Ras/MAPK Pathway Confers Basement Membrane Dependence upon Endoderm Differentiation of Embryonic Carcinoma Cells*

The formation of extraembryonic endoderm is one of the earliest steps in the differentiation of pluripotent cells of the inner cell mass during the early stages of embryonic development. The primitive endoderm cells and the derived parietal and visceral endoderm cells gain the capacity to produce collagen IV and laminin. The deposition of these components results in the formation of basement membrane and epithelium of the endoderm, with polarized cells covering the inner surface of the blastocoels. We used retinoic acid-induced endoderm differentiation of stem cell-like F9 embryonic carcinoma cells to study the role of the Ras pathway and its regulation in the formation of the visceral endoderm. Upon endoderm differentiation of F9 cells induced by retinoic acid, c-Fos expression, the downstream target of the Ras pathway, is suppressed by uncoupling Elk-1 phosphorylation/activation to MAPK activity. However, attachment to matrix gel greatly enhances the activation of MAPK in endoderm cells but not in undifferentiated F9 cells. Enhanced MAPK activation as a result of contact with basement membrane is able to compensate for reduced Elk-1 phosphorylation and c-Fos expression. We conclude that endoderm differentiation renders the activation of the Ras pathway basement membrane dependent, contributing to the epithelial organization of the visceral endoderm.

In multicellular organisms, individual cells communicate with each other to maintain the harmony and homeostasis of the organism. One means of communication is through the release of soluble and diffusible factors such as hormones and growth factors, which bind the cell surface or nuclear receptors and trigger intracellular signaling. Direct physical contact mediated by cell surface receptors between cell-cell and cell-matrix are another kind of communication. The cell surface events transmit into the cell interior through cascades of biochemical reactions known as signal transduction, leading to modification of cellular enzymatic activities and gene expression. In the multicell structure, depending on the positioning cues provided by cell-cell and cell-matrix contacts, a particular cell type may differently interpret the signal of a diffusible factor, leading to the modification of the intracellular signaling pathway and resulting in an integrated cellular response.

The Ras/MAPK1 pathway is a major intracellular signaling pathway involved in cell proliferation, differentiation, and tumorigenicity (1–3). Investigation of mammalian cells in culture and model organisms have established the Ras/MAPK pathway; in responding to growth factor binding to cognate cell surface receptors, the small G protein, Ras, is activated by the exchange of bound GDP for GTP (2, 3). Activated Ras binds and recruits Raf-1 to the cell surface. A cascade of kinases, Raf-1, MEK, and MAPK (or Erk), is sequentially phosphorylated and activated (4–6). Activated MAPK can then translocate into the nucleus to phosphorylate transcription factors to modulate gene expression. One common example is that MAPK phosphorylates and activates the transcription factor Elk-1 (7–9). Subsequently, phosphorylated/activated Elk-1 binds the c-fos promoter and allows transcriptional activation of c-fos, an immediate early response gene (10, 11). Although it is not essential in gene knockout mice studies (12–14), c-Fos is thought to have an important role in cell cycle progression, cell differentiation, and tumorigenicity (15–19).

The effects of cell-cell and cell-matrix contacts have been recognized and analyzed in cell culture (22–25). In epithelia, the cells are often organized by a sheet of basement membrane composed of a scaffold composed mainly of collagen IV and laminin (27–29). The cells located in the stroma are in contact with an extracellular matrix composed of proteins such as fibronectin, collagen I, collagen III, etc. (26). It has been found that NIH3T3 fibroblasts attached to a fibronectin substratum, compared with cells in suspension, are much more efficient in transmitting the Ras/MAPK signal (20–25). The regulated step in cell attachment is the activation of Raf-1 by Ras, because tyrosine phosphorylation and Ras activation are not affected, and Raf-1, MEK, and MAPK activation are much stronger in adherent than in suspended cells (21, 22).

Thus far, most of the cell culture studies of cell-matrix interaction on signaling have used fibroblasts as models (20–25). However, the profound effects of basement membrane contact on growth, death, and differentiation of epithelial cells have

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§ The abbreviations used are: MAPK/Erk, mitogen-activated protein kinase/extracellular-signal regulated kinase; MEK, MAPK/Erk kinase; Dab2, Disabled-2; E5, embryonic day 5; LRP, low density lipoprotein receptor-related protein; est, expressed sequence tag; MOPS, 4-morpholinepropanesulfonic acid; SPARC, secreted protein, acidic and rich in cysteine.

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been recognized (27–31). The presence and intactness of basement membrane are dynamically regulated by altering synthesis and degradation and have important roles in development (28, 29) and in physiological processes such as mammary gland involution (32, 33), and ovarian surface rupture during ovulation (34–37). Analysis of cell–basement membrane contact on cellular signaling is lacking, probably because of the lack of proper models for epithelial cells and basement membrane in cultures. Most of the established cell lines of epithelial origin have not been able to faithfully mimic the in vivo properties of interaction with basement membrane because they have already undergone changes to become independent of the basement membrane during the process of adapting to tissue culture conditions (38).

Here, we used the F9 embryonic carcinoma cells as a model to investigate the Ras/MAPK signaling pathway and its regulation by basement membrane. F9 cells are a well characterized teratocarcinoma line derived from tumors of the gonads (testes). F9 cells are undifferentiated, with characteristics resembling those of stem cells in early embryos, and have been widely used to study early embryonic development and retinoic acid regulation (39–45). Induced by retinoic acid, F9 cells undergo differentiation into visceral endoderm cells, an epithelial cell type in the early embryo (40, 41). We found that, accompanying epithelial differentiation of the F9 cells, the regulation of the Ras/MAPK pathway is altered (46, 47); the activation of MAPK and Elk-1 is uncoupled, and MAPK activation is enhanced by contact with basement membrane to compensate for the inefficiency in Elk-1 activation and c-Fos expression. Thus, the Ras/MAPK pathway is altered in F9 cell differentiation so that the cells become basement membrane-dependent.

**EXPERIMENTAL PROCEDURES**

**Materials**—Retinoic acid (all-trans-retinoic acid) and purified collagen I and IV were purchased from Sigma. Tissue culture supplies were obtained from Fisher. Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from Mediatech (Herndon, VA); Matrigel was obtained from BD Pharmingen. TRIzol reagent, purified fibronectin, purified laminin, 100× antibiotic-antimycotic solution, Lipo-fectAMINE, and serum-free Opti-MEM I medium were purchased from Invitrogen; the ECL SuperSignal West Dura extended duration substrate immunodetection reagents were purchased from Pierce; Hybridization solution came from IntegenX (Purchase, NY); positively charged nylon membranes were from Roche Molecular Biochemicals; and [α-32P]dCTP was from PerkinElmer Life Sciences. All other general chemicals and supplies, including MeSO4, ethanol, isopropanol, and agarose, were from Sigma or Fisher and were reagent grade or higher.

**Cell Culture**—F9 mouse embryonic carcinoma cells were purchased from American Type Culture Collection (ATCC). F9 cells were cultured on gelatin-coated tissue culture plates in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, and 1× antibiotic-antimycotic solution. The plates were coated with a auto-clarified 0.1% gelatin solution overnight at 4 °C and then washed three times with phosphate-buffered saline prior to use. All-trans-retinoic acid was added to cells from a 1 μM stock solution in MeSO4. Control cultures contained an equal volume of MeSO4 alone. Usually, retinoic acid was added 24 h after plating of cells. Cell growth was determined by tripling counting with a hemacytometer or by measuring the MTT assay (Promega). The results of the MTT assay agreed well with those of the cell counting.

**Matrix Gel**—Matrigel was diluted 1:3 with Dulbecco’s modified Eagle’s medium with 10% heat-inactivated serum on ice, and the solution was added to each well of 96-well plates (10 μl each) or 6-well dishes (250 μl each) to coat the surface. The gel in tissue culture ware was then incubated for 1 h at 37 °C to solidify it prior to plating the F9 cells.

The coating of tissue culture plates with purified components of basement membrane was done according to the manufacturer’s suggestion. Briefly, plates were precoated with phosphate-buffered saline containing collagen I (10 μg/ml), collagen IV (2.5 μg/ml), laminin (20 μg/ml), or fibronectin (20 mg/ml) for 4 h at 37 °C or 4 °C overnight to coat the plates with basement membrane proteins. The plates were then washed once with warm phosphate-buffered saline before the cells were plated.

**DNA Expression Array**—The cDNA microarray chips were prepared at the Fox Chase Cancer Center Facility according to standard protocol. Briefly, 15,552 mouse cDNA fragments from NIA-15K library (National Institutes of Health) were amplified by polymerase chain reaction (PCR), purified by isopropanol precipitation, and resuspended in 50% MeSO4 at a concentration of 150 ng/μl. Arrays were spotted on a GeneMachine Omnimgrid arrayer (GeneMachine, San Carlos, CA) using polylysine-coated glass slides. Slides were baked for 3 h at 80 °C in a vacuum oven, cross-linked in UV light (90 mJ) in a Stratalinker (Stratagene, La Jolla, CA), and processed as described in the MGuide (smgm.stanford.edu/pbrown/mguide/index.html).

**Northern Blot Analysis**—Total RNA was isolated from F9 cell monolayers according to the TRIzol method (Invitrogen). RNA was separated at 1% agarose gels containing 7% formaldehyde and 20 μM MOPS buffer, transferred to positive charged nylon membranes using 2× SSC buffer, and fixed by baking. DNA probes are as follows: mouse cDNA for hp15 2.5 kBSA plasmid inserts DNA fragments were ligated into pUC19 plasmid containing collagen IV α1, and laminin A subunit (Lama-1 gene). All of the cDNA were obtained from ATCC and were sequence-verified and characterized prior to use. DNA probes were labeled with [α-32P]dCTP using a random prime labeling kit (Amer sham Biosciences). Hybridization

**Antibodies and Western Blot Analysis**—Total RNA was isolated from F9 cell monolayers according to the TRIzol method (Invitrogen). RNA was separated at 1% agarose gels containing 7% formaldehyde and 20 μM MOPS buffer, transferred to positive charged nylon membranes using 2× SSC buffer, and fixed by baking. DNA probes are as follows: mouse cDNA for hp15 2.5 kBSA plasmid inserts DNA fragments were ligated into pUC19 plasmid containing collagen IV α1, and laminin A subunit (Lama-1 gene). All of the cDNA were obtained from ATCC and were sequence-verified and characterized prior to use. DNA probes were labeled with [α-32P]dCTP using a random prime labeling kit (Amer sham Biosciences). Hybridization

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RESULTS

Induction of Epithelial Differentiation of F9 Embryonic Carcinoma Cells by Retinoic Acid—The visceral endoderm is one of the earliest epithelial structures formed in mammalian embryonic development (49). As shown schematically in Fig. 1 for an E5.0–E5.5 mouse embryo, an epithelial structure known as primitive endoderm in earlier stages and as visceral endoderm (VE) upon further differentiation is derived from the stem cell-like cells of the inner cell mass (ICM) and organized by a sheet of basement membrane (BM) into a single-layer simple epithelium. F9 embryonic carcinoma cells exhibit the properties of embryonic stem cells and can be induced into visceral endoderm-like cells by retinoic acid in culture (40–42). Here, we have characterized the differentiation of F9 cells as a model for the study of epithelial cell signal transduction. The expression of collagen IV and laminin, components of the epithelial basement membrane, is initiated in monolayer F9 cell cultures upon the addition of retinoic acid (Fig. 2A). Using a probe for laminin A subunit (Lama-1 gene), a single message is weakly detectable in untreated F9 cells but is greatly elevated by day 3 of retinoic acid treatment (Fig. 2A). Collagen IV expression is absent in undifferentiated F9 cells; by day 3 of the retinoic acid treatment, two messengers were detected using collagen IV α1 as a probe (Fig. 2A). Disabled-2 (Dab2, two spliced forms for p96 and p67), a marker for visceral endoderm, is expressed by day 4 of retinoic acid induction.

The global change in gene expression of F9 cells was investigated by DNA expression array, comparing mRNA from control cultures and 4-day retinoic acid treatment (Fig. 2B, Table I). In our limited 15,000-mouse gene DNA expression array, only subtle global changes were observed. The gene with the highest increase in expression upon retinoic acid treatment was identified to be procollagen type IV, α1 gene. At an upper cut-off point of +2.4 (5-fold increase), 27 entries were obtained (Table I), containing six uncharacterized est sequences (not shown). The most dramatic increases are in collagen IV α1 (+46-fold), laminin α1 (+16-fold), and γ1 (+14-fold), and laminin B1 (+10.6-fold). Many of these proteins specify the epithelial phenotype of the differentiated cells (Table I). The maximal decrease of gene expression detected is −2.4–4.6 (11.0-fold) for an est sequence. At the lower end of expression cut-off at −2.4 (6-fold) or more fold of decrease, 143 entries containing 125 non-annotated ests were obtained (not shown). The 18 known genes are shown (Table I). The upper and lower cut-offs were selected so that the changes in all of the identified genes are likely to be above the background fluctuations. Other than the dramatic increases in the expression of the components of basement membrane, we recognized no additional remarkable feature in the expression pattern related to cell adhesion or Ras/MAPK signaling. It should be noted that the unknown entries represent est sequences that may belong to the same gene. Also, known visceral endoderm markers exhibit only moderate changes in the DNA array expression assay, including Dab2 (+4.6-fold), GATA-6 (+2.8-fold), lrp-1 (2.8-fold), and lrp-2 (or megalin, +3.0-fold).

Notably, epithelium-related genes such as collagen IV α1, all three forms of laminins, lrp-1, lrp-2, and SPARC were induced (Fig. 2B, Table I). All integrin subtypes, α1,6 and β1,4,5,6, exhibit some increase, up to 3.5-fold, in differentiated cells. Other significant changes are increases in cathepsin L and low density lipoprotein receptor and decreases in myosin heavy chain. Generally, the expression profile of F9 cells changes to adapt to those of epithelial cell types upon retinoic acid treatment, with the most remarkable change being the expression of collagen IV and laminin.

Retinoic Acid Suppresses F9 Cell Growth and Restoration of Growth by Basement Membranes—As shown previously (46, 47), retinoic acid suppresses F9 cell growth. Nonetheless, we found that cell growth suppression can be reversed by growing the differentiated cells on a layer of Matrigel (Fig. 3A). The Matrigel, however, has no effects on the growth of undifferentiated F9 cells. In a preliminary survey, no individual or combination of components of the basement membrane appears to be able to stimulate the growth of the differentiated cells as...
Alteration of expression profile induced by retinoic acid in F9 embryonic carcinoma cells determined by DNA expression array

TABLE I

Selected genes that exhibit 2.3 (5.0-fold) or greater increase in expression or 2.6 (6-fold) or greater decrease in expression are listed.

| UniGene accession no. | Name                                      | 2^n   | -Fold changes |
|-----------------------|------------------------------------------|-------|---------------|
| AW559158             | Procollagen, type IV, α1                 | 5.5246| +46.0         |
| AW537108             | Laminin, α1                              | 4.0469| +16.5         |
| AW546272             | Laminin, γ1                              | 3.8371| +14.4         |
| AW537016             | Cathepsin L                              | 3.8414| +13.3         |
| AW550296             | Amyloid β (A4) precursor protein         | 3.4918| +11.3         |
| AW556156             | Laminin B1 subunit 1                    | 3.3771| +10.4         |
| AW541488             | β-2 microglobulin                        | 3.0772| +8.4          |
| AU040903             | Calbindin-D9K                             | 3.0124| +8.1          |
| AW536238             | Embigin                                  | 2.9979| +8.0          |
| AW558008             | Calumenin                                | 2.9495| +7.7          |
| AW539363             | Nucleoside-binding protein 1             | 2.8114| +7.0          |
| AW552122             | Tissue factor pathway inhibitor          | 2.7542| +6.7          |
| AW550653             | Collagen-binding protein 1               | 2.5918| +6.0          |
| AW556371             | Dlxin-1                                  | 2.5888| +6.0          |
| AU029098             | Plasminogen activator, tissue            | 2.5012| +5.7          |
| AW557574             | lrp-1                                    | 2.463 | +5.5          |
| AW553366             | Lysosomal membrane glycoprotein 2        | 2.4117| +5.3          |
| AW536169             | SPARC                                    | 2.4067| +5.3          |
| AW558222             | Ne23-Mg                                  | 2.6166| -6.1          |
| AU041515             | GPI-anchored protein                     | -2.6255| -6.2         |
| AW549987             | Gene-rich cluster, B gene                | -2.656 | -6.3          |
| AU016784             | Solute carrier family 16                 | -2.6597| -6.3          |
| AA049924             | Interleukin-1 receptor-associated kinase | -2.6612| -6.3          |
| AW547618             | Weakly similar to msk54                  | -2.8746| -6.4          |
| C78930               | Stromal cell derived factor 4            | -2.6912| -6.4          |
| AU047515             | LIM motif-containing protein kinase 2     | -2.705 | -6.5          |
| L00919               | F30a/musProt4.1                          | -2.7127| -6.6          |
| AU024101             | Interferon-dependent positive acting      | -2.7208| -6.6          |
|                     | transcription factor 3y                 |       |               |
| C79911               | Y-box protein MSY2                       | -2.739 | -6.7          |
| AU431349             | Ribosomal protein, mitochondria, S7      | -2.7547| -6.7          |
| C79918               | Serine proteinase Omi                   | -2.765 | -6.7          |
| AU041888             | Oxysterol 7α-hydroxylase (Cyp27A1)       | -2.7964| -6.9          |
| C17645               | Highly similar to KIAA1017 protein      | -2.7999| -7.0          |
| AU043470             | Similar to Ubiquitin/60S fusion protein   | -2.8486| -7.2          |
| C80049               | Similar to myosin heavy chain            | -2.8648| -7.3          |
| AU041890             | Similar to putative G-binding protein    | -2.9128| -7.5          |
| AU041891             | Developmentally regulated repeat element-containing transcript 3 | -3.0925| -8.5          |

Table 1:

F9 cells were treated with 1 μM retinoic acid or Me2SO solvent control for 4 days. Total RNA was isolated first, and mRNA was purified subsequently. The mRNA was used to produce fluorescent probes and hybridize to DNA expression array as described under “Experimental Procedures.” Selected genes that exhibit 2.3 (5.0-fold) or greater increase in expression or 2.6 (6-fold) or greater decrease in expression are listed. GPI, glycoprotein inositol.

while Matrigel (Fig. 3B). The Matrigel alters the morphology of the F9 cells as compared with cells grown on plastic (Fig. 3C). On a plastic surface, F9 cells treated with retinoic acid for 4 days were well separated and dispersed compared with nontreated cells, which appeared tightly packed and physically connected. F9 cells on Matrigel, however, are even more rounded up, and retinoic acid treatment results in spreading of the cells. Nevertheless, Matrigel does not induce retinoic acid-independent F9 cell differentiation as determined by the expression of differentiation marker such as GATA-4 (not shown) and Dab2 (Fig. 3D). In this representative experiment (Fig. 3D), culturing F9 cells on Matrigel did not induce differentiation, as indicated by the lack of Dab2 expression. Retinoic acid-induced differentiation is associated with the expression of Dab2, and growth on Matrigel also did not inhibit differentiation of F9 cells.

**Matrigel Contact Restores c-Fos Expression and Elk-1 Phosphorylation/Activation in Differentiated F9 Cells—** Differentiation of F9 cells by retinoic acid leads to suppression of serum-stimulated c-Fos expression (Fig. 4A), consistent with earlier results (46, 47) indicating that retinoic acid uncouples MAPK activation and c-Fos expression accompanying cell growth suppression. As in the case of cell growth restoration, attachment of the differentiated F9 cells to Matrigel also restores serum-stimulated c-Fos expression in the differentiated F9 cells (Fig. 4B).

The restoration of c-Fos expression was found to be the result of recovering of Elk-1 phosphorylation (Fig. 5). In retinoic acid-treated F9 cells, Elk-1 phosphorylation/activation is inhibited (Fig. 5A) despite strong activation of MAPK as previously reported (46, 47). Consistently, Elk-1 phosphorylation is recovered in differentiated F9 cells plated on Matrigel (Fig. 5B), although Matrigel alone has no activating capacity because Elk-1 is not significantly phosphorylated prior to serum addition (Time 0), and Matrigel does not increase Elk-1 activation in undifferentiated F9 cells (Fig. 5B). Therefore, attachment to Matrigel restores Elk-1 phosphorylation and thus c-Fos expression in retinoic acid-induced F9 endodermal differentiation.

**Matrigel Activates MAPK in Differentiated but Not in Undifferentiated F9 Cells—** We next examined the activation of MAPK during retinoic acid differentiation. Consistent with previous reports (46, 47) and despite suppression of c-Fos expression, retinoic acid-induced differentiation does not suppress serum-stimulated MAPK activation (Fig. 6A). Occasionally, we even observed that MAPK activation by serum was enhanced in retinoic acid-treated cells, although the phosphorylation of Elk-1 or c-Fos expression is relatively inefficient. One possibility is that the enhanced MAPK activation probably is due to the synthesis and deposition of basement membrane around the differentiated cells. Usually, we differentiated the F9 cells with retinoic acid for 4 days and then collected the cells by trypsin digestion and reseeded them on new tissue culture plates prior to serum stimulation. To examine this idea of basement membrane deposition on culture plates, we grew F9...
cells with or without retinoic acid continuously on plastic to high cell density and stimulated the cells with serum without changing plates (to allow accumulation of basement membrane deposition). These cells exhibited a much stronger MAPK activation with retinoic acid than without retinoic acid (Fig. 6B). The kinetics of serum-stimulated MAPK were observed consistently to be slightly delayed in differentiated F9 cells on Matrigel than in undifferentiated cells (Fig. 6B). With a stronger
MAPK activation in these cells, c-Fos expression is restored to nearly the same level as undifferentiated cells, although the efficiency of MAPK to induce c-Fos expression is still relatively low in differentiated compared with undifferentiated cells.

Retinoic acid treatment greatly suppressed c-Fos expression when cells were plated on plastic plates (Fig. 7A), consistent with earlier results (46, 47). Plating and culturing of the retinoic acid-differentiated cells directly on Matrigel, however, drastically increased the activation of MAPK (Fig. 7B). Despite a much stronger MAPK activation in differentiated cells on Matrigel (increase to 5-fold at the 30-min time point), c-Fos expression was increased compared with the cells without Matrigel but was still slightly lower (0.85-fold) than that of the undifferentiated cells (Fig. 7B). In the parallel control experiment of F9 cells grown on plastic (Fig. 7A), retinoic acid greatly suppressed c-Fos expression (20-fold lower) without significantly altering MAPK activation. Additionally, the Matrigel had no significant effect on MAPK activation in undifferentiated F9 cells (compare Fig. 6, panels A and B), ruling out the possibility that the enhanced activation by Matrigel is caused by contaminating growth factors in the Matrigel preparation. In conclusion, retinoic acid-differentiated cells acquire sensitivity for MAPK activation in response to attachment to Matrigel.

**DISCUSSION**

The retinoic acid-induced differentiation of F9 embryonic carcinoma cells from stromal cells of the inner cell mass to visceral endoderm cells with epithelial properties, can be used as a model for the analysis of epithelium-basement membrane interaction. It is anticipated that cellular signaling would be modified as a result of altered gene expression during F9 cell differentiation. Retinoic acid induces the expression of laminin, collagen IV, and Dab2 (Fig. 8). The promoter of the laminin gene contains retinoic acid responsive element (52) and may be induced directly by retinoic acid. GATA-4 and GATA-6 factors induce expression of collagen IV through the GATA-6 transcription factor (50). Dab2 mediates the effect of retinoic acid on the uncoupling of serum and growth factors stimulated c-Fos expression and MAPK activation, and cell growth suppression (24). On the other hand, laminin and collagen IV form basement membrane, and the basement membrane sensitizes MAPK activation. As a result, the Ras/MAPK pathway confers basement membrane dependence.

**Fig. 6. Effect of culture condition on MAPK activation in F9 cells.** A. F9 cells treated with or without retinoic acid (RA; 1 μM) for 4 days were harvested and replated on plastic culture dishes. The cells were cultured overnight (16 h) with (NS, nonsynchronized) or without serum and then were stimulated with 10% fetal bovine serum (FBS) for 0 (−) to 15 (+) min. Total cell lysates were harvested and assayed for phospho-Erk (P-Erk) and actin by Western blotting. B. F9 cells were cultured continuously on the same culture dishes with or without retinoic acid for 5 days. Following a 16-h culture without serum, the cells were stimulated with 10% serum and then were stimulated with 10% fetal bovine serum (FBS) for 0–90 min. Total cell lysates were harvested at each time point and assayed for c-Fos expression, MAPK activation (P-Erk), and actin determined by Western blotting.

**Fig. 7. Effect of Matrigel on MAPK phosphorylation/activation in F9 cells.** F9 cells treated with or without retinoic acid (RA; 1 μM) for 4 days were replated on plastic dishes (A) or on Matrigel (B). The cells were cultured overnight (16 h) without serum and then stimulated with 10% fetal bovine serum (FBS) for 0–90 min. Total cell lysates were harvested at each time point and assayed for c-Fos expression, MAPK activation (P-Erk), and total MAPK (Erk).

**Fig. 8. Schematic representation of Ras/MAPK signal pathway regulation in the epithelial differentiation of F9 embryonic carcinoma cells.** In embryonic carcinoma cells, retinoic acid (RA), induces laminin (Lam) expression directly (23) and induces expression of Dab2 and collagen IV (Col IV) through the GATA-6 transcription factor (50). Dab2 mediates the effect of retinoic acid (RA) on the uncoupling of serum and growth factors (GF) stimulated c-Fos expression and MAPK activation, and cell growth suppression (24). On the other hand, laminin and collagen IV form basement membrane, and the basement membrane sensitizes MAPK activation. As a result, the Ras/MAPK pathway confers basement membrane dependence.
tion of these two separate differentiation-associated alterations results in the activation of c-Fos expression, the downstream target of Ras/MAPK pathway, to become basement membrane-dependent. As a result, following visceral endoderm differentiation, the serum- and growth factor-activated Ras/MAPK/Erlk1/c-Fos pathway and cell growth depend on attachment to basement membrane (Fig. 8). Therefore, the Ras/MAPK pathway acts to ensure the growth advantage of epithelial cells attached to the basement membrane, contributing to the organization of visceral endoderm epithelium along a sheet of basement membrane.

Presumably, Dab2 expression contributes to the growth-suppressive activity of retinoic acid in F9 cells in culture by suppressing c-Fos expression, disassociated from MAPK activation. Dab2 can suppress c-Fos expression in other epithelial cell types besides endoderm cells (53). Uncoupling of MAPK activation and c-Fos expression was observed in other scenarios such as the expression of α-synuclein (54), KSR (kinase suppressor of Rass55, 56), and Gab2 (57). The mechanism for the uncoupling of MAPK activation and Elk-1 phosphorylation by Dab2 is not yet clear. Cellular endocytic trafficking is likely to play a role in transporting and regulating the convergence and disassociation of the kinase MAPK and the substrate Elk-1, first because Dab2 is known to associate with megalin (58) and myosin VI (59, 60), and additionally, all three proteins, Dab2 (61), megalin (62), and myosin VI (63), are thought to participate in the endocytic transport of membrane vesicles and attached signaling molecules (including MAPK and Elk-1). Furthermore, endocytosis and cellular trafficking are known to regulate cellular signaling (64, 65).

The mechanism for the effect of basement membrane contact on MAPK activation presumably involves integrins. The engagement of integrin is known to activate the Ras/MAPK pathway in NIH3T3 fibroblasts (20, 24, 25). In fibroblasts, attachment of the cells to a surface as opposed to suspension, is sufficient for MAPK activation (20–25). Unlike fibroblasts, differentiated F9 cells appear to require contact with an intact basement membrane, rather than with just the surface or individual components of the basement membrane, to enhance MAPK activation. Possibly, the collaboration between subtypes of integrins specific for binding to collagen IV and laminin mediates the MAPK activation. Alternatively, other basement membrane-binding cell surface receptors such as megalin and LRP may be involved. We found that the activity to enhance MAPK activation can be mimicked by Matrigel but not by individual or a combination of purified components, including collagen IV, laminin, and fibronectin. It is possible that other minor basement membrane component(s) such as SPARC in Matrigel contributes to the activity in enhancing MAPK activation. Alternatively, the mixing of purified individual components in vivo is not able to mimic fully the biochemical structure; hence the activity of the basement membrane. Matrigel preparation, on the other hand, may be able to preserve basement membrane properties constituted by the components thereof.

The uncoupling of c-Fos expression from MAPK activation is mediated by Dab2 in both visceral endoderm cells (46, 47) and other epithelial cells of adult tissues such as breast (53) and ovary (66). Thus, it is likely that the role of Dab2 regulation of the Ras/MAPK pathway in epithelial organization is not unique to visceral endoderm cells but is common to other epithelial cells. Dab2 is often lost in epithelial tumor cells (67, 68), and its loss correlates with the transformation of the epithelial cells to become basement membrane-independent and disorganized (66, 69). Previously, Dab2 has been proposed to function in epithelial cell positional organization (51, 66). The current conclusion that Ras/MAPK signaling is basement membrane-dependent provides a mechanism for the role of Dab2 in epithelial cell positional organization and underlines the critical role of Dab2 expression loss in the epithelial cell transformation to become basement membrane-independent in tumorigenicity.

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