Regulation of laminin gene expression in preimplantation mammalian development by COUP-TF

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Abstract

The deposition of a basement membrane by primitive endoderm cells is necessary for polarization of the epiblast and formation of the proamniotic cavity in periimplantation mammalian embryos. The synthesis of this basement membrane is in turn dependent upon laminin expression. Here we have used embryoid bodies derived from mouse embryonic stem cells to investigate the molecular mechanisms controlling laminin gene expression in differentiating primitive endoderm cells. We demonstrate that COUP-TFs are dramatically up-regulated in primitive endoderm cells, and that these factors are sufficient to induce expression of *LAMB1* and *LAMC1* genes. These results indicate that COUP-TFs play a hitherto unsuspected role in early embryonic development by regulating basement membrane deposition.

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

1. Introduction

Basement membranes (BMs) play fundamental roles during development where they regulate the cell survival, proliferation and differentiation necessary for organogenesis, and they are necessary for the maintenance of cells in adult tissues (Colognato and Yurchenco, 2000). Despite their importance, little is known about the mechanisms regulating the deposition of BMs, although clearly such mechanisms are essential for the development and adult life of multicellular animals. Previously, using mouse embryonic stem (ES) cells, we targeted the *LAMC1* gene encoding the laminin γ1 subunit which resulted in the inhibition of assembly and secretion of this ubiquitous BM component (Smyth et al., 1999). Analysis of the early embryonic lethality resulting from this knock-out and the development of embryoid bodies (EBs) derived from *LAMC1-/-* ES cells showed that BM deposition in vivo and in vitro had been prevented (Smyth et al., 1999). Thus, laminin is necessary for the self-assembly of other BM components into BMs (Colognato and Yurchenco, 2000) and hence the factors regulating laminin gene expression play a crucial role in BM deposition.

The first BM necessary for embryonic development is that deposited between the primitive endoderm (PrE) and epiblast of preimplantation blastocysts, and is necessary for both polarization of epiblast cells and proamniotic cavity formation (Murray and Edgar, 2000). The laminin and other proteins that assemble to form this BM are synthesized by the PrE cells (Dziadek and Timpl, 1985), and there is a dramatic up-regulation in the expression of the genes coding for laminin subunits concomitant with PrE cell differentiation (Kleinman et al., 1987). Although there are a few reports describing the *cis*- and *trans*-acting factors that can influence laminin gene expression in a variety of cells, it remains unknown which transcription factors are involved in the induction of the laminin gene expression during early embryogenesis, and hence are crucial to the subsequent development of the embryo-proper.

EBs derived from spontaneously differentiating ES cells and retinoic acid-induced differentiating F9 embryonal carcinoma (EC) cells have been used extensively as model systems to analyze events occurring during peri-implantation development (Robertson, 1987; Niwa et al., 2000). Retinoic acid stimulates the expression of many genes in F9 cells, including *LAMB1* and *LAMC1* which code for laminin β1 and γ1 subunits, respectively, and those coding for the transcription factors vHNF1 and COUP-TFs I and II, two orphan members of the nuclear hormone receptor superfamily (Ogawa et al., 1988; Wang and Gudas, 1988; Kuo et al., 1991; Ben-Shushan et al., 1995). While *LAMB1*, *LAMC1* and *vHNF1* are also known to be expressed in PrE cells (Cereghini, 1996; Smyth et al., 1999), the expression profile *of COUP-TFs I* and *II* in either pre-implantation development or differentiating EBs has not previously been analyzed.

We show here that the expression of *COUP-TF*s *I* and *II* are induced in the PrE cells of pre-implantation embryos and ES cell-derived EBs. Consistent with a role for COUP-TFs in the induction of laminin genes, their induction precedes that of *LAMB1* and *LAMC1*. In addition, we show by transfection studies that both COUP-TFI and COUP-TFII can up-regulate expression of *LAMB1* and *LAMC1* genes. Thus, the increased expression of *COUP-TFs* in differentiating PrE cells can mediate the laminin expression necessary for subsequent embryonic development dependent upon BM formation.

2. Results (Figures at end of MS)

2.1. Morphological differentiation of primitive endodermal cells

The peripheral cells of day 1 EBs were morphologically indistinguishable from the inner core cells, but by day 2 the outer cells had elongated to resemble PrE cells, having a spindle-shaped morphology (Nadijcka and Hillman, 1974) (Fig. 1A,B). Further analysis showed that the PrE cells, which stain intensely with toluidine blue (Hogan and Tilly, 1978), had formed a cohesive layer at the periphery of day 2 EBs (Fig. 1C). Transmission electron microscopy was used to confirm that these cells had the typical ultrastructure of PrE cells (Fig. 1D), characterised by their spindle-shaped morphology, the presence of sparse microvilli and an abundance of electron-dense secretory vesicles and rough endoplasmic reticulum (Nadijcka and Hillman, 1974).

2.2. Biochemical differentiation of primitive endodermal cells

Using an antibody that recognises all 3 chains of laminin (Kücherer-Ehret et al., 1990), we found that no laminin immunoreactivity was present in undifferentiated ES cells, but following 2 days of suspension culture, a minority of peripheral cells had begun to express laminin (Fig. 2A-D). By day 4, anti-laminin-1 immunohistochemistry indicated the presence of a sheet-like BM between the outer endoderm and inner core cells of the EB (Fig. 2G,H).

Whole-mount in situ hybridization showed that the PrE cells of day 2 EBs displayed intense *COUP-TF1*, *COUP-TF-II* and *LAMB1* expression (Fig. 3A-C) in addition to the expression of *LAMC1* previously reported (Smyth et al., 1999). To confirm that PrE cells also expressed *COUP–TFs* at the equivalent stage in vivo, we performed whole-mount in situ hybridization on E3.5 pre-hatched blastocysts, at which stage a layer of PrE cells is

discernible on the surface of the inner cell mass (Nadijcka and Hillman, 1974). We found that *COUP-TFI* was expressed by these cells (Fig. 3D). RT-PCR analysis was also used to show that both *COUP-TFs* were expressed at this stage of development (results not shown).

To investigate further the kinetics of expression of *LAMB1*, *LAMC1* and *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 were induced by day 2 (Fig. 4A). However, whereas the levels of both *COUP-TFs* were maximal at day 2, those of the laminin genes were not maximal until day 4 (Fig. 4A).

2.3. Transient cell transfections with COUP-TF

We wished to establish if COUP-TFI and/or II was sufficient to induce expression of *LAMB1* and *LAMC1* genes. To this end, we transiently transfected cells with vectors containing *COUP-TFI*, *COUP-TFII*, or *GFP* downstream of the CMV promoter. The *GFP* vector, in addition to providing us with a control, also allowed us to monitor the transfection efficiency. RT-PCR analysis showed that cells transfected with the control vector did not express *COUP-TFI*, *COUP-TFII* or *LAMC1*, and expressed only very low levels of *LAMB1* (Fig. 4B,C). Expression of either *COUP-TF*, however, resulted in induction of both *LAMC1* and *LAMB1* (Fig. 4B,C). Thus, both COUP-TFI and COUP-TFII are sufficient to induce the expression of these genes coding for subunits of laminin-1.

3. Discussion

The up-regulation of laminin expression in PrE cells is necessary for the subsequent development of the embryo (Smyth et al., 1999; Murray and Edgar, 2000). In this report we show that the transcription factors *COUP-TFI* and *COUP-TFII* are dramatically up-regulated in the PrE cells of both E3.5 blastocysts and EBs prior to the expression of *LAMB1* and *LAMC1*, and furthermore, either COUP-TF is sufficient to induce the transcription of *LAMB1* and *LAMC1*.

Previous work has shown that *COUP-TFI-/-* mice die shortly after birth as a result of a defect in development of the glossopharyngeal nerve (Qiu et al., 1997), whereas *COUP-TFII -/-* mice die prior to E10 and have defects in cardiogenesis (Pereira et al., 1999). However, these proteins are very similar and it has been demonstrated that they are able to transactivate the same target genes (Power and Cereghini, 1996). Furthermore, the *COUP-TFs* are expressed in overlapping domains (Qiu et al., 1994), indicating that there is likely to be functional redundancy during development. Thus, the expression of both *COUP-TFI* and *COUP-TFII* in PrE cells and the ability of both gene products to induce laminin gene expression is consistent with the observation that knockout of either factor alone results in a later phenotype, rather than causing defective PrE cell differentiation.

Insight into PrE differentiation has been gained with F9 EC cells, which unlike ES cells, require RA to induce laminin expression and differentiation by activation of the Ras-MAP kinase pathway (Verheijen et al., 1999). However, the immediate early genes responsible for laminin expression that are induced by this pathway remain to be established. Our demonstration that *COUP-TFs* are up-regulated in PrE cells and induce expression of *LAMB1* and *LAMC1* suggests that these transcription factors might be immediate targets of the Ras-MAPK pathway. Earlier reports investigating the control of *LAMB1* and *vHNF1* expression have shown that despite the presence of a RA response element (RARE) in the 5' upstream

region of these two genes, neither is directly induced by RA (Wang and Gudas, 1988; Cereghini et al., 1992). However, *vHNFI* is transactivated by COUP-TFI and COUP-TFII (Power and Cereghini, 1996), which are known to be directly induced by RA in EC cells (Qiu et al., 1996). Thus, given that COUP-TFs can positively regulate transcription by binding to RAREs (Thomassin et al., 1996), then it is likely that COUP-TFs regulate *LAMB1* expression in PrE cells by binding to the RARE present in the promoter region of this gene. Although little is known about the regulation of *LAMCI*, it has been established that a critical element in the promoter is a GC rich region, which is a consensus binding site for SP1 (Levavasseur et al., 1996; Lietard et al., 1997). Recent work has shown that COUP-TFs can positively regulate transcription by binding to SP1 (Pipaon et al., 1999) indicating that this mechanism may operate to regulate *LAMC1* expression

It has recently been shown that activation of the Ras-MAPK pathway via the adaptor protein Grb2 is necessary for the expression of laminin by PrE cells (Cheng et al., 1998). Furthermore, the phenotype of *FGF-4-/-*, *FGFR-2-/-* and *Grb2-/-* embryos are strikingly similar (Feldman et al., 1995; Arman et al., 1998; Cheng et al., 1998), suggesting that the activation of FGFR-2 by FGF-4 might result in Grb2 binding to FGFR-2, which in turn activates the Ras-MAP kinase pathway necessary for PrE differentiation. While it remains to be established if the induction of *COUP-TFs* is involved in the Grb2-Ras-MAPK pathway (Cheng et al., 1998), it is interesting to note that both drk (downstream of receptor kinase, the homologue of Grb2) and seven-up (the homologue of COUP-TF1) are involved in specifying the fate of the outer photoreceptors R1/R6 and R3/R4 in the *drosophila* eye (Begemann et al., 1995). However, because both FGF-4 and FGFR-2 are ubiquitously expressed by blastocyst and ES cells (Niswander and Martin, 1992; Arman et al., 1998), then additional mechanisms must operate so that laminin expression is selectively activated in the peripheral cells of the inner cell mass and EBs derived from ES cells. One distinguishing feature of the peripheral

cells is their polarization. Thus, the restricted distribution of E-cadherin to the baso-lateral domains of the peripheral cells and consequent changes in their cytoskeleton (Kadokawa et al., 1989) may affect activation of the pathway (for example, see (Iwasaki et al., 2000).

In conclusion, our results show that COUP-TF I and II are both expressed in primitive endodermal cells and that they can both regulate the expression of genes coding for laminin subunits. Thus, this redundancy of expression of the COUP-TFs indicates that they play a hitherto unexpectedly early role in the control of basement membrane deposition and hence are important for subsequent embryonic development.

4. Experimental procedures

4.1. Cell culture

Undifferentiated ES cells were cultured in the presence of LIF (Gibco-BRL) on mitomycin-treated STO feeder cells in gelatinized tissue culture dishes as previously described (Smyth et al., 1999). To make EBs, ES cells were allowed to aggregate in the absence of LIF by suspension culture in bacterial petri dishes (Murray and Edgar, 2000). For in situ hybridization, EBs were fixed for 1 h with 4% (wt/vol) paraformaldehyde and dehydrated through a series of graded methanols and stored at –20°C. For immunostaining, EBs were fixed for 1 h with 4% (wt/vol) paraformaldehyde, soaked in 15% (wt/vol) sucrose overnight at 4°C and then incubated in 7.5% (wt/vol) gelatin:15% (wt/vol) sucrose for 1 h at 37°C. Aliquots were pipetted onto gelatin blocks and allowed to set at room temperature before mounting in OCT cryofixative (Dako), cooled by liquid nitrogen-isopentane and storage at –80°C. For transmission electron microscopy and toluidine blue staining, EBs were fixed for 1 h in 2% (wt/vol) glutaraldehyde: 4% (wt/vol) paraformaldehyde and prepared as described previously (Robinson, 1982).

4.2. Mouse embryos

Embryos were obtained by mating random-bred CD1 mice. Noon on the day of plug was taken as 0.5 days postcoitum (p.c.). For whole-mount in situ hybridization, blastocysts were flushed from the uterus at 3.5 days p.c., washed in PBS, fixed for 15 min with 4% (wt/vol) paraformaldehyde, washed three times in PBS and dehydrated through a series of graded methanols before storage at –20°C.

4.3. Immunostaining

Ten μm cryostat sections were stained with rabbit anti-EHS laminin antisera (1/5000) which recognizes all three subunits of laminin type-1 (Smyth et al., 1999). The secondary antibody was TRITC-conjugated swine anti-rabbit IgG at 1/100 dilution (Dako). The sections were mounted in fluorescent mounting medium (Dako) and photographed using a Leitz RM22 fluorescent microscope. Attached ES cells were permeabilised by inclusion of 0.1% (vol/vol) Triton X100 in all solutions.

4.4. Whole-mount in situ hybridization

The cDNA used as templates for probe production was reverse transcribed from total RNA extracted from day 2 EBs. 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, annealing temperature 57^{o}C. Nucleotides 431-1051 of mouse *COUP-TFII* cDNA (Qiu et al., 1994) were amplified with GCAAGAGCTTCTTCAAGCG and GCTTCTCCACTTGCTCTTGG, annealing temperature 54^{o}C. The PCR fragments were cloned into the T-Easy vector (Promega) and transcribed with T7 or SP6 RNA polymerases using digoxigenin-UTP (Boehringer Mannheim) for sense or antisense probes. The 903bp *Bgl*II-*Eco*RI fragment of mouse *LAMB1* cDNA (Barlow et al., 1984) was sub-cloned into the *Bam*H1-*Eco*RI sites of pGEM1 (Promega) and transcribed with T7 or SP6 RNA polymerases using digoxigenin-UTP (Boehringer Mannheim) for sense or antisense probes, respectively. Whole-mount in situ hybridization was performed as previously described (Leibl et al., 1999). For EBs, frozen sections were prepared for analysis and photographed using a Leitz RM22 microscope. Embryos were analyzed and photographed in whole-mount.

4.5. Reverse transcription-PCR

Total RNA was extracted using guanidinium isothiocyanate and reverse transcribed using Superscript ^{TM} II (GIBCO BRL). Primers for *COUP-TFI* and *COUP-TFII* were those used for riboprobe synthesis (see above). *LAMB1* primers were GCAGACACAACACCAAAGGC and TGTACCCATCACAGATCCCG; product size 344bp, annealing temperature 56°C. *LAMC1* primers were CAGCCTTGTCCCTGCC and AGGCTTTGCATTTGTCG; product size 339bp, annealing temperature 56°C. *GAPDH* primers were GGTGAAGGTCGGAGTCAACGG and GGTCATGAGTCCTTCCACGAT; product size 520bp, annealing temperature 54^{o}C. Semi-quantitative RT-PCR was performed as previously described to determine mRNA levels relative to that of *GAPDH* (Squitti et al., 1999).

4.6. Transient transfections

Mouse STO cells were maintained in DMEM (Gibco-BRL) containing 10% (wt/vol) fetal bovine serum (Gibco-BRL). One day before transfection, cells were plated at 50% confluency on gelatinized 3.5 cm tissue culture dishes. Cells were transfected overnight 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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Figures

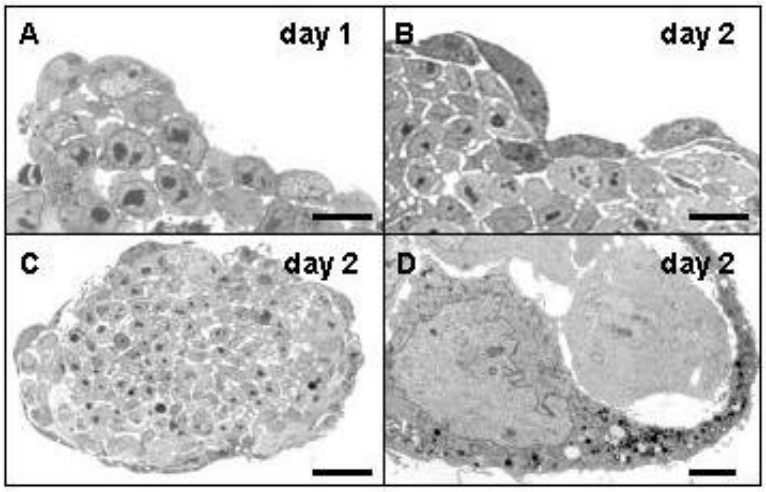

Fig. 1. The differentiation of primitive endoderm cells in EBs. (*A*-*C*) Toluidine blue-staining of resin-embedded sections shows that at day 1, peripheral cells are indistinguishable form the core cells of the EB (*A*). By day 2, peripheral cells have acquired the characteristic morphology of PrE cells (*B*), and form a layer of darker cells at the surface (*C*). (*D*) Transmission electron microscopy shows that the peripheral cells of day 2 EBs have the ultrastructural characteristics of PrE cells; note the electron dense secretory vesicles. Bars: 10 μm (*A*); 15 μm (*B*); 30 μm (*C*); 1.5 μm (*D*).

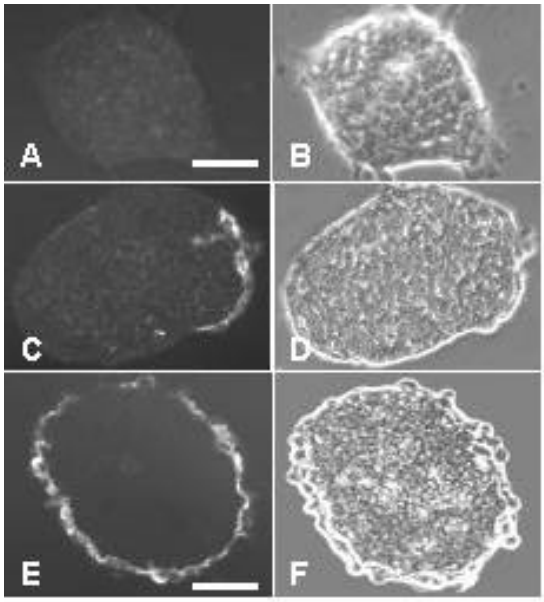

Fig. 2. Expression of laminin-1 by the primitive endoderm cells of differentiating EBs. (*A, C, E*) Immunofluorescence staining with anti-laminin-1 polyclonal antibody: (*A*) undifferentiated ES cells grown for 2 days on gelatinized tissue culture plastic; (*C*) section of day 2 EB showing laminin expression in some of the peripheral cells; (*E*) section of day 4 EB showing the presence of a sheet-like BM between the outer endoderm and inner core cells of the EB. (*B, D, F*) Bright-field images of (*A, C, E*), respectively. Bars: 50 μm (*A-D*); 60 μm (*E, F*).

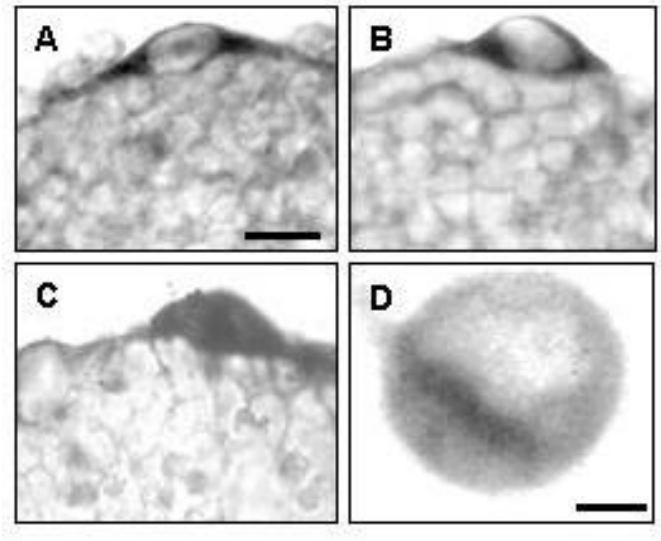

Fig. 3. Expression of *COUP-TFs* in primitive endoderm cells. Whole-mount in situ hybridization. (*A-C*) Day 2 EBs hybridized with *COUP-TF1* probe (*A*), *COUP-TFII* probe (*B*), *LAMB1* probe (*C*), shows expression of all three genes in cells with the characteristic spindle-shaped morphology of PrE cells. (*D*) Whole-mount image of E3.5 mouse blastocyst hybridized with *COUP-TFI* probe shows expression in the PrE cells, which are positioned at the surface of the inner cell mass. Bars: 10 μm (*A-C*); 40 μm (*D*).

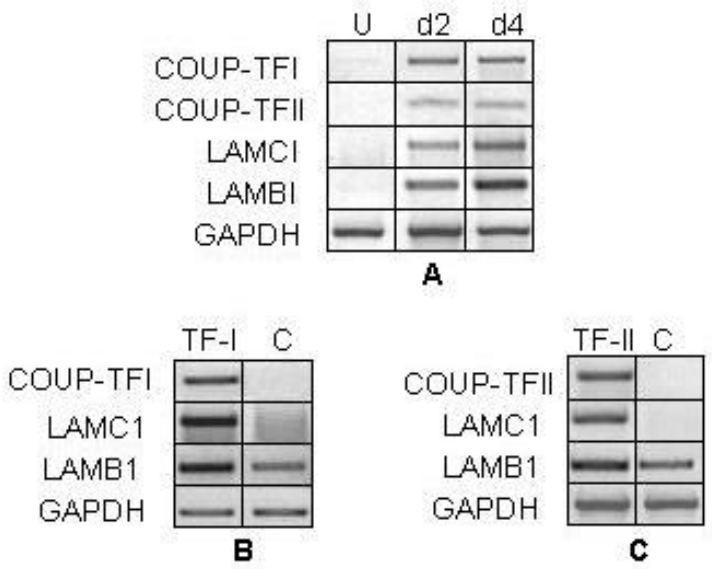

Fig. 4. RT-PCR analysis of *COUP-TFs*, *LAMB1* and *LAMC1* expression. (*A*) undifferentiated ES cells (U), day 2 and day 4 EBs; *GAPDH* is shown as a loading control. (*B*) Cells transfected with *COUP-TFI* (TF-I) or *GFP* control vector (C), shows induction of *LAMB1* and *LAMC1* by *COUP-TFI*; (*C*) RT-PCR analysis of cells transfected with *COUP-TFII* (TF-II) or *GFP* control vector (C), shows induction of *LAMB1* and *LAMC1* by *COUP-TFII*. *GAPDH* is shown as a loading control.