits in the absence of γ1 in *LAMC1−/−* EBs. Rabbit antiperlecan antibodies were raised against recombinant perlecan domain III3 (Schulze et al., 1995). Incubations with primary antibodies were carried out overnight in 1% (vol/vol) goat serum in PBS at room temperature, and the sections were washed three times in PBS. The secondary antibody was TRITC-conjugated swine anti-rabbit IgG (Dako), which was applied in 1% (vol/vol) goat serum in PBS at room temperature for 2 h, and the sections were washed three times in PBS. For the detection of fragmented DNA on EB frozen sections, the TUNEL method was used as described previously (Smyth et al., 1999). Sections were mounted in fluorescent mounting medium (Dako) and photographed using a Leitz fluorescence microscope. All digital images were prepared with Adobe Photoshop 5.

### Reverse Transcription–PCR (RT-PCR) Analysis of mRNA

Total RNA was extracted from *LAMC1+/−* and *LAMC1−/−* ES cells or EBs using guanidinium isothiocyanate (Chomczynski and Sacchi, 1987), and reverse-transcribed using Superscript™ II RT (GIBCO BRL). For undifferentiated ES cells and day 2 EBs, whole populations were used, but for day 10 EBs, 10–15 cavitated *LAMC1+/−* EBs and an equal number of *LAMC1−/−* EBs were selected using phase-contrast microscopy. α-Fetoprotein (*AFP*) primers were those used for riboprobe synthesis (see below), and *BMP4* and *FGF-5* primers were as described previously (Johansson and Wiles, 1995). *GAPDH* primers were as follows: forward (5′-GGTGAAGGTCGGAGTCAACGG-3′) and reverse (5′-GGTCATGAGTCCTTCCACGAT-3′; product size, 520 bp). Semi-quantitative RT-PCR was performed as previously described to determine mRNA levels relative to that of GAPDH (Squitti et al., 1999).

### Whole-mount In Situ Hybridization

A sequence containing nucleotides 309–770 of mouse *AFP* cDNA (Tilghman et al., 1979) was amplified by PCR with forward primer (5′-ACATCAGTGTCTGCTGGCAC-3′) and reverse primer (5′-ACCGAGTTTCCTTGGCAACAC-3′), from cDNA reverse-transcribed from total RNA extracted from day 10 EBs. The PCR fragment was cloned into the T-Easy™ Vector (Promega) and transcribed with T7 or SP6 and digoxigenin-UTP for sense or antisense probes. Whole-mount in situ hybridization was performed as previously described (Leibl et al., 1999).

## Results and Discussion

Fig. 1 shows a schematic diagram of the organization of cells and BM during the periimplantation stages of mouse development.

### Cavitation Fails in LAMC1 −/− EBs Despite the Presence of VE Cells

Histological analysis of *LAMC1+/−* control EBs, which are able to synthesize BMs and have a wild-type phenotype (Smyth et al., 1999), showed that they cavitated in suspension culture as expected (Fig. 2 a). In contrast, the

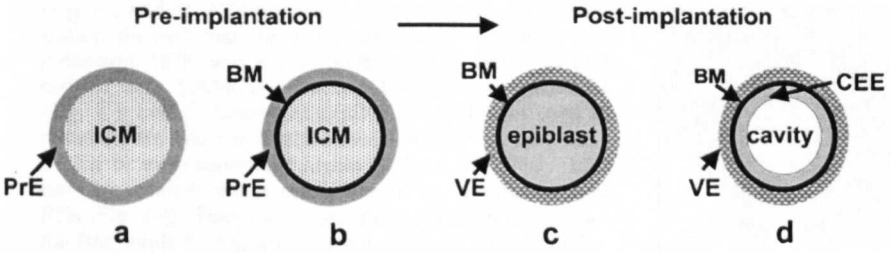
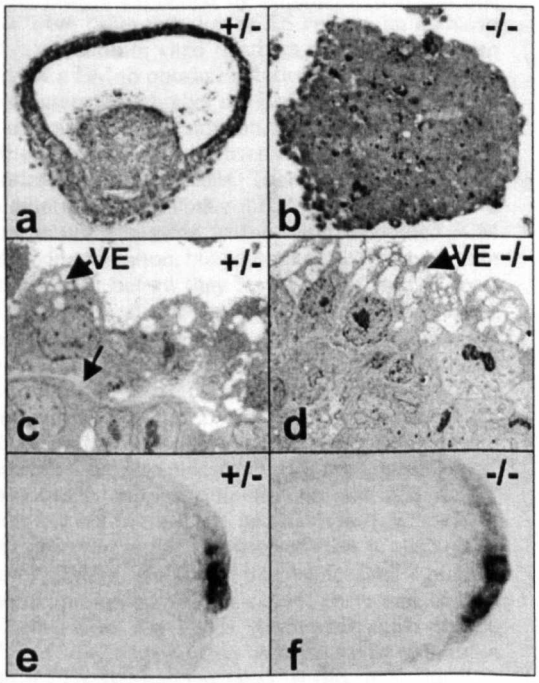
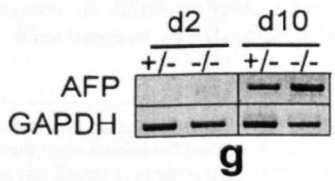



*Figure 1.* Schematic diagram showing the peri-implantation stages of mouse development. (a) Shortly after blastocyst formation, the cells on the surface of the ICM differentiate to become primitive endodermal cells. (b) The primitive endodermal cells deposit a basement membrane (BM). (c) After implantation, the primitive endodermal cells in contact with the BM differentiate to become visceral endoderm (VE) cells, and the remaining ICM cells differentiate to become epiblast cells. (d) The epiblast cells in contact with the BM polarize to form the columnar epiblast epithelium (CEE), and the unpolarized epiblast cells in the center undergo programmed cell death, giving rise to the proamniotic cavity.

*LAMC1−/−* EBs failed to form a cavity (Fig. 2 b). Furthermore, EM revealed that in addition to the lack of deposition of a BM (Smyth et al., 1999), a columnar ectodermal epithelium (CEE) failed to form in the *LAMC1−/−* EBs (see Fig. 3 e). Despite these differences, EM also showed that cells with the morphological characteristics of VE, namely apical vacuoles and microvilli, were present at the periphery of both *LAMC1−/−* and *LAMC1+/−* EBs



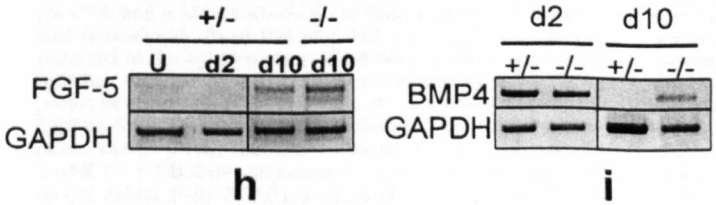




*Figure 2.* VE cell differentiation and cavitation in EBs. (a and b) Toluidine blue–stained frozen sections of EBs after 7.5 d in suspension culture show that *LAMC1+/−* EBs had cavitated by this time (a), whereas the *LAMC1−/−* EBs failed to cavitate (b). (c and d) EM shows differentiation of cells with the morphological characteristics of VE in both *LAMC1+/−* (c) and *LAMC1−/−* (d) EBs. However, a BM is only present in the *LAMC1+/−* control EBs (c, white arrow). (e and f) Whole-mount in situ hybridization for the VE marker *AFP* shows positive cells at the periphery of both *LAMC1+/−* (e) and *LAMC1−/−* (f) EBs. (g and i) RT-PCR analysis; *GAPDH* mRNA is shown as a loading control. (g) *AFP* mRNA is expressed late in both *LAMC1+/−* and *LAMC1−/−* EBs. (h) Only a trace of *FGF-5* mRNA is detectable in undifferentiated *LAMC1+/−* ES cells (U) and day 2 (d2) EBs, but *FGF-5* is induced by day 10 (d10) in both *LAMC1−/−* and control EBs; the double band results from splice variants (Johansson and Wiles, 1995). (i) *BMP4* mRNA is expressed in both *LAMC1−/−* and control EBs at day 2; but, by day 10, levels are greatly reduced in the controls. VE, visceral endoderm.

(Fig. 2, c and d). Additionally, whole-mount in situ hybridization showed that the VE marker *AFP* (Dziadek and Adamson, 1978) was expressed in some of the peripheral cells of both *LAMC1−/−* and *LAMC1+/−* control EBs (Fig. 2, e and f). Semi-quantitative RT-PCR was used to demonstrate that the relative levels of *AFP* mRNA were similar or even somewhat higher in the *LAMC1−/−* EBs than in controls while being absent in undifferentiated EBs (Fig. 2 g). Taken together, these results indicate that the BM, while having no apparent effect on VE cell differentiation, is necessary for the previously reported regulation of PCD by endodermal cells (Coucouvanis and Martin, 1995).

### *Epiblast Cell Differentiation in LAMC1−/− EBs*

Several lines of evidence have been presented suggesting that VE regulates epiblast cell differentiation. For example, disruption of the VE-specific gene *Evx1* inhibits epiblast cell differentiation (Spyropoulos and Capecchi, 1994), and factors expressed by an endodermal cell line can induce the differentiation of ES cells to an epiblast-like cell population in vitro (Rathjen et al., 1999). Given the need for a BM to obtain cavitation of the epiblast, we wished to determine whether the BM was necessary for all aspects of epiblast differentiation, or, alternatively, if the role of the BM was more restricted to being a requirement for polarization of CEE cells. Therefore, RT-PCR was used to determine the relative mRNA levels of *FGF-5*, a gene that is not expressed in the undifferentiated ICM cells before implantation, but subsequently is turned on in epiblast cells just before they become polarized to form the CEE (Haub and Goldfarb, 1991). The results show that the profile of *FGF-5* expression in EBs mimics that seen in vivo; only trace amounts of *FGF-5* mRNA were present in undifferentiated *LAMC1+/−* ES cells and at early time points during EB differentiation, whereas the levels increased at later time points (Fig. 2 h). *BMP4* mRNA levels were also investigated, as this signaling molecule is normally expressed in early epiblast cells before cavitation, but not in the CEE, and has been implicated in the PCD observed in EC cell-derived EBs (Coucouvanis and Martin, 1999). We found that while *BMP4* mRNA levels were initially similar in *LAMC1−/−* and control *LAMC1+/−* EBs, the levels were maintained in the *LAMC1−/−* EBs, whereas they were markedly reduced in the control EBs that had cavitated by this time (Fig. 2 i). The maintenance of *BMP4* mRNA expression in the *LAMC1−/−* EBs, together with the *FGF-5* data, indicates

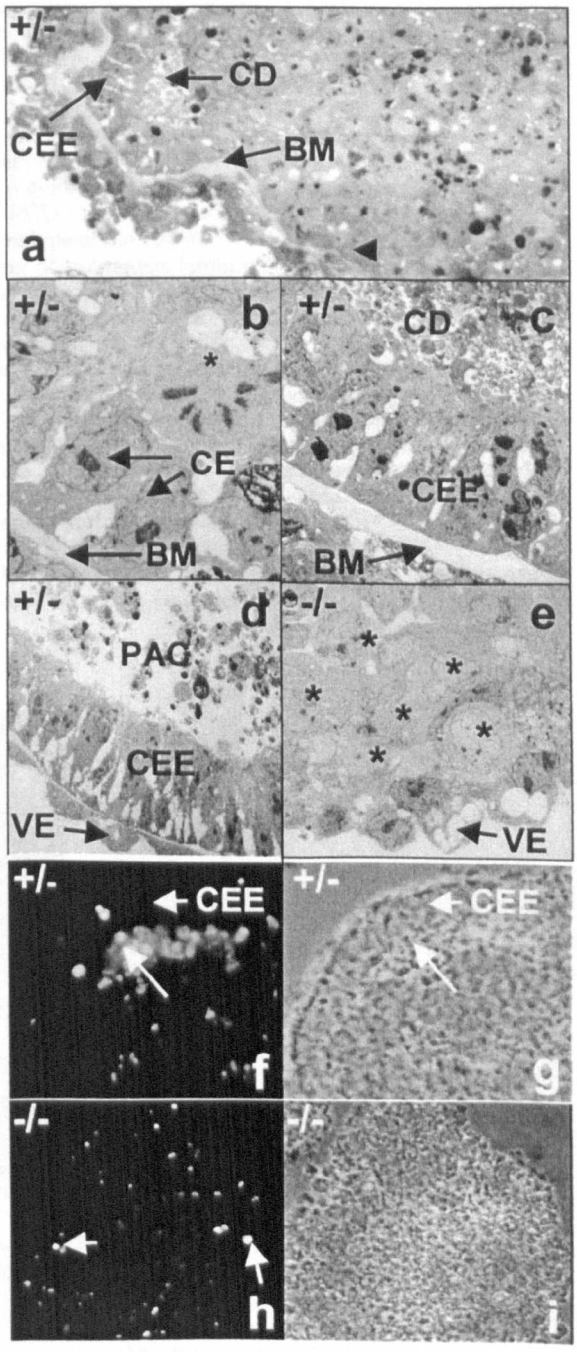



*Figure 3.* Epiblast cell polarization and PCD in EBs. (a) Toluidine blue–stained resin-embedded section of a day 6 *LAMC1+/−* EB shows that cell debris is present only at the apical surface of the CEE and is absent where the epiblast cells remain unpolarized (arrowhead). (b–d) EM of *LAMC1+/−* EBs. (b) The cell (asterisk) at the apical surface of the two columnar ectodermal cells has rounded up and become vacuolated by day 5; (c) a pocket of cell debris (CD) is present at the apical surface of the CEE on day 6; (d) as the debris is removed at day 7.5, the proamniotic cavity (PAC) becomes evident. (e) EM of day 7.5 *LAMC1−/−* EB shows unpolarized epiblast cells (asterisks) and no cell debris. (f–h) TUNEL analysis after 6 d: in *LAMC1+/−* EBs, a cluster of TUNEL-positive cells (f, arrow) is present at the apical surface of the CEE. (g) Phase-contrast image of f. Only a few randomly scattered TUNEL-positive cells (h, arrows) are present in *LAMC1−/−* EBs. (i) Phase-contrast image of h. Note that the BM in these EBs does not have a *lamina lucida*, which is consistent with previous reports of its absence in some EBs and in Reichert's membrane (Martin et al., 1977; Inoue et al., 1983). BM, basement membrane; CE, columnar epiblast cell; CEE, columnar epiblast epithelium; CD, cell debris; PAC, proamniotic cavity; VE, visceral endoderm.

that the BM has no apparent effect on initial epiblast cell differentiation. The development of epiblast cells is considered to be an obligatory intermediate step in the differentiation of ES cells into embryonic cell lineages (Rathjen et al., 1999). Thus, the fact that myoblasts, endothelial and neuronal cells can differentiate from *LAMC1−/−* ES cells (Smyth et al., 1999) also indicates that the BM is not required for at least some aspects of epiblast cell differentiation, although it is necessary for the polarization of cells to form the CEE.

### *Relationship between PCD, Epiblast Cell Polarization and BMs*

In control *LAMC1+/−* EBs, we found that the first stage of cavitation involved a loss of cell–cell contact between the polarized CEE cells and the cells positioned at their apical surface (Fig. 3 b). Subsequently, small pockets of cell debris could be identified at the apical surface of the CEE (Fig. 3 c), and, finally, a cavity became evident as the debris was phagocytosed by the cells of the CEE (Fig. 3 d). During cavitation, cell debris was restricted to the apical surface of the CEE, and was never observed in the vicinity of the unpolarized epiblast cells (Fig. 3 a). TUNEL analysis of control and age-matched *LAMC1−/−* EBs showed that whereas only randomly scattered TUNEL-positive cells were present in the *LAMC1−/−* EBs (Fig. 3 h), clusters of TUNEL-positive cells were observed exclusively at the apical surface of the CEE in control EBs (Fig. 3 f). Thus, there is a precise correlation between the development of the CEE and PCD.

To demonstrate that the BM was responsible for both the PCD and polarization of the CEE cells, the mutant phenotype of the *LAMC1−/−* EBs was rescued by the addition of exogenous laminin type-1. This resulted in the deposition of a BM-like sheet defined by anti–laminin type-1 and antiperlecan immunoreactivity between the outer endoderm and inner core cells of *LAMC1−/−* EBs (Fig. 4, a–d). In addition, CEE cells were found aligned on this sheet, and cells at the apical surface of the CEE cells had either detached or had undergone PCD, thereby forming a cavity (Fig. 4 e). The indirect effect of the BM on the PCD in the epiblast indicates either that the CEE is responsible for inducing PCD of those cells positioned at its apical surface, or, alternatively, the PCD was induced by a VE cell–derived molecule with restricted diffusion and whose synthesis was dependent upon contact of VE cells with the BM. To decide between these alternative hy-

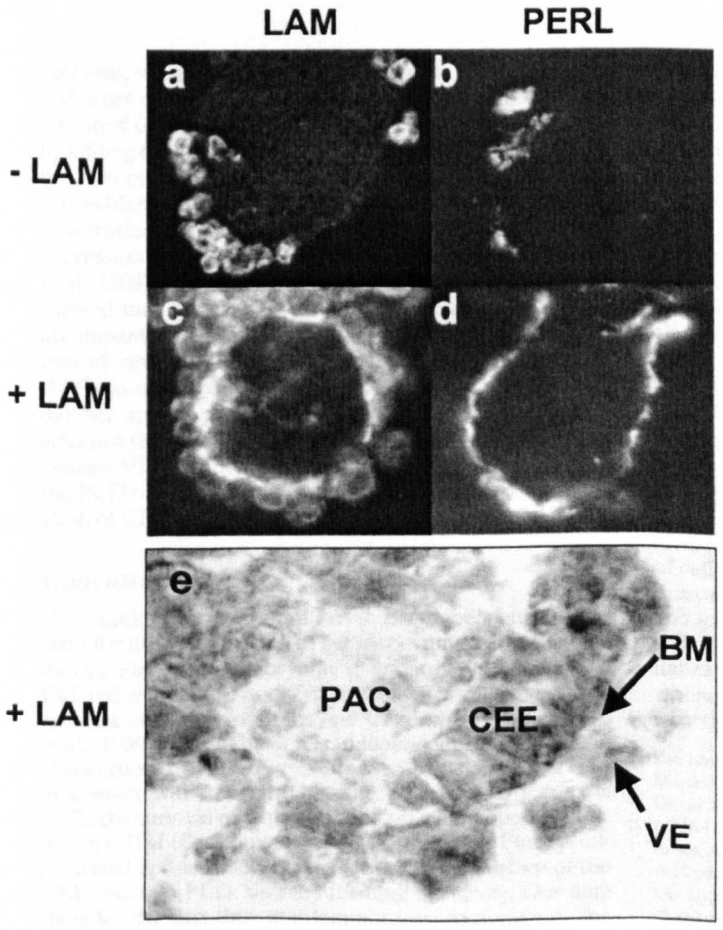



*Figure 4.* Rescue of *LAMC1−/−* EBs by the addition of exogenous laminin. (a–d) Immunofluorescence staining for laminin (a and c) and perlecan (b and d) in *LAMC1−/−* EBs after 2 d of culture: without (a and b) and with the addition of laminin type-1 (c and d). (e) Toluidine blue–stained frozen section of *LAMC1−/−* EB after laminin addition shows that the CEE and PAC develop. BM, position of the basement membrane–like deposition of laminin and perlecan; CEE, columnar epiblast epithelium; PAC, proamniotic cavity; VE, visceral endoderm.

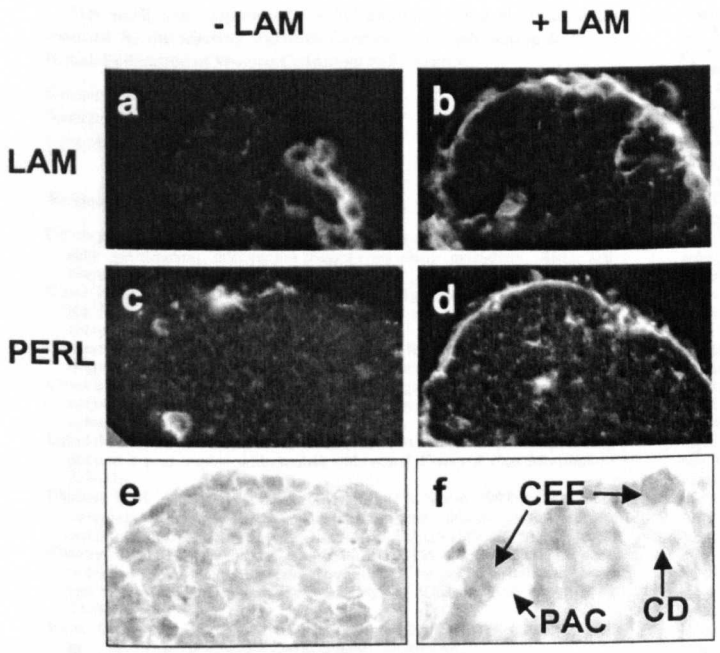



*Figure 5.* Laminin addition to *LAMC1*+/− control EBs accelerates the deposition of a basement membrane, leading to epiblast polarization and cavitation. (a–d) Immunofluorescence staining for laminin (a and b) and perlecan (c and d) in control EBs after 2 d culture: without (a and c) and with addition of laminin type-1 (b and d). (e and f) Toluidine blue–stained frozen sections of day 2 control EBs grown without (e) or with (f) the addition of type-1 laminin. Note the formation of the CEE and a proamniotic-like cavity on the addition of laminin. CD, cell debris; CEE, columnar epiblast epithelium; PAC, proamniotic cavity.

potheses, we made use of the observation that a complete BM is not observed in wild-type *LAMC1*+/− EBs until after day 4 of differentiation (results not shown). However, by adding exogenous laminin at the start of differentiation, the rate of BM deposition was accelerated so that a BM was evident by day 2 of differentiation (Fig. 5, a–d). This observation supports our previous conclusion that laminin expression is the rate-limiting step in BM deposition (Smyth et al., 1999). In addition, histological analysis of these EBs showed that the early deposition of a BM-like sheet was accompanied by premature CEE formation and the initiation of cavitation (Fig. 5 f). However, the expression of *AFP* was unaffected by the absence of a BM (Fig. 2 f) and did not appear prematurely in laminin-treated EBs (results not shown). Thus, although the differentiation of the mature VE phenotype occurs independently of the BM, the PCD of epiblast cells is closely linked to the differentiation of CEE cells, which in turn is dependent upon a BM.

## *Conclusions*

Our results demonstrate a novel BM-dependent mechanism for the coordination of cellular events leading to cavitation. First, extra-embryonic endodermal cells deposit a BM and also induce undifferentiated ICM cells to become epiblast cells (Spyropoulos and Capecchi, 1994; Rathjen et al., 1999). This induction is independent of the BM as it also occurs in *LAMC1*−/− EBs. Second, the epiblast cells in contact with the BM become polarized to form the CEE, the survival of which is then dependent upon contact with the BM (Coucouvanis and Martin, 1995). Finally, unpolarized epiblast cells that lie at the apical surface of the CEE undergo PCD, thereby forming the cavity. Our data show for the first time that laminin (and consequently the BM) could be both a death signal (acting indirectly) and a survival signal (acting directly). While the mechanism responsible for this indirect BM-dependent PCD remains to be determined, the fact that it is observed directly at the apical surfaces of newly polarized CEE cells is consistent with the involvement of a CEE-derived factor whose diffusion is highly restricted or, alternatively, a cell–cell contact phenomenon within the epiblast.

During organogenesis in later development, cavity formation occurs in many tissues including the exocrine glands (Hieda and Nakanishi, 1997), lungs (Schuger et al., 1995), mammary glands (Humphreys et al., 1996) and kidneys (Coles et al., 1993). In the submandibular gland and lung, both initially derived from solid masses of cells, there is a strong association between development of a continuous BM, epithelialization, and cavity formation (Schuger et al., 1995; Hieda and Nakanishi, 1997). Although the mechanism of cavity formation has not been investigated in most cases, PCD followed by phagocytosis of cell debris by the epithelial cells recently has been demonstrated in mammary gland (Humphreys et al., 1996) and kidney development (Coles et al., 1993). Therefore, it is likely that the ability of BMs to coordinate both epithelialization and cell death is used throughout development whenever a lumen or cavity is to be created from a solid structure.

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# Appendix 2

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Gene expression pattern

# Regulation of laminin and COUP-TF expression in extraembryonic endodermal cells

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**Abstract**

Laminin expression and the subsequent deposition of a basement membrane by primitive endoderm cells is necessary for early mammalian development. We demonstrate that the transcription factors *COUP-TF I* and *II* are up-regulated in primitive endoderm cells faster than *LAMB1* and *LAMC1*, and that either COUP-TF is sufficient to induce expression of these laminin genes. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords*: Laminin; *LAMB1*; *LAMC1*; Primitive endoderm; COUP-TF; Basement membrane

## 1. Results and discussion

The basement membrane (BM) deposited between the primitive endoderm (PrE) and epiblast of mammalian blastocysts regulates the differentiation of epiblast cells and proamniotic cavity formation (Coucouvanis and Martin, 1995; Smyth et al., 1999; Murray and Edgar, 2000). The laminin and other proteins that form this BM are synthesized by the PrE cells (Dziadek and Timpl, 1985), but the factors regulating laminin expression in PrE cells as they differentiate from embryonic stem (ES) cell precursors are unknown. Insight into PrE differentiation has been gained with F9 embryonal carcinoma (EC) cells, which unlike differentiating ES cells, require retinoic acid (RA) to induce laminin expression by activation of the Ras-MAP kinase pathway (Verheijen et al., 1999). However, the immediate early genes induced by RA that are necessary for its indirect regulation of laminin expression remain to be established (Wang and Gudas, 1988). While it has been demonstrated that retinoic acid can induce expression of the genes coding for the COUP-TF transcription factors in EC cells (Ben-Shushan et al., 1995), the expression of *COUP-TFs I* and *II* in differentiating PrE cells and their relationship to laminin gene expression has not been investigated.

Embryoid bodies (EBs) composed of differentiating ES cells have been used to investigate endodermal cell differentiation (Robertson, 1987). The peripheral cells of day 1 EBs were morphologically indistinguishable from the inner core cells (Fig. 1A), but by day 2 the outer cells had elongated to resemble PrE cells (Fig. 1B), displaying a spindle-shaped morphology and intense staining with toluidine blue (Hogan and Tilly, 1978; Nadijcka and Hillman, 1974). Additionally, by day 2 these cells had begun to express laminin immunoreactivity (Fig. 1D). Whole-mount in situ hybridization confirmed laminin gene expression at this time (Fig. 2C), and also demonstrated that the PrE cells expressed high levels of *COUP-TFI* (Fig. 2A) and *COUP-TF-II* (Fig. 2B). In order to verify that the *COUP-TFs* are also expressed in vivo, RT-PCR was used to show that both *COUP-TFI* and *COUP-TFII* mRNAs are present in E3.5 blastocysts (Fig. 3B), at which stage a layer of PrE cells has been shown to have developed on the surface of the inner cell mass (Nadijcka and Hillman, 1974). Furthermore, whole-mount in situ hybridization localized *COUP-TFI* expression to this position (Fig. 2D).

To investigate the kinetics of expression of the genes coding for laminin subunits and the COUP-TFs, we performed RT-PCR analysis on undifferentiated ES cells, day 2 and day 4 EBs. None of these genes were expressed in undifferentiated ES cells, but they were induced in PrE cells by day 2 (Fig. 3A). However, whereas the levels of both *COUP-TFs* were maximal at day 2, those of the laminin genes increased further by day 4 (Fig. 3A).

Given that the maximal expression of COUP-TFs are expressed prior to the genes coding laminin subunits, we wished to determine if these transcription factors can regulate laminin gene expression. Although it has been shown

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PII: S0925-4773(00)00554-2

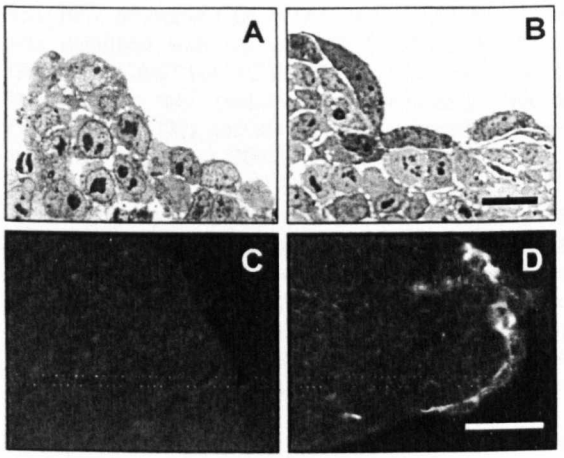


Fig. 1. Differentiation of primitive endoderm cells in EBs. (A,B) Toluidine blue-staining shows peripheral cells with PrE morphology on day 2. (C,D) Immunofluorescence with anti-laminin-1 polyclonal antibodies shows laminin expression on day2. Bars: 10 μm (A,B); 50 μm (C,D).

that knockouts of individual *COUP-TFs* result in later phenotypes and do not apparently affect PrE cell differentiation (Pereira et al., 1999; Qiu et al., 1997), our demonstration that both *COUP-TFs* are expressed in PrE cells (Fig. 2A,B) and the fact that they are able to transactivate the same target genes (Power and Cereghini, 1996) indicates that they may have redundant effects. RT-PCR analysis showed that cells transfected with a *GFP* control vector did not express *COUP-TFI*, *COUP-TFII* or *LAMC1*, and expressed only very low levels of *LAMB1* under these

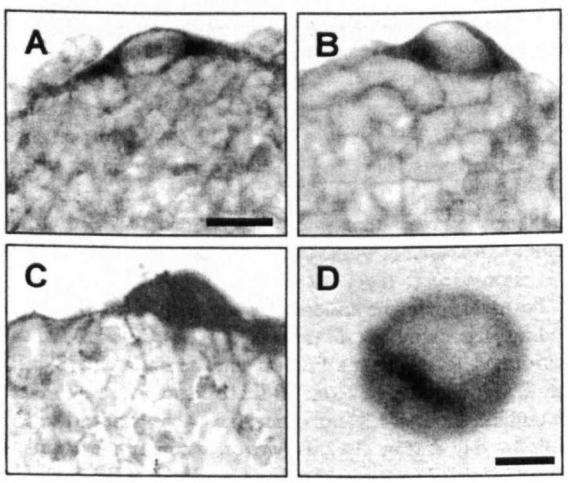


Fig. 2. Expression of *COUP-TFs* in primitive endoderm cells. (A–C) In situ hybridization of day 2 EBs with *COUP-TF1* probe (A), with *COUP-TFII* probe (B), with *LAMB1* probe (C). (D) Whole-mount image of E3.5 mouse blastocyst hybridized with *COUP-TFI* probe shows expression in the location of PrE cells at the surface of the inner cell mass. Bars: 7 μm (A–C); 70 μm (D).

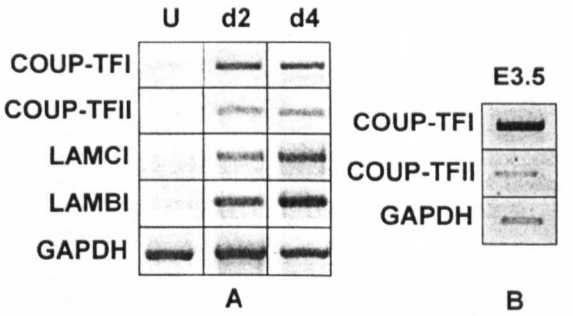


Fig. 3. RT-PCR analysis of *COUP-TF*, *LAMB1* and *LAMC1* expression. (A) Undifferentiated ES cells, day 2 and day 4 EBs; (B) E3.5 blastocyst. *GAPDH* is shown as a loading control.

culture conditions (Fig. 4A,B). However, transfection with vectors expressing either *COUP-TFI* (Fig. 4A) or *COUP-TFII* (Fig. 4B) both resulted in induction of *LAMC1* and *LAMB1* (Fig. 4A,B). Thus, both COUP-TFI and COUP-TFII are sufficient to induce the expression of the genes coding for the $\beta 1$ and $\gamma 1$ subunits of laminin-1. In conclusion, our results show that COUP-TF I and II are both expressed in primitive endodermal cells and that the expression of genes coding for laminin subunits can be stimulated by either COUP-TF.

## 2. Materials and methods

Undifferentiated ES cells and EBs were cultured as previously described (Murray and Edgar, 2000). Embryos were obtained by mating random-bred CD1 mice. Noon on the day of plug was taken as 0.5 days postcoitum. Laminin immunostaining was performed as previously described (Murray and Edgar, 2000).

The sequence comprising nucleotides 1269–1838 of mouse *COUP-TFI* cDNA (Jonk et al., 1994) was amplified by PCR with the primers AGCCATCGTGCTATTCACG and TTCTCACCAGACACGAGGTC, and nucleotides

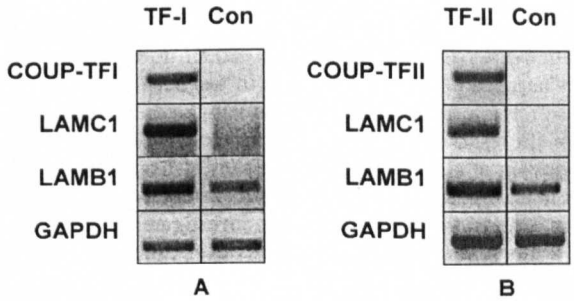


Fig. 4. Induction of laminin gene expression by COUP–TFs. RT-PCR analysis of cells transfected with *COUP-TFI* (A) or *COUP-TFII* (B) and *GFP* control vectors (Con), showing induction of *LAMB1* and *LAMC1* by either *COUP*. *GAPDH* is shown as a loading control.

431–1051 of mouse *COUP-TFII* cDNA (Qiu et al., 1994) was amplified with GCAAGAGCTTCTTCAAGCG and GCTTCTCCACTTGCTCTTGG. Whole-mount in situ hybridization was performed as previously described (Leibl et al., 1999), and the RT-PCR is described in detail in Murray and Edgar (2000).

For transfection studies, mouse STO cells were plated at 50% confluency 1 day prior to transfection of the monolayer by the calcium phosphate coprecipitation procedure using 2 μg of plasmid in 750 μl of culture medium. RNA was extracted for RT-PCR analysis on the 5th day following transfection. The full-length coding sequences of mouse *COUP-TFI* and *COUP-TFII* (donated by Ming-Jer Tsai, Baylor College of Medicine) were sub-cloned from pBluescript II KS (Stratagene) into the *Eco*RI-*Xho*I sites of pcDNA3 (Invitrogen). The control vector was mitochondrial *GFP* in the *Eco*RI-*Xho*I sites of pcDNA3.

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# Appendix 3

# Regulation of the differentiation and behaviour of extra-embryonic endodermal cells by basement membranes

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## SUMMARY

**Both the extracellular matrix and parathyroid hormone-related peptide (PTHrP) have been implicated in the differentiation and migration of extra-embryonic endodermal cells in the pre-implantation mammalian blastocyst. In order to define the individual roles and interactions between these factors in endodermal differentiation, we have used embryoid bodies derived from *Lamc1*$^{-/-}$ embryonic stem cells that lack basement membranes. The results show that in the absence of basement membranes, increased numbers of both visceral and parietal endodermal cells differentiate, but they fail to form organised epithelia. Furthermore, although parietal endodermal cells only migrate away from control embryoid bodies in the presence of PTHrP, they readily migrate from *Lamc1*$^{-/-}$ embryoid bodies in the absence of PTHrP, and this migration is unaffected by PTHrP. Thus, the basement membrane between epiblast and extra-embryonic endoderm is required for the proper organisation of visceral and parietal endodermal cells and also restricts their differentiation to maintain the population of primitive endodermal stem cells. Moreover, this basement membrane inhibits migration of parietal endodermal cells, the role of PTHrP being to stimulate delamination of parietal endodermal cells from the basement membrane rather than promoting migration per se.**

Key words: Basement membrane, Laminin, Cell migration, Parathyroid hormone-related peptide, Endoderm, Embryonic development

## INTRODUCTION

Prior to implantation of the mammalian blastocyst, cells at the periphery of the inner cell mass (ICM) that line the blastocoel cavity differentiate to become primitive endoderm, depositing a basement membrane (BM) between themselves and the remaining undifferentiated cells of the ICM (Nadijcka and Hillman, 1974; Smyth et al., 1999). The primitive endoderm cells that remain attached to this BM differentiate to become visceral endoderm (VE) cells, whereas those lying adjacent to the trophectoderm differentiate to become parietal endoderm (PE) cells which migrate over the blastocoelic surface of the trophectodermal BM (Enders et al., 1978; Gardner and Papaioannou, 1975). As they migrate on this pre-existing BM, PE cells secrete large amounts of laminin and other BM components that are incorporated into Reichert's membrane, a thick BM that acts as a barrier between maternal and embryonic environments, thus forming the parietal yolk sac (Salamat et al., 1995).

The mechanisms that regulate differentiation and behaviour of the extra-embryonic endoderm cells are only now becoming apparent. VE cell differentiation has been shown to be dependent on the presence of factor(s) derived from undifferentiated embryonic stem (ES) cells (Lake et al., 2000). It is likely that one such factor is the VE-inducing molecule, bone morphogenic protein (BMP)4, which is expressed in undifferentiated ES cells but subsequently is downregulated in differentiating columnar epiblast cells (Coucouvanis and Martin, 1999), the development of which is dependent on the BM (Coucouvanis and Martin, 1995; Murray and Edgar, 2000). Conversely, in conditions where only differentiated epiblast cells are present, more PE cells have been shown to develop (Lake et al., 2000), suggesting that PE cell differentiation is a default pathway that will be indirectly enhanced by the BM. However, apart from this indirect mechanism, previous work using F9 embryonal carcinoma cells has shown that PE cell differentiation can be directly promoted by parathyroid hormone related peptide (PTHrP) (Chan et al., 1990; van de Stolpe et al., 1993). Furthermore, PTHrP has been demonstrated to stimulate the migration of PE cells from ICM explants (Behrendtsen et al., 1995). Taken together, these observations indicate that PE cell differentiation is likely to be influenced by both direct and indirectly acting factors that, in turn, may be regulated by the BM. The relative importance of these factors and their interactions remain to be established.

In order to analyse the functions of BMs during embryogenesis, we have previously targeted the mouse *Lamc1* gene, the mutation of which results in early embryonic lethality (Smyth et al., 1999). The *Lamc1* gene encodes the laminin γ1 subunit, which is found in most if not all laminin isoforms expressed during early embryogenesis (see references in Colognato and Yurchenco, 2000). Consequently, in the absence

of the γ1 subunit, no BMs were deposited in these embryos because mature trimeric laminin molecules fail to be assembled and secreted from the cells (Smyth et al., 1999). Analysis of the embryos at different stages showed that the blastocysts died very shortly after implantation, at which time it became apparent that the parietal yolk sac had failed to develop (Smyth et al., 1999). The failure of yolk sac development is likely to be a consequence of abnormal PE cell differentiation and/or migratory behaviour, but it remains to be established which is compromised by the lack of the BM.

The availability of embryoid bodies (EBs) derived from *Lamc1*$^{-/-}$ mouse embryonic stem (ES) cells offers a unique system to define the reasons for the defects in endodermal cell differentiation that result from the lack of BM. EBs represent a well-established in vitro model for investigating peri-implantation development (Robertson, 1987). In a sequence of events that closely resembles development in vivo, cells positioned at the periphery of EBs differentiate into primitive endodermal cells which in turn differentiate into either VE or PE cells (Robertson, 1987). The results of the present analysis using EBs demonstrate that while the initial differentiation of primitive endodermal cells is unaffected by the lack of a BM, the organisation of VE and PE cells is disrupted in its absence. Furthermore, more VE and PE cells differentiated from primitive endodermal cells in *Lamc1*$^{-/-}$ EBs, indicating that the BM helps maintain the population of primitive endodermal stem cells. The PE cells of *Lamc1*$^{-/-}$ EBs migrate more readily on laminin and fibronectin substrates than those differentiating in control EBs which require PTHrP in order to initiate migration, suggesting not only that the major role of PTHrP is to permit PE cell delamination from the BM, but also that loss of contact with the BM is responsible for enhanced PE cell differentiation.

## MATERIALS AND METHODS

### ES cell and EB culture

The production of R1 mouse *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ ES cells has been described in detail previously (Smyth et al., 1999). The clone of *Lamc1*$^{+/-}$ ES cells used here as a control has a wild-type phenotype and was that used previously for the construction of healthy heterozygous germline animals (Smyth et al., 1999). The absence of clonal artefacts in the *Lamc1*$^{-/-}$ cells was confirmed by rescue of phenotype by addition of laminin type-1 (Sigma) to developing EBs (Murray and Edgar, 2000). The cells were cultured on mitomycin-treated STO feeder cells in gelatinised 3.5 cm tissue culture dishes. The culture medium was DMEM (Gibco-BRL) supplemented with 15% (vol/vol) ES grade foetal bovine serum (FBS) (Gibco-BRL), 0.1 mM β mercaptoethanol, 1 mM L-glutamine, 0.1 mM non-essential amino acids and 1000 U/ml of LIF (ESGRO; Gibco-BRL). ES cells were subcultured on STO feeder layers every 2 days.

To make EBs, ES cells were trypsinised, triturated and split 1:10 by replating in bacterial petri dishes, under which conditions the cells remained in suspension and formed aggregates. The EB culture medium was as above, except that LIF was omitted and the FBS content was reduced to 10% (vol/vol). For immunostaining, EBs were fixed for 1 hour with 4% (wt/vol) paraformaldehyde, washed three times in PBS and soaked in 15% (wt/vol) sucrose overnight at 4°C. The samples were then incubated in 7.5% (wt/vol) gelatin:15% (wt/vol) sucrose for 1 hour at 37°C. 100 μl aliquots were pipetted onto a block of gelatin and allowed to set at room temperature, after which they were mounted onto cork discs with OCT cryofixative (Dako) and frozen in liquid nitrogen-cooled isopentane before storage at –80°C. For Toluidine Blue staining, EBs were fixed for 1 hour in 2% (wt/vol) glutaraldehyde:4% (wt/vol) paraformaldehyde and prepared as described previously (Robinson, 1982).

Endodermal cell counts were performed on single, Toluidine Blue-stained sections obtained at the largest diameter from serial section series of 15-33 *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ EBs (numbers for individual experiments are given in the figure legends). The sections were viewed with bright field illumination on a Leitz RM22 microscope. EBs that were growth-retarded or that had formed multiples were omitted from the analysis. Primitive endodermal cells were identified as thin, spindle-shaped cells that stained intensely with Toluidine Blue and formed a continuous monolayer on the surface of the EBs (Nadijcka and Hillman, 1974; Hogan and Tilly, 1978). These cells were clearly distinguishable from PE cells, which were thicker, more rounded and only loosely associated with the surface of the EBs (Casanova and Grabel, 1988; Enders et al., 1978). Cells with this appearance lacked apical vacuoles, distinguishing them from VE cells, and were shown in other experiments to display strong laminin immunoreactivity, consistent with their identity as PE cells. Results are presented ± s.e.m., the significance of differences being assessed by unpaired *t*-test; $P<0.05$ was regarded as significant.

For analysis of PE migration, EBs were cultured in suspension for 4 days to allow PE cells to differentiate, and then plated on tissue culture plastic, fibronectin or laminin substrates for various times and fixed with 4% paraformaldehyde (wt/vol) for 30 minutes. The fibronectin substrate was prepared by coating tissue culture chamber slides (GIBCO BRL) with gelatin and allowing fibronectin to adsorb from medium containing FBS (Grabel and Watts, 1987). The laminin substrate was prepared by pipetting a 200 μl aliquot of either 10 μg/ml or 100μg/ml laminin (Sigma) in phosphate buffered saline (PBS) onto a tissue culture chamber slide. The slide was left at room temperature for 30 hours to allow the laminin to adsorb to the plastic. 100 nM PTHrP 1-34 (a gift from Dr J. A. Gallagher, Department of Human Anatomy, University of Liverpool) was added to the culture medium at the same time as EBs were plated onto the tissue culture slides.

### Immunostaining

Cryostat sections (10 μm) were blocked with 10% (vol/vol) goat serum (GS) in PBS, which was applied for 1 hour and then aspirated before application of the primary antibody. The primary antibodies used were rabbit anti-EHS laminin, which recognises all three subunits of laminin type 1, at 1/5000 dilution (Kücherer-Ehret et al., 1990); rabbit anti-mouse α-feto-protein (AFP) serum at 1/200 dilution (ICN Biomedicals); and rat TROMA-3 diluted 1/2 (a gift from N. Smyth, Department of Biochemistry, University of Cologne), which recognises the mouse PE cell specific cytokeratin Endo C (Boller and Kemler, 1983). Incubations with primary antibodies were carried out overnight in 1% (vol/vol) GS in PBS in a humidified atmosphere at room temperature and the sections were then washed three times in PBS. For immunostaining with anti-EHS laminin and anti-AFP, the secondary antibody was TRITC-conjugated swine anti-rabbit IgG at 1/100 dilution (Dako); for TROMA-3, the secondary antibody was biotinylated rabbit anti-rat IgG at 1/50 dilution (Dako). Secondary antibodies were applied in 1% (vol/vol) GS in PBS at room temperature for 2 hours and the sections were then washed three times in PBS. Streptavidin conjugated with fluorescein isothiocyanate (FITC) (Amersham) at 1/50 dilution in 1% (vol/vol) GS in PBS was applied to TROMA-3 sections at room temperature for 2 hours and the sections were then washed three times in PBS. Sections were mounted in fluorescent mounting medium (Dako) and photographed using a Leitz RM22 fluorescent microscope.

### Reverse transcription-PCR

Total RNA was extracted from *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ EBs using guanidium isothiocyanate (Chomczynski and Sacchi, 1987), and reverse transcribed using Superscript™ II (Gibco BRL). For day 2

and day 6 EBs, whole populations were used, but for day 10 EBs, 10-15 cavitated *Lamc1*$^{+/-}$ EBs and an equal number of *Lamc1*$^{-/-}$ EBs were selected using phase contract microscopy. Primers for HNF4α1 (Nakhei et al., 1998), perlecan (Miller et al., 1997), and vHNF1 and GATA-4 (Duncan et al., 1997) were as described previously. GAPDH primers were 5′ggtgaaggtcggagtcaacgg3′ (forward) and 5′ggtcatgagtccttccacgat3′ (reverse); product size, 520 bp; annealing temperature, 54°C. Lamb1 primers were 5′gcagacacaacaccaaaggc3′ (forward) and 5′tgtacccatcacagatcccg3′ (reverse); product size, 344 bp; annealing temperature, 56°C. AFP primers were 5′acatcagtgtctgctggcac3′ (forward) and 5′agcgagtttccttggcaacac3′ (reverse); product size, 461 bp; annealing temperature, 54°C. Semi-quantitative RT-PCR was performed as previously described to determine mRNA levels relative to those of GAPDH (Squitti et al., 1999).

## RESULTS

### Extra-embryonic endodermal cell differentiation in *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ embryoid bodies

After 2 days of suspension culture, light microscopy of Toluidine Blue-stained resin-embedded sections showed that *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ control EBs were indistinguishable, in both cases spindle-shaped primitive endodermal cells that stained intensely with Toluidine Blue (Hogan and Tilly, 1978) formed a monolayer on the surface (Fig. 1A,B). This is typical of the organisation of these cells in the blastocyst (Nadijcka and Hillman, 1974). There were no obvious differences in the overall growth characteristics of *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs.

By day 6, the primitive endodermal cells had begun to diverge into two distinct morphological types in both *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs (Fig. 1C-F). Type 1 were highly polarised with large apical vacuoles (Fig. 1C,D), characteristic of VE cells (Morini et al., 1999), whereas type 2 cells were not as tightly associated, and were smaller and lacked large apical vacuoles (Fig. 1E,F), consistent with the appearance of PE cells (Casanova and Grabel, 1988; Enders et al., 1978). It has been shown previously that VE cells express low levels of BM components (Behrendtsen et al., 1995; Hogan and Tilly, 1981), and consequently the BM underlying these cells in *Lamc1*$^{+/-}$ EBs was not visible with light microscopy (Fig. 1C). In contrast, PE cells express very high levels of BM components (Behrendtsen et al., 1995; Hogan et al., 1984), and so the BM-like extracellular matrix underlying these cells in *Lamc1*$^{+/-}$ EBs tended to be extremely thick (Fig. 1E), resembling Reichert's membrane (Inoue et al., 1983). However, extracellular matrix material was present as discrete deposits in *Lamc1*$^{-/-}$ EBs and did not form a continuous Reichert's membrane-like structure (Fig. 1F), owing to the lack of secreted laminin (Smyth et al., 1999).

At day 6, the VE cells of *Lamc1*$^{+/-}$ EBs were arranged in an epithelial monolayer at the periphery of the EB (Fig. 1C). The VE cells of *Lamc1*$^{-/-}$ EBs, however, while being located at the periphery, formed disorganised groups in which the basal surfaces of the cells were orientated towards each other (Fig. 1D), rather than being aligned parallel to the core of the EB as they were in *Lamc1*$^{+/-}$ EBs. The PE cells of *Lamc1*$^{+/-}$ EBs were arranged one to two cells deep overlying the thick BM that separated them from the inner core cells of the EB (Fig. 1E). In contrast, the PE cells of *Lamc1*$^{-/-}$ EBs usually formed multilayers at the periphery and were frequently

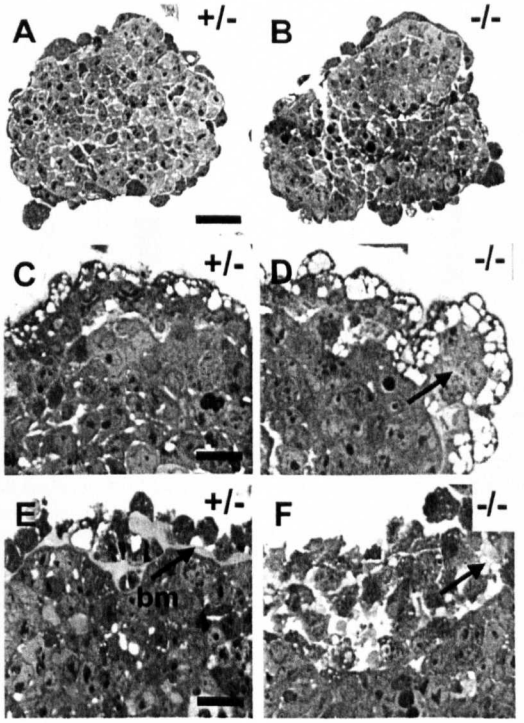


**Fig. 1.** Extra-embryonic endodermal cell differentiation in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs. Genotypes of EBs are as indicated. (A-F) Toluidine Blue-stained sections. (A,B) Day 2 EBs; the primitive endodermal cells form a layer of intensely stained cells at the periphery. Note that at this stage *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs are indistinguishable. (C,D) Day 6 EBs show localised VE cell differentiation with characteristic apical vacuoles; arrow in D indicates a group of VE cells where the basal surfaces are orientated towards each other rather than parallel to the surface of the EB. (E,F) Day 6 EBs show localised differentiation of smaller PE cells; arrow in E shows a continuous BM-like sheet of extracellular matrix material in the *Lamc1*$^{+/-}$ EB, whereas only discrete deposits were apparent the *Lamc1*$^{-/-}$ EB (arrow in F). bm, basement membrane. Scale bars: in A, 30 μm in A,B; in C, 10 μm in C-F.

associated with discrete deposits of extracellular matrix material (Fig. 1F).

To determine if the absence of compartmentalisation between endodermal cells and remaining inner core cells of the BM-deficient *Lamc1*$^{-/-}$ EBs affected the numbers of endodermal cells, we counted these cells in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs at days 2 and 10 (Fig. 2A). At both time-points endodermal cells could be clearly distinguished from the inner core cells of the EBs because of their intense staining with Toluidine Blue (Hogan and Tilly, 1978). The results showed that there was no significant difference ($P>0.05$, unpaired *t*-test) between the numbers of extra-embryonic endodermal cells in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs at either day 2 or day 10 (Fig. 2A). To substantiate this result, semi-quantitative RT-PCR was used to estimate the relative expression levels of *Gata4* and *vHNF1* mRNAs. Both these genes are expressed in primitive, visceral and parietal endodermal cells (Arceci et al., 1993; Barbacci et al., 1999; Soudais et al., 1995), so they are an appropriate

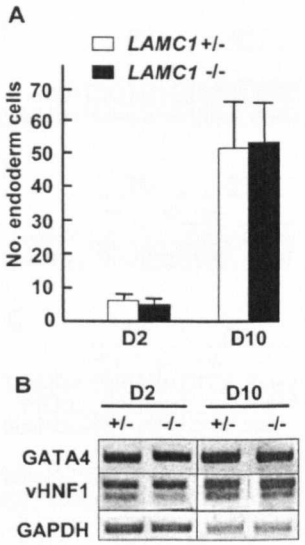


**Fig. 2.** Analysis of extra-embryonic endodermal cell differentiation in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs. (A) The numbers of extra-embryonic endodermal cells were counted in 1 μm Toluidine Blue-stained sections of day 2 and day 10 *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs. In the day 2 samples, $n$=22 for *Lamc1*$^{-/-}$ EBs, and $n$=28 for *Lamc1*$^{+/-}$ EBs. In the day 10 samples, $n$=20 for *Lamc1*$^{-/-}$ EBs, and $n$=15 for *Lamc1*$^{+/-}$ EBs. Error bars represent the standard error of the mean. There is no significant difference between the total numbers of extra-embryonic endodermal cells in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs at day 2 or at day 10 ($P$>0.05; unpaired $t$-test). (B) RT-PCR analysis; GAPDH is shown as a loading control. The levels of *Gata4* and *vHNF1* mRNA in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs are similar at both days 2 and 10; the double band observed with the *vHNF1* primers results from splice variants (Cereghini et al., 1992).

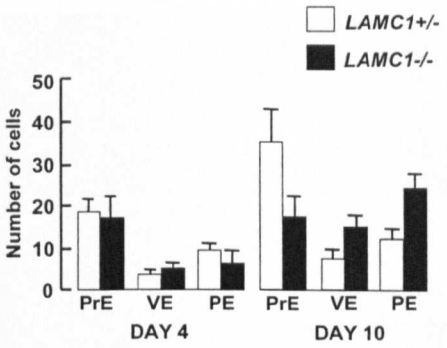


**Fig. 3.** Quantitative analysis of primitive, visceral and parietal endodermal cells in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs at days 4 and 10. The number of primitive endodermal (PrE), VE and PE cells were counted in 1 μm Toluidine Blue-stained sections of *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs. Cells were classified on the basis of morphology (see Fig. 1); for both day 4 *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs, $n$=33; error bars represent the s.e.m. There is no significant difference between the number of primitive endodermal (PrE), VE and PE cells in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs at day 4. (For all classes of endoderm, $P$>0.05; unpaired $t$-test.) For day 10 *Lamc1*$^{-/-}$ EBs, $n$=20; for day 10 *Lamc1*$^{+/-}$ EBs, $n$=15. By day 10, the numbers of primitive endodermal (PrE), VE and PE cells are significantly different between *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs. (PrE, $P$<0.001; VE, $P$<0.02; PE, $P$<0.02; unpaired $t$-test.)

indicator for all extra-embryonic endodermal cell lineages. The results showed that the expression levels of these genes in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs were similar both at day 2 and day 10 (Fig. 2B). Thus, the absence of a BM in *Lamc1*$^{-/-}$ EBs did not appear to affect the overall levels of extra-embryonic endodermal cell differentiation.

## Differentiation of VE and PE cells from primitive endodermal cells is enhanced in *Lamc1*$^{-/-}$ EBs

Although the BM did not affect the overall numbers of extra-embryonic endodermal cells, we wished to establish if it had any specific effect on the number of VE and/or PE cells that differentiated form primitive endodermal cells. Accordingly, the numbers of VE and PE cells were determined in age-matched *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs by scoring them on the basis of their morphological characteristics as described above. At day 4, no significant difference ($P$>0.05, unpaired $t$-test) was observed in the numbers of primitive endodermal, VE or PE cells in *Lamc1*$^{-/-}$ relative to *Lamc1*$^{+/-}$ EBs (Fig. 3). By day 10, however, the numbers of both VE and PE cells were significantly increased ($P$<0.02) in *Lamc1*$^{-/-}$ relative to *Lamc1*$^{+/-}$ EBs (Fig. 3). Given that there was no difference between the overall number of extra-embryonic endodermal cells in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs (Fig. 2A), then the higher numbers of VE and PE cells observed in *Lamc1*$^{-/-}$ EBs is consistent with an increased differentiation of primitive endodermal cells into both these lineages. This hypothesis is supported by the observation that the number of primitive endodermal cells was significantly greater ($P$<0.001) in the *Lamc1*$^{+/-}$ EBs compared with *Lamc1*$^{-/-}$ EBs (Fig. 3).

To establish enhanced VE cell differentiation definitively, we performed immunostaining with the VE cell-specific marker, AFP. We found that there were more AFP-positive cells in 10 day *Lamc1*$^{-/-}$ EBs than in the *Lamc1*$^{+/-}$ EBs (Fig. 4A,E), consistent with the increased numbers of cells with VE-like morphology. To confirm that the increase in differentiation of primitive endoderm to VE cells had not occurred at the expense of PE cell differentiation, we performed immunostaining with TROMA-3 monoclonal antibodies that recognise the PE-specific cytokeratin Endo C (Boller and Kemler, 1983). We found that there were also more TROMA-3-positive cells in the *Lamc1*$^{-/-}$ compared with *Lamc1*$^{+/-}$ EBs, and that the organisation of the *Lamc1*$^{-/-}$ cells was disrupted compared with *Lamc1*$^{+/-}$ controls (Fig. 4C,G).

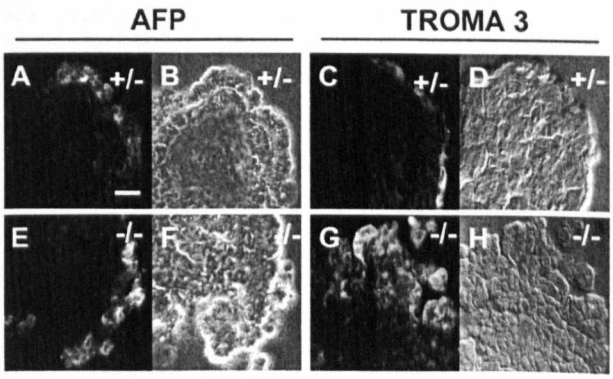


**Fig. 4.** Immunostaining of visceral and parietal endodermal cell markers in sections of day 10 *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ EBs. Genotypes of EBs are as indicated. (A,E) Immunofluorescence staining for AFP; (B) bright field image of A; (F) bright field image of E; (C,G) Immunofluorescence staining with the monoclonal antibody TROMA-3; (D) Bright field image of C; (H) Bright field image of G. Scale bar: 25 μm.

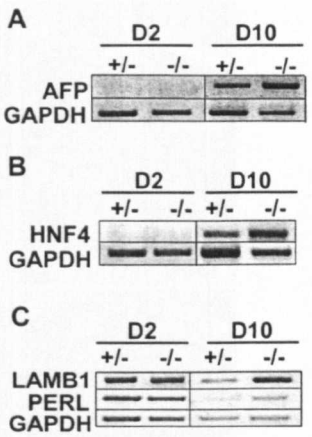


**Fig. 5.** Semi-quantitative RT-PCR analysis of visceral and parietal cells markers in *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ EBs. Genotypes and age of EB samples are as indicated. GAPDH is shown as a loading control. (A) *Afp* is not expressed in *Lamc1*$^{-/-}$ or *Lamc1*$^{+/-}$ EBs at day 2, but is detectable by day 10, with higher mRNA levels in the *Lamc1*$^{-/-}$ EBs. (B) *HNF4α1* is not expressed in *Lamc1*$^{-/-}$ or *Lamc1*$^{+/-}$ EBs at day 2, but is detectable by day 10, with higher mRNA levels in the *Lamc1*$^{-/-}$ EBs. (C) *Lamb1* and *perlecan* are expressed at equivalent levels in *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs at day 2, but by day 10 mRNA levels are higher in *Lamc1*$^{-/-}$ compared with *Lamc1*$^{+/-}$ EBs.

Semi-quantitative RT-PCR was used to estimate the relative mRNA levels of *AFP* and another VE cell-specific marker HNF4α1 (Nakhei et al., 1998). As expected, these genes were not expressed in either EB type at day 2, before any VE cells had differentiated (Fig. 5A,B), but at later time-points the levels of both mRNAs were increased in *Lamc1*$^{-/-}$ compared with *Lamc1*$^{+/-}$ EBs, consistent with there being more VE cells in the *Lamc1*$^{-/-}$ EBs (Fig. 5A,B). In contrast to the VE markers, there were relatively high levels of expression of *Lamb1* and *perlecan* mRNAs at day 2 (Fig. 5C), reflecting the expression of BM components by primitive endodermal cells. However, the relative levels of *Lamb1* and perlecan mRNAs were higher in *Lamc1*$^{-/-}$ EBs than controls at later time points (Fig. 5C), consistent with greater numbers of PE cells.

## PE cell migration from *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ control EBs

Although the failure of parietal yolk sac development in *Lamc1*$^{-/-}$ embryos (Smyth et al., 1999) could have been caused by either a defect in PE cell differentiation or PE cell migration, the above experiments show that PE cells do differentiate in the absence of a BM. Thus, the effect of the BM on PE migration was next examined. To do this, *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs were cultured for 4 days in suspension, and then allowed to attach to different tissue culture substrates. This time-point was chosen because although PE cells have started to differentiate at day 4, there was no significant difference in the numbers of these cells between the *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs, which may have affected their migration characteristics (Fig. 3). Furthermore, we have shown previously that formation of the columnar epiblast epithelium and proamniotic cavity normally begins in *Lamc1*$^{+/-}$ EBs from day 5 (Murray and Edgar, 2000). Hence, by using day 4 EBs to assay PE cell migration, we were able to avoid any influence of the columnar epiblast epithelium on the migration of these cells.

After 30 hours of attachment to a fibronectin substrate, there was no evidence of cell migration from control EBs (Fig. 6A,B). However, after 3 days culture, some migration of PE cells (defined by intracellular laminin immunoreactivity and morphology) was noted (Fig. 6C,D), consistent with the timing of PE cell migration on fibronectin substrates previously reported (Behrendtsen et al., 1995). In contrast, after 30 hours of attachment to fibronectin, extensive PE cell migration from *Lamc1*$^{-/-}$ EBs had already occurred (Fig. 6E,F; Fig. 7). It has previously been demonstrated that the addition of laminin inhibits PE cell migration over fibronectin substrates (Behrendtsen et al., 1995). Thus, the PE cell migration from *Lamc1*$^{-/-}$ EBs noted in the present experiments could be due to the absence of laminin secreted from these cells. To test this hypothesis, EBs were plated directly onto laminin substrates. Counts of migrating PE cells confirmed that little migration from *Lamc1*$^{+/-}$ control EBs occurred on either fibronectin or

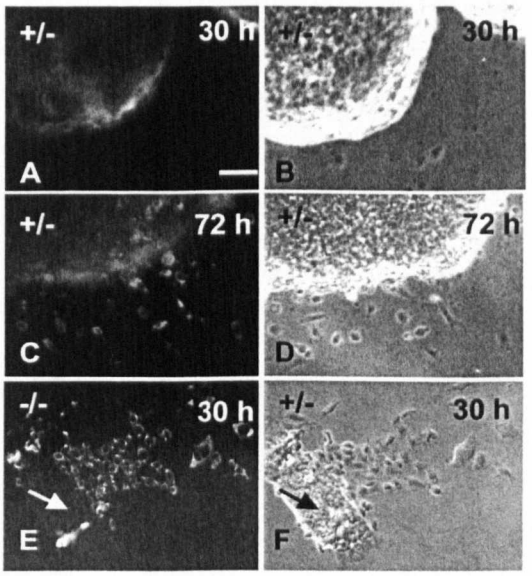


**Fig. 6.** PE cells migrate from *Lamc1*$^{-/-}$ EBs at an earlier time-point than from *Lamc1*$^{+/-}$ EBs. (A,C) Immunofluorescence staining for laminin in *Lamc1*$^{+/-}$ EBs cultured for 30 hours (A) and 3 days (C) on a fibronectin substrate. (B) Bright field image of A; (D) bright field image of C. (E) Immunofluorescence staining for laminin in *Lamc1*$^{-/-}$ EBs grown for 30 hours on fibronectin; note that the core cells of the EBs (arrow) do not express laminin. (F) Bright field image of E. Scale bar: 50 μm.

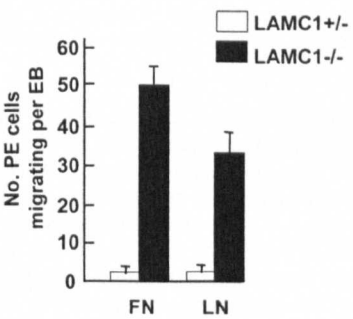


**Fig. 7.** Quantitative analysis of PE cell migration on fibronectin and laminin substrates. *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs were either cultured on fibronectin (FN) or laminin (LN). Note that significantly more PE cells migrate from *Lamc1*$^{-/-}$ EBs grown on FN compared with laminin (LN) ($P<0.01$; Student's *t*-test). Data shown are from one representative experiment. Error bars represent the standard deviation.

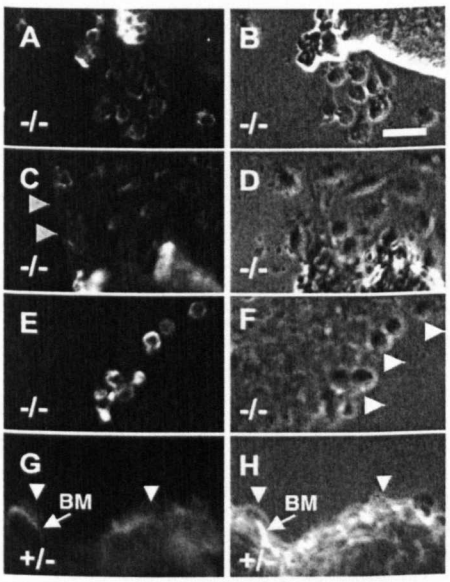


**Fig. 8.** Laminin is a permissive substrate for PE cell migration from *Lamc1*$^{-/-}$ EBs. (A,C,E) Immunofluorescence staining for laminin in *Lamc1*$^{-/-}$ EBs grown for 30 hours on a substrate coated with 10 μg/ml laminin (A), a substrate partially coated with 100 μg/ml laminin (C) and tissue-culture plastic (E); arrowheads in C depict the limit of the laminin substrate as detected by the anti-laminin antibodies; (B,D,F) Bright-field images of A,C,E, respectively. (G) Immunofluorescence staining in 30 hour outgrowths of *Lamc1*$^{+/-}$ EBs shows absence of delamination of PE cells (arrowheads) from the BM, in contrast to that seen in F (arrowheads). (H) Bright field image of G. Scale bar: 30 μm.

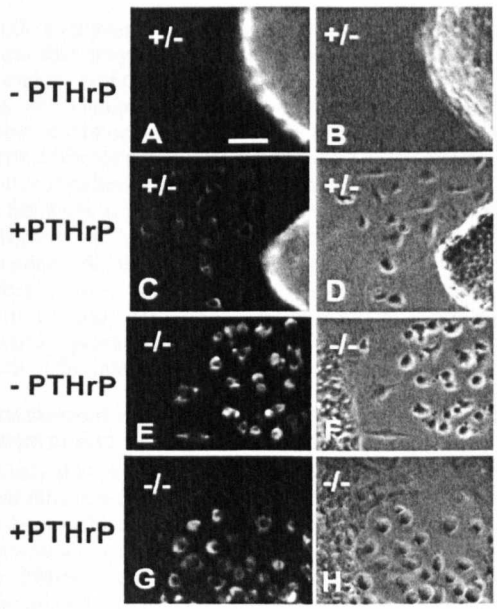


**Fig. 9.** PTHrP is necessary for PE cell migration from *Lamc1*$^{+/-}$ EBs. (A,C) Immunofluorescence staining for laminin in *Lamc1*$^{+/-}$ EBs grown for 30 hours on a laminin substrate without PTHrP (A) or in the presence of 100 nM PTHrP (C). (B,D) Bright field images of A and C, respectively. (E,G) Immunofluorescence staining for laminin in *Lamc1*$^{-/-}$ EBs grown for 30 hours on a laminin substrate without PTHrP (E) or in the presence of PTHrP (G). (F,H) Bright field images of E,G, respectively. Note that the lower levels of intracellular laminin in the PE cells from *Lamc1*$^{+/-}$ EBs (C) compared with those migrating from *Lamc1*$^{-/-}$ EBs (E,G) is due to rapid laminin secretion following trimer assembly. Scale bar: 30 μm.

laminin substrates after 30 hours culture (Fig. 7). Surprisingly, extensive PE cell migration from *Lamc1*$^{-/-}$ EBs was observed on both fibronectin and laminin substrates at this time point (Fig. 7; Fig. 8A,B), although the migration was lower ($P<0.01$, unpaired *t*-test) on laminin compared with that on fibronectin (Fig. 7), consistent with previous observations (Behrendtsen et al., 1995). Significantly, it was noted that PE cell migration from *Lamc1*$^{-/-}$ EBs stopped abruptly at the limit of the laminin substrate (Fig. 8C-D), indicating that laminin is a permissive substrate for PE cell migration, whereas tissue culture plastic is not. Taken together, these observations indicate that the absence of secreted laminin is unlikely to explain the rapid migration of PE cells from *Lamc1*$^{-/-}$ EBs and an alternative explanation is required.

## Role of the basement membrane and PTHrP on PE cell delamination

Before displaying migratory behaviour, it is clear that PE cells must first detach from the EB. In order to differentiate between PE cell delamination and migration, we made use of the observation that tissue culture plastic is not a permissive substrate for PE cell migration from *Lamc1*$^{-/-}$ EBs (Fig. 8C). Thus, when plated directly onto tissue culture plastic, it was found that although the PE cells were unable to migrate away from *Lamc1*$^{-/-}$ EBs, nevertheless they were able to detach from the EBs and adhere to the substrate (Fig. 8E,F). In *Lamc1*$^{+/-}$ EBs, on the other hand, the PE cells remained firmly attached to the EB showing an absence of both delamination and migration (Fig. 8G,H). These observations suggest that attachment to the BM present in control EBs is the major factor inhibiting PE cell migration on laminin or fibronectin substrates.

It has previously been shown that PTHrP promotes PE cell migration on a variety of substrates, including fibronectin and laminin (Behrendtsen et al., 1995). In the light of the above observations, we wished to determine if the action of PTHrP was on delamination rather than a stimulation of migration per se. Thus, *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs were cultured for 30 hours on laminin substrates with or without the addition of PTHrP. The results demonstrate that while PTHrP was necessary for PE cell migration from control EBs (Fig. 9C,D), as previously reported (Behrendtsen et al., 1995), the addition of PTHrP to cultures of *Lamc1*$^{-/-}$ EBs did not make any appreciable difference to PE cell migration (Fig. 9E-H). Determination of cell numbers confirmed that while PTHrP did not affect PE cell migration from *Lamc1*$^{-/-}$ EBs, the number of migratory cells remained lower ($P<0.01$, unpaired *t*-test) in *Lamc1*$^{+/-}$ in the presence of PTHrP than in *Lamc1*$^{-/-}$ EBs (Fig. 10). Given the lack of effect of PTHrP on PE cell migration in BM-deficient *Lamc1*$^{-/-}$ EBs where no delamination is required, these results suggest that the major effect of PTHrP in *Lamc1*$^{+/-}$ EBs is to induce delamination from the BM.

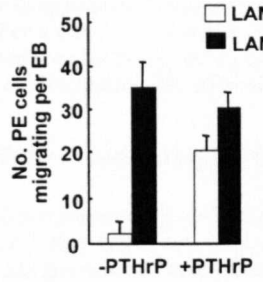


**Fig. 10.** Quantitative analysis of the effects of PTHrP on PE cell migration. *Lamc1*$^{-/-}$ and *Lamc1*$^{+/-}$ EBs were cultured for 30 hours on laminin substrates either with or without 100 nM PTHrP. The number of PE cells migrating from *Lamc1*$^{-/-}$ EBs is significantly greater than the number migrating from *Lamc1*$^{+/-}$ EBs in both the presence ($P<0.01$) and absence ($P<0.01$) of PTHrP (Student's *t*-test). The data shown are from one representative experiment. Error bars show the standard deviations from the means.

## DISCUSSION

In this study we have used EBs to analyse the factors influencing extra-embryonic endodermal cell differentiation and behaviour, in order to explain the peri-implantation lethality caused by the absence of BMs in *Lamc1*$^{-/-}$ mouse embryos (Smyth et al., 1999). The results show that while the initial differentiation of primitive endodermal cells was unaffected by the absence of BMs in *Lamc1*$^{-/-}$ EBs, the BM affects the subsequent development of VE and PE cells by regulating their numbers and organisation. Furthermore, analysis of EB outgrowths showed that PE cells migrated more readily from *Lamc1*$^{-/-}$ EBs than from controls, which required PTHrP in order to permit delamination of the PE cells from the BM. Thus, the failure of parietal yolk sac development in vivo (Smyth et al., 1999) was not caused by failure of PE cell differentiation or intrinsic migratory ability, but is likely to have been due to the absence of their normal trophectodermal BM substrate.

### The relationship between basement membranes and the initial differentiation of primitive endodermal cells

The laminin and other BM components deposited between primitive endodermal cells and the rest of the ICM or embryoid body are produced by the primitive endodermal cells themselves (Cheng et al., 1998). Indeed, the increased expression of laminin and other BM components is one of the earliest indications of primitive endodermal cell differentiation, which occurs in EBs several days before deposition of a BM (Grover et al., 1983; Murray and Edgar, 2000). It is therefore not surprising that the present results demonstrate the initial differentiation of endodermal cells to be independent of the BM. It has recently been shown that increased steady-state levels of the transcription factor Oct3/4 result in the differentiation of ES cells into primitive endodermal cells (Niwa et al., 2000). However, the intercellular signalling mechanisms that regulate Oct3/4 expression are unknown, and the relationship between Oct3/4 and laminin expression is currently under investigation (manuscript in preparation).

### Effects of the basement membrane on organisation of endodermal cells

The endodermal cells that formed in *Lamc1*$^{-/-}$ EBs differed from controls in being unable to form an organised epithelium, although the presence of apical vacuoles in the VE cells clearly indicates that they were polarised. These observations agree with earlier work on BM-deficient dystroglycan-null EBs (Henry and Campbell, 1998) in demonstrating that VE cell polarisation can occur in the absence of the BM. However, the VE cells differentiating in β1-integrin-null EBs not only fail to form an epithelium but also fail to polarise (Aumailley et al., 2000; Stephens et al., 1993), indicating that the requirement of β1-integrins for VE cell polarity may involve mechanisms independent of their interactions with BM ligands. This hypothesis is supported by the observation that cadherin:catenin complexes are unable to maintain the subcortical cytoskeleton characteristic of polarised cells in the absence of β1 integrins (Wang et al., 1999).

### The basement membrane inhibits the differentiation of visceral and parietal endodermal cells

It is likely that the absence of the BM has indirect effects on VE cell differentiation, owing to its role in the development of the epiblast, which has been shown to be dependent on the BM (Coucouvanis and Martin, 1995; Murray and Edgar, 2000). While BMP4 is expressed in undifferentiated ES cells, it is downregulated in columnar epiblast cells (Coucouvanis and Martin, 1999). In the absence of the BM, failure of columnar epiblast development is accompanied by increased levels of BMP4 (Murray and Edgar, 2000). Because BMP4 in turn induces VE cell differentiation (Coucouvanis and Martin, 1999; Gu et al., 1999; Sirard et al., 1998; Yang et al., 1998), absence of the BM would be expected to result in the increased numbers of VE cells described here.

The present results show that not only VE cell differentiation but also that of the PE cells is enhanced in *Lamc1*$^{-/-}$ EBs. PE differentiation from primitive endodermal cells has been suggested to occur as a default pathway in the absence of VE differentiation factor(s) produced by ES cells (Lake et al., 2000). This notion is supported by observations that PE differentiation is enhanced at the expense of VE differentiation in *Actr1a*$^{-/-}$ and *Smad4*$^{-/-}$ mutants, implicating the involvement of ES cell-derived BMP4 (Gu et al., 1999; Sirard et al., 1998; Yang et al., 1998). However, given the increased numbers of VE cells in *Lamc1*$^{-/-}$ EBs, then the increased differentiation of PE cells reported here is unlikely to have been due to an indirect effect of the BM on epiblast development.

PE cell differentiation has been shown to be directly stimulated by trophectodermal cells and/or the PTHrP which they secrete (Behrendtsen et al., 1995; van de Stolpe et al., 1993). However, PE cells differentiate in EBs that lack a trophectoderm (Doetschman et al., 1985), and PTHrP receptor-null embryos progress normally through this stage of development (Lanske et al., 1996), indicating that PTHrP is not essential for PE cell differentiation and that alternative mechanisms must be responsible. A very early hypothesis has been made, suggesting that PE cell differentiation occurs owing to loss of contact with the BM, as a result of overcrowding caused by continuing proliferation of primitive endodermal cells (Gardner, 1982). The present experiments support this hypothesis by showing that more PE cells do indeed develop in EBs lacking a BM. However, we also demonstrate that early differentiating PE cells have the ability to migrate away from the remaining cells of *Lamc1*$^{-/-}$ EBs without the need for additional factors such as PTHrP. Thus,

the increased numbers of PE cells appearing at later time points in *Lamc1*$^{-/-}$ EBs could be either a direct consequence of lack of BM contact and/or alternatively could be caused by the loss of lateral inhibition if early differentiating PE cells are not restrained by the BM.

### Interactions of BM and PTHrP in regulating PE cell migration

When presented with permissive substrates, PE cells migrated much more readily from *Lamc1*$^{-/-}$ than from *Lamc1*$^{+/-}$ control EBs. Using ICM explants, it has previously been shown that laminin substrates reduce PE cell migration (Behrendtsen et al., 1995). However, a lack of secreted laminin from *Lamc1*$^{-/-}$ cells was not responsible for enabling migration, as the PE cells migrated readily on exogenous laminin substrates. In order to explain these observations, it should be noted that, prior to migration, PE cells must normally delaminate from the BM deposited between extra-embryonic endoderm and the epiblast. Thus, any factors regulating delamination are likely to play a crucial role in affecting the migration of PE cells.

Previous work has suggested that PTHrP stimulates PE cell differentiation and migration (Behrendtsen et al., 1995; van de Stolpe et al., 1993), and the present results agree with this observation by showing that PTHrP increases the number of migrating PE cells from control EBs. However, PTHrP did not increase PE cell migration from *Lamc1*$^{-/-}$ EBs, indicating that the main effect of PTHrP is to promote PE cell delamination from the primitive endodermal BM. It is known that the binding of PTHrP to the type I PTHrP receptor activates protein kinase C (PKC)-coupled signal transduction pathways (Abou-Samra et al., 1992). Furthermore, activation of PKC has recently been shown to regulate intracellular β1 integrin trafficking (Ng et al., 1999). A similar relocalisation of β1 integrins caused by PTHrP-induced PKC activation may therefore be involved in PE cell delamination from the BM, thereby allowing migration to proceed.

### The early lethality of Lamc1$^{-/-}$ embryos

The fact that PE cells differentiate in BM-deficient *Lamc1*$^{-/-}$ EBs indicates that the failure of parietal yolk sac formation in vivo (Smyth et al., 1999) is likely to be caused by defective PE cell migration. Indeed, both *Lamc1*$^{-/-}$ and integrin β1-null mouse embryos lack BMs, and it has been reported that cells with the intense laminin immunoreactivity characteristic of PE cells accumulate on the surface of these ICMs shortly after implantation (Smyth et al., 1999; Stephens et al., 1995). Because outgrowth analysis showed that PE cells derived from *Lamc1*$^{-/-}$ EBs have no migratory defect, then the failure of PE cells to form the parietal yolk sac in *Lamc1*$^{-/-}$ embryos probably results from the absence of the trophectodermal BM, which is the normal substrate for migrating PE cells (Leivo et al., 1980; Salamat et al., 1995; Wartiovaara et al., 1979).

The only known function of PE cells is to synthesise Reichert's membrane, which acts as a filtrative layer between the embryonic and maternal environments (Gardner, 1983). However, despite the formation of trophectodermal and primitive endodermal BMs in dystroglycan-null mutants, Reichert's membrane fails to develop, although the PE cells do migrate (Williamson et al., 1997). Notably, dystroglycan-null mutants form apparently normal egg cylinders and do not die until after E7.5. The fact that embryonic lethality caused by to the lack of PE cell migration in *Lamc1*$^{-/-}$ and β1 integrin-null embryos occurs prior to E6.5 indicates that the death of these embryos may result from events other than the failure to deposit Reichert's membrane. Very recently it has been demonstrated that the ability to interact with extracellular matrices via β1 integrin receptors can regulate gene expression in endodermal cells (Aumailley et al., 2000). It is therefore possible that a defect in gene expression caused by the lack of basement membranes also contributes to the lethality seen in *Lamc1*$^{-/-}$ embryos.

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# Appendix 4

# The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF)

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**LIF (leukaemia inhibitory factor) is commonly used to maintain mouse embryonic stem cells in an undifferentiated state. These cells spontaneously differentiate when allowed to aggregate in the absence of LIF, forming embryoid bodies in which early embryonic cell lineages develop. Using embryoid bodies cultured in the presence and absence of LIF, we show that although LIF inhibited the development of visceral and parietal endodermal cells, it did not affect the differentiation of the primitive endodermal cell precursors of these extraembryonic cell lineages. Furthermore, deposition of the basement membrane produced by the primitive endodermal cells, which separates them from the remaining cells of the embryoid body, still occurred. The differentiation of primitive ectodermal cells and their progeny was inhibited by LIF, as evidenced by the lack of expression of *FGF-5*, muscle and neuronal markers. However, cavitation of the embryoid body and maintenance of the cells in contact with the primitive endodermal basement membrane as an epiblast epithelium still occurred normally in the presence of LIF. These results indicate that cavitation and formation of the epiblast epithelium are regulated by mechanisms distinct from those controlling the differentiation of epiblast cell lineages. Furthermore, although epithelium formation and cavitation do not require the differentiation of visceral endodermal cells, the results are consistent with the hypothesis that the primitive endodermal basement membrane is sufficient to induce the epithelialization of undifferentiated embryonic stem cells necessary for cavitation.**

*Key words:* basement membrane; embryoid body; embryonic stem cell; endoderm; epithelium; primitive ectoderm.

## Introduction

Shortly before implantation of the mammalian embryo, the inner cell mass (ICM) of the blastocyst consists of a small group of undifferentiated cells, separated from a layer of primitive endoderm by a basement membrane (BM) [30]. The primitive endoderm cells give rise to two extraembryonic cell lineages, the parietal and visceral endoderm [15]. Parietal endoderm cells migrate over the blastocoelic surface of the trophectodermal BM [12], secreting large amounts of BM components that form Reichert's membrane of the parietal yolk sac [30]. In contrast, the primitive endoderm cells that remain attached to the BM of the ICM differentiate to become visceral endoderm cells [14], while the remaining ICM cells differentiate to become the epiblast, or primitive ectoderm (see [19]).

Initially, the ICM cell to epiblast cell transition consists of an alteration in the profile of expressed genes including the up-regulation of *FGF5* [16] and down-regulation of *BMP4* [9], but is not accompanied by any obvious morphological differentiation [28]. A few hours later, however, the epiblast cells in contact with the BM become polarized to form the columnar epiblast epithelium, while cells at the centre of the epiblast undergo programmed cell death (PCD), thereby giving rise to the proamniotic cavity [8].

In addition to the established role of the primitive

endodermal BM in the survival of columnar epiblast cells [8], we have shown recently that this BM is also necessary for the polarization of epiblast cells and the PCD leading to cavitation [22]. However, the BM did not appear to affect the regulation of gene expression during epiblast cell differentiation because the changes in expression of *FGF-5* and *BMP4* occurred normally in its absence [22]. Thus, while it is clear that epiblast cell polarization is not necessary for the developmental changes in gene expression, it remains unknown if the biochemical differentiation of these cells is necessary for their polarization and subsequent programmed cell death to form the proamniotic cavity.

Embryoid bodies (EBs) derived from differentiating mouse embryonic stem (ES) cells have been used extensively as a model system to investigate the early stages of mammalian development [29]. To examine the relationship between epiblast cell differentiation and epithelium formation, we have here inhibited the differentiation of epiblast cells in ES cell-derived EBs by adding leukaemia inhibitory factor (LIF) to the culture medium. In agreement with an earlier report, we show that in the presence of LIF, primitive endoderm cells can still differentiate [31] and they deposit a BM. However, the further differentiation of these cells into visceral and parietal endodermal cell lineages is inhibited. Moreover, although LIF is known to block the biochemical differentiation of epiblast cells [31], we found that LIF did not block formation of a proamniotic-like cavity, which was invariably lined by a columnar epithelial layer. Our results demonstrate that the primitive endodermal BM is sufficient for both the formation of a columnar epithelium and subsequent cavitation by ES cells. The relationships between the effects of LIF on the differentiation of endodermal and epiblast cells are discussed.

## Results

*Primitive endoderm cells differentiate in the presence of LIF*

Light microscopy analysis of toluidine blue stained frozen sections showed that after 2 days of suspension culture, cells with the characteristic spindle-shaped morphology of primitive endoderm cells [25] had differentiated at the periphery of EBs grown both in the absence and in the presence of LIF (Fig. 1A,B). Furthermore, immunostaining with the primitive endodermal marker laminin-1 [6], showed that cells displaying positive immunoreactivity were beginning to appear at the periphery of day 2 EBs, both with and without LIF (Fig. 1C,D). Both spindle shaped morphology and laminin immunoreactivity were observed in >70% EBs, irrespective of the presence of LIF. By day 10, anti-laminin-1 immunohistochemistry indicated the presence of a sheet-like BM between the outer endoderm and inner core cells of approximately 50-60% of EBs

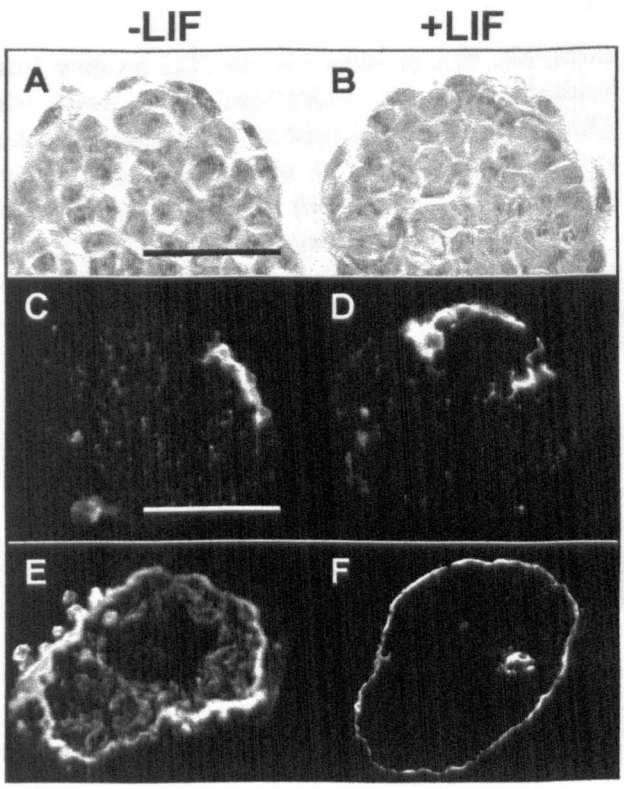


**Fig. 1.** Primitive endoderm cells develop and deposit a BM in the presence of LIF. LIF status is as indicated. **A,B** Toluidine blue-stained frozen sections of day 2 EBs show the presence of cells with the spindle-shaped morphology characteristic of primitive endoderm at the periphery. **C-F** Immunofluorescence staining for laminin in sections of: **C,D** day 2, and **E,F** day 10 EBs. Scale bars: 15μm, (A,B); 30μm (C-F).

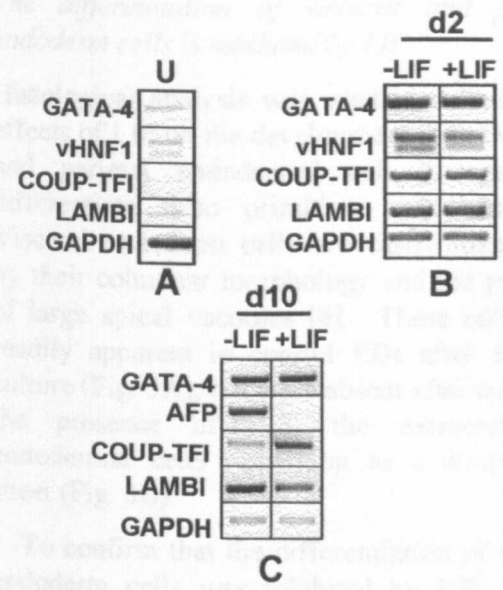


**Fig. 2.** LIF affects extraembryonic endoderm-specific gene expression. Semi-quantitative RT-PCR analysis of EBs grown in the absence and presence of LIF. LIF status is as indicated. *GAPDH* is shown as a loading control. **A,B** Expression levels of *GATA-4*, *vHNF1*, *COUP-TFI* and *LAMB1* in: **A** undifferentiated ES cells, and **B** day 2 EBs. **C** Expression levels of *GATA-4*, *AFP*, *COUP-TFI* and *LAMB1* at day 10; note the absence of *AFP* expression in the +LIF sample. The double band observed with the *vHNF1* primers results from splice variants [5].

cultured both with and without LIF (Fig. 1E,F). However, in the presence of LIF the BM was thin and regular, reminiscent of the thin BM initially deposited by primitive endodermal cells (Fig. 1F), whereas in controls the BM was more irregular and generally much thicker, reminiscent of Reichert's membrane (Fig. 1E).

To further analyze the effect of LIF on the differentiation of extraembryonic endoderm, we used RT-PCR to determine the expression levels of *GATA4* and *vHNF1*, which are expressed by all extraembryonic endodermal cells [1, 2, 32]. We found that while *GATA4* and *vHNF1* were virtually undetectable in undifferentiated ES cells (Fig. 2A), the expression of these genes, together with that of *LAMB1* (encoding the laminin β1 subunit), was up-regulated by day 2 both in the presence and absence of LIF (Fig. 2B). After 10 days culture, while comparable levels of both *GATA4* (Fig. 2C) and *vHNF1* expression (not shown) were found both with and without LIF, the presence of LIF did affect the levels of expression of the primitive endoderm-specific marker, *COUP-TF1* [23], which was maintained at high levels and not down-regulated as in the controls without LIF (Fig. 2C). Furthermore, *LAMB1*, which is expressed in primitive endoderm cells and is normally further up-regulated in their parietal endoderm cell derivatives [17], was lower after 10 days with LIF than in its absence (Fig. 2C). Strikingly, in the presence of LIF, the expression of the gene coding for the visceral endodermal cell marker alpha-fetoprotein (*AFP*) [11] was totally inhibited (Fig. 2C). Taken together, these results suggest that while LIF does not affect the initial differentiation of primitive endodermal cells, it does influence their subsequent development in culture by suppressing the appearance of parietal and visceral endoderm-specific markers.

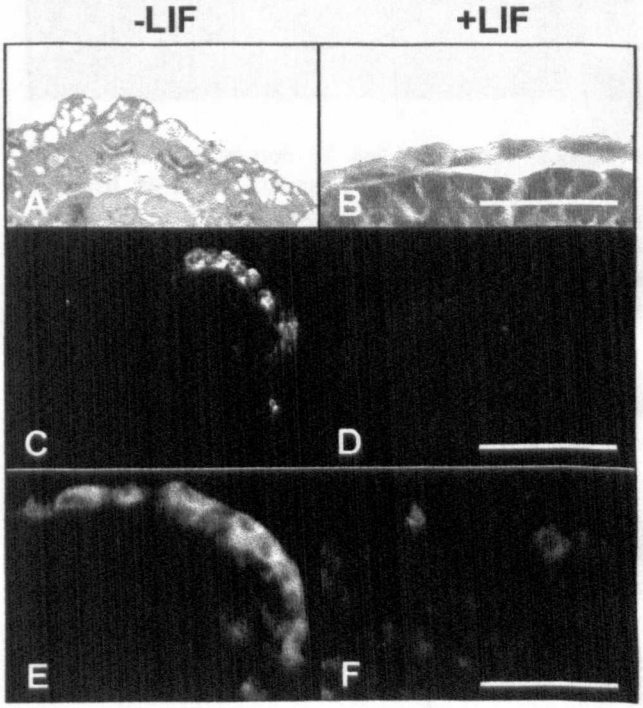


**Fig. 3.** LIF inhibits visceral and parietal endoderm cell differentiation. LIF status is as indicated and EBs are day 10. **A,B** Toluidine blue-stained frozen sections show cells with the typical morphological characteristics of visceral endoderm cells are present only in controls (**A**). **C,D** Immunofluorescence staining for the visceral endoderm cell-specific marker, AFP. **E,F** Immunofluorescence staining for the parietal endoderm cell-specific marker, Endo C. Scale bars: 6μm (A,B); 30μm (C,D); 20μm (E,F).

*The differentiation of visceral and parietal endoderm cells is inhibited by LIF*

Histological analysis was used to delineate the effects of LIF on the development of the visceral and parietal endodermal cell lineages that differentiate from primitive endoderm cells. Visceral endoderm cells are easily identifiable by their columnar morphology and the presence of large apical vacuoles [4]. These cells were readily apparent in control EBs after 10 days culture (Fig. 3A), but were absent after culture in the presence of LIF, the extraembryonic endodermal cells remaining as a simple thin sheet (Fig. 3B).

To confirm that the differentiation of visceral endoderm cells was inhibited by LIF, and to determine the effect of LIF on the differentiation of parietal endoderm cells, we performed immunostaining for alpha-fetoprotein (AFP), and the parietal endodermal cell-specific marker Endo C [3]. In contrast to the majority (>60%) of control EBs, neither AFP (Fig. 3C,D) nor Endo C-expressing cells (Fig. 3E,F) were detected in any EBs cultured for 10 days with LIF. Thus, these results are consistent with those from analysis of gene expression in indicating that the differentiation of primitive endoderm cells to visceral and parietal endoderm is inhibited by LIF.

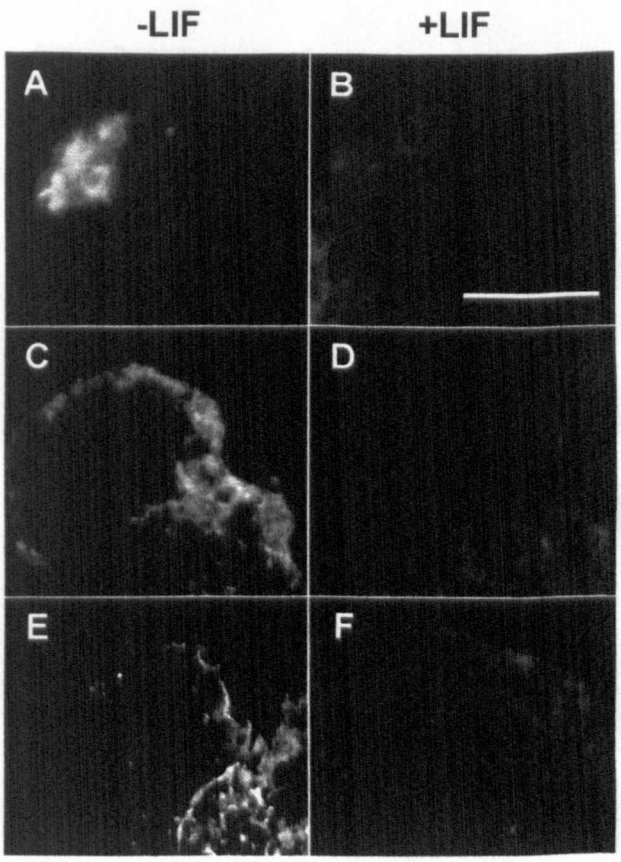


**Fig. 4.** Differentiation of embryonic cell lineages is inhibited by LIF. LIF status is as indicated and EBs are day 10. **A,B** Immunofluorescence staining for α smooth muscle actin. **C,D** Immunofluorescence staining for skeletal myosin. **E,F** Immunofluorescence staining for neurofilament. Scale bar: 30μm (A-F).

*Epiblast cell differentiation but not cavitation is inhibited by LIF*

To demonstrate the effects of LIF on epiblast cell differentiation, immunostaining for α smooth muscle actin, skeletal muscle myosin and neurofilament protein was performed on EBs grown with or without LIF for 10 days. The results show that whereas cells displaying positive immunoreactivity for α smooth muscle actin, skeletal muscle myosin and neurofilament protein appeared in approximately 50% of controls (Fig. 4A,C,E), none were detectable in any of the EBs cultured in the presence of LIF (Fig. 4B,D,F). Because the development of epiblast cells is an obligatory step in the differentiation of ES cells into embryonic lineages [28, 31], then the absence of mesodermal and ectodermal derivatives is evidence that the differentiation of epiblast cells is inhibited by LIF. To confirm inhibition of epiblast cell differentiation by LIF, we carried out semi-quantitative RT-PCR to determine the effect of LIF on *FGF-5* expression in the EBs. *FGF-5* is not expressed in the undifferentiated stem cells of the inner cell mass before implantation, but is subsequently induced in epiblast cells just before they become polarized to form the columnar epiblast epithelium [16]. The results show that while the expression of *FGF-5* is negligible in EBs at day 2, the presence of LIF blocks the up-regulation of *FGF-5* expression that normally occurs during subsequent culture (Fig. 5E). Taken together, these results support those of

previous workers in demonstrating that LIF blocks the biochemical differentiation of epiblast cells [31]. Additionally, the morphology of EBs differed depending on exposure to LIF.

Thus, in the absence of LIF irregular EBs were found after 10 days culture in which outgrowths of cells and accumulations of cells within the EBs were clearly evident (Fig. 5A), whereas in the presence of LIF the EBs were regular and spherical (Fig. 5B). However, histological analysis of day 10 EBs showed that they formed a columnar epithelium in the presence of LIF (Fig. 5D) similar to that developing in controls (Fig. 5C). Significantly, cavitation was also observed in both sample-types (Fig. 5C,D). The proportion of EBs that cavitated in the presence of LIF (59.6% ± 8.1 s.e.m., n=49) was not significantly different from controls (51.3% ± 4.4 s.e.m., n=45; $p > 0.1$, Student's t test). Non-cavitating EBs in both groups failed to develop a columnar epithelium. The results thus indicate that the development of undifferentiated ES cells into columnar epithelial cells and the subsequent cavitation of the epiblast require neither the biochemical differentiation of the epiblast cells nor the presence of visceral or parietal endoderm cells (see Fig. 6).

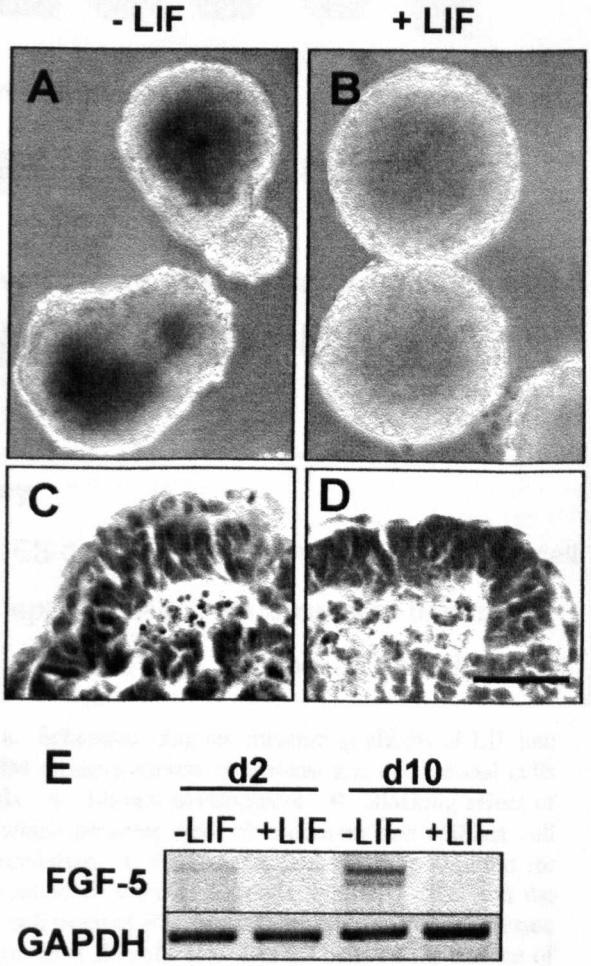


**Fig. 5.** Morphology and differentiation of embryoid bodies cultured for 10 days in the absence and presence of LIF. LIF status is as indicated. **A,B** Phase-contrast pictures of representative embryoid bodies. Note that in the absence of LIF the embryoid bodies are irregular and that outgrowths of cells and internal accumulations of cells are evident. **C,D** Toluidine blue-stained frozen sections. **E** RT-PCR analysis of *FGF-5* expression. *GAPDH* is shown as a loading control. Note that *FGF-5* expression is undetectable in day 2 EBs but by day 10, while this gene is up-regulated in controls, it remains undetectable in the +LIF samples. The double band results from splice variants *FGF-5* [18]. Scale bar: 6μm (C,D).

## Discussion

In this study we have made use of the ability of LIF to block specific aspects of ES cell differentiation in EBs. The results show that although LIF inhibits both epiblast cell differentiation and the development of both visceral and parietal endodermal cells, it blocks neither the ability of ES cells to form an epithelium in response to a basement membrane nor the cavitation which ensues.

*The effect of LIF on the differentiation of extraembryonic endodermal cells*

Consistent with an earlier report [31], we found that the differentiation of primitive endoderm cells at the periphery of EBs was unaffected by LIF, as assayed by histological and gene expression analyses. In addition, however, we have been able to show here that the further differentiation of primitive endoderm cells into visceral and parietal lineages is effectively blocked by LIF.

It has previously been shown that the levels of the α-fetoprotein VE marker were reduced after culture in the presence of LIF, indicative of only a

partial inhibition of VE cell differentiation [31]. However, it was also reported that the extent of this inhibition is dependent on the ES cell line used [31]. The mouse R1 ES cell line employed in the present experiments displayed a total inhibition of VE and PE differentiation after LIF treatment, thus allowing us to use EBs derived from these cells to determine the effects of specific types of extraembryonic endodermal cells on epiblast differentiation.

It is likely that LIF has distinct mechanisms of actions influencing visceral and parietal endoderm differentiation. Thus, LIF is a member of the interleukin-6 family of cytokines that bind to the gp130 receptor, resulting in activation of both the Jak/Stat and Ras/MAPK signal transduction pathways in ES cells [13, 27]. As the Ras/MAPK pathway must be inactivated for parietal endoderm cell differentiation [37], then the stimulation of this pathway by LIF would be expected to inhibit parietal cell differentiation.

In contrast, the differentiation of visceral endoderm cells is stimulated by BMP4, which is expressed by undifferentiated ES cells [9, 21]. It is known that LIF activates Stat1 and Stat3 in ES cells via Jak [26], and it has recently been shown that activated Stat1 antagonises signaling of TGFβ family members like BMP4 [36]. Thus, activation of the Jak/Stat1 pathway in ES cells by LIF would be expected to interfere with BMP4 signalling, thereby preventing visceral endoderm development. This hypothesis is supported by the observation that BM components are also known to activate the Jak/Stat pathway [35], and that the presence of a BM in EBs reduces the number of primitive endodermal cells differentiating into VE [24].

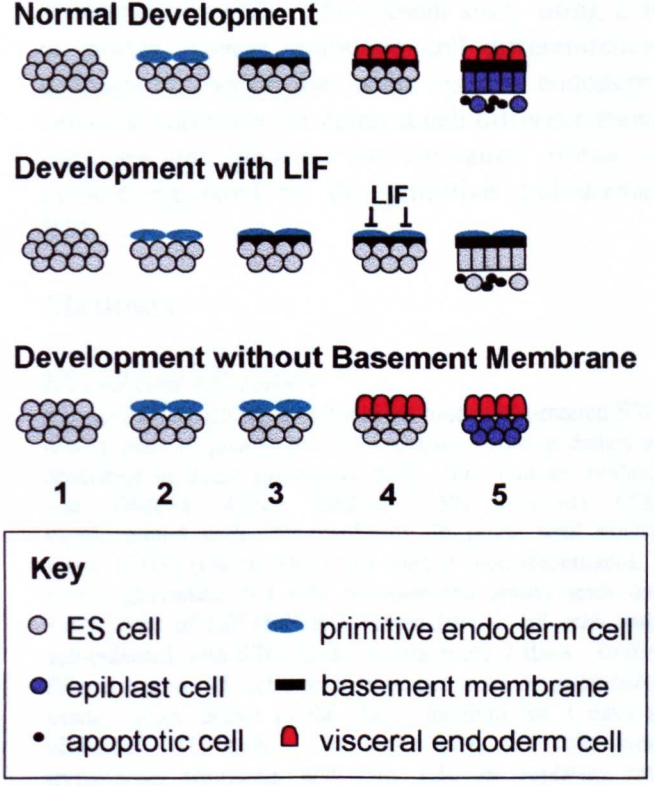


**Fig. 6.** Schematic diagram illustrating effects of LIF and the BM on development of epiblast and endodermal cells in EBs. **A** Normal development. **B** Blocking effect of LIF which prevents visceral endoderm and epiblast cell differentiation. **C** Role of the BM which is required for polarization of internal ES cells. Initially, EBs and the inner cell mass of E3.0 blastocysts consist of an aggregate of equivalent ES cells (1). The ES cells on the surface of the aggregate differentiate to become primitive endoderm cells that begin to express extra-cellular matrix molecules (2). Subsequently, the primitive endoderm cells deposit a BM (3). Concomitant with this, undifferentiated ES cells within the aggregate secrete factors that induce primitive to visceral endoderm cell differentiation. (4) The visceral endoderm cells secrete unidentified factors that induce ES to epiblast cell differentiation, while the primitive endodermal BM induces the epiblast cells in contact with it to become polarized to form the columnar epiblast epithelium. (5) The unpolarized epiblast cells at the surface of the columnar epiblast epithelium detach and undergo programmed cell death to form the proamniotic cavity.

*The differentiation of epiblast cells*

It has been shown in an earlier report that LIF blocks epiblast cell differentiation in EBs, as assayed by the absence of expression of the epiblast cell marker *FGF-5* and lack of mesodermal derivatives [31]. Our results are in keeping with these observations, in that we also found no evidence of *FGF-5* expression or of the appearance of mesodermal or ectodermal derivatives in EBs exposed to LIF. However, despite the expected lack of differentiation, the ES cells aligned against the primitive endodermal BM formed a columnar epithelium

followed by cavitation. This specific inhibitory action of LIF is in direct contrast to the role of the BM, which while being unnecessary for epiblast cell differentiation as reflected by *FGF-5* expression and the development of embryonic cell lineages, is required for columnar epithelium formation and cavitation [22]. Thus the processes of biochemical differentiation and epithelium formation by ES cells are distinct, not interdependent and controlled by different regulatory mechanisms (see Fig. 6). Furthermore, apparently undifferentiated ES cells in EBs have the inherent ability to form an epithelium with subsequent cavitation if presented with a BM.

While the data presented here are consistent with epithelium formation by ES cells being dependent upon the BM deposited by primitive endodermal cells [22], the mechanism by which LIF blocks the biochemical differentiation of the ES cells remains unclear. However, there is good evidence that VE cells regulate epiblast cell differentiation because disruption of the visceral endoderm-specific gene *Evx1* inhibits the differentiation of epiblast cells [33]. Moreover, it has recently been demonstrated that factors expressed by an endodermal cell line can induce the differentiation of ES cells to an epiblast-like cell population [28].

Significantly, this differentiation in response to endoderm-derived factors was reported not to be inhibited by LIF [28], consistent with the hypothesis that LIF's ability to inhibit epiblast differentiation is caused indirectly by its blockade of visceral endoderm cell differentiation. It has been suggested previously that visceral endoderm cells secrete a factor that induces epiblast cells to undergo programmed cell death, with the exception of those aligned against the primitive endodermal BM which selectively supports their survival [8]. Our recent work, however, has shown that visceral endoderm cells are not sufficient to induce programmed cell death in BM-deficient EBs [22]. Moreover, premature BM deposition accelerates programmed cell death and early cavitation, prior to visceral endoderm cell differentiation [22]. The present study using LIF to inhibit visceral endoderm cell differentiation supports the notion that while visceral endoderm cells are necessary for epiblast cell differentiation, they are not required for cavitation which is instead regulated by the primitive endodermal BM.

## Methods

### *ES cell and EB culture*

R1 mouse ES cells were cultured on mitomycin-treated STO feeder cells in gelatinized 3.5 cm tissue culture dishes as described in detail previously [22]. The culture medium was DMEM (Gibco BRL) / 5% (vol/vol) $CO_2$, supplemented with 15% (vol/vol) ES grade fetal bovine serum (FBS) (Gibco BRL), 0.1 mM β mercaptoethanol, 1 mM L-glutamine, 0.1 mM non-essential amino acids and 1,000 U/ml of LIF (ESGRO; Gibco BRL). ES cells were sub-cultured onto STO feeder layers every 2 days. Before EB formation, ES cells were passaged once on gelatinized tissue culture dishes in the above medium for 2 days to eliminate STO cells. To make EBs, ES cells were trypsinized, triturated, and split 1:10 by replating into bacterial petri dishes, under which conditions the cells remained in suspension and formed aggregates. The EB culture medium was as above, except that for control samples, LIF was omitted. The results shown are representative of three separate populations of EBs grown either in the absence or in the presence of LIF. For histological analysis, some 20-30 sections and 15-20 sections from each population were examined after days 2 and 10 of culture, respectively.

### *Immunostaining*

EBs were fixed for 1 h with 4% (wt/vol) paraformaldehyde, washed three times in phosphate buffered saline (PBS) and soaked in 15% (wt/vol) sucrose overnight at 4°C. The samples were then incubated in 7.5% (wt/vol) gelatin:15% (wt/vol) sucrose for 1 h at 37°C. 100 μl aliquots were pipetted onto a block of gelatin and allowed to set at room temperature, following which they were mounted onto cork discs with OCT cryofixative (DAKO) and frozen in liquid nitrogen-cooled isopentane before storage at –80°C prior to sectioning. Ten μm cryostat sections were blocked with 10% (vol/vol) goat serum (GS) in PBS for 1 hour. The primary antibodies used were rabbit anti-EHS laminin that recognizes all three subunits of laminin, at 1/5000 dilution [20]; rabbit anti-mouse α-feto-protein (AFP) serum at 1/200 dilution (ICN Biomedicals); rat TROMA-3 at 1/2 dilution (a gift from N. Smyth, Department of Biochemistry, University of Cologne) that recognizes the mouse parietal endoderm cell-specific cytokeratin Endo C [3]; rabbit anti-bovine neurofilament (a gift from D. Moss of this Department) at 1/1000 dilution; rabbit anti-mouse skeletal myosin at 1/20

dilution (Sigma); monoclonal anti-α smooth muscle actin (mouse IgG2a isotype; Sigma) at 1/5000 dilution. Incubations with primary antibodies were carried out overnight in 1% (vol/vol) GS in PBS in a humidified atmosphere at room temperature and the sections were then washed three times in PBS. For rabbit primary antibodies, the secondary antibody was TRITC-conjugated swine anti-rabbit IgG at 1/100 dilution (Dako); for TROMA-3, the secondary antibody was biotinylated rabbit anti-rat IgG at 1/50 dilution (DAKO); for mouse α smooth muscle actin, the secondary antibody was FITC-conjugated rabbit anti-mouse IgG at 1/40 dilution (Dako). Secondary antibodies were applied in 1% (vol/vol) GS in PBS at room temperature for 2 hours and the sections were then washed three times in PBS. Streptavidin conjugated with fluorescein isothiocyanate (Amersham) at 1/50 dilution in 1% (vol/vol) GS in PBS was applied to TROMA-3 sections at room temperature for 2 hours and the sections were then washed three times in PBS. Sections were mounted in fluorescent mounting medium (Dako) and photographed using a Leitz RM22 fluorescent microscope.

### *RT-PCR*

Total RNA was extracted from undifferentiated ES cells or EBs using guanidinium isothiocyanate [7], and reverse transcribed using Superscript ™ II RT (GIBCO BRL). For undifferentiated ES cells and day 2 EBs, whole populations were used, but for day 10 EBs, 10-15 control EBs and an equal number of EBs grown in the presence of LIF were selected. Primers for *FGF-5* [18], *GAPDH* and *AFP* [22], and *vHNF1* and *GATA-4* [10] were as described previously. *LAMB1* primers were gcagacacaacaccaaaggc (forward) and tgtacccatcacagatcccg (reverse), product size 344bp, annealing temperature 56°C. *COUP-TF1* primers were agccatcgtgctattcacg (forward) and ttctcaccagacacgaggtc (reverse), product size 569bp, annealing temperature 57°C; Semi-quantitative RT-PCR was performed as previously described to determine mRNA levels relative to that of *GAPDH* [34].

## Acknowledgements

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# Appendix 5

# 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 γ1 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 α1 subunit did occur, but these were not deposited in the extracellular matrix. These results show that the laminin γ1 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 α2 subunit (merosin) can result in autosomal forms of muscular dystrophy (Helbling-Leclerc et al., 1995), and targeted disruption of the laminin β2 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 γ1 is one of the earliest expressed laminin subunits which, together with the α1 and β1 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 trophectodermal epithelium (Dziadek and Timpl, 1985; Thorsteinsdottir, 1992). Furthermore, γ1 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 γ1 subunit has instead the other known member of this subunit family, γ2 (Kallunki et al., 1992). However, the γ2 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 (Champliaud 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 γ1 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). $5 \times 10^6$ cells were transfected by electroporation with 25 μg of linearized construct 1 and colonies selected for resistance to G418 at 380 μg/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 cells 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 α1 raised against recombinant domain IVa (Schulze et al., 1996); anti-laminin γ1 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 GCGCATCAGAACACTCAACG (sense) and CAAGGGTGGTCATCATAAGG (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 GATAACTGTCAGCACAACACC (sense) and GTGAAGTAGTAACCGGACTCC (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 $5 \times 10^3$ 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 [$^{35}$S]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 1-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

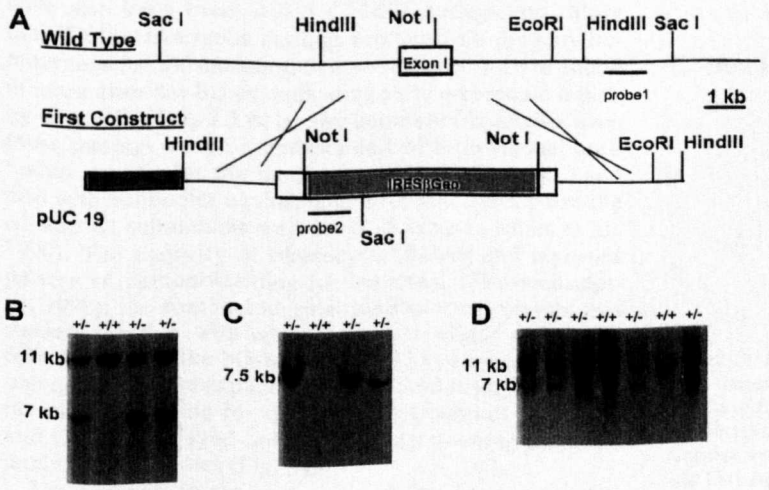


*Figure 1.* Homologous recombination in the *LAMC1* gene of ES cells and germline transmission in mice. (A) Restriction map including the first exon of the *LAMC1* gene and the targeting construct used to disrupt the gene. (B) SacI-digested DNA from ES clones analyzed with probe 1, showing appearance 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.

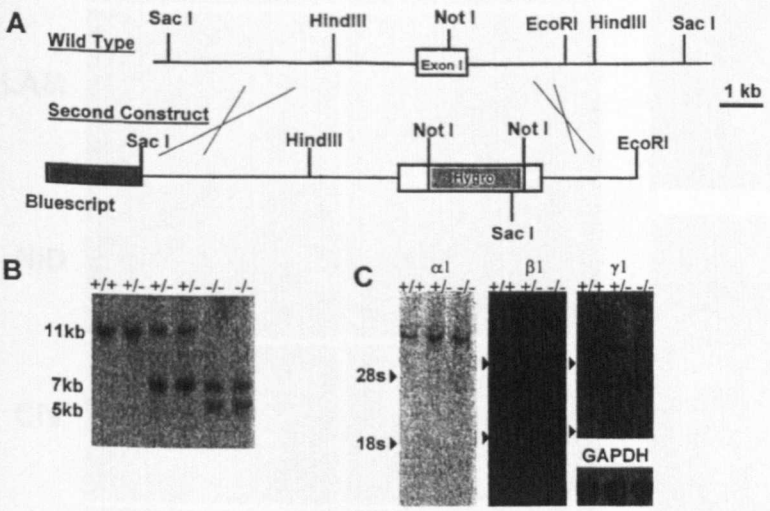


*Figure 2.* Analysis of ES cells lines generated by the homologous replacement of the second *LAMC1* allele. (A) Restriction map of the second targeting construct used to disrupt the remaining *LAMC1* allele by insertion of a hygromycin resistance cassette. (B) Southern blot analysis with probe 1 of 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 *LAMA1*(α1), *LAMB1*(β1), and *LAMC1*(γ1). The GADPH 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 γ1 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 α1 and β1 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 γ1 subunit (Fig. 3 A) and also with antibodies against laminin-1 which recognize the α1 and β1 subunits as well as γ1 (Kücherer-Ehret et al., 1990). The majority of blastocysts showed the reported pattern of immunostaining for laminin-1 (Thorsteinsdottir, 1992): the trophectodermal 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 γ1 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 γ1 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 γ1 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-

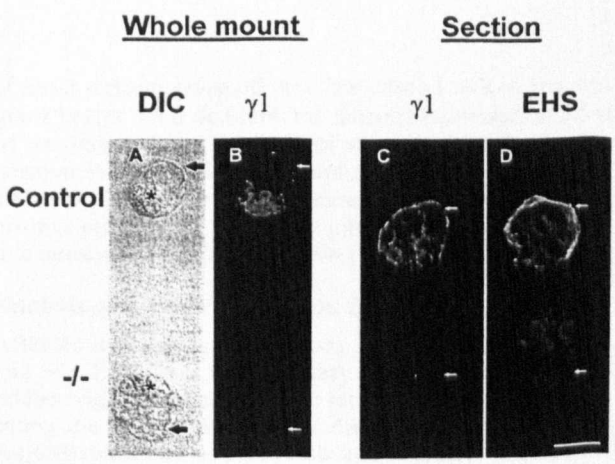


*Figure 3.* Appearance of 3.5 d pc preimplantation blastocysts from heterozygote matings. (A and B) Whole mount micrographs of the same pair of blastocysts. (C and D) Frozen 7-μm serial sections. A shows the appearance of the blastocysts using Normarski optics. B and C are stained with γ1 subunit antibodies and D is stained with antibodies against laminin-1. Arrows, location of the trophectoderm; asterisks, inner cell mass. Embryos lacking laminin γ1 immunoreactivity can expand to form blastocysts of normal appearance but lack the trophectodermal basement membrane. Immunoreactivity of the other laminin subunits is intracellular, the cells showing cytoplasmic staining (D). Bars, 50 μm.

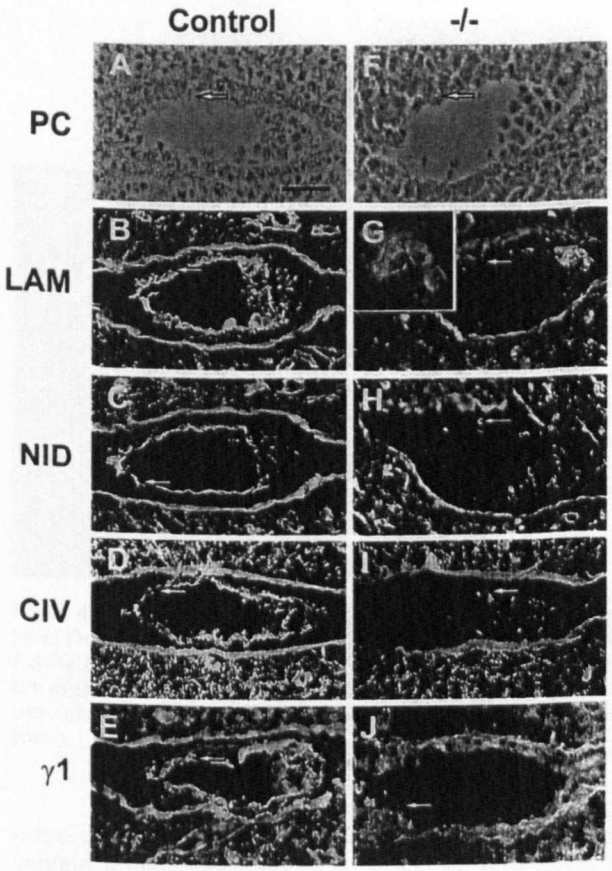


*Figure 4.* Immunofluorescence staining for basement membrane components in frozen sections of 4.5 d pc embryos in utero. (A–E) Wild-type or heterozygous embryos; (F–J) −/− Embryos, as defined by lack of laminin γ1 immunoreactivity (J). (A and F) Phase–contrast photomicrographs. Immunostaining was performed with antibodies directed against: laminin-1 (B and G); nidogen (C and H); collagen type IV (D and I); laminin γ1 subunit (E and J). In wild-type or heterozygous embryos, all antibodies show staining under the trophectoderm (arrows) and within the inner cell mass. Occasionally strongly staining parietal endoderm cells can be seen migrating over the trophectoderm (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 trophectoderm in these embryos (arrows), although the maternal basement membrane underlying the uterine epithelium is clearly visible in all cases. Bar, 50 μm.

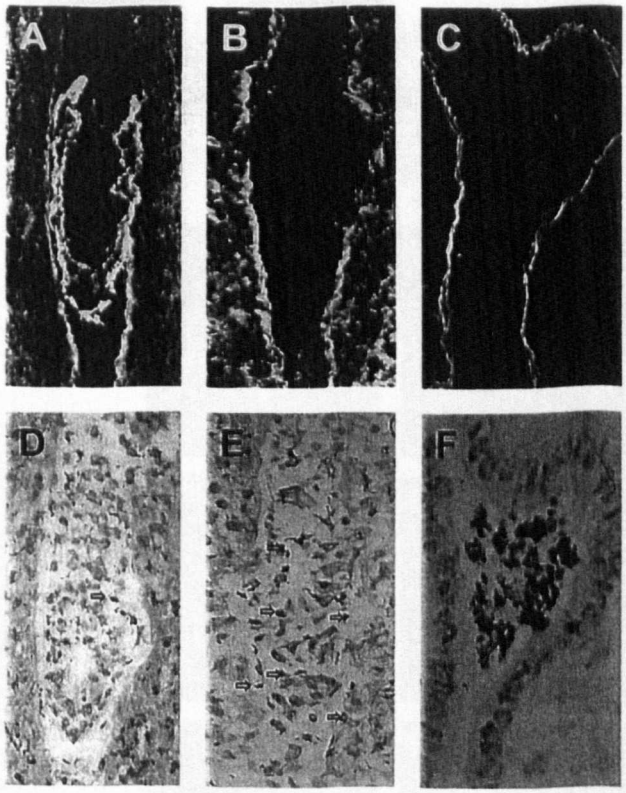


*Figure 5.* Appearance of 5.5 d pc embryos in utero. Frozen sections of embryos were immunostained with anti–laminin γ1 antibodies to show: (A) a control embryo staining for γ1 (either +/+ or +/−); (B and C) −/− embryos lacking γ1 staining. The γ1-negative embryonic cells had lost recognizable structure, large aggregates of cells being present (E and F). TUNEL staining revealed low numbers of cells with fragmented DNA in serial sections from γ1-positive embryos (D), whereas γ1-negative embryos displayed either increased (E) or intense (F) staining.

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

Of the 80 decidua examined at 5.5 d pc from heterozygous matings, 28 contained laminin γ1–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 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 γ1-positive embryos (Fig. 5 D), and the laminin γ1–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,

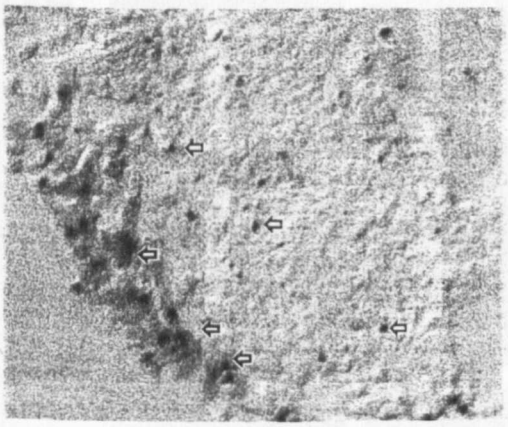

*Figure 6.* LacZ staining of +/− embryoid bodies after 48 h of culture. The lacZ reaction product is localized to cells in which the *LAMC1* promoter is active are found mainly around the periphery of the embryoid body, although a few scattered weakly positive cells were also seen in the center of the 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 γ1 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 α1 and β1 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 γ1 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),

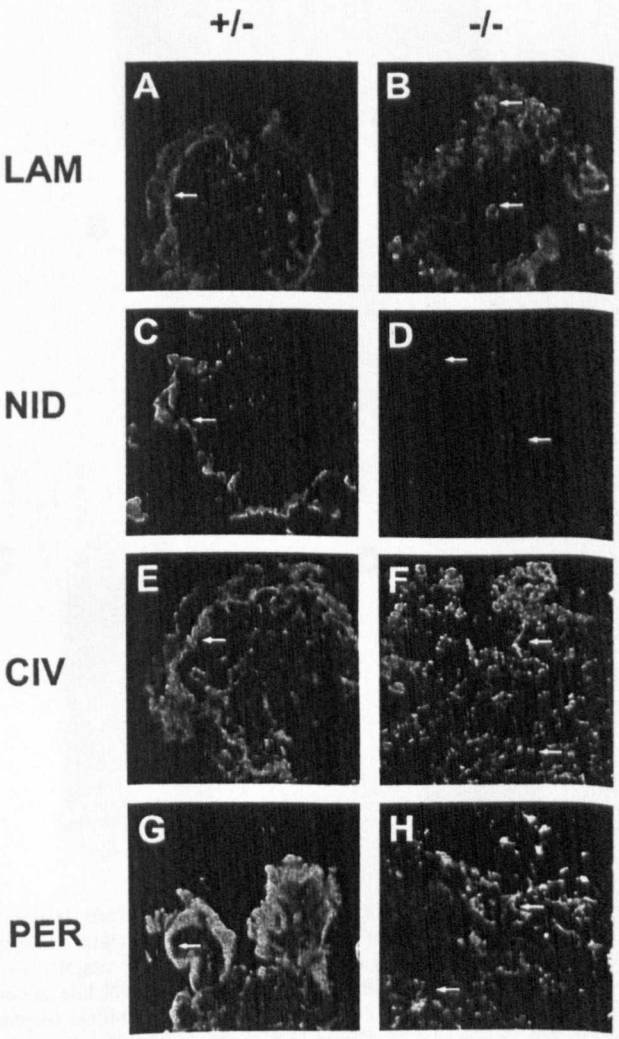


*Figure 7.* Immunofluorescence staining for basement membrane components in embryoid bodies after 7 d of culture. The primary polyclonal antibodies used were: anti–laminin-1 (A and B); anti-nidogen (C and D); anti-collagen type IV (E and F); and anti-perlecan (G and H). Genotypes of the ES cells are 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 [$^{35}$S]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

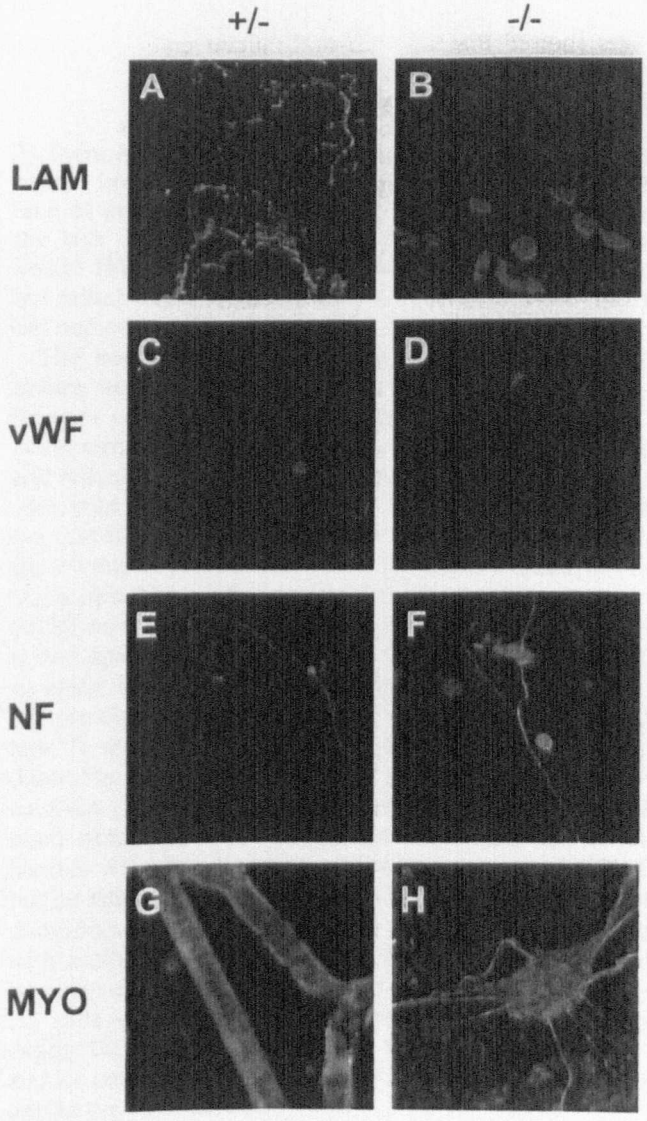


*Figure 8.* Immunofluorescence staining for laminin and cell-specific markers in +/− and −/− ES cells after 21 d of culture under differentiating conditions. The primary polyclonal antibodies used were: anti–laminin-1 (A and B); anti–von Willebrand factor (C and D); anti-neurofilament 200 subunit (E 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).

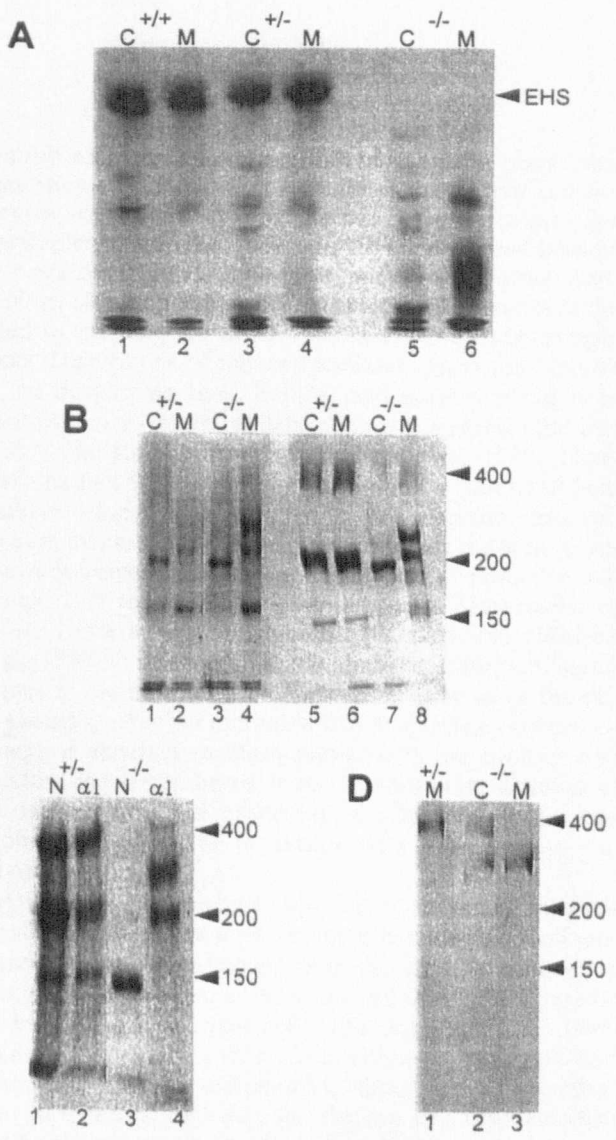


*Figure 9.* Embryoid bodies lacking the laminin γ1 subunit fail to produce intact laminin but do secrete a truncated α1 subunit and free nidogen. (A) Immunoprecipitation with anti–laminin-1 antibodies and 3% SDS-PAGE under nonreducing conditions of $^{35}$S-labeled laminin from cell extracts (1, 3, and 5) and media (2, 4, and 6) of embryoid bodies +/+ (1 and 2), +/− (3 and 4) and −/− (5 and 6) for the γ1 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 α1 subunit (2 and 4) and nidogen (1 and 3). (D) Anti–laminin α1 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.

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 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 α1 subunit-specific antibodies directed against the IVa domain. In addition to the 400-kD α1 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 α1 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 α1 subunit and nidogen, they are not associated as normal (Fig. 9 C, lanes 1 and 2), consistent with the absence of the laminin γ1 subunit in the −/− ES cells.

## Discussion

We have used homologous recombination to target one or both of the *LAMC1* alleles coding for the laminin γ1 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 trophectodermal 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 γ1 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 γ1–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 trophectodermal 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 trophectodermal 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 α1 or β1 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 trophectodermal 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 β1 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 β1-containing integrin receptor (Coucouvanis and Martin, 1995). At 5.5 d pc we were unable to find anything other than disrupted laminin γ1–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 γ1 subunit being re-

quired for the formation of α2/γ1-containing laminin isoforms that are necessary for the maintenance of myotubes.

### *Covalent Laminin Trimers Fail to Form in the Absence of the γ1 Subunit*

In the absence of the γ1 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 α1 subunit to form a triple coiled-coil α-helix with preassembled βγ 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 α1 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 α1 subunit. A similar but incomplete cutting of the laminin α1 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 α1 subunit adopts a conformation laying it open to cleavage (Yurchenco et al., 1997). Although the truncated α1 subunit in the −/− ES cell medium described here was noncovalently associated with a protein band of 200 kD, the fact that the α1 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 γ1 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 α1, and β1 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 between 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 β1 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.

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