# Distinct GATA6- and laminin-dependent mechanisms regulate endodermal and ectodermal embryonic stem cell fates

**Li Li[1], Esther Arman[1], Peter Ekblom[2], David Edgar[3], Patricia Murray[3] and Peter Lonai[1,*]**

[1]Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel
[2]Department of Cell and Molecular Biology, Lund University, Box 94, S-221 00, Lund, Sweden
[3]Department of Human Anatomy and Cell Biology, University of Liverpool, Liverpool L69 3G3E, UK
*Author for correspondence (e-mail: peter.lonai@weizmann.ac.il)

*Accepted 20 August 2004*

Development 131, 5277-5286
Published by The Company of Biologists 2004
doi:10.1242/dev.01415

## Summary

**This study investigates the establishment of alternative cell fates during embryoid body differentiation when ES cells diverge into two epithelia simulating the pre-gastrulation endoderm and ectoderm. We report that endoderm differentiation and endoderm-specific gene expression, such as expression of laminin 1 subunits, is controlled by GATA6 induced by FGF. Subsequently, differentiation of the non-polar primitive ectoderm into columnar epithelium of the epiblast is induced by laminin 1. Using GATA6 transformed *Lamc1*-null endoderm-like cells, we demonstrate that laminin 1 exhibited by the basement membrane induces epiblast differentiation and cavitation by cell-to-matrix/matrix-to-cell interactions that are similar to the in vivo crosstalk in the early embryo. Pharmacological and dominant-negative inhibitors reveal that the cell shape change of epiblast differentiation requires ROCK, the Rho kinase. We also show that pluripotent ES cells display laminin receptors; hence, these stem cells may serve as target for columnar ectoderm differentiation. Laminin is not bound by endoderm derivatives; therefore, the sub-endodermal basement membrane is anchored selectively to the ectoderm, conveying polarity to its assembly and to the differentiation induced by it. Unique to these interactions is their flow through two cell layers connected by laminin 1 and their involvement in the differentiation of two epithelia from the same stem cell pool: one into endoderm controlled by FGF and GATA6; and the other into epiblast regulated by laminin 1 and Rho kinase.**

Key words: FGF signalling, GATA6, GATA4, Basement membrane, Polarization, Early development, ES cells

## Introduction

The inner cell mass (ICM) of preimplantation and early postimplantation mammalian embryos contain cells ancestral to the entire individual, which undergo extensive morphological change prior to gastrulation. In the blastocyst and early egg cylinder the ICM consists of an aggregate of non-polar stem cells, which before gastrulation undergo epithelialization and cavitation, creating a pseudostratified columnar epithelium that surrounds a central cavity similar to the proamniotic canal of the early embryo (Coucouvanis and Martin, 1995). The pseudostratified columnar epithelium or epiblast attaches to the sub-endodermal basement membrane (BM). This polarized epithelium allows intermingling of clonal derivatives and is thought to be necessary for gastrulation (Coucouvanis and Martin, 1995; Gardner and Cockroft, 1998). Much is known about the role of endoderm to ectoderm signalling in anteroposterior patterning of the early embryo (Beddington and Robertson, 1998). The establishment of major elements of the amniote body plan during gastrulation has been also studied in detail (Tam and Behringer, 1997). However, the mechanism that precedes these changes and transforms the non-polar primitive ectoderm into the columnar polar epiblast is little understood.

Embryonic stem (ES) cell derived embryoid bodies (EBs) are similar to the egg cylinder embryo, but, in contrast to it, they can be grown in large quantities, providing a useful model for early embryogenesis. In their classical paper, Coucouvanis and Martin set out the mechanism of EB differentiation as a model for pregastrulation development and tube formation by cavitation. EBs have an external endoderm that is similar to the primitive or visceral endoderm of the embryo and is separated from the inner columnar ectoderm by a basement membrane (BM). Using a genetically undefined spontaneous mutation, which fails to form the columnar ectoderm layer, it was proposed that cavitation is regulated by two signals: one emanating from the outer endoderm layer was thought to be responsible for the apoptotic signal/s of cavitation; the second, originating in the BM, was considered necessary for the maintenance and survival of the columnar ectoderm (Coucouvanis and Martin, 1995).

The work to be described here started as a study of the role of FGF signalling in EB differentiation and lead to questions regarding BM assembly that were investigated using ES cells that express truncated Fgfr2 cDNA as a dominant-negative mutation (Chen et al., 2000). We reported that ES cells expressing dnFgfr fail to develop the two characteristic cell layers of the EB. They display a homogenous aggregate of non-polar cells and form no endoderm or ectoderm-like elements, but survive for weeks during cultivation (Chen et al., 2000).

We observed that EBs formed by dnFgfr ES cells fail to synthesize laminin and collagen IV isotypes, which supply the protein network of the BM. Co-cultivating wild-type and dnFgfr ES cells rescued EB differentiation, suggesting that an FGF-controlled extracellular substance, subsequently identified as laminin 1, is required for epiblast differentiation. Exogenously added laminin 1 partially rescued the EB phenotype and induced epithelial transformation, demonstrating that laminin 1 produced by the endoderm (Hogan, 1980; Leivo et al., 1980) is necessary and sufficient to induce epiblast polarization (Li et al., 2001a).

Other reports also demonstrated that laminin 1 is required for EB differentiation. Targeted disruption of β1-integrin, which inhibits laminin α1 synthesis, interferes with epiblast differentiation (Aumailley et al., 2000). Disruption of *Lamc1* encoding laminin γ1, one of the three polypeptides of the laminin 1 heterotrimer, leads to a similar phenotype (Smyth et al., 1999). Significantly, defective epiblast differentiation caused by loss of either gene was rescued by exogenously added laminin 1 (Murray and Edgar, 2000), which in turn could be inhibited by the E3 fragment of laminin α1 containing the heparin and sulfatide binding site of the LG4 globular domain of the laminin α1-chain (Li et al., 2002). Recognising the potential importance of these findings for understanding epithelial differentiation and early development, we assumed that it would help their analysis if we defined the succession and main intermediates of EB differentiation.

In the present study, we aimed to obtain a comprehensive view of the developmental interactions that precede gastrulation. To achieve this, several specific questions had to be answered. Is FGF signalling required for the differentiation of both epithelia and the pattern of their arrangement in the EB, or for only an initial step that is necessary for later events? Defective FGF signalling could be partially restored by exogenous laminin 1 (Li et al., 2001a). The next question is can the same effect be obtained by laminin 1 presented by the BM in a physiological cell-matrix interaction? It was also important to determine whether laminin affects the stem cell directly, or whether it activates precursors after they reached a specific stage of FGF dependent differentiation. To answer these questions, we used mutant and wild-type ES cell lines, and studied their behaviour as an effect of chemical inhibitors and co-cultivation experiments between mutant and wild-type cells.

## Materials and methods

### Cell culture and stable transfections

Wild-type ROSA 11 (R11) and AB.2.2 ES cells, as well as the R11-derived dnFgfr, 1C6 ES cell line have been described before (Chen et al., 2000). *Lamc1*$^{+/-}$ and *Lamc1*$^{-/-}$ ES cells have been described by Smyth et al. (Smyth et al., 1999). GATA6 or GATA4 cDNA controlled by the pCAG-IP promoter were gifts from Fujikura et al., and the transfections were carried out as previously described (Fujikura et al., 2002). GATA6 cDNA was ligated also into the pEF-BOS plasmid, containing the EF-1α promoter (Mizushima and Nagata, 1990) and used to transform R11 and 1C6 cells with results similar to those with pCAG-IP. Dominant-negative ROCK, which is controlled by pEF-BOS, was a kind gift from S. Narumiya (Kyoto, Japan).

Embryoid bodies were cultured as described before (Chen et al., 2000). Briefly, ES cells grown on irradiated embryonic fibroblasts were harvested by trypsinization and plated on tissue culture plates for two consecutive 2 hour periods to remove feeder cells. They were then were incubated overnight, when the aggregates formed were removed and transferred to bacteriological plates to be grown as suspension without the addition of LIF. GATA6 and GATA4 transformed cells were grown in ES cell medium, without LIF. Medium was changed every second day. Mixed cultures between GATA transformed ES cells and dnFgfr or *Lamc1* mutant ES cells were prepared by mixing single cell suspensions. Cells from each cell line ($2\times10^6$) were mixed in a 9 cm bacteriological plate and grown as described before (Li et al., 2001a).

### Cytology

For morphological detail paraformaldehyde fixed JB4 plastic embedded 1-4 μm sections were stained with Toluidine Blue. For chimera experiments, the ES cell pellet was stained for β-galactosidase and the sections were counterstained with neutral red. Confocal analysis was as described before (Li et al., 2001a).

### Expression studies

Western blotting was as described before (Chen et al., 2000). Microarray data were from an unpublished study using Affymetrix MG-U74Av2 chips. Total RNA was isolated by the RNAzol B of Tel-Test from 1C6 dnFgfr and R11 wild-type embryoid bodies at culture day 0, 2 and 4. cRNA for hybridization and scanning was prepared as described by others (Clark et al., 2000). The following genes and accession numbers of the probe sets were used to detect them: *Gata4*, M98339; *Gata6*, U51335; *Rac1*, X57277; *Cdc42*, L78074; *RhoC*, X80638; *Fog1*, AF0066492; *HNF3b*, L10409; *Sox17*, D49473. Each analysis was repeated twice.

### Antibodies

Monoclonal antibody MAB200, which is specific for LG4 of laminin α1 was described before (Kadoya et al., 1995). Laminin 1 antibody was from Sigma (L9393); collagen IV antibody (AB756); antibody to perlecan (MAB #1948) and antibody to ZO1 (Mab 1520) were from Chemicon International. Antibodies to ROCKI and II were from BD Transduction Laboratories (611136 and 610623). Fluoresceinated phalloidin was from Sigma-Aldrich (P-1951); Cy-3- or FITC-labelled secondary antibodies were from Jackson ImmunoResearch Laboratories.

### Microscopy and image analysis

For bright-field images a Nikon E800 microscope with a Nikon DXM1200 digital camera and a 20× (N.A., 0.75) or a 40× (D.A., 0.95) objective was used. Confocal microscopy was in a Zeiss LSM5 instrument using a 20× (N.A., 0.5) or 40× (N.A., 1.20) objective. The images were processed by Photoshop 5.5 or 7.0.

## Results

### Endoderm differentiation is activated by GATA6 under FGF control

Previous results have suggested that differentiation of both epithelia of the EB may require FGF signalling (Chen et al., 2000). Now we wished to establish when is this effect initiated and whether it continues throughout EB differentiation. To do this, we inhibited FGF signalling using the pharmacological inhibitor SU5402 (Mohammadi et al., 1997). In our EB protocol, the feeder cells used to propagate ES cells are removed by adhesion to tissue culture plates. Then the ES cell aggregates are harvested and transferred to bacteriological plates to form EBs in suspension. The day of transfer to the bacteriological plate is defined as day 0. We found that SU5402 applied from day 0 until the end of the culture period strongly inhibited EB differentiation (Fig. 1A). To test whether FGF

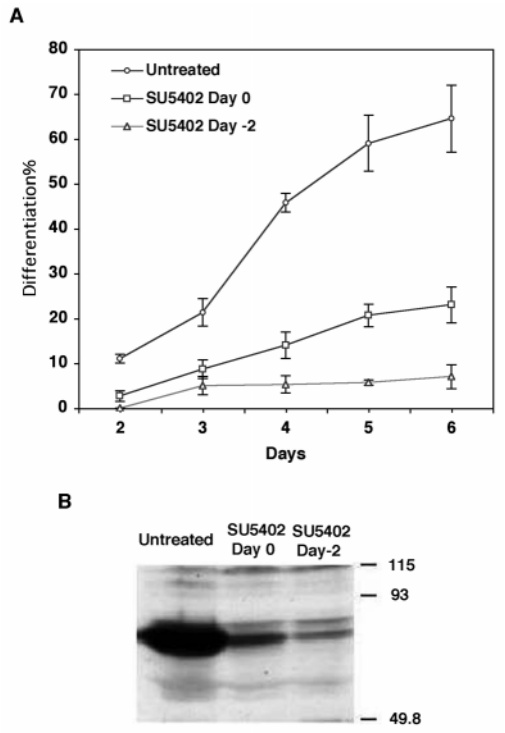


**Fig. 1.** FGF signalling is required for early EB differentiation. (A) EB differentiation is most sensitive to the Fgfr inhibitor SU5402 when added at the start or before (day –2) ES cell aggregation. (B) Western blot. α-Fetoprotein expression shows the effect of SU5402 on visceral endoderm differentiation. Differentiation was defined as the percent of EBs exhibiting two epithelia and cavitation, as seen in plastic-embedded Toluidine Blue stained sections.

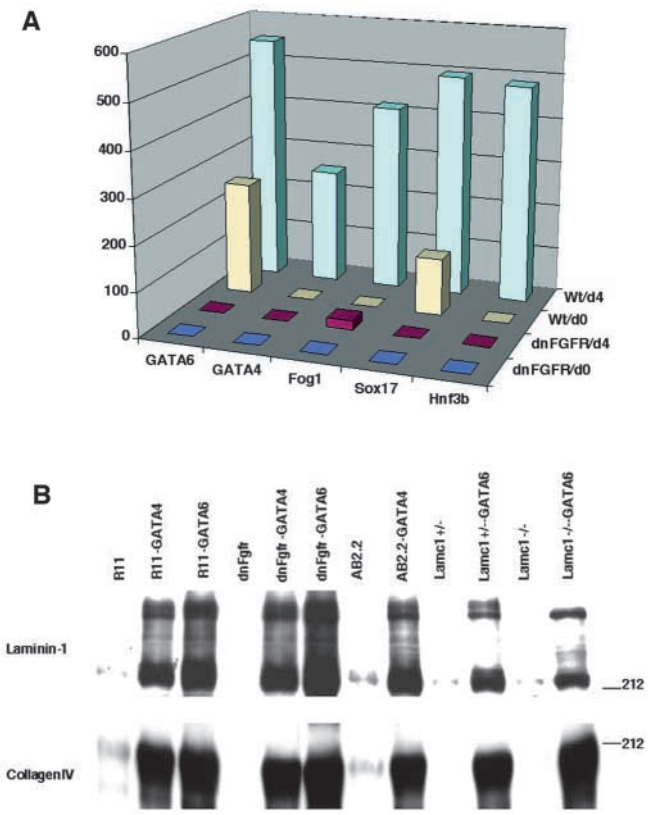


**Fig. 2.** GATA4 and GATA6 are under FGF control and overexpress BM proteins. (A) Effect of the dnFgfr mutation on Fgf, Fgfr and GATA factor expression; data from a DNA microarray experiment. Units are kDa. (B) GATA6 or GATA4 greatly enhances synthesis of laminin 1 and collagen IV even in dnFgfr and *Lamc1* mutant ES cells (western blot).

signalling is required even earlier, we studied the effect of SU5402 at day –2, i.e. well before the ES cells are harvested for EB differentiation. This early treatment resulted in even stronger inhibition of differentiation (Fig. 1A), which was also apparent in reduced expression of α-fetoprotein, a marker of the mature visceral endoderm (Fig. 1B). Hence, FGF signalling is required very early in EB development, prior to any overt differentiation of the ES cell aggregates.

Next, we investigated the expression of early endoderm genes as a function of FGF signalling. Comparing mRNA levels from a DNA microarray analysis (L.L. and P.L., unpublished) of wild-type and dnFgfr ES cell cultures revealed that loss of FGFR function inhibits a number of endoderm specific transcription factors. Expression of GATA4, GATA6, *Fog1* (a gene associated with GATA factors), *Sox17* and *Hnf3b*, were all strongly downregulated in the dnFgfr mutant compared with wild type (Fig. 2A).

Expression of GATA4 and GATA6 controlled by strong promoters induces endoderm-like differentiation and the transcription of numerous endoderm-specific genes, including laminin β1 (Fujikura et al., 2002). To determine whether the GATA factors behave as intermediaries of FGF signalling, we transfected control, *Lamc1*-null and heterozygous as well as dnFGFR ES cells with GATA4 or GATA6 cDNA controlled by the pCAGI (Fujikura et al., 2002) or pEF-BOS promoter (Mizushima and Nagata, 1990). Western blotting of extracts from these stable cell lines with antibodies to laminin 1 or type IV collagen revealed significant increase in the 400 kDa laminin α1 and in the 200 kDa laminin β1 and γ1 polypeptides, as well as in the close to 200 kDa collagen IV chains (Fig. 2B). Significantly, GATA6 transformed dnFgfr ES cells also responded with increased accumulation of laminin 1 and collagen IV (Fig. 2B) chains. It follows that once GATA4 or GATA6 is activated, FGF signalling is not required to induce laminin and type IV collagen synthesis, which indicates that GATA6 expression is downstream of FGF signalling, but once expressed it is sufficient to induce endoderm differentiation.

## GATA factor-transformed endoderm-like cells secrete BM proteins

Most GATA6 or GATA4 transformed ES cell clones displayed endoderm like epithelial morphology, although the periphery of larger colonies contained a few fat cell-like and neuron-like elements (not shown). The endoderm-like cells underwent further differentiation from primitive endoderm-like cells containing BM proteins in their cytoplasm to cells surrounded by extracellular BM components similar to the visceral endoderm. Staining with antibody to laminin α1 or collagen IV revealed that these proteins are retained in the cytoplasm during the first days of culture (Fig. 3A). Extracellular

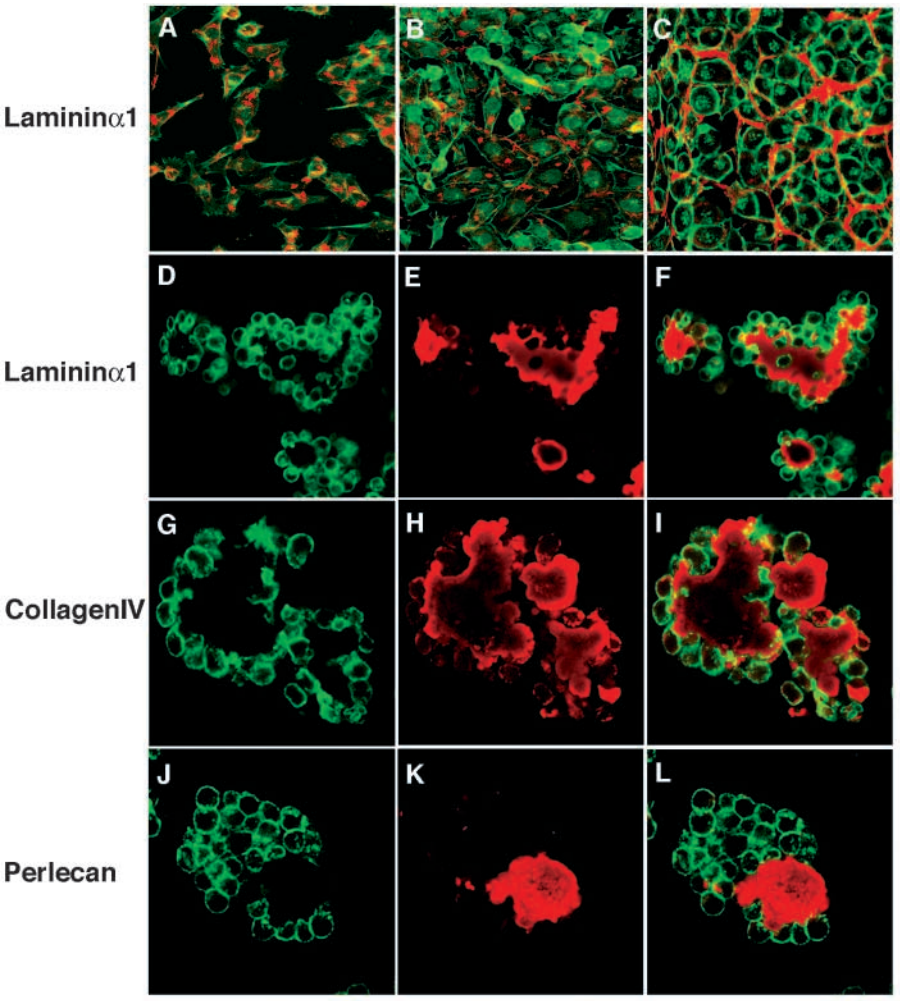


**Fig. 3.** GATA6 transfected endoderm-like derivatives of ROSA11 ES cells grown as adherent cultures (A-C) and as cysts in suspension cultures (D-L) (confocal images). (A-C) During the first day of culture, the cells accumulate laminin α1 in the cytoplasm (A), which, by day 3 (B) to 4 (C), is secreted to the extracellular space. (D,G,J) Fluoresceinated phalloidin; (E,H,K) BM proteins (Cy3), see left side for antibody used; (F,I,L) merged images.

secretion became apparent by the third day (Fig. 3B) and by the fourth day, the BM proteins formed a lattice surrounding groups of endoderm-like cells, which did not contain cytoplasmic laminin 1 or collagen IV (Fig. 3C) [although while both laminin and collagen IV could be detected in the culture supernatant by western blotting (not shown)]. Suspension cultures of GATA6-transformed endoderm-like cells grown on bacteriological dishes formed cysts, which after 3-4 days of incubation were filled with a mixture of laminin α1, collagen IV and perlecan (Fig. 3D-L). It follows that GATA6 transformed endoderm-like cells synthesize and secrete large amounts of multiple BM components. It is worth noting that the α1LG4-specific antibody, used to detect laminin α1, did not stain the surface of mature GATA4/6 transformed visceral endoderm-like cells (Fig. 3C,F), suggesting that these cell do not exhibit the appropriate laminin receptor or laminin anchorage site.

### Epithelial transformation of ES cells by a cell-to-matrix-matrix-to-cell interaction

Our data suggest that FGF signalling is required before overt endoderm differentiation, presumably to induce GATA6 expression and sub-endodermal BM deposition. It has been shown previously that exogenously added laminin 1 can induce epiblast epithelialization (Murray and Edgar, 2000; Li et al., 2001a). Considering the importance of this observation for understanding epiblast differentiation and epithelialization in general, we wished to gain direct evidence that the sub-endodermal basement membrane mediates the interaction leading to epiblast epithelialization.

1C6 dnFgfr ES cells expressing the β-galactosidase reporter were co-cultivated with β-galactosidase-negative GATA4-transformed endoderm-like cells of AB2.2 origin. After 5 days of culture, the EBs were fixed, stained for β-galactosidase, embedded in plastic and counterstained with neutral red (Fig. 4A-D). Wild-type AB2.2 cells developed into cystic EBs with external endoderm and inner columnar ectoderm (Fig. 4A). β-Galactosidase-positive cells of the dnFgfr mutant (1C6) line exhibited no signs of endoderm or ectoderm differentiation (Fig. 4B). The endoderm-like wild-type AB2.2-GATA4 cells, formed cysts bordered by a single endoderm layer with no obvious epiblast differentiation (Fig. 4C). By contrast, co-cultivation of 1C6 and AB2.2-GATA4 cells resulted in chimeric EBs with β-galactosidase-negative endoderm derived from the GATA transformed cell line and β-galactosidase-positive columnar ectoderm derived from the dnFgfr mutant 1C6 cell line (Fig. 4D).

Our results demonstrated that GATA-transformed ES cells can rescue epiblast differentiation, nevertheless we could not exclude if this was due to a product or products other than

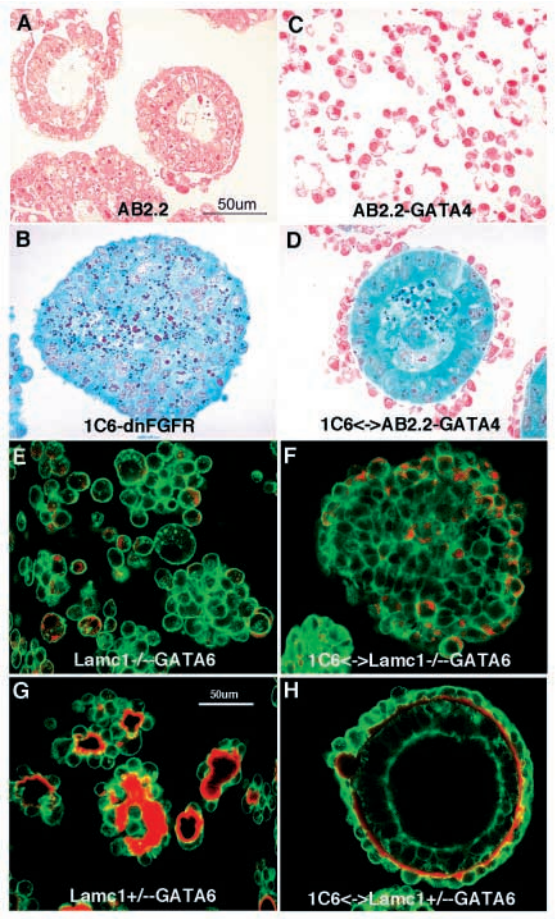


**Fig. 4.** Genetic evidence for the role of laminin 1 in the cell-to-matrix interaction required for epiblast polarization. Upon co-cultivation, GATA4 transformed endoderm-like ES cells rescue the dnFgr mutant (A-D). The GATA6 transferred homozygous *Lamc1* mutant, in contrast to the heterozygote (E-H), does not accumulate BM proteins and cannot rescue dnFgfr cells. (A-D) Plastic-embedded sections; (E-H) β-galactosidase and Neutral Red staining. (E-H) Whole-mount confocal microscopy: green, phalloidin (a marker for F-actin); red, laminin α1. (A) AB2.2, wild-type EBs; (B) dnFgfr EB expressing β-galactosidase; (C) GATA4 transformed AB2.2 cells; (D) co-cultivation of dnFgfr (1C6) ES cells with GATA4 transformed AB2.2. (E) GATA6-transformed *Lamc1*$^{-/-}$ cells. *Lamc1*$^{-/-}$ GATA6 cells form aggregates with minimal BM protein expression. (F) Co-cultivation of *Lamc1*$^{-/-}$ GATA6 cells with dnFgr cells does not lead to phenotypic rescue. (G) *Lamc1*$^{+/-}$ GATA6 cells form cysts containing BM proteins. (H) *Lamc1*$^{+/-}$ GATA6 cells rescue differentiation of 1C6. Scale bars: 50 μm.

laminin 1. A genetic test analyzed this problem. GATA6 transformed *Lamc1*$^{-/-}$ ES cells (Smyth et al., 1999) were co-cultured with dnFgfr mutant 1C6 cells (Fig. 4E-H). *Lamc1*$^{-/-}$ cells are unable to form a basement membrane (Smyth et al., 1999). In accordance with the mutant defect, these cells failed to rescue the dnFgfr mutant (Fig. 4E,F). By contrast, the heterozygote with one normal *Lamc1* allele did rescue 1C6 differentiation (Fig. 4G,H). Numerically, 70-80% of the wild type, but not more than 3% of 1C6 aggregates underwent differentiation. During co-cultivation with the GATA6 transformed *Lamc1* heterozygote 100% of the EB reached epiblast differentiation and cavitation, while GATA6 transformed *Lamc1*$^{-/-}$ cells produced less than 4% differentiation in their dnFgfr partner. It was also apparent from these results that epiblast differentiation is coupled with polarization of the newly formed epithelium. As Fig. 4H shows, the apical domain of the epiblast facing the central cavity exhibited strong F-actin accumulation, as demonstrated by increased phalloidin staining. We therefore conclude that GATA4/6-mediated FGF signalling is necessary and sufficient for laminin expression by endodermal cells, which can interact with the juxtaposed stem cells in a cell-to-matrix interaction to induce epithelialization and polarization of the epiblast.

## Pluripotent stem cells are the target for epiblast epithelialization

Pluripotent ES cells are induced directly to an endoderm-like phenotype by forced GATA4 or GATA6 expression (Fujikura et al., 2002). Although cystic embryoid bodies frequently contain non-polar stem cell-like elements, it was not clear whether the columnar ectoderm of the epiblast develops from the same stem cell pool that gives rise to the endoderm. To address this issue, we used dnFgfr ES cells of the 1C6 line. Although these cells do not undergo EB differentiation, when aggregated with four-cell stage wild-type embryos and transplanted into pseudopregnant females they developed into most cell types in the chimeric embryo (Fig. 5). When such dnFgfr ES cells were treated with 30-40% conditioned medium from GATA6 transformed *Lamc1*$^{+/-}$ cells, 70-80% of the resulting EBs differentiated into single cavitated columnar epithelia without an endoderm layer (Fig. 6A). By contrast, no differentiation could be observed when supernatants derived from GATA6 transformed *Lamc1*$^{-/-}$ cells were used (Fig. 6B). It follows that ectodermal epithelialization and cavitation is due to endodermal laminin 1 and its target cells are undifferentiated pluripotent ES cells.

EBs developing from GATA supernatant treated dnFgfr ES cells exhibit a columnar monolayer surrounding the central cavity and bind laminin 1 to their basal domain (Fig. 6A). This indicated that the columnar cells exhibit laminin receptors. To demonstrate whether laminin receptors are also displayed by undifferentiated ES cells, we first showed that ES cells do not synthesize laminin 1. Fig. 6C demonstrates no laminin α1 staining of ES cells to which no GATA6 cell supernatant was added, whereas its addition to the same cells was easily detected on the membrane of most if not all ES cells (Fig. 6D), indicating that undifferentiated ES cells exhibit laminin receptors necessary for BM-induced epithelialization of the primitive ectoderm.

## Endoderm and epiblast polarization follows distinct ROCK-dependent mechanisms

Both ectodermal and endodermal epithelialization of the EB involves extensive cytoskeletal rearrangements. To investigate this aspect of EB development, our attention was turned to the Rho family of small GTPases that are important mediators of cytoskeletal and cell shape changes (Etienne-Manneville and Hall, 2002). To obtain preliminary information, we inspected data from a DNA microarray experiment (L.L. and P.L., unpublished) that compared wild-type and dnFgfr EB cultures (Fig. 7A). All three major members of the Rho family were detected in the developing EB. Rac1 and Cdc42 transcripts

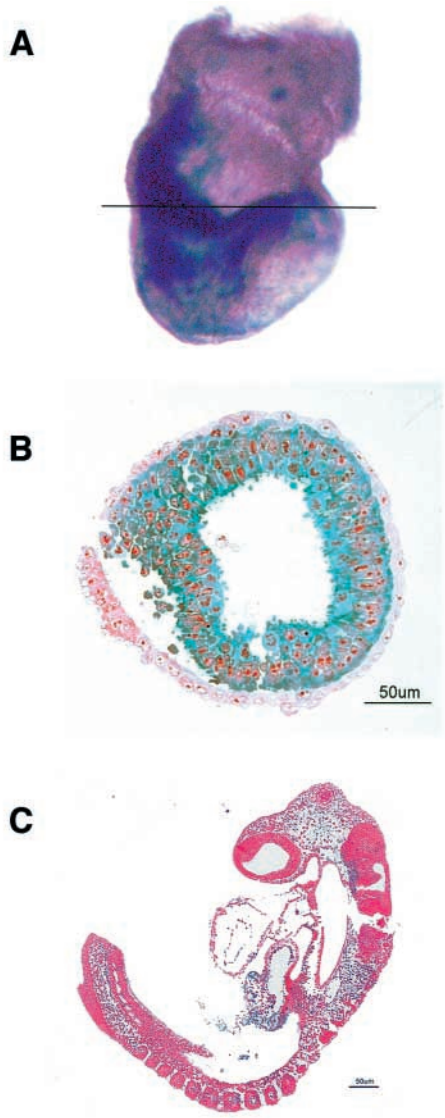


**Fig. 5.** Cells of the dnFgfr ES cell line1C6 colonize most lineages of the early embryo when introduced as chimera aggregated with wild-type cells. (A-C) β-Galactosidase staining indicates derivatives of the 1C6 line. (A) 7.5 dpc embryo, whole mount. (B) 7.5 dpc cross-section at the level of line in A. (C) 9.5 dpc embryo sagittal section. (B,C) Plastic embedded sections. Scale bars: 50 μm.

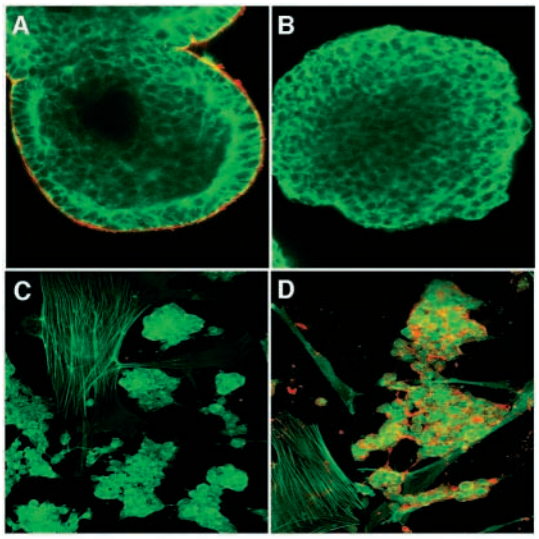


**Fig. 6.** Pluripotent stem cells and their ectoderm derivatives exhibit laminin receptors. (A-D) Confocal images. (A) Day 4, 1C6 dnFgfr ES cells activated with *Lamc1*$^{+/-}$ GATA6 culture supernatant. Stained with antibody to laminin α1. The single layer of columnar cells covered by laminin α1 (red). (B) 1C6 dnFgfr ES cells incubated with *Lamc1*$^{-/-}$ GATA6 culture supernatant harvested between day four and day five of culture and stained with antibody to laminin α1. Differentiation and laminin α1 staining are both absent. (C) Wild-type ROSA11 ES cells on feeder layer stained with antibody to laminin α1 and phalloidin (green). See fibroblast in left upper quarter. There is a lack of laminin staining. (D) Wild-type ROSA11 ES cells on feeder layer treated with ROSA11-GATA6 culture supernatant for 6 hours and stained with antibody to laminin α1 (red) and phalloidin (green). There is laminin deposition in the membrane of all ES cells.

were displayed at similar levels in undifferentiated ES cells and during EB development, showing no obvious dependence on FGF signalling. By contrast, the C isotype of Rho was most prominently expressed in the wild type with its peak at day 4, when epiblast polarization takes place (Fig. 7A).

To test the involvement of *RhoC*, we inhibited ROCK, the Rho kinase, which mediates the function of Rho isotypes (Maekawa et al., 1999). Two pharmacological inhibitors, Y-27632 (Ishizaki et al., 2000) and H-1152 (Ikenoya et al., 2002), and an ES cell line stably expressing a Rho-binding negative construct (dnROCK) (Ishizaki et al., 1997) were used. Both the pharmacological inhibitors and dominant-negative ROCK inhibited epiblast differentiation without affecting endoderm development. Transmission electron microscopy revealed that although the wild-type epiblast formed well-organized columnar ectoderm (Fig. 7B), Y-27632-treated EBs exhibited disorganized unconnected stem cells in the interior of the EB (Fig. 7E), while the external endoderm layer seemed to be intact, as in the untreated control. Adhesion structures were evident in the endoderm (Fig. 7C) and untreated ectoderm (Fig. 7D), but in treated cultures only the endoderm displayed such connections (Fig. 7F).

Immunofluorescence analysis revealed the familiar structure of wild-type cavitated EBs with robust F-actin accumulation at the apical domain of the columnar cells (Fig. 8A). The morphology of defective ES cell differentiation induced by ROCK inhibitors (Fig. 8B-D) could be separated into two groups. They exhibited either an interior aggregate of round non-polar stem cells (Fig. 8B,C), or if any cavitation took place, non-polar, non-columnar cells surrounded the cavity and no significant F-actin accumulation could be observed in their apical domain (Fig. 8B,D). In this latter group cavitation could be observed without epithelialization. Both variants displayed well-formed subendodermal BMs as detected by antibodies to laminin α1 (Fig. 8A,B), collagen IV (Fig. 8C) or perlecan (not shown). According to these findings, ROCK is required for epiblast polarization, but is not essential for endoderm differentiation. This assumption was supported by similar expressions of the tight junction protein ZO-1 characteristic for the endoderm of the EB, in the lateral aspect of both wild type

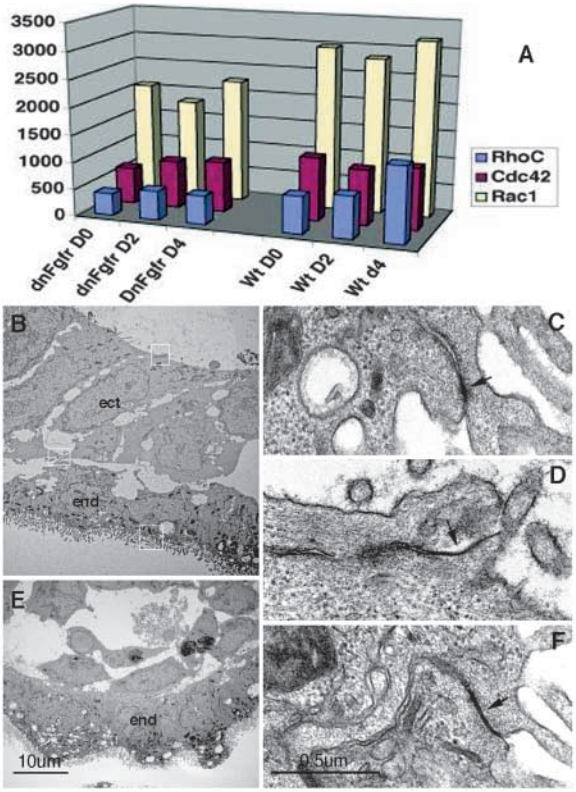


**Fig. 7.** RhoC and ROCK are involved in epiblast differentiation. (A) Results of DNA microarray analysis reveals that Rac1 and Cdc42 are expressed throughout EB differentiation and may not be dependent on FGF signalling. However, expression of RhoC is higher in the wild type than in the dnFgfr mutant and has its maximum at day 4, when epiblast differentiation is at its peak. (B-F) Transcription electronmicroscopy. (B-D) Wild type. (B) Overview; (C) adhesion structure in endoderm; (D) adhesion structure in the epiblast. (E,F) Y-27632 treatment of wild type. (E) Overview; (F) adhesion structure in endoderm. Abbreviations: end, endoderm; ect, ectoderm or epiblast. Scale bars: in E, 10 μm for B,E; in F, 0.5 μm for C,D,F. Arrows in C,D,F indicate adhesion structures. White square in B indicates the position of the adhesion structure in D.

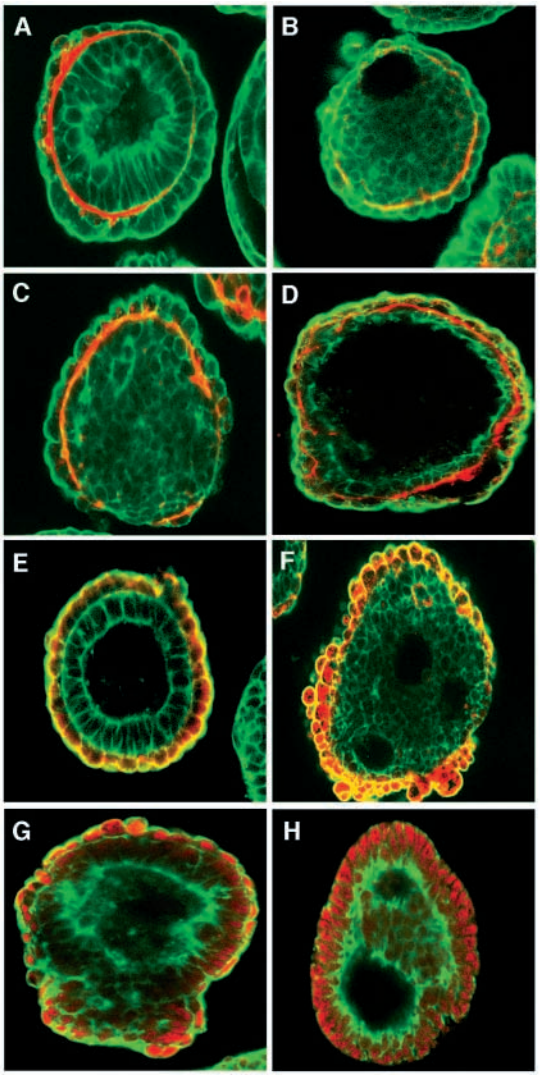


**Fig. 8.** ROCK activity is required for epiblast differentiation and polarization. Confocal microscopy. Green, phalloidin staining detecting F-actin. Red, specific protein signal. (A) Wild-type (untreated) BM is detected by antibody to laminin α1. There is F-actin accumulation in the apical domain of the epiblast. (B) Y-27632 treatment, note the lack of columnar ectoderm and partial cavitation without F-actin accumulation. BM detected by laminin α1 antibody. (C) H-1152 treatment, antibody to collagen IV, (D) EB formed by ES cells expressing dnROCKII. Note flat internal cells without significant F-actin accumulation in the apical domain. (E,F) ZO-1 staining. The pattern is similar in the wild-type endoderm (E) and in H-1152-treated EB (F). (G,H) ROCKII staining. (G) Wild type; (H) EB formed by dnFgfr derived GATA supernatant treated ES cells. There is F-actin accumulation in the epical epiblast domain and a lack of endoderm with strong ROCKII staining in H.

(Fig. 8E) and H1152-treated endoderm (Fig. 8F). It follows that the cytoskeletal rearrangement of the endoderm and ectoderm is under separate and distinct control.

Further insight to the separation of ROCK function in the two epithelia was obtained by studying its expression. ROCK I (not shown) and ROCK II were strongly expressed in the endoderm but less in the epiblast (Fig. 8G). Separation of ROCK function in the two epithelia was analysed using GATA cell supernatant activated EBs, which exhibit no endoderm differentiation. Fig. 8H shows that the columnar epithelium of these modified EBs displayed strong ROCK II expression, suggesting that ROCK expression is controlled by separate mechanisms in the endoderm and ectoderm.

## Discussion

In this report, we have investigated the series of consecutive events that lead from primary ES cell aggregates to cavitated EBs distinguished by an extra-embryonic endoderm-like outer and a columnar, epiblast-like inner epithelium. As an experimental system to elucidate interactions between the endoderm and primitive ectoderm we used GATA4- or GATA6-transformed endoderm-like cells co-cultivated with mutant ES cell lines. This system demonstrated that GATA4 and GATA6 transform ES cells into functional extra-embryonic endoderm

that deposits a BM, which in turn mediates epiblast polarization. We found that GATA transformed cells synthesize and later secrete laminin 1 and collagen IV into the culture supernatant, which could be used to rescue epiblast differentiation. Genetic evidence of laminin γ1 null ES cells demonstrated the specificity of mutant rescue. This experimental system thus recreated the physiological BM-mediated interaction and permitted to separate endoderm and epiblast differentiation according to their respective FGF/GATA6 and laminin/Rho kinase-dependent mechanisms.

### Endoderm differentiation is induced by FGF and GATA4/6 signalling

Endoderm differentiation depends on FGF signalling, as demonstrated by the targeted disruption of *Fgf4* (Wilder et al., 1997). *Fgf4* is expressed in the ICM and contributes to the maintenance of the endoderm (Goldin and Papaioannou, 2003), where the multiple FGF receptors that read its signals are localized (Chen et al., 2000). Expression of GATA4 and GATA6, where GATA4 is regulated by GATA6, is controlled by FGF signalling (Morrisey et al., 1998). Nevertheless, the immediate downstream elements of FGF signalling are insufficiently understood in EB differentiation. In vitro evidence suggests that most FGF dependent signals go through *Frs2a*, a docking protein (Lax et al., 2002), which communicates with the *Grb2* adaptor. Interestingly although null mutants of *Fgf4* (Feldman et al., 1995) or *Grb2* (Cheng et al., 1998) die with defective endoderm development shortly after implantation, *Frs2a* null embryos survive till advanced gastrulation (Hadari et al., 2001), indicating that FGF signalling may exhibit unique characteristics in the early embryo. Analysis of signal transduction in dnFgfr ES cells revealed that PI3K-Akt/PKB rather than MAPK-ERK signalling is affected by defective FGF activity (Chen et al., 2000). In agreement, we also found that constitutively active Akt/PKB enhances endoderm development and the synthesis of laminin and collagen IV isotypes, indicating that the PI3K-Akt/PKB pathway predominates in FGF-dependent endoderm differentiation (Li et al., 2001b).

We showed here that GATA6 is an intermediary of FGF signalling. GATA6, which is transcribed already in the ICM (Koutsourakis et al., 1999), behaves as a master gene for endoderm differentiation (Fujikura et al., 2002). GATA6 activates the synthesis of all three polypeptide chains of laminin 1, which together with collagen IV, nidogen and perlecan assemble into the sub-endodermal BM. We found that GATA factors induce endoderm differentiation and BM assembly even in dnFgfr ES cells, indicating that once activated, these transcription factors induce endoderm differentiation independently from FGF signalling. Because endoderm differentiation requires GATA6 (Morrissey et al., 1998) and because cysts of GATA6 transformed cells contain only endoderm-like elements, we conclude that GATA factors are required and sufficient to induce endoderm development and deposition of the subendodermal BM.

Additional elements of this pathway are the transcription factors COUP-TFs I and II, which are upregulated by GATA4/6 during endoderm development (Fujikura et al., 2002) and induce *Lamc1* and *Lamb1* expression (Murray and Edgar, 2001). It follows that minimal elements of this interaction are, sequentially, *Fgf4* (Wilder et al., 1997), multiple Fgfr (Chen et al., 2000), PI3K and AKT/PKB (Chen et al., 2000; Li et al., 2001b), GATA6 and GATA4 (Fujikura et al., 2002), COUP-TFs I and II (Murray and Edgar, 2001), as well as the genes encoding the three polypeptide chains of laminin 1.

Evidence mainly from the Kemler laboratory demonstrates that E-cadherin is also required for early EB differentiation. E-cadherin-null ES cells fail to aggregate, do not form a normal ectoderm and do not undergo EB differentiation (Larue et al., 1994). Therefore, E-cadherin-dependent ES cell aggregation may be a prerequisite for the restriction of FGF signalling to the outer cells of the developing EB. E-cadherin is connected to the β-catenin-GSK3-wnt pathway (Huber et al., 1996). Patterning events involving cadherin-Wnt/β-catenin interactions have been shown to be controlled by FGF signalling (Ciruna and Rossant, 2001; Kawakami et al., 2001).

### Directional cell-to-matrix, matrix-to-cell signalling activates epiblast polarization

There is strong evidence for the epithelialization of ES cells by exogenous laminin 1 (Murray and Edgar, 2000; Li et al., 2001a; Li et al., 2002). Here, we demonstrate that laminin 1 can induce epiblast differentiation as part of the BM that mediates the physiological interaction of the endoderm with the epiblast. We also show that while laminin 1 binds to ES cells and their ectodermal derivatives, it does not associate with the primitive endoderm. Thus, the cell-binding domains of the laminin α1 chain determine the location of the subendodermal BM by interacting with their receptors displayed by the stem cells localized below the endoderm layer (Li et al., 2002). This therefore defines the direction of laminin-mediated signalling, thereby determining the topographical relationship of endoderm and ectoderm.

Besides inducing epiblast polarization, the BM affects the simple two-cell layer pattern of the EB and egg cylinder embryo. As cell-to-matrix interactions take place through direct contact, epithelialization of residual stem cells is precluded, and a single epiblast monolayer develops from cells immediately adjacent to the BM. It has been proposed that the residual stem cells are removed by programmed cell death induced by factors derived from the endoderm, to form a central cavity (Coucouvanis and Martin, 1995). Investigation of the role of BMP signalling in cavitation indicated that BMP2 synthesized in the endoderm, and BMP4 in the primitive ectoderm can both contribute to cavitation, although BMP4 is expressed only for a short period (Coucouvanis and Martin, 1999). Our data indicate that cavitation and columnar ectoderm differentiation does not require the endoderm, provided that exogenous laminin 1 is presented. It is therefore possible that the developing ectoderm itself secretes the necessary apoptotic factors, such as BMP4, although inhibition of ROCK activity uncouples cavitation from full epithelialization of the primitive ectoderm and argues that cavitation may be either not different from necrosis, or it might be due to mechanical separation of the columnar ectoderm from the residual stem cells. This issue requires further study.

### Rho kinase is required for epiblast polarization

Dominant-negative ROCK abolishes epiblast polarization without affecting endoderm differentiation, suggesting that it may be regulated separately in the two cell lineages. This assumption was supported by observing that ROCK expression

and epiblast polarization does not require the endoderm for the laminin-induced differentiation of dnFgfr ES cells. Although ROCK is required for the epithelialization of the primitive ectoderm, it is not sufficient to induce this process, as suggested by our observation that dominant-active ROCK does not rescue dnFgfr differentiation (L.L. and P.L., unpublished). Although in the epiblast ROCK activity may be induced by laminin, in the endoderm it appears to be under FGF control and the resistance of endodermal differentiation to ROCK inhibition is consistent with the possibility that RAC1 or Cdc42, which are co-expressed in the endoderm, may have a role in endodermal differentiation.

Separation of endoderm and epiblast differentiation has been repeatedly observed in this study. We show that FGF signalling is required for endoderm differentiation but not for epiblast polarization, which is independently induced by laminin 1 of the sub-endodermal BM. The two lineages are also distinguished by laminin binding. ES cells and their ectodermal derivatives bind laminin, while the primitive and visceral endoderm do not, which defines the direction of laminin-induced differentiation. It follows that the extra-embryonic and embryonic epithelium of the EB and egg cylinder embryo develop by distinct mechanisms, which are connected by the inductive activity of the laminin component of their common BM. Future research will have to clarify whether other epithelial transitions are also controlled by laminin-dependent mechanisms.

We thank Drs Masayuki Yamamoto for permitting to use his GATA cDNAs and Dr Hitoshi Niwa for providing these cDNAs in the pCAG-IP vector. We also thank Prof. S. Narumiya for the dominant-negative ROCK construct. This study was in part supported by the Israel Science Fund administered by the Israel Academy of Science (to P.L.).

## References

**Aumailley, M., Pesch, M., Tunggal, L., Gaill, F. and Fassler, R.** (2000). Altered synthesis of laminin 1 and absence of basement membrane component deposition in β1 integrin-deficient embryoid bodies. *J. Cell Sci.* **113**, 259-268.

**Beddington, R. S. and Robertson, E. J.** (1998). Anterior patterning in mouse. *Trends Genet.* **14**, 277-284.

**Chen, Y., Li, X., Eswarakumar, V. P., Seger, R. and Lonai, P.** (2000). Fibroblast growth factor (FGF) signaling through PI 3-kinase and Akt/PKB is required for embryoid body differentiation. *Oncogene* **19**, 3750-3756.

**Cheng, A. M., Saxton, T. M., Sakai, R., Kulkarni, S., Mbamalu, G., Vogel, W., Tortorice, C. G., Cardiff, R. D., Cross, J. C., Muller, W. J. et al.** (1998). Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. *Cell* **95**, 793-803.

**Ciruna, B. and Rossant, J.** (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev. Cell* **1**, 37-49.

**Clark, E. A., Golub, T. R., Lander, E. S. and Hynes, R. O.** (2000). Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406**, 532-535.

**Coucouvanis, A. and Martin, G. R.** (1995). Signals for death and survival, a two step mechanism for cavitation in the vertebrate embryo. *Cell* **83**, 279-287.

**Coucouvanis, E. and Martin, G. R.** (1999). BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. *Development* **126**, 535-546.

**Etienne-Manneville, S. and Hall, A.** (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.

**Feldman, B., Poueymirou, W., Papaioannou, V. E., DeChiare, T. M. and Goldfarb, M.** (1995). Requirement of FGF4 for postimplantation mouse development. *Science* **267**, 246-249.

**Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki-Ji, J. and Niwa, H.** (2002). Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev.* **16**, 784-789.

**Gardner, R. L. and Cockroft, D. L.** (1998). Complete dissipation of coherent clonal growth occurs before gastrulation in mouse epiblast. *Development* **125**, 2397-2402.

**Goldin, S. N. and Papaioannou, V. E.** (2003). Paracrine action of FGF4 during periimplantation development maintains trophectoderm and primitive endoderm *Genesis* **36**, 40-47.

**Hadari, Y. R., Gotoh, N., Lax, I. and Schlessinger, J.** (2001). Critical role of FRS2 alpha in FGF receptor signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **98**, 8578-8583.

**Hogan, B. L. M.** (1980). High molecular weight extracellular proteins synthesized by endoderm cells derived from mouse teratocarcinoma cells and normal extraembryonic membranes. *Dev. Biol.* **76**, 275-285.

**Huber, O., Bierkamp, C. and Kemler, R.** (1996). Cadherins and catenins in development. *Curr. Opin. Cell Biol.* **8**, 685-691.

**Ikenoya, M., Hidaka, H., Hosoya, T., Suzuki, M., Yamamoto, N. and Sasaki, Y.** (2002). Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. *J. Neurochem.* **81**, 9-16.

**Ishizaki, T., Naito, M., Fujisawa, K., Maekawa, M., Watanabe, N., Saito, Y. and Narumiya, S.** (1997). p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.* **404**, 118-124.

**Ishizaki, T., Uehata, M., Tamechika, I., Keel, J., Nonomura, K., Maekawa, M. and Narumiya, S.** (2000). Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol. Pharmacol.* **57**, 976-983.

**Kadoya, Y., Kadoya, K., Durbeej, M., Holmvall, K., Sorokin, L. and Ekblom, P.** (1995). Antibodies against domain E3 of laminin-1 and integrin α6 subunit perturb branching epithelial morphogenesis of the embryonic submandibular gland, but by different modes. *J. Cell Biol.* **129**, 521-534.

**Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C. and Izpisua Belmonte, J. C.** (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* **104**, 891-900.

**Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F.** (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126**, 723-732.

**Larue, L., Ohsugi, M., Hirchenhain, J. and Kemler, R.** (1994). E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc. Natl. Acad. Sci. USA* **91**, 8263-8267.

**Lax, I., Wong, A., Lamothe, B., Lee, A., Frost, A., Hawes, J. and Schlessinger, J.** (2002). The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. *Mol. Cell* **10**, 709-719.

**Leivo, I., Vaheri, A., Timpl, R. and Wartiovaara, J.** (1980). Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* **76**, 100-114.

**Li, S., Harrison, D., Carbonetto, S., Fassler, R., Smyth, N., Edgar, D. and Yurchenco, P. D.** (2002). Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation. *J. Cell Biol.* **157**, 1279-1290.

**Li, X., Chen, Y., Scheele, S., Arman, E., Haffner-Krausz, R., Ekblom, P. and Lonai, P.** (2001a). FGF signaling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body. *J. Cell Biol.* **153**, 811-822.

**Li, X., Talts, U., Talts, J. F., Arman, E., Ekblom, P. and Lonai, P.** (2001b). Akt/PKB regulates laminin and collagen IV isotypes of the basement membrane. *Proc. Natl. Acad. Sci. USA* **98**, 14416-14421.

**Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. and Narumiya, S.** (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* **285**, 895-898.

**Mizushima, S. and Nagata, S.** (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**, 5322.

**Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J.** (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.

**Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S.** (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579-3590.

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

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

**Smyth, N., Vatansever, H. 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.

**Tam, P. P. L. and Behringer, R. R.** (1997). Mouse gastrulation, the formation of a mammalian body plan. *Mech. Dev.* **68**, 3-25.

**Wilder, P. J., Kelly, D., Brigman, K., Peterson, C. L., Nowling, T., Gao, Q. S., McComb, R. D., Capecchi, M. R. and Rizzino, A.** (1997). Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. *Dev. Biol.* **192**, 614-629.