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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′-AGCGAGTTTCCTTGGCAACAC-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

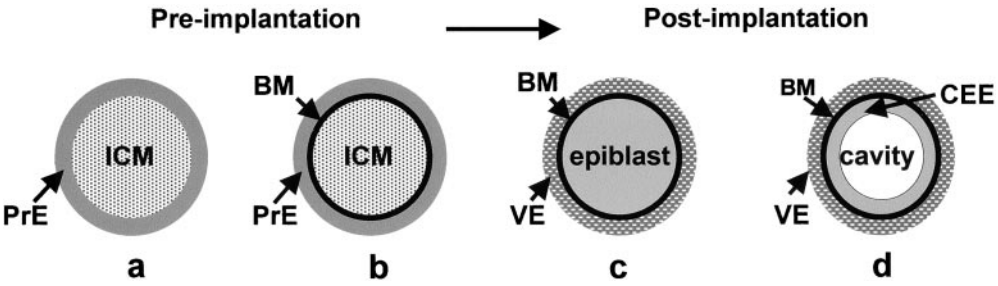

Figure 1. Schematic diagram showing the peri-implantation stages of mouse development. (a) Shortly after blastocyst formation, the cells on the surface of the ICM differentiate to become primitive endodermal cells. (b) The primitive endodermal cells deposit a basement membrane (BM). (c) After implantation, 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

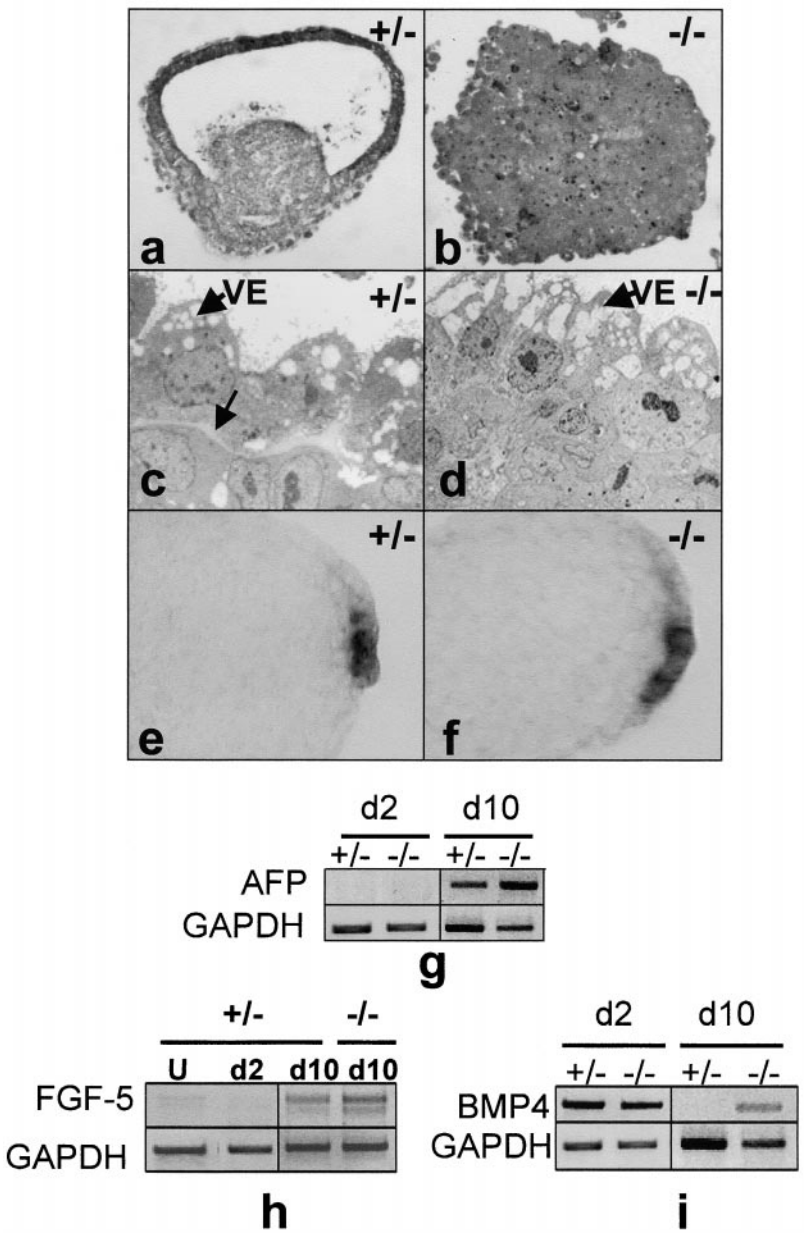

Figure 2. VE cell differentiation and cavitation in EBs. (a and b) Toluidine blue–stained frozen sections of EBs after 7.5 d in suspension culture show that *LAMC1*+/− EBs had cavitated by this time (a), whereas the *LAMC1*−/− EBs failed to cavitate (b). (c and d) EM shows differentiation of cells with 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

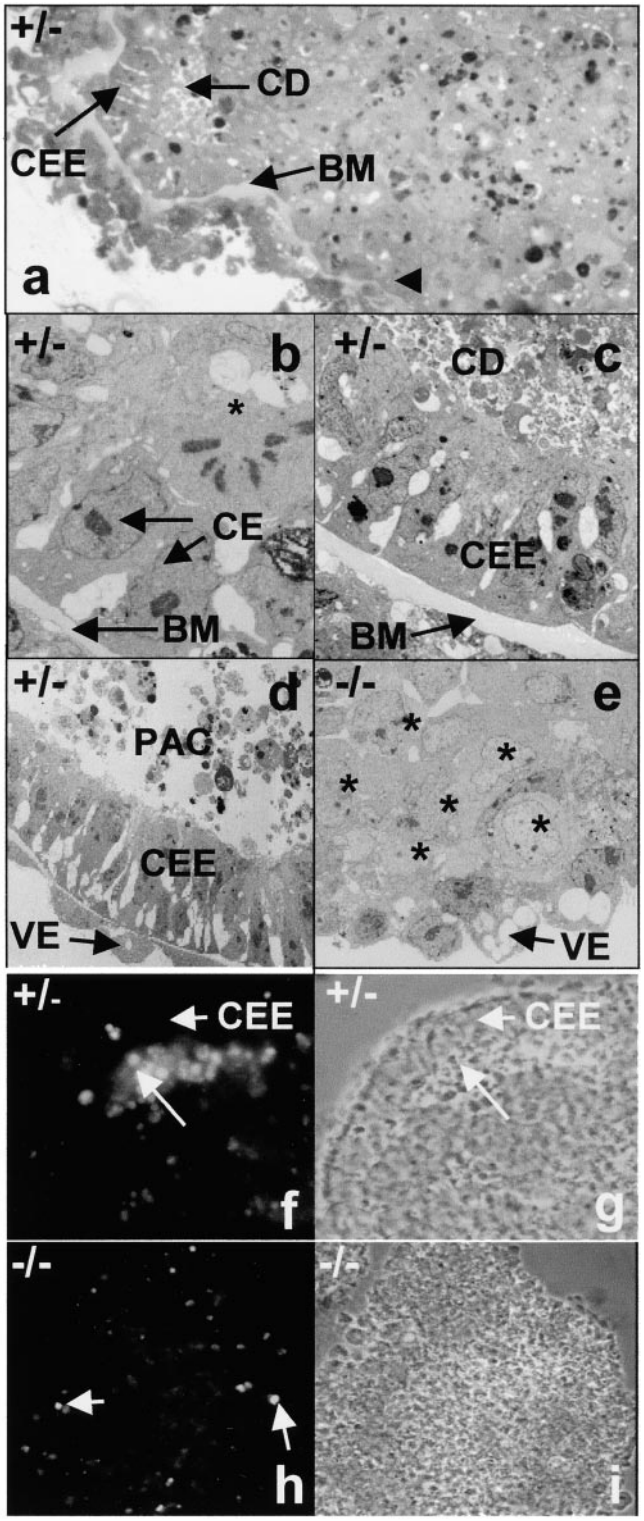

Figure 3. Epiblast cell polarization and PCD in EBs. (a) Toluidine blue–stained resin-embedded section of a day 6 *LAMC1*+/− EB shows that cell debris is present only at the apical surface of the CEE and is absent where the epiblast cells remain unpolarized (arrowhead). (b–d) EM of *LAMC1*+/− EBs. (b) 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-

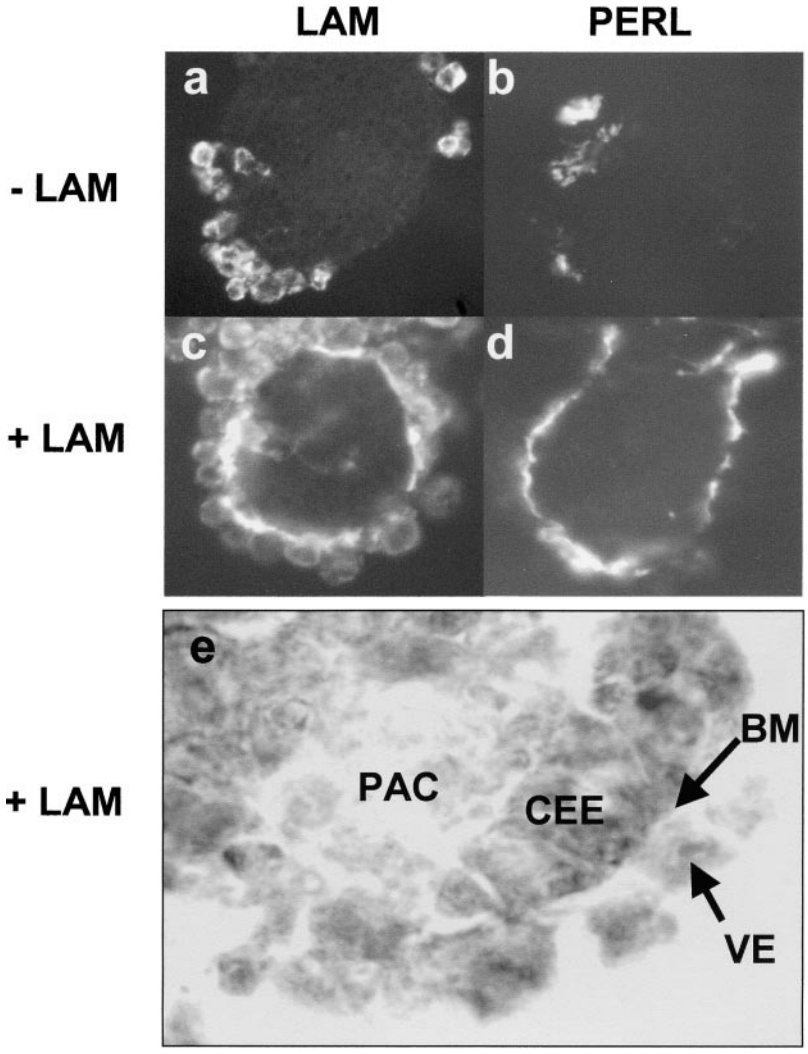

Figure 4. Rescue of *LAMC1*−/− EBs by the addition of exogenous laminin. (a–d) Immunofluorescence staining for laminin (a and c) and perlecan (b and d) in *LAMC1*−/− EBs after 2 d of culture: without (a and b) and with the addition of laminin type-1 (c and d). (e) Toluidine blue–stained frozen section 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.

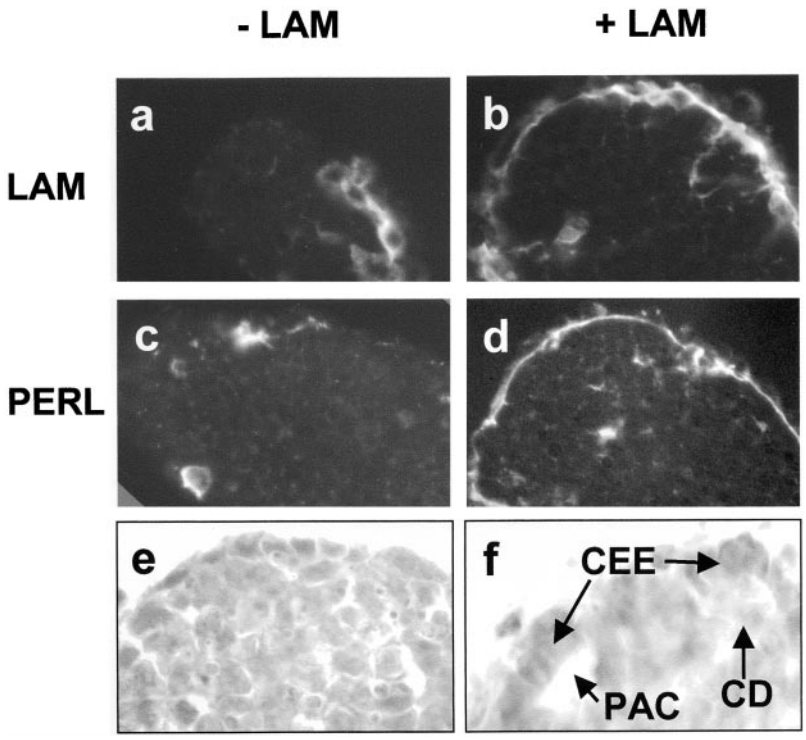

Figure 5. Laminin addition to *LAMC1*+/− control EBs accelerates the deposition of a basement membrane, leading to epiblast polarization and cavitation. (a–d) Immunofluorescence staining for laminin (a and b) and perlecan (c and d) in control EBs after 2 d culture: without (a and c) and with addition 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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