

Fibroblast Growth Factor Signaling and Basement Membrane Assembly Are Connected during Epithelial Morphogenesis of the Embryoid Body

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Abstract. Fibroblast growth factors and receptors are intimately connected to the extracellular matrix by their affinity to heparan sulfate proteoglycans. They mediate multiple processes during embryonic development and adult life. In this study, embryonic stem cell-derived embryoid bodies were used to model fibroblast growth factor signaling during early epithelial morphogenesis. To avoid redundancy caused by multiple receptors, we employed a dominant negative mutation of *Fgfr2*. Mutant-derived embryoid bodies failed to form endoderm, ectoderm, and basement membrane and did not cavitate. However, in mixed cultures they displayed complete differentiation induced by extracellular products of the normal cell. Evidence will be presented here that at least one of these products is the basement membrane or factors connected to it. It will be shown that in the mutant, collagen IV and

laminin-1 synthesis is coordinately suppressed. We will demonstrate that the basement membrane is required for embryoid body differentiation by rescuing columnar ectoderm differentiation and cavitation in the mutant by externally added basement membrane proteins. This treatment induced transcription of *Eomesodermin*, an early developmental gene, suggesting that purified basement membrane proteins can activate inherent developmental programs. Our results provide a new paradigm for the role of fibroblast growth factor signaling in basement membrane formation and epithelial differentiation.

Key words: FGF signaling • basement membrane • epithelial differentiation • early development • embryoid bodies

Introduction

Fibroblast growth factors (FGFs)¹ and their tyrosine kinase receptors (FGFR) are involved in multiple physiological and pathological processes. They are important throughout embryonic development as well as in angiogenesis and wound healing. FGFR mutations cause defective bone and craniofacial development and are associated with cancer (Wilkie, 1997; Cappellen et al., 1999). A common denominator of these multiple activities links the FGF system with the extracellular matrix (ECM) and with epithelial mesenchymal interactions.

Various FGF isotypes bind heparan sulfate proteoglycans (HSPGs) of the ECM and basement membrane (BM). A molecular mechanism for these interactions was revealed by crystallographic evidence, showing that FGFR dimers and their ligands form a specific groove for HSPG binding. This triple complex of FGF, FGFR, and HSPG represent the FGF signaling unit (Plotnikov et al., 1999). Additional important signaling molecules, such as *wnt* and *hedgehog*, also have affinity to HSPGs (for a review see Perrimon and Bernfield, 2000). Therefore, the HSPG-bearing ECM and BM may serve as platforms for growth factor signaling.

Localization and binding specificity of FGF and FGFR indicate that they signal across cell sheets separated by the BM. Splice variants of *Fgfr2* and other FGFR loci display specific localization. So-called “b-type” splicing alternatives are expressed in epithelia, whereas “c-type” receptors are expressed in the mesenchyme (Orr-Urtreger et al., 1993). Most FGF are bound either by b or by c type receptors (Ornitz et al., 1996). Due to their localized expression

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¹Abbreviations used in this paper: BM, basement membrane; ECM, extracellular matrix; ES, embryonic stem; FGF, fibroblast growth factor; HSPG, heparan sulfate proteoglycan; *mEomes*, mouse *Eomesodermin*; RT, reverse transcription.

and binding specificity, epithelial FGFRs interact with mesenchymal ligands, whereas mesenchymal receptors recognize epithelial FGFs. This coordination between transcriptional localization and binding specificity may have developed to promote epithelial mesenchymal interactions.

Genetic evidence for FGF-mediated epithelial mesenchymal interactions was obtained in limb development. The b variant of *Fgfr2* is expressed in the epithelium of the apical ectodermal ridge, whereas its ligand, Fgf10, in the progress zone mesenchyme and its loss of function mutations display similar phenotypes (Arman et al., 1999; Seckine et al., 1999; De Moerlooze et al., 2000). Additional functional connections of the FGF system with the ECM are associated with morphogenic cell migration. FGFR homologues are required for trachea and mesoderm migration in *Drosophila* (Beiman et al., 1996) and for sex myoblast migration in *Caenorhabditis elegans* (DeVore et al., 1995), whereas in the gastrulating mouse embryos, *Fgfr1* is responsible for cell migration through the primitive streak (Ciruna et al., 1997). Further molecular connection between FGFRs and the ECM was based on the aggregation of integrins with FGFR at sites of intracellular phosphorylation (Miyamoto et al., 1996).

A series of data on epithelial differentiation join these findings and argue for a functional role of the BM. Evidence derives from differentiation induction by BM components (for a review see Ashkenas et al., 1996), from inhibition of differentiation by antibodies specific to BM proteins and their receptors (Klein et al., 1988; Durbeek et al., 1995; Kadoya et al., 1995; Schuger et al., 1995) and from the targeted mutagenesis of genes encoding them (Fassler et al., 1995; Stephens et al., 1995; Williamson et al., 1997; Murray and Edgar, 2000).

We were interested in a further exploration of the link between FGF signaling and the ECM. It was recently demonstrated that truncated *Fgfr2* cDNA expressed in embryonic stem (ES) cells inhibits their differentiation and abrogates FGF signaling through the phosphatidylinositol (PI)-3 kinase–Akt/PKB pathway (Chen et al., 2000). The experiments to be described here investigate the cellular mechanism of this dominant negative mutation. We will demonstrate that ES cells expressing the truncated *Fgfr2* cDNA can recognize an extracellular differentiation signal produced by wild-type cells. We will also show that loss of FGFR function abrogates laminin-1 and collagen IV synthesis and that externally added laminin-1 or Matrigel can rescue ectoderm differentiation and cavitation. Our data collectively suggest that FGF signaling contributes to the regulation of BM formation. To our knowledge, these data suggest a previously unrecognized type of connection between FGF signaling and the ECM and offer testable paradigms for the mechanism of epithelial morphogenesis.

Materials and Methods

Embryoid Bodies

Mutant ES cell clones were derived from the ROSA11 line, expressing the β -geo cassette. As a control, ROSA11, or its ancestor the AB2.2 ES cell line (a gift of A. Bradley, Anderson Cancer Center, Houston, Texas) that has no reporter, were cultured as described previously (Chen et al., 2000). In brief, undifferentiated stem cells were seeded first on tissue culture plates for 24 h to remove residual feeder cells. The cell clumps formed

were then detached by pipetting and transferred to bacteriological dishes. The day of transfer of primary aggregates was denominated as day 0.

Dominant Negative Mutation

Fgfr2 cDNA was truncated downstream of the transmembrane domain (from nucleotide 650 to 2,069) and was controlled by the EF-1a promoter. Details of the construct and selection of high expressing clones was as described (Chen et al., 2000).

Teratomas

129/Pas mice were injected subcutaneously with 5×10^6 wild-type or mutant ES cells. Teratocarcinomas were dissected after 2–3 wk of growth, when their diameter reached 0.5–1.5 cm.

Cytology and Histology

For semithin sections, embryoid bodies were washed twice in PBS, fixed in 4% paraformaldehyde at 4°C overnight, and after dehydration in ethanol were embedded in JB-4 resin (Polysciences, Inc.). 1–4- μ m sections were cut with a glass knife. For cytology, the sections were stained with toluidine blue. In cell mixing experiments, the embryoid bodies were prefixed and stained for β -galactosidase and the sections were counterstained with neutral red. Teratocarcinomas were fixed in Bouin fixative, embedded in Paraplast, and the sections were stained with hematoxylin and eosin. Microphotography was with a ZEISS Axiomat microscope. Films were scanned and figures were prepared with Photoshop 5.5 software.

Treatment with Externally Added BM Proteins

Matrigel (growth factor reduced; Becton Dickinson) was added in solution for the first day of culture. Matrigel, which in general is used as a concentrated semisolid gel, was here used as a 4% (vol/vol) solution with the floating ES cell aggregates. Purified laminin-1–nidogen complex (a gift of Dr. R. Timpl, Max-Planck Institute for Biochemistry, Martinsried, Germany) was added to the cultures for the first day in a concentration of 50–100 μ g/ml. Collagen IV was from Sigma-Aldrich (C0543). It was used at a final concentration of 3.0 μ g/ml that approximates its concentration in Matrigel.

Antibodies

The MAB371 dystroglycan specific antibody was a gift from Chemicon International. Antibody specific to fibronectin was from Sigma-Aldrich (F3648), to integrin- α 6 and integrin- β 1 from BD PharMingen (MAB GoH3 and CD29, respectively), to perlecan from Chemicon International (MAB no. 1948), and to collagen IV from Chemicon International (AB756P). The nidogen-1 antibody was a gift of Dr. R. Timpl (Martinsried, Germany). The laminin- α 1–specific Mab200 antibody was described previously (Kadoya et al., 1995). Cy-3–labeled secondary antibodies were from Jackson ImmunoResearch Laboratories. Fluoresceinated phalloidin was from Sigma-Aldrich (P1951).

Confocal Analysis

Embryoid bodies were washed twice in PBS and fixed in cold 4% paraformaldehyde for 20 min at room temperature. After washing twice with PBS, fixed embryoid bodies were permeabilized with 1% Triton X-100 and 10% BSA in PBS for 10 min at room temperature, followed by repeated washing in PBS. Antibodies were diluted in PBS and 3% BSA. The same solution was used for blocking (30 min). The primary antibody was added in microdrops for 1 h, followed by five washes in PBS containing 3% BSA. Incubation with Cy-3–labeled secondary antibodies or with fluoresceinated phalloidin was for 30 min, followed by five washes. Stained embryoid bodies were pipetted into a well cut into a Perspex microscope slide that was covered at one side by a coverslip. Mineral oil was layered on the suspension and the embryoid bodies were observed, as whole mounts, in a Bio-Rad Laboratories 1024 confocal inverted microscope. Each embryoid body was inspected through multiple optical sections.

RNA Blot Hybridization

Embryoid bodies or ES cells were recovered from culture and washed in PBS. Lysis and RNA extraction was with the RNeasy kit (QIAGEN). 10 μ g total RNA was denatured in glyoxal and separated on a denaturing gel. Posthybridization wash was in $0.1 \times$ SSC at 68°C. The following DNA

probes were used: for laminin- α 1, a 532-bp fragment (residues 313–835), and for collagen α 1 (IV), a 400-bp fragment (residues 3,254–3,653) was synthesized by reverse transcription (RT)-PCR. Laminin- β 1, dystroglycan, fibronectin, perlecan, and nidogen-1 probes have been used as described (Ekblom et al., 1990; Ibraghimov-Breskovnaya et al., 1992; Talts et al., 1995; Costell et al., 1999).

In Situ Hybridization

Nonradioactive in situ hybridization was according to established methods (Conlon and Herrmann, 1992) with small modifications. In brief, embryoid bodies were collected, washed in PBS, and fixed in cold 4% paraformaldehyde for 2 h. After short dehydration in methanol, they were stored at -20°C . The reaction was started by rehydration to increase permeability. Protease treatment for vHNF1 and mouse *Eomesodermin* (*mEomes*) was with 2 $\mu\text{g}/\text{ml}$ proteinase K for 10 min. For in situ hybridization with vHNF (Barbacci et al., 1999), a cDNA fragment (residues 948–1,354) was synthesized by RT-PCR. The cDNA probe for *Brachyury* was from B. Herrmann (MPI for Immunobiology, Freiburg, Germany), for *ptc* from M. Scott (Stanford University, Stanford, CA), for *Gli-1* from A. Joyner (NYU Medical Center, New York, NY), for *cripto-1* from A. Simeone (International Institute of Genetics and Biophysics, Naples, Italy), for BMP4 and BMP2 from G. Martin (University of California, San Francisco, San Francisco, CA), and for *mEomes* from J. Rossant (Lumenfeld Institute, Toronto, Ontario, Canada). Hybridization conditions, such as the duration of protease treatment, dehydration, and hybridization with “sense” transcripts, were fine-tuned on wild-type embryoid bodies before the detailed experiment. After color reaction, embryoid bodies were quickly dehydrated in ethanol and embedded in plastic. 5–10- μm sections were counterstained with neutral red.

RT-PCR

Total RNA was isolated from ES cells or embryoid bodies with RNazol B (Tel-Test, Inc.). RT-PCR was performed with the Titan RT-PCR System (Boehringer). Denaturation was for 30 s at 94°C , annealing for 30 s at 50 – 60°C , and synthesis for 1 min at 72°C . Primers, annealing temperature, and cycles for β -HI globin were: AGTCCCATGGAGTCAAAGA and CTAAGGAGACCTTTGCTCA with 40 cycles at 55°C ; for Flk1, GTG-GATCTGAAAAGACGC and CATTCTTCTCCGATAGG with 40 cycles at 50°C ; and for kit ligand (KL), GACTGTGTGCTCTCTTCAAC and CTTGCAAAACCTCCAGAGTC with 45 cycles at 50°C .

Results

Truncated *Fgfr2* cDNA Abrogates Differentiation of Both Cell Layers of the Embryoid Body

In a previous study, *Fgfr2* cDNA, truncated downstream of the transmembrane exon and controlled by the EF-1a promoter, was introduced into ROSA11 ES cells that contain the β -galactosidase reporter (Chen et al., 2000). Wild-type cells developed into cystic embryoid bodies (Fig. 1 A), whereas ES cell clones expressing the truncated, dominant negative FGFR cDNA formed compact bodies that failed to cavitate (Fig. 1 B). To obtain morphological detail, embryoid bodies were embedded in plastic and toluidine blue-stained semithin sections were prepared. Wild-type embryoid bodies displayed two cell layers (Fig. 1 C). A visceral endoderm-like polar surface epithelium covered the internal epiblast-like columnar ectoderm and the two cell layers, separated by a BM, surrounded a well-defined cavity (Fig. 1 D). At the start of differentiation, both wild-type and mutant ES cells formed aggregates. The aggregates expanded during the first 2 d of culture, and on the surface of the wild-type embryoid body endoderm appeared on the third day. Columnar ectoderm and the first signs of cavitation manifested at late day 3 or 4, and cavitation became complete by day 6. After aggregates have been formed, mutant-derived embryoid bodies ceased

to differentiate further and did not form columnar ectoderm (Fig. 1, E and F). Mutant embryoid bodies grew and survived for 6 or 8 d, after which they declined. Larger mutant embryoid bodies developed necrotic foci, but no cavitation took place (Fig. 1 F).

As a molecular parameter of visceral endoderm differentiation, we assayed vHNF1 transcription by in situ hybridization. Variant HNF1 is an early visceral endoderm marker (Barbacci et al., 1999) that was transcribed by wild-type, but not by mutant-derived embryoid bodies (Fig. 2, A and B). This result joined previous data on additional visceral endoderm markers, such as HNF4, *Evx1*, and α -fetoprotein, which were also suppressed in our mutant (Chen et al., 2000).

Mature cystic embryoid bodies undergo embryonic hemopoiesis and vasculogenesis (Risau et al., 1988). We investigated whether the dominant negative FGFR mutant will undergo this line of differentiation. RT-PCR analysis revealed that neither embryonic globin nor *Flk-1*, or stem cell factor (*kit* ligand), were transcribed in the mutant, whereas they were copiously expressed by the wild-type (Fig. 2 C). It follows that morphologically undifferentiated mutant embryoid bodies cannot contribute to gastrulation-associated gene expression.

To investigate whether the dominant negative mutation has in vivo effects, ES cells were transplanted subcutaneously into strain 129 mice. Wild-type ES cells formed teratomas containing cartilage (Fig. 3 A), polar epithelium (Fig. 3 B), and muscle (Fig. 3 C), whereas the mutant grew into undifferentiated tumors, save a few scattered epithelial vessels (Fig. 3 D). It follows that defective FGF signaling deeply abrogates ES cell differentiation both in vitro and in vivo.

The Mutant Can Recognize a Differentiation Factor, but Cannot Produce It

We prepared mixed cultures to investigate the cellular mechanism of our mutation. This arrangement allowed testing whether the mutant defect resides within the mutant cell or outside of it in products secreted by its wild-type neighbors. Single cell suspensions of various ES cell lines were mixed at the onset of culture. Two wild-type ES cell lines, the β -galactosidase-positive ROSA11 (Fig. 4 A) and the β -galactosidase-negative AB2.2 clone (Fig. 4 B), and one mutant cell line (Fig. 4 C), the β -galactosidase-positive, 1C3 clone (Chen et al., 2000), were used. We made two kinds of chimeric embryoid bodies. As control, the two wild-type cell lines colonized both cell layers to similar extent (Fig. 4 D). In the experimental chimera, which contained β -galactosidase-labeled mutant and unlabeled wild-type cells, the mutant colonized both cell layers and mutant-derived fragments were detectable in the central, apoptotic cavity (Fig. 4 E). Almost 80% of the experimental embryoid bodies contained differentiated mutant cells (Table I). It follows that the mutant, if it receives an extracellular signal from its wild-type neighbors, can differentiate into both endoderm and ectoderm and participates in apoptotic cavitation.

To investigate the radius of action of this extracellular signal (or signals), wild-type and mutant ES cells were grown separately until primary aggregates formed and

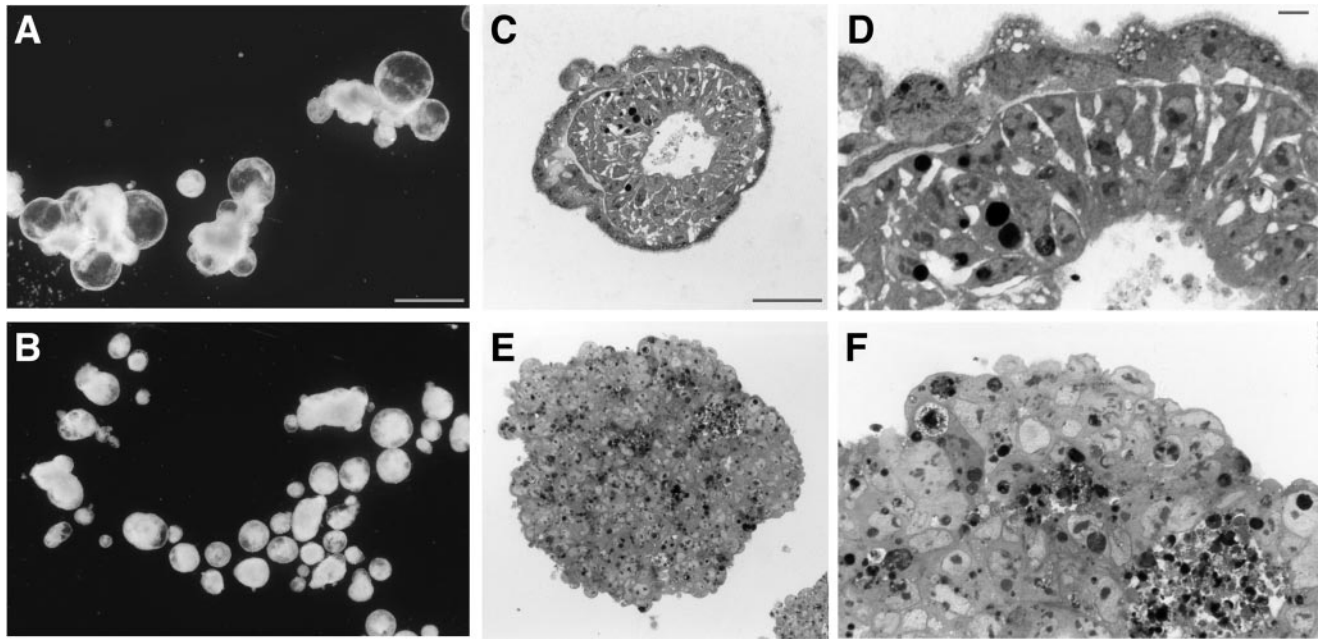


Figure 1. The dominant negative FGFR mutation abrogates embryoid body differentiation. Cellular morphology of embryoid bodies. Comparison between wild-type (A, C, and D) and mutant (B, E, and F). (A and B) Stereomicroscope view; (C–F) semithin sections. The mutant does not undergo ectoderm and endoderm differentiation and does not cavitate. Bars: (A and B) 75 μm ; (C–F) 25 μm .

only then were mixed and incubated for five more days. This arrangement limited the contact between wild-type and mutant cells. Counting fused aggregates revealed that only a third contained a few scattered differentiated mutant cells (Fig. 4 F and Table II). We conclude that to be effective, the extracellular factor had to be close to the mutant cell or make actual contact with it.

These results are interpreted to suggest that the mechanism of the dominant negative FGFR mutation is “non-cell autonomous.” That is, the mutant cannot produce, but can recognize an extracellular signal that is secreted by the wild-type. We assumed that this factor is a cell adhesion structure, or an element of ECM. First, E-cadherin, an adhesion receptor of embryonic cells, was investigated. E-cadherin is required for compaction of the mouse morula and for ES cell aggregation at the onset of embryoid body differentiation (Larue et al., 1994). However, there was no significant difference in E-cadherin expression between wild-type and mutant. Next, we sought indications for the involvement of the integrin family and investigated the activation of focal adhesion kinase (FAK), one of the cytoplasmic protein kinases of the integrin pathway (Schlaepfer and Hunter, 1998). When no significant difference was found, we decided to explore whether the dominant negative mutation affects the BM.

Table I. The Dominant Negative Mutant Cannot Make, but Can Recognize a Differentiation Factor Produced by the Wild-Type

Mixed embryoid bodies	No. embryoid bodies counted	Percentage
With differentiated mutant cells	90	76.9
With undifferentiated mutant cells	27	23.1
Total	117	100.0

The FGFR Mutation Inhibits Laminin-1 and Collagen IV Expression

We first compared the localization and expression of individual BM-related proteins in wild-type and mutant embryoid bodies by confocal immunofluorescence microscopy. Three groups of BM-associated proteins were investigated. Among the receptors of collagen IV and laminin-1, dystroglycan (Fig. 5, A and B), integrin- $\alpha 6$ (Fig. 5, E and F), and integrin- $\beta 1$ were studied. BM-associated proteins were detected by antibodies to fibronectin (Fig. 5, C and D), nidogen-1 (Fig. 5, G and H), or perlecan (Fig. 5, K and L). Network-forming proteins of the BM were visualized by anti-laminin- $\alpha 1$ (Fig. 5, I and J) or anti-collagen IV (Fig. 5, M and N). Morphological detail was supplied by fluoresceinated phalloidin, which detects the actin cytoskeleton.

Laminin- $\alpha 1$, nidogen-1, and fibronectin localized in the BM of wild-type embryoid bodies as early as the second day of culture (Fig. 5, O–R). These proteins, as well as collagen IV (Fig. 5 M) and perlecan (Fig. 5 K), concentrated

Table II. The Differentiation Signal Acts Across a Short Distance

Embryoid bodies containing mutant cells	Total	State of mutant cells in the embryoid body	
		Differentiated	Undifferentiated
Nonfused	145	0	145 (100%)
Fused	86	29 (33.7%)*	57 (66.3%)
Total	231		

Primary aggregates (5–20 cells) of mutant and wild-type ES cells were mixed and grown for 6 d and embryoid bodies containing LaZ-positive differentiated or undifferentiated cells were counted.

*Not >1–5 differentiated mutant cells were found in these fused embryoid bodies.

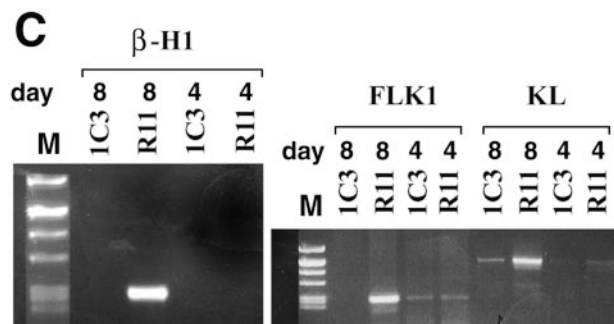
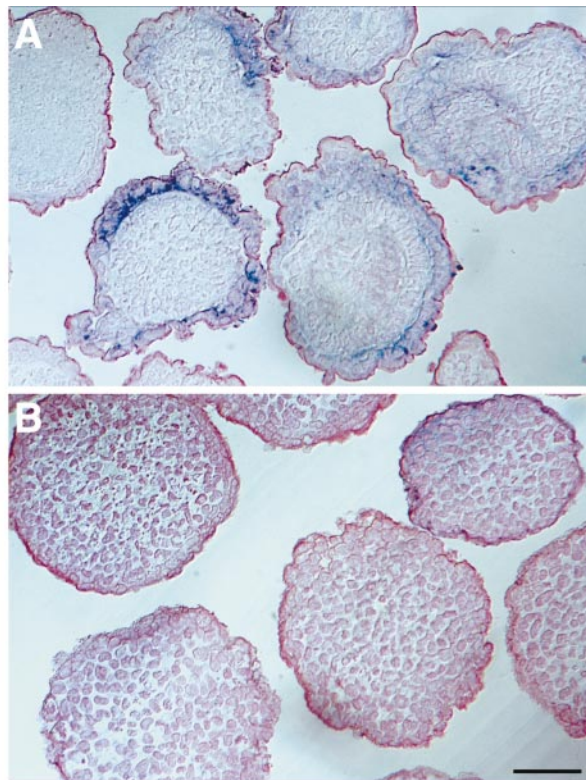


Figure 2. The dominant negative mutation abolishes lineage-specific markers. (A and B) In situ hybridization with vHNF1 (A, wild type; B, mutant). Bar: 50 μ m. Note the lack of vHNF1 transcription in the mutant. (C) RT-PCR analysis, β -H1 embryonic globin; Flk1, vascular endothelial growth factor (VEGF) receptor; KL, kit ligand; IC3, a dominant negative ES cell clone; R11, ROSA11 wild-type ES cells; M, marker. Note that mutant cultures transcribe these hemopoietic and angiogenic markers at very low levels.

in the BM, between the endoderm and ectoderm. Dystroglycan was detected both in the endoderm and ectoderm (Fig. 5 A). Integrin- α 6 localized exclusively to the ectoderm (Fig. 5 E), whereas integrin- β 1 was found in the surface endoderm (not shown). Hence, laminin receptors were present in both cell layers of the wild-type embryoid body. In contrast to wild-type, no BM could be detected in mutant embryoid bodies and the specific proteins, such as integrin- α 6 (Fig. 5 F), dystroglycan (Fig. 5 B), fibronectin (Fig. 5 D), nidogen-1 (Fig. 5 H), and perlecan (Fig. 5 K), were all distributed as a diffuse, punctuate label.

The central finding of this investigation was that two network-forming proteins of the BM, laminin- α 1 (Fig. 5 J) and collagen IV (Fig. 5 N), were either undetectable, or gave only weak signals in mutant-derived embryoid bodies. The results suggest that due to defective FGF signaling, the expression of laminin- α 1 and the collagen IV isoforms was selectively lost as detected by our antibodies, whereas several other BM-related proteins remained expressed, albeit in a disorganized manner. It follows that intact FGF signaling may be required for the expression of these essential network-forming components of the BM.

To obtain additional evidence, transcription of BM components was investigated by RNA blot hybridization. Perlecan, fibronectin, nidogen-1, and dystroglycan transcripts were detectable in both mutant and wild-type ES cells and embryoid bodies (Fig. 6). However, laminin- α 1, laminin- β 1, and collagen α 1 (IV) transcripts were absent in the mutant, or they were detectable only in greatly reduced amounts. It follows that loss of FGFR function leads to the coordinate loss of laminin-1 and collagen α 1 (IV) expression.

Partial Rescue of the Dominant Negative Mutation by Matrigel and Laminin-1

We showed above that the FGFR defect of embryoid body differentiation is non-cell autonomous and coincides with the loss of network-forming BM proteins. We next asked whether the BM, or purified BM proteins, are required for embryoid body differentiation. The answer to this question was sought in the effect of externally added BM proteins on wild-type and mutant embryoid bodies (Fig. 7, A and B).

First, growth factor-reduced Matrigel, a commercial preparation of BM proteins, was used. Matrigel at a concentration of 4% (vol/vol), was added for the first 24 h. After an additional 5 d, close to 70% of Matrigel-treated mutant embryoid bodies (25–80%, in eight different experiments) formed a continuous columnar ectoderm layer with normal, but somewhat smaller cavities than the wild-type (Fig. 7 C and Table III), whereas without Matrigel no differentiation took place (Fig. 7 A). Similar results, although at somewhat lower frequency, were obtained when 50–100 μ g/ml of a purified laminin-1–nidogen complex (Paulsson et al., 1987) was added to the cultures (Table III). Laminin-1–treated cells, similarly to Matrigel treatment, grew to embryoid bodies, displayed columnar ectoderm on their surface, and 15–30% underwent cavitation but developed no visceral endoderm (Fig. 7 E and Table III). In contrast to Matrigel and laminin-1 treatment, collagen IV had no effect (not shown).

The laminin-1 preparation used here is a highly purified protein fraction (Paulsson et al., 1987; Fässler et al., 1995), and the Matrigel used was depleted in growth factors. Nevertheless, to exclude the role of growth factor contamination, the mutant was treated with insulin, EGF, FGF2, and PDGF-BB in concentrations far exceeding possible contamination in both preparations (Table IV). However, this had no effect as judged by morphological criteria.

It was unexpected that both Matrigel and laminin-1–treated mutant embryoid bodies failed to form visceral endoderm (Fig. 7, C and E). As a control, wild-type cultures were also treated with Matrigel or laminin-1. The results

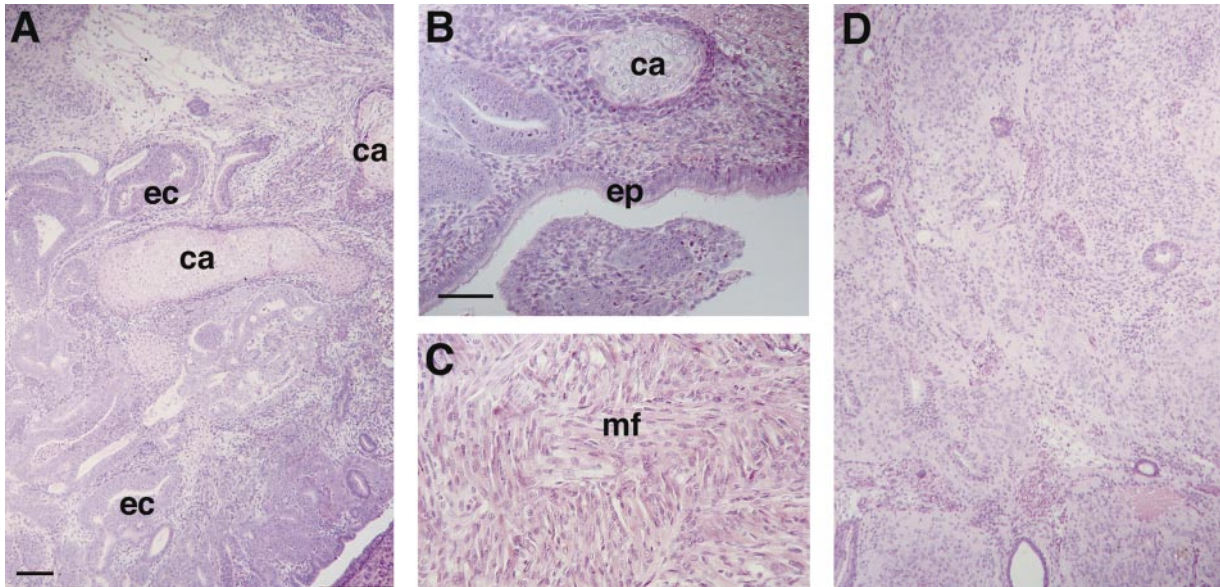


Figure 3. The FGFR mutation abrogates differentiation in vivo. (A–C) Wild-type and (D) mutant-derived teratomas. Bars: (A–D) 50 μm . Mutant teratomas undergo little or no differentiation (D), whereas the wild-type forms cartilage (A), ciliar epithelium (B), and muscle (B). ca, cartilage; ec, ectoderm; ep, ciliated epithelium; mf, muscle fiber.

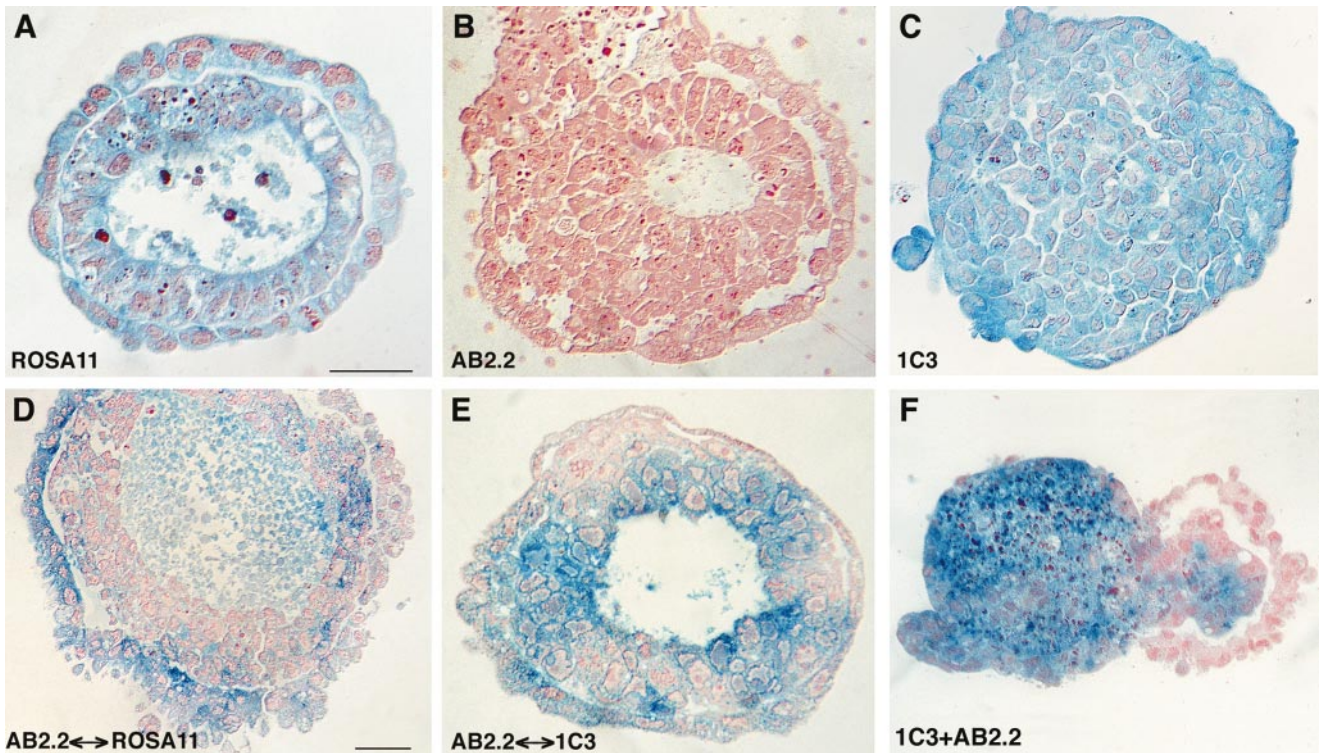


Figure 4. Mutant cells can recognize, but cannot produce an extracellular differentiation factor. Embryoid bodies grown for 6 d from (A–C) individual cell lines or from (D–F) mixed ES cell cultures. Origin of embryoid bodies: (A) β -galactosidase-positive wild-type ROSA 11 cells; (B) β -galactosidase-negative, AB2.2 wild-type cells; (C) 1C3, a ROSA 11-derived, dominant negative mutant clone; (D) control chimera made of ROSA11 and AB2.2 cells; and (E) experimental chimera grown from mutant, 1C3, and wild-type, AB2.2 cells. Note that the mutant colonizes both cell layers and its fragments are localized in the central cavity. (F) Fused embryoid body from a culture of mixed (ROSA11 and 1C3) aggregates. In this arrangement, no or little rescue of the dominant negative mutant could be observed. Bars: (A–F) 25 μm .

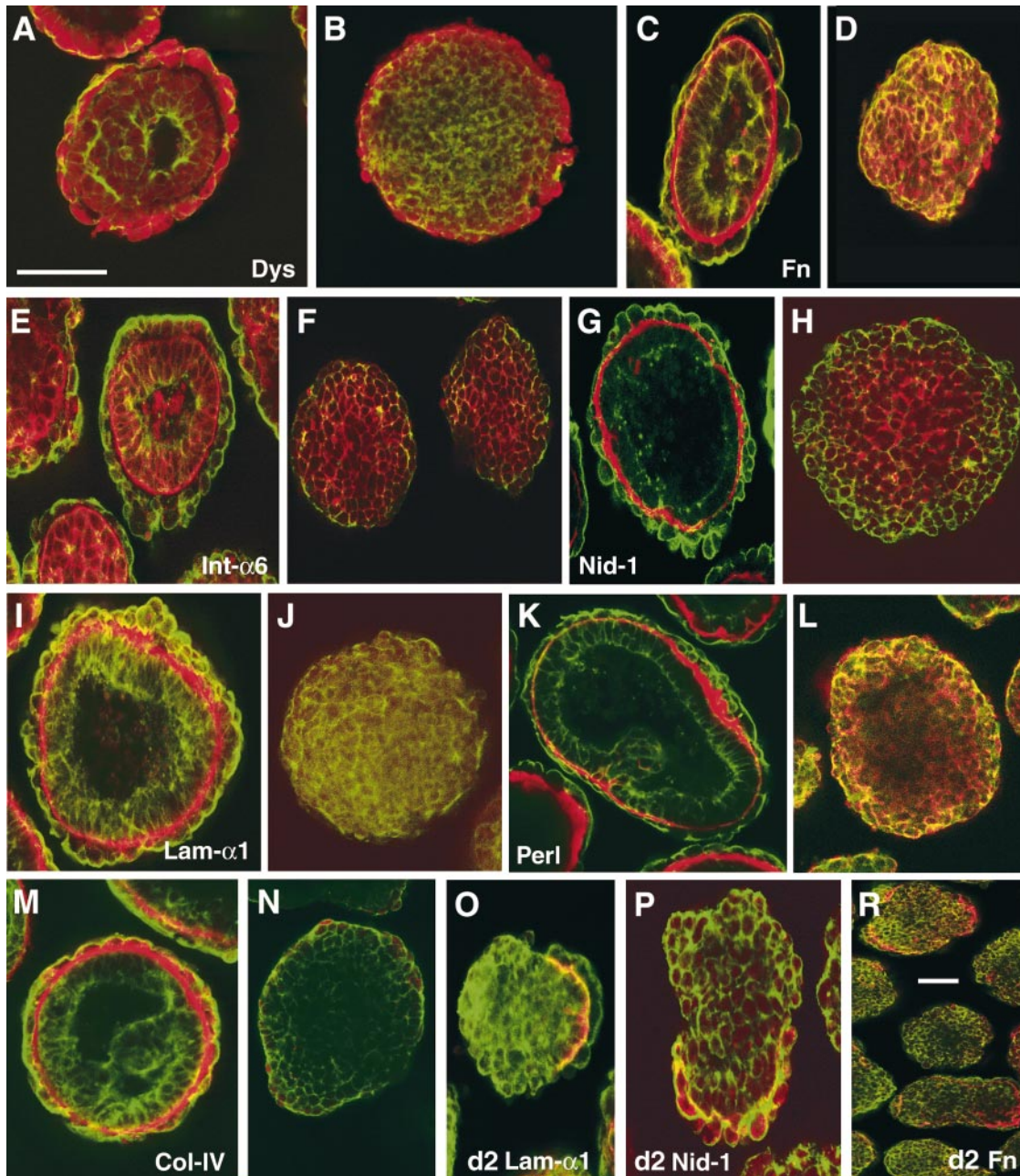


Figure 5. Localization of BM proteins in wild-type and mutant embryoid bodies. (A–N) Mature embryoid bodies after 7 d of culture. (O–R) Early embryoid body differentiation (2 d of culture). Note that expression of specific proteins, in O–N, coincides with the inception of differentiation. (A–N) The first picture of each pair of panels shows a wild-type, whereas the second shows a mutant embryoid body stained with the same reagent. Green false color: fluoresceinated phalloidin; red false color: specific proteins. Note that mutant embryoid bodies (B, D, F, H, and L) express all BM proteins with the exception of laminin- α 1 (J) and collagen IV (N). Lack of BM and punctuated, diffuse label of BM receptors and BM-associated proteins in the mutant is in contrast to the absence of laminin- α 1 (J) and collagen IV (N). Dys, dystroglycan; Fn, fibronectin; int- α 6, integrin- α 6; Nid-1, nidogen-1; Lam- α 1, laminin- α 1; Perl, perlecan; Col-IV, collagen IV. Bars: 40 μ m.

were similar to those with the mutant, as both formed columnar ectoderm and cavitated normally, but no visceral endoderm could be distinguished (Fig. 7, D and F). Lack of visceral endoderm differentiation by wild-type cells was unusual, because these cells are constitutively capable of visceral endoderm formation.

For the time being, more than one interpretation is possible for this phenomenon. Previous data suggest that FGF

signaling is required for endoderm differentiation (Wilder et al., 1997; Arman et al., 1998). It is also possible that the sub-endodermal BM is a default signal for columnar ectoderm differentiation, and once the single endoderm layer has been formed, it inhibits further endoderm differentiation. In addition, the particular externally added BM proteins might have been inhibitory to the visceral endoderm. Although further studies will have to clarify this problem, our results clearly

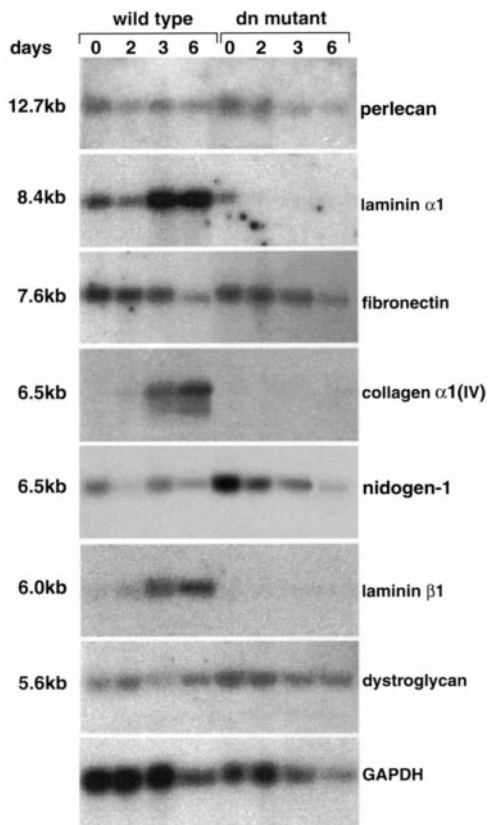


Figure 6. Transcription of genes encoding BM proteins. RNA blot analysis. Wild-type or double-negative (dn) mutant ES cells and embryoid bodies were harvested at day 0, 2, 3, and 6 of culture and subjected to RNA extraction and filter hybridization. The blot was hybridized in succession to the DNA probes indicated at the right. Laminin- α 1, laminin- β 1, and collagen α 1 (IV) are not or only weakly expressed in the mutant, whereas perlecan, fibronectin, nidogen-1, and dystroglycan are transcribed to similar extent in wild-type and mutant cultures.

show that BM proteins can rescue ectoderm differentiation and cavitation, even when FGF signaling is defective.

BM Proteins Activate a Normal Embryonic Differentiation Program

Besides Matrigel, which contains several BM proteins, a highly purified laminin-1–nidogen complex also rescued embryoid body differentiation. To explain how a purified complex of two proteins can reconstitute the role of the complex BM, we assumed that laminin-1 was bound by precursors of the ectoderm. Laminin-1 then could activate its receptors and facilitate the assembly of BM components synthesized by the mutant. This assumption was supported by the finding that nidogen-1 (Fig. 8 A), perlecan (Fig. 8 B), and fibronectin (Fig. 8 C) formed an overlapping layer along the columnar ectoderm of laminin-treated mutant embryoid bodies (Fig. 8). Next, we investigated whether partial rescue by BM proteins activates genes that are involved in early mammalian development.

Differentiation of mutant ES cells into columnar ectoderm model early epiblast differentiation. This step was the salient event of the BM protein–induced partial rescue of our mutation. To find molecular evidence beyond mor-

Table III. Partial Rescue of Differentiation by Matrigel and by the Laminin-1–Nidogen Complex

Cells	Treatment	Percentage of differentiation	No. counted
Wild-type	–	74	237
Mutant	–	4	434
Mutant	4% Matrigel for 24 h	67	124
Mutant	50 μ g/ml laminin-1 for 24 h	30	164

Table IV. 100–10,000-fold Excess of Growth Factors Is Insufficient to Rescue the Dominant Negative FGFR Mutant

Growth factors	Approximate concentration in growth factor–reduced Matrigel	Growth factor added to mutant (ng/ml)	Difference of concentration*
FGF2	0.004 pg/ml	25	6.25×10^3
EGF	20 pg/ml	10	5×10^2
Insulin	0.2 ng/ml	20	1×10^2
PDGF-BB	0.00 ng/ml	20	2×10^4

*Growth factors were added to mutant cultures at day 0 and their concentration was maintained throughout the culture until day 5. No signs of differentiation could be observed as a result of these treatments.

phological observations, first transcription of embryonic globin, *flk-1*, and *kit* ligand (stem cell factor) was examined. However, Matrigel failed to induce these genes (not shown). This was not surprising, if we consider that rescued embryoid bodies developed no endoderm. Therefore, interaction between extraembryonic ectoderm and visceral endoderm, which is required for embryonic hemopoiesis and angiogenesis, could not take place.

Next, genes involved in early mammalian development were studied. Recent data associate important patterning events with the visceral endoderm (Beddington and Robertson, 1999). Expression of several early developmental genes, such as *Brachyury*, *ptc*, *Gli-1*, *cripto-1*, *Otx-2*, bone morphogenetic protein (BMP)4, and BMP2, was undetectable, or unchanged after Matrigel treatment. This also may have been due to loss of the normal interaction between visceral endoderm and epiblast. However, Matrigel treatment did induce the transcription of a T-box gene, *Eomesodermin* (Fig. 9).

Eomesodermin is the earliest pan-mesodermal marker of *Xenopus* embryogenesis. It activates several mesodermal transcription factors and its overexpression leads to the formation of a partial secondary axis (Ryan et al., 1996). Its mammalian orthologue, *mEomes*, is first expressed in the trophectoderm and later in its derivative the extraembryonic ectoderm. During gastrulation, it is transcribed in the primitive streak, in the nascent mesoderm, and in the anterior visceral endoderm (Ciruna and Rossant, 1999). In wild-type embryoid bodies, *mEomes* was expressed by the columnar ectoderm (Fig. 9, A and B), whereas in the undifferentiated mutant no *mEomes* transcripts could be detected (Fig. 9 C). Importantly, however, *mEomes* was activated in the ectoderm of Matrigel-treated mutant embryoid bodies that passed a certain level of differentiation (Fig. 9, D–F). In the three experiments testing *mEomes* expression, 30–40% of the Matrigel-treated embryoid bodies underwent overt differentiation and around a third to one-

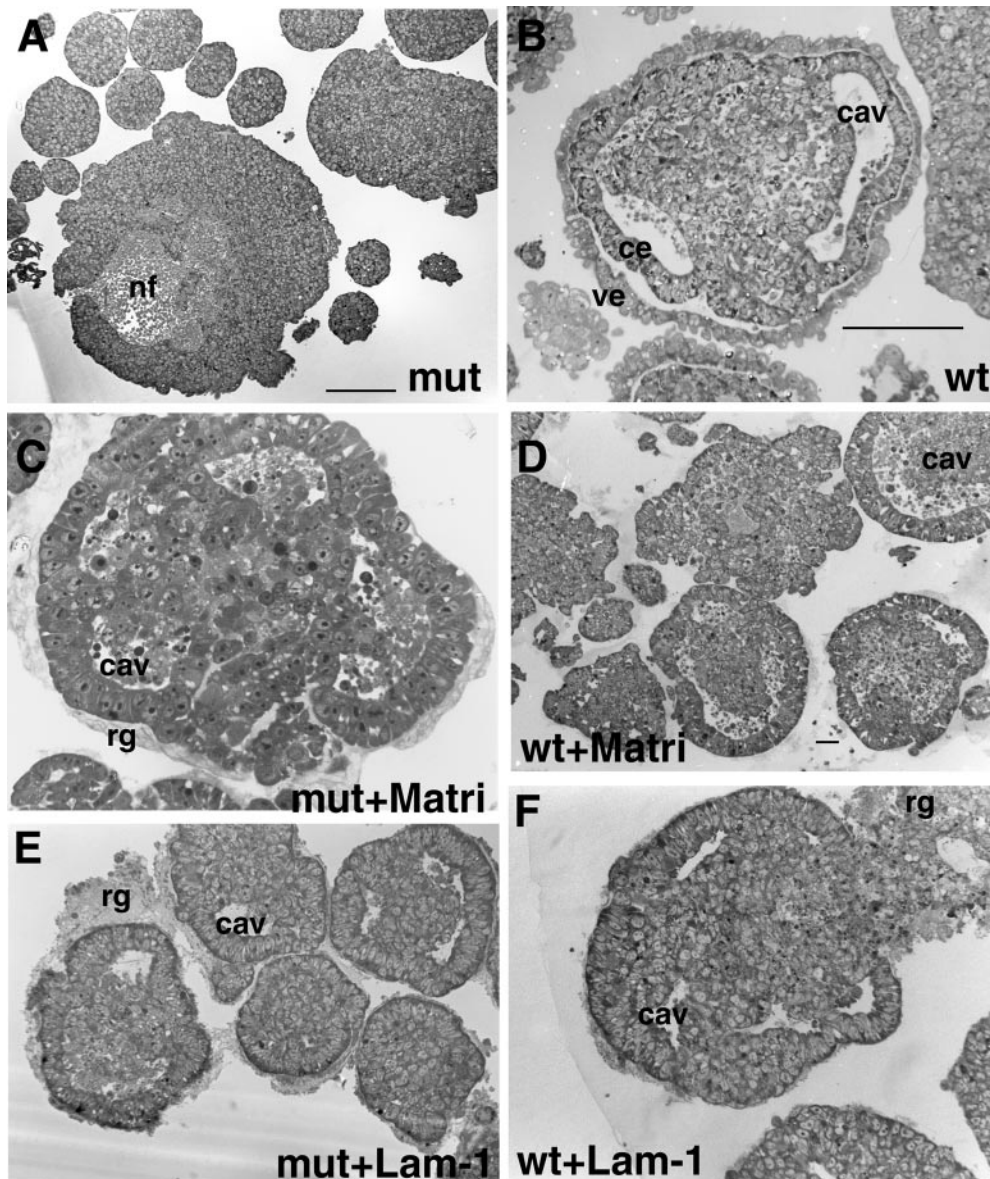


Figure 7. Partial rescue of the mutant by Matrigel or by purified laminin-1–nidogen complex. (A) Untreated mutant (mut); (B) untreated wild-type (wt); (C and D) mutant and wild-type, treated with Matrigel (Matri); and (E and F) mutant and wild-type, treated with laminin-1 (Lam-1)–nidogen complex. Note that BM protein–treated mutant (C and E) and wild-type (D and F) embryoid bodies cavitate and form columnar ectoderm, but both fails to form visceral endoderm. Control and untreated mutant harvested after 6 d of culture. Matrigel- and laminin-treated cultures harvested at day 7. cav, cavitation; ce, columnar ectoderm; nf, necrotic focus; rg, residual gel (Matrigel or laminin). Bars: (A, B, C, D, and F) 40 μ m.

half contained numerous *mEomes*-positive cells. Gene expression was restricted to internal cells developing into columnar ectoderm surrounding a nascent (Fig. 9, D and E) or more advanced central cavity (Fig. 9 F).

Discussion

The Mutant Defect Resides in an Extracellular Product of FGF Signaling

We studied the role of FGF signaling in embryoid body differentiation by introducing a truncated FGFR cDNA into ES cells. This dominant negative mutation was preferred to isotype-specific gene targeting, because ES cells homozygous for mutant alleles of *Fgfr1* or *Fgfr2* have the potential to differentiate (Deng et al., 1994; and our unpublished results). Dominant negative FGFR mutations have broader effects than mutations of individual FGFR isotypes, because truncated FGFR monomers can inhibit multiple FGFR isotypes through heterodimeriza-

tion (Ueno et al., 1992). It is of relevance that in a previous study we detected transcripts of all four FGFR in differentiating embryoid bodies (Chen et al., 2000).

Our dominant negative FGFR mutation abolished differentiation of both cell layers of the embryoid body, suggesting by extrapolation that FGF signaling is required for the differentiation of the first embryonic cell lineages (Chen et al., 2000). The decisive observation of this study was that if mutant ES cells came in contact with extracellular products of differentiating wild-type cells, they could contribute to both cell layers of the embryoid body and participated in cavitation. It follows that an extracellular FGFR-dependent product or products are involved in the induction of embryoid body differentiation. The results presented here identify at least one of these products with the subepithelial BM. The following lead to these conclusions: (a) the mutation inhibited laminin-1 and collagen α 1 (IV) synthesis; (b) purified laminin-1 rescued ectoderm differentiation and cavitation; and (c) externally added BM proteins activated a program of early vertebrate development.

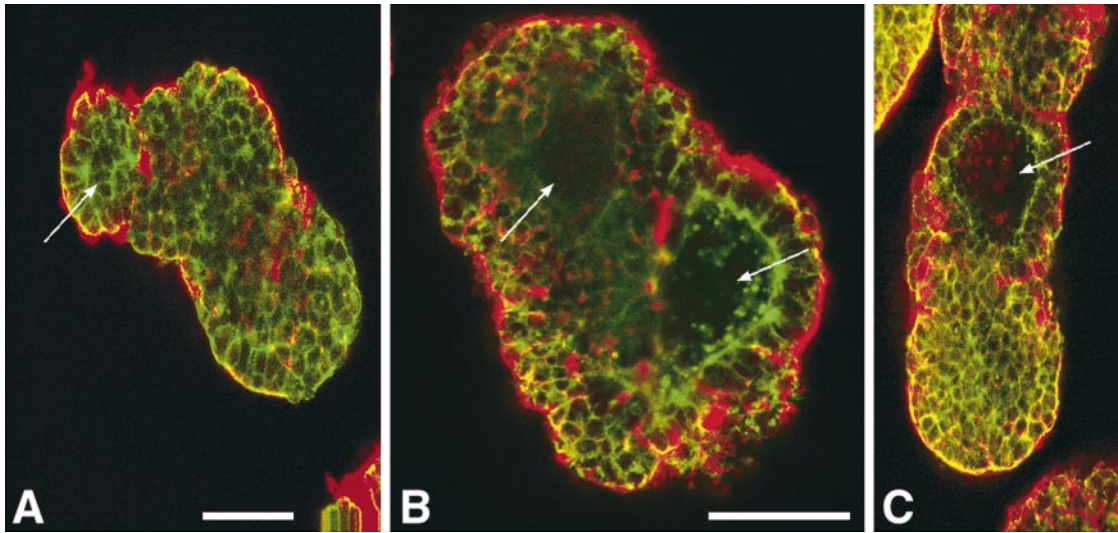


Figure 8. BM assembly on the surface of rescued mutant embryoid bodies. 1C3 mutant embryoid bodies were treated with purified laminin-1–nidogen complex, as described in the text and Materials and Methods. Treated embryoid bodies were stained on the sixth day of culture with phalloidin (green) and the ECM-specific antibodies (red). Specific staining: (A) nidogen-1, (B) perlecan, and (C) fibronectin. Arrows point to cavity formation and to the adjacent columnar ectoderm. Note that the BM-related proteins are situated on the surface of the embryoid body, with no visible external endoderm. Bars: (A and C) 40 μm ; (B) 60 μm .

Defective Laminin-1 and Collagen IV Synthesis in the Mutant

Investigating the molecular defect of the mutation revealed that mutant-derived embryoid bodies form no BM. Nevertheless, several BM proteins and their receptors were expressed by the mutant, albeit in a disorganized manner, whereas laminin-1 and collagen IV were absent at

both the protein and mRNA levels. Laminin-1 and collagen IV are the network-forming elements of the BM (Colognato and Yurchenco, 2000). As the BM is required for epithelial differentiation (see Introduction), loss of differentiation in our mutation was likely to be due to loss of BM assembly.

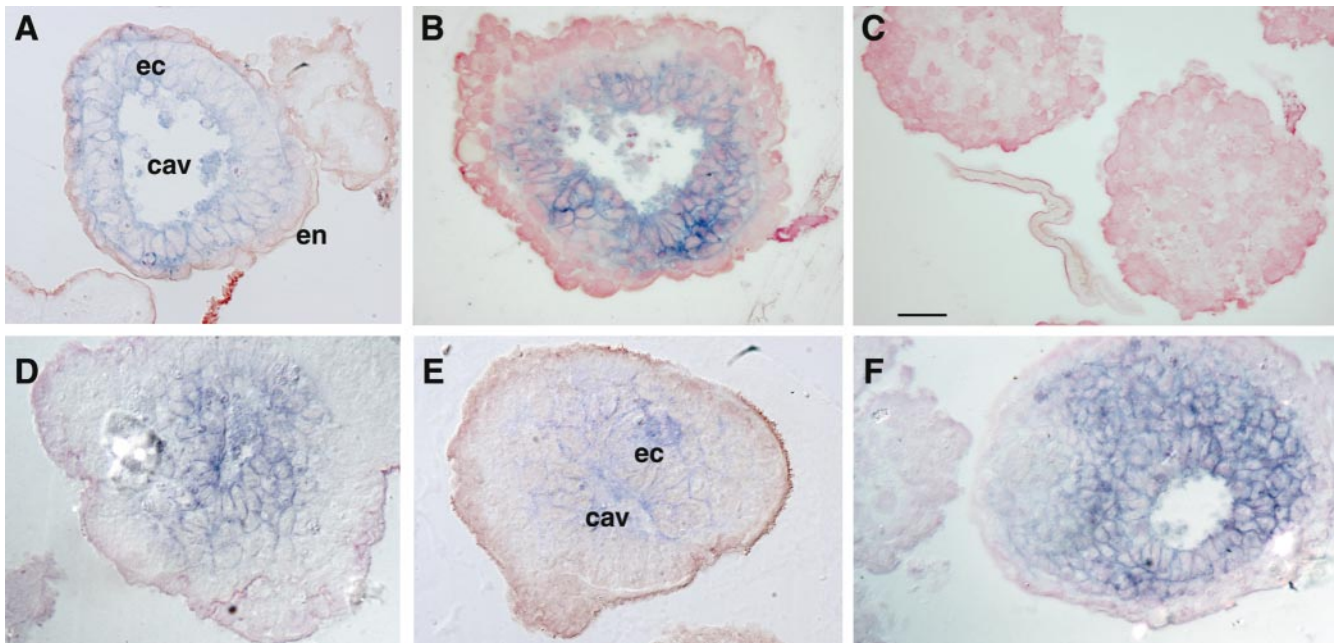


Figure 9. Externally added Matrigel induces *Eomesodermin* transcription. In situ hybridization with *mEomes*. (A and B) Wild-type; (C) dominant negative mutant; (D–F) Matrigel-treated mutant. Note that both wild-type and rescued mutant embryoid bodies express *mEomes* in the columnar ectoderm and in internal cells that presumably differentiate into columnar ectoderm, whereas no *mEomes* transcription can be detected in the untreated mutant. The figures derive from three experiments, harvested at day 6. The frequency of Matrigel-induced differentiation was between 30 and 40% and overt *mEomes* expression was detected in the more advanced third or half. cav, cavity; ec, columnar ectoderm; en, visceral endoderm. Bar: 25 μm .

Coordinate loss of laminin-1 and collagen-IV synthesis was reminiscent of their coordinate induction by retinoic acid in F9 teratocarcinoma cells (Kleinman et al., 1987), or by myogenic factors in differentiating myoblasts (Kroll et al., 1994). It follows that abrogation by loss of FGFR function and induction by physiological inducers may affect similar mechanisms. FGF-induced laminin synthesis in neuroepithelium cultures also points in this direction (Drago et al., 1991). However, the pathway between FGF signaling, laminin-1, and collagen IV synthesis is yet to be described. As the FGF system and both laminins and collagen IV have multiple isoforms, this appears to be a daunting task. Our ongoing effort to clarify the connection of FGF signaling with BM assembly relies on previous results suggesting that our mutant embryoid bodies are defective in Akt/PKB activation (Chen et al., 2000). Preliminary results indicate that constitutively active PI-3 kinase p110 or Akt/PKB increase laminin- γ 1 and collagen- α 1 (IV) transcription.

Partial Rescue of Ectoderm Differentiation by BM Proteins

Rescue of epithelial differentiation in mixed cultures by extracellular components of FGF signaling, or by addition of BM proteins to mutant ES cells, both pointed to the ECM's role in epithelial differentiation. Numerous studies demonstrate that BM proteins are required for epithelial differentiation. Antibodies specific to the dystroglycan binding site of laminin- α 1 inhibit epithelial differentiation during kidney and salivary gland development (Durbeej et al., 1995; Kadoya et al., 1995). Loss of function mutations of dystroglycan (Williamson et al., 1997), integrin- β 1 (Fassler et al., 1995; Stephens et al., 1995), and laminin- γ 1 (Smyth et al., 1999) cause early postimplantation lethality. ES cells homozygous for these mutations form embryoid bodies with defective visceral endoderm. However, they still express certain signs of differentiation (Henry and Campbell, 1998; Aumailley et al., 2000). Murray and Edgar showed that embryoid bodies derived from laminin- γ 1^{-/-} ES cells have no detectable BM, do not undergo ectoderm differentiation and cavitation, but display a degree of visceral endoderm differentiation (Murray and Edgar, 2000). It is possible that compound mutations of more than one BM proteins should result in even more severe defects.

This study describes two experimental arrangements that rescued the dominant negative FGFR mutation. In mixed cultures, mutant-derived ES cells contributed to both cell layers, whereas externally added BM proteins induced ectoderm differentiation, but seemed to inhibit visceral endoderm formation. The most significant difference between rescue by wild-type cells or by external BM proteins is that wild-type cells in mixed cultures sustain FGF signaling, which is absent in cultures of BM protein-treated mutant cells. Hence, we conclude that FGF signaling supplies additional differentiation signals beyond those involved in BM assembly. Indeed, FGF signaling is required for primitive endoderm differentiation, which is the precursor of the visceral endoderm (Wilder et al., 1997; Arman et al., 1998).

Taking these data together, one can outline a series of

steps leading to embryoid body differentiation and early embryonic development. Aggregation of ES cells and the compaction of the morula are controlled by the E-cadherin- β -catenin pathway (Larue et al., 1994). Endoderm differentiation in ES cell aggregates or blastocysts requires FGF signaling (Wilder et al., 1997; Arman et al., 1998). As embryoid bodies express multiple FGFR (Chen et al., 2000), this is likely to be a complex process. FGF signaling activates the genes encoding laminin-1 and collagen IV in the differentiating endoderm, leading to BM assembly and the induction of ectoderm differentiation. The ectoderm anchors in the subendodermal BM and cells that fail to attach to it undergo apoptotic cavitation (Cocouvanis and Martin, 1995). Besides its structural and antiapoptotic effects, the subendodermal BM may contribute to differentiation in two nonexclusive ways, via its own signaling receptors, and/or as a platform presenting signaling molecules to the receptors of adjacent cells.

External BM Proteins Activate *mEomes*, a Gene Active in Mesoderm Induction

Laminin-1-treated embryoid bodies display several overlapping BM proteins on their surface. Their binding is likely to be connected to the rearrangement of receptors in the differentiating ectoderm. It follows that signaling through receptors for laminin-1 may induce changes conducive to increased BM protein synthesis, receptor expression, and BM assembly.

Beyond the morphological detection of BM assembly and epithelial differentiation, we sought evidence for the activation of normal embryonic differentiation by externally added BM proteins. Among several markers, which were expressed constitutively in mutant and wild-type and presumably required interaction between the visceral endoderm and the ectoderm, *Eomesodermin*, a pan-mesodermal marker, was activated as the result of BM protein-induced ectoderm differentiation. *mEomes* is expressed in the trophoctoderm-derived extraembryonic ectoderm of the mouse embryo (Ciruna and Rossant, 1999), and it is later involved in embryonic hemopoiesis and vasculogenesis. Transcription of *mEomes* in ES cell-derived embryoid bodies, which contain no trophoctoderm derivatives, may be connected to the embryoid body's propensity for hemopoiesis and vasculogenesis. Activation of this pan-mesodermal gene in ES cell-derived embryoid bodies corresponds to the epithelialization of the early epiblast into the columnar ectoderm of the egg cylinder embryo. It follows that *mEomes* expression induced by BM proteins in the ectoderm of the embryoid body belongs to the programs of early vertebrate development.

The central finding emerging from this study is that FGF signaling is required for the expression of laminin-1 and collagen- α 1 (IV), which contribute to BM assembly and as a consequence to ectoderm differentiation. Similar processes may be important for the formation of early embryonic cell layers and for cell to matrix interactions during organogenesis and other physiological and pathological processes that require FGF signaling and the assembly or modification of the BM.

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