

Targeted Deletion of $\beta 1$ Integrins in F9 Embryonal Carcinoma Cells Affects Morphological Differentiation but not Tissue-specific Gene Expression

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Abstract. The integrin superfamily of heterodimeric transmembrane adhesion receptors mediates many cell-cell and cell-matrix interactions whose functions are believed to be critical for normal morphogenesis and differentiation. By eliminating the $\beta 1$ integrin gene through homologous recombination, we have assessed the role of the $\beta 1$ integrin family in the F9 embryonal carcinoma model for endodermal differentiation. F9 cells were unexpectedly found to maintain three copies of the $\beta 1$ gene and complete elimination required three sequential rounds of targeting to generate triple knockout lines ($\beta 1$ TKO).

Elimination of the $\beta 1$ integrin family of adhesion receptors from F9 cells resulted in reduced adhesion to fibronectin, laminin and collagen, but strongly enhanced adhesion to vitronectin. The absence of $\beta 1$ integrins did not promote significant compensatory up-regulation of either $\beta 3$ or $\beta 5$ subunits, both of which are known to act as vitronectin receptors when as-

sociated with αv . The loss of $\beta 1$ integrins severely affected morphological differentiation when the $\beta 1$ -deficient cells were induced to differentiate to either parietal or visceral endoderm. Parietal endoderm derived from $\beta 1$ -deficient cells retained a rounded morphology and migrated poorly on both fibronectin and vitronectin. Visceral endoderm derived from $\beta 1$ -deficient cells were also unable to form a normal, confluent epithelial monolayer; instead, a non-contiguous layer containing clumps of disorganized cells was observed. However, loss of $\beta 1$ integrins did not interfere with induction by differentiating agents of tissue-specific gene products for either visceral or parietal endoderm. These results suggest that $\beta 1$ integrins mediate morphological differentiation (migration and epithelial formation) but not tissue-specific gene expression in induced F9 cells, and that these two processes are not necessarily linked in this system.

THE proper temporal and spatial differentiation of embryonic cells is a complex process that requires signals initiated by interactions with other cells and with extracellular matrix (ECM).¹ Cells receive information from ECM through cell surface receptors. Of particular importance are the integrins, a family of heterodimeric, transmembrane receptors that interact with both ECM ligands and intracellular components, including cytoskeleton-associated proteins (Damsky and Werb, 1992; Hynes, 1992). Studies using function-perturbing reagents (e.g., antibodies) have indicated that integrins containing the $\beta 1$ subunit, in particular, communicate signals that help to regulate the terminal

differentiation of several cell types, including myoblasts, keratinocytes, and mammary epithelium (Menko and Boettiger, 1987; Streuli et al., 1991; reviewed by Adams and Watt, 1993). Such studies have also suggested that integrins influence morphogenetic events such as amphibian gastrulation, chick neural crest cell migration, trophoblast outgrowth and invasion, and neurite outgrowth (Bronner-Fraser, 1986; Boucault et al. 1984; Sutherland et al., 1993; Tomaselli et al., 1987). Strong support for a critical role for integrins in morphogenesis is also found from analyses of mutations in integrin complexes present in *Drosophila* (Leptin et al., 1989; Bogaert et al., 1987) in which muscle attachment sites were observed to be highly abnormal. However, studies suggesting a role for $\beta 1$ integrins in differentiation in higher organisms are indirect, as there are no known naturally occurring $\beta 1$ gene mutations of any kind in such organisms.

F9 embryonal carcinoma cells present a simple and tractable system for assessing the role of $\beta 1$ integrins in cell adhesion, embryonic morphogenesis, and cellular differentiation. These cells can differentiate to two types of cells: visceral

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1. **Abbreviations used in this paper:** α FP, α fetoprotein; DKO, double knock-out; ECM, extracellular matrix; hyg, hygromycin; neo, neomycin; PE, parietal endoderm; RA, retinoic acid; SKO, single knock-out; SSEA, stage-specific embryonic antigen; TKO, triple knock-out; tPA, tissue plasminogen activator; VE, visceral endoderm.

(VE) and parietal (PE) endoderm, which display distinctive morphologies and express tissue-specific differentiation markers (Hogan and Taylor, 1981). F9 cells can be induced to form a single-cell layer of polarized epithelium (VE) around a core of stem cells by culturing them as aggregates in suspension for 7–10 d in the presence of retinoic acid (RA). This epithelium is characterized by the loss of the stem cell marker SSEA-1 (stage-specific embryonic antigen-1) (Solter and Knowles, 1978; Casanova and Grabel, 1988), induction of alpha fetoprotein (α FP), and the concomitant organization of a basement membrane rich in laminin and collagen IV (Hogan and Taylor, 1981; Hogan et al., 1983; Grover et al., 1983b). The differentiation of VE in F9 cells mimics developmental events in vivo, in which a columnar epithelium with an organized basement membrane forms around an undifferentiated mass of cells (the inner cell mass, from which the embryo proper is derived), which then produces α FP as its histotypic gene product (Dziadek and Adamson, 1978). Previous studies have shown that addition of high levels of exogenous laminin, or antibodies against laminin, to F9 aggregates inhibits normal VE morphogenesis and also prevents synthesis of α FP, suggesting a critical role for ECM and ECM receptors in influencing gene expression as well as morphological differentiation (Grover et al., 1983a).

The alternative differentiation pathway to parietal endoderm is induced by treatment of F9 cells with RA and dibutyryl cyclic AMP (dbcAMP) (Strickland et al., 1980). Under these conditions, refractile, highly migratory SSEA-1-negative cells are generated (Strickland et al., 1980; Marotti et al., 1982; Imada et al., 1990) that express elevated levels of collagen IV, laminin, and tissue plasminogen activator (tPA). As in the case of VE, the behavior and biochemical properties of F9-derived PE mimic those properties of PE in the mouse embryo, in which cells detach from the periphery of the stationary endoderm layer that covers the inner cell mass, migrate along the inner surface of the trophoblast and egg cylinder, and synthesize the proteins characteristic of F9-derived PE. Previous work (Grabel and Watts, 1987) has suggested that PE differentiation, as well as migration, may be influenced by fibronectin via the fibronectin receptor ($\alpha 5\beta 1$) since outgrowth and differentiation of PE cells from embryonal carcinoma cell aggregates was greatly enhanced on fibronectin, and could be blocked with RGD peptides or anti-fibronectin receptor antibodies. These data, again, suggest a contribution of cell-ECM interactions in regulating both cell behavior and differentiation.

$\beta 1$ integrin protein is present throughout early mouse development, and several of its associated α -subunits show regulated expression during stages when rapid changes in tissue organization, cellular adhesion, and differentiation are occurring (Sutherland et al., 1993), suggesting that integrins may be involved in these important processes. However, the specific functional roles that $\beta 1$ integrins play during murine embryogenesis remain ill-defined due to the lack of function-perturbing reagents, the inaccessibility of the embryo to experimental manipulation, and the lack of naturally occurring genetic mutations in the $\beta 1$ integrin gene in vertebrates. Therefore, to directly address the role of $\beta 1$ integrins in early development, $\beta 1$ integrin expression was completely eliminated in F9 embryonal carcinoma cells through homologous recombination. This approach has made possible the analysis of integrin function in differentiation as well as in several

morphogenetic processes (epithelialization, cell migration, and organization of basement membrane) in an accessible, well-studied model for endoderm differentiation.

Materials and Methods

Cell Culture

Parental F9, and integrin $\beta 1$ single (SKO), double (DKO) and triple (TKO) knockout F9 cell lines were routinely cultured in 10% FCS in DMEM containing 4.5 g/l glucose, plus 84 μ M 2-mercaptoethanol (β ME), 100 μ g/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamine.

For differentiation studies, cells were cultured in Ham's F12:DMEM 1:1, supplemented with penicillin/streptomycin, glutamine and 10% FCS, as above. Differentiation to PE was carried out by culturing parental F9, or the knockout lines, in suspension on bacteriological-grade plastic. After 3 d in culture the cells typically formed tight balls of undifferentiated cells ("bods") which were then cultured for an additional 5 d in wells coated with 5 μ g/ml fibronectin or 10 μ g/ml vitronectin in the presence of 10^{-7} M RA and 10^{-3} M dbcAMP. PE could also be induced in monolayer culture by culturing F9 or knockout cells directly on tissue culture plastic in the presence of RA and dbcAMP for 5 d. When indicated, PE differentiation was carried out in serum-free differentiation medium (Ham's F12:DMEM 1:1 supplemented with 0.1% Mito plus, a serum supplement that contains some growth factors, but no attachment factors [Collaborative Research, Bedford, MA] and RA or RA/dbcAMP). VE was generated by maintaining F9 and TKO bods in suspension for 7–9 d in the presence of 5×10^{-8} M RA.

Vector Construction

To isolate $\beta 1$ integrin genomic clones, a genomic pEMBL3 library from adult Balb/c mouse liver (Clontech, Palo Alto, CA) was probed with an 875 bp BstNI fragment from the 5' region of a mouse $\beta 1$ integrin cDNA clone (Tominaga, 1988). Two positive clones were identified, subcloned into Bluescript II KS (Stratagene, La Jolla, CA) and mapped to locate intron/exon boundaries. A 6-kb EcoRI/BamHI fragment spanning the first three coding exons of the $\beta 1$ integrin gene was further subcloned into Bluescript (referred to as pBS $\beta 1$ -6.0) and used in construction of positive/negative (Mansour et al., 1988) targeting vectors. Recombinant PCR (Higuchi, 1990) (see Table I for primer sequences and PCR conditions) was used to insert a promoterless neo' cassette (derived from pMC1neopA, Stratagene) in frame immediately downstream of the $\beta 1$ integrin AUG start site. This was accomplished by designing special PCR primers (Table I) which would amplify a 510-bp region of the $\beta 1$ integrin gene between the 5' EcoRI site and the AUG start site and would add a 3' overhanging end homologous to the first 16 bp of the neomycin gene. Similarly, the entire coding region of pMC1neopA including the poly A tail (925 bp) was amplified using primers which would add a 5' overhanging end homologous to the last (most 3') 16 bp of the $\beta 1$ integrin PCR fragment. When the products of these two amplifications were combined in the "recombinant" PCR step, the $\beta 1$ 3' and neomycin 5' overhanging ends annealed and provided a novel "fused" template which could be amplified in its entirety using the left $\beta 1$ and the right neomycin primers. This fusion then generated a 1,435-bp $\beta 1$ integrin/neo fusion product which was digested with EcoRI/BamHI and ligated back into the original 6-kb $\beta 1$ integrin plasmid (pBS $\beta 1$ -6.0) digested with EcoRI/BclI. To confer negative as well as positive selection capability to the targeting vector, a 1.5-kb Xba-BamHI fragment (spanning the HSV-tk gene) of pIC19R/MC1-tk (gift of M. Capecchi, University of Utah, Salt Lake City, UT) was inserted in the BamHI site at the 3' end of the $\beta 1$ integrin insert thus generating the complete pBS $\beta 1$ /neo/tk targeting vector. Following correct gene targeting, the neomycin insertion prevents synthesis of $\beta 1$ transcripts while neomycin transcripts are generated from the active, endogenous $\beta 1$ integrin promoter, thus conferring resistance to the drug G418. In contrast, the HSV-tk gene is lost during homologous recombination, rendering targeted clones insensitive to FIAU (1-[2-deoxy, 2-fluoro-b-D-arabinofuranosyl]-5 iodouracil; Bristol-Myers Squibb, Wallingford, CT).

The hygromycin resistance-containing vector was constructed in a similar manner using recombinant PCR to fuse the promoterless hygromycin-resistance cassette (derived from pGK-hyg, gift of R. Mortenson, Brigham and Women's Hospital, Boston, MA) just downstream of the $\beta 1$ integrin AUG start site (Table I). In this case, PCR primers were designed to generate a 455-bp $\beta 1$ integrin fragment with an EcoRI/ClaI overhang at the 5' end (for future cloning purposes), and at the 3' end, just downstream of the AUG start site, an overhang homologous to the first 16 bp of the hygromycin-resistance gene was added. A short 279-bp hygromycin-resistance fragment

Table I. Primers Used for PCR Amplifications

β 1/neo recombinant PCR:

β 1 primers: left 5' ATTAACCCTCACTAAAG 3' (=T3 primer)
right 5' CAATGGCCGATCCCATCTATAACAGAAAATG 3'
neo primers: left 5' TTTTCTGTTATAGATGGGATCGGCCATTG 3'
right 5' GGATAACAATTTACACA 3'

β 1/hyg recombinant PCR:

β 1 primers: left 5' CCGAATTCATCGATATGTTTTAGCCTGAGGAG 3'
right: 5' GTTCAGGCTTTTCATCTATAACAGAAAATG 3'
hyg primers: left 5' CATTCTGTTATAGATGAAAAGCCTGAAC 3'
right 5' CAATAGGTCAGGCTCTCG 3'

PCR reactions were carried out in 50 μ l final volume of the following mixture: 0.01 M Tris, pH 8.3, 0.05 M KCl, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 10 pM oligonucleotide primers, 0.5 U Taq 1 polymerase (AmpliTaq, Perkin-Elmer-Cetus Corp.), and 1 μ g plasmid template DNA. Temperature cycling was as follows: 94°C for 1 min, 48°C for 2 min, and 72°C for 3 min, 25 cycles.

was amplified, beginning at its AUG start site (bp 210 of pGK-hyg) and ending just downstream (bp 479) of an EcoRI site located at bp 456. The PCR primers were designed such that a 5' overhang homologous to the last 16 bp of the β 1 PCR fragment would be added to the hyg fragment. When the two PCR products were combined in the "recombinant" PCR step and amplified with the left β 1 primer and the right hyg primer, a 779-bp β 1/hyg fragment was generated and cloned into pCR (Invitrogen, San Diego, CA) according to the manufacturer's instructions. This pCR- β 1/hyg plasmid was then digested with EcoRI and the 779 bp β 1/hyg fragment was ligated back into pGK-hyg to generate an intact hygromycin-resistance gene with 455 bp of the β 1 integrin sequence added upstream of the AUG start site. This pGK- β 1/hyg plasmid was then digested with ClaI/BglII and ligated into pBS β 1-6.0. The HSV-tk gene was cloned into pBS β 1-6.0/hyg as described for pBS β 1-6.0/neo to generate the final pBS β 1-hyg/tk targeting vector.

Transfection and Selection of Targeted Clones

4 \times 10⁷ F9 cells were electroporated with 25 μ g/ml pBS β 1/neo/tk or pBS β 1/hyg/tk targeting vector in PBS at 280 V, 880 μ F in a BRL electroporator (GIBCO-BRL, Gaithersburg, MD). Electroporated cells were plated into 20 100-mm dishes and selection was imposed with either 350 μ g/ml active G418 (GIBCO-BRL) and 0.2 μ M FIAU (Bristol-Meyers Squibb) or with 300 μ g/ml hygromycin B (Calbiochem Corp., La Jolla, CA) and 0.2 μ M FIAU 24 h after plating. After 14–18 d, resistant colonies were picked and eventually expanded, frozen and DNA isolated for genomic-Southern blots.

To knock out the third copy of the β 1 integrin gene, β 1 double knock-outs (DKOs) were plated at 2.5 \times 10⁴ cells/100-mm dish and cultured for three weeks in an elevated concentration (3.5 mg/ml) of active G418. For all selections, cells were fed every other day.

Southern Blotting

10 μ g of DNA from each resistant colony was digested overnight with PvuII and separated on 0.8% agarose gels. After transfer to Duralon membranes (Stratagene) in 10 \times SSC (1.5 M sodium chloride, 0.15 M sodium citrate), blots were hybridized with a random primed 220-bp Eco/Sal fragment of the integrin gene located just 5' of the region encompassed by the targeting vector. Specific activity of the labeled fragment was >10⁹ cpm/ μ g DNA. Blots were hybridized in QuikHyb (Stratagene) overnight at 45°C and washed several times in 2 \times SSC, 0.1% SDS at room temperature followed by a 15–30 min wash in 0.5 \times SSC, 0.1% SDS at 50°C. Blots were exposed to x-ray film with intensifying screens for 24–48 h.

Antibodies

Antibodies against integrins were obtained from the following sources: rabbit anti- β 1 cytoplasmic domain from S. Carbonetto (McGill University, Montreal); rabbit anti- β 3 cytoplasmic domain from James Gailit (SUNY at Stony Brook, NY); rabbit anti- β 4 cytoplasmic domain from V. Quaranta (Scripps Research Foundation, La Jolla CA); rabbit anti- β 5 cytoplasmic domain from D. Cheresch (Scripps Research Foundation, La Jolla, CA); rabbit anti- α 3 cytoplasmic domain from R. Hynes (MIT, Cambridge, MA); hamster monoclonal anti-mouse α 5 from Pharmingen (San Diego, CA); and rat monoclonal anti-mouse α 6 (GoH3) from A. Sonnenberg (Netherlands Cancer Institute, Amsterdam). Antibodies against PE and VE differentiation markers were obtained from the following sources: mouse monoclonal

anti-stage-specific embryonic antigen (SSEA)-1, National Institute of Child Health and Human Development, Developmental Studies Hybridoma Bank (Baltimore, MD); anti-EHS laminin, E-Y Labs (San Mateo, CA); anti- α FP, ICN Biomedicals (Cleveland, OH). Secondary antibodies against mouse, rat, hamster, and rabbit IgG, conjugated to fluorescein or rhodamine were obtained from Jackson Immunoresearch (West Grove, PA).

Immunoprecipitation

Control and PE induced F9 and TKO monolayers in T75 flasks were grown to 85% confluence, labeled with 10 ml of 1 μ g/ml NHS-LC-Biotin (Pierce, Rockford, IL) in PBS on ice for 90 min, washed twice with PBS containing 50 mM glycine followed by incubation in PBS-glycine for 15 min. Cells were then lysed with 1 ml NP-40 lysis buffer (0.1 Tris-HCl, pH 7.5 containing 1% NP-40, 2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml Leupeptin, 4 μ g/ml Pepstatin A, and 100 U of Aprotinin) and triturated intermittently for 30 min. Insoluble materials were removed by centrifugation at 1,300 g for 5 min at 4°C, and then at 13,000 g for 20 min at 4°C. Each ml of supernatant was precleared by two sequential 1 h incubations with 10 μ l of packed, unconjugated Protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ). Lysates were analyzed for protein content using the BCA method (Pierce) and aliquoted to ensure that equal amounts of protein were used. Immunoprecipitations were carried out on 100–500 μ g lysate protein from induced and control F9 and TKO, depending on the antibody. Samples were incubated with the appropriate anti-integrin subunit sera at 1:50–1:100 dilution for 1 h at 4°C. The goal was to have an antibody:antigen ratio such that all antigen was precipitated with a single round of primary antibody. Immune complexes were then incubated with 100 μ l Protein A-Sepharose CL-4B beads at 4°C for 2 h. After removing the beads, the supernatant was incubated with an additional 100 μ l beads for 1 h to ensure that all immune complexes were precipitated. The Sepharose beads with bound immune complexes were washed sequentially with the following buffers: 1. HSA reagent (16 mM potassium phosphate, 0.6 M NaCl, pH to 7.4); 2. MDB reagent (16 mM potassium phosphate, 3 mM SDS, 0.05% NP-40, 300 mM NaCl, pH 8.3); 3. SA reagent (16 mM potassium phosphate, 300 mM NaCl, pH 7.4); 4. 20 mM Tris-HCl, pH 8.3. Samples were fractionated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose. The nitrocellulose was blocked with Blotto (5% Carnation nonfat dry milk, 3% BSA, 0.1% Tween-20 in PBS) and incubated for 1 h in streptavidin-conjugated HRP (Amersham, Arlington Heights, IL). Biotinylated proteins were visualized using enhanced chemiluminescence (Amersham). Quantitation of cell surface integrin expression was measured by densitometry using the program Image 1.49 (Wayne Rasband, NIH Research Services Branch). Images of immunoprecipitated proteins visualized by enhanced chemiluminescence were captured, scanned and the background subtracted. TKO values were expressed as a percentage of the F9 values to normalize the data.

Immunofluorescence

For immunofluorescence of control and PE monolayers of F9, SKO, and TKO, cells were plated on fibronectin-coated (5 μ g/ml) or vitronectin-coated (10 μ g/ml) glass coverslips (11 mm in wells of 24-well tissue culture plates; Corning Inc., Corning, NY) in serum-containing medium, with or without RA and dbcAMP. After 5 d, cells were rinsed with PBS and fixed and permeabilized for 5 min with acetone at –20°C. Coverslips were then incubated overnight in 1% BSA in PBS to prevent nonspecific antibody binding, then incubated for 1 h with primary antibody, washed five times

with PBS over 15 min, incubated for 30 min with rhodamine or fluorescein-conjugated secondary antibody, rinsed five times over 15 min with PBS and mounted in gelvatol containing *p*-phenylene diamine (Sigma Chemical Co., St. Louis, MO), to prevent quenching of fluorescence signal.

To localize antigens in control and differentiated bods, samples were rinsed in PBS, fixed overnight in ethanol:acetic acid 99:1, embedded in paraffin, sectioned (10- μ m sections) and mounted on poly-L-lysine-coated slides. Paraffin was removed with two changes of xylene and sections were rehydrated. Slides were then blocked with 0.2% BSA in PBS for 30 min and incubated for 1 h with rabbit anti-EHS laminin, rabbit anti- α FP, or mouse anti-SSEA-1 antibodies diluted in PBS. Samples were washed, incubated with secondary antibody and processed as described above.

Cell Adhesion Assay

Wells of 96-well plates (Corning Inc.) were incubated overnight at 4°C with 100 μ l ECM ligands diluted in PBS (vitronectin, GIBCO-BRL, 10 μ g/ml; collagen type IV, Collaborative Research, Inc., 10 μ g/ml; laminin, Collaborative Research Inc., 10 μ g/ml; and fibronectin, GIBCO-BRL, 5 μ g/ml). Negative control wells were incubated with 0.2% BSA. Wells were washed with PBS, and blocked with 100 μ l 0.2% BSA in PBS for 1 h, followed by three washes with PBS. Cells were harvested with 0.05% trypsin/0.02% versene and washed in serum free DMEM containing 0.2% Soybean Trypsin Inhibitor (SBTI; Sigma Chemical Co.). Cells were plated at a density of 1.6×10^4 in 100 μ l. For some experiments, GpenRGDSPCA, GRGDSP or GRGESP peptides (Telios Pharmaceuticals, La Jolla, CA) were included in the plating medium at 0.1 mg/ml. To achieve an even distribution of cells, plates were spun at 3,500 g for 10 s and incubated at 37°C. Cells on laminin, collagen, and vitronectin were incubated for 3 h while cells on fibronectin were incubated for 15 min. Incubation times were chosen which permitted attachment of the parental F9 cells. After incubation, plates containing cells on fibronectin were shaken on an orbital shaker at 300 rpm for 30 s followed by three washes with PBS. Wells with cells plated on laminin, collagen, and vitronectin were washed three times with PBS. At the end of the washes, no cells remained adhered to the BSA-coated wells. Cell adherence was quantitated using a protein quantitation kit, BCA (Pierce), by adding 100 μ l of BCA reagent to each well, incubating for 30 min at 37°C and then reading on an Elisa Plate Reader (Molecular Devices Corp., Menlo Park, CA) at 562 nm.

Migration Assay

F9 and β 1 TKO were grown in suspension for 3 d to form undifferentiated bods. They were washed in PBS to remove the serum, and then incubated in serum-free differentiation medium (DMEM:F12, 1:1, with 0.1% Mito plus and 10^{-7} M RA and 10^{-5} M dbcAMP) in 24-well dishes coated with 10 μ g/ml vitronectin or fibronectin. After 5 days of outgrowth, wells were photographed. Outgrowth was calculated on the photographs by directly measuring the distance of outgrowth from the edge of the bods in eight directions and calculating the average of the measurements. 8–10 bods were measured for each condition in each of two separate experiments.

Substrate Gel Zymography

To determine the presence of tissue plasminogen activator (tPA), a marker for PE differentiation, 10% SDS-polyacrylamide gels containing 1 mg/mL α -casein (Sigma Chemical Co.) and 13 μ g/mL human lys-type plasminogen (American Diagnostica, Greenwich, CT) were prepared. F9 and TKO cells were grown in 60-mm tissue culture dishes for 5 d in serum-free differentiation medium. Conditioned medium was harvested, spun at 600 g for 5 min and frozen at -70°C . 5 μ g protein per lane was analyzed by SDS-PAGE. SDS was removed by incubation of the gel for 1 h at room temperature in 50 mM Tris, pH 7.5, containing 2.5% Triton X-100. Gels were then rinsed five times with incubation medium (50 mM Tris, 10 mM CaCl_2 , 0.02% sodium azide, pH 7.6–8.0) and incubated for an additional 14 h at 37°C to permit protease digestion of the substrate. Gels were then stained for 15 min with Coomassie blue and destained with a mixture of 10% acetic acid and 10% methanol until the cleared bands were visible and the stacking gel was completely destained.

RNA Isolation

RNA was isolated from 7 d VE (RA-treated) and control (untreated) bods according to the method of Chomczynski and Sacchi (1987). Essentially, bods were washed twice in PBS, resuspended in 1 ml solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl,

0.1 M β -mercaptoethanol), 1 ml phenol:chloroform (24:1), and 0.1 ml 2 M sodium acetate pH 4.7. The suspension was mixed thoroughly, spun at 10,000 g for 20 min and the aqueous layer removed to a fresh tube. RNA was precipitated for 30–60 min at -20°C with 1 ml isopropyl alcohol and collected by centrifugation at 10,000 g for 20 min. The pellet was resuspended in 200 μ l of solution D, reprecipitated with an equal volume of isopropyl alcohol, chilled to -20°C and again collected by centrifugation, rinsed with 70% ethanol, resuspended in water, and the concentration determined by UV spectroscopy at A_{260} .

Northern Blot Analysis

20 μ g of RNA from each sample was run on a 1.2% agarose gel containing 6.3% formaldehyde and $1\times$ MOPS (20 mM MOPS, 5 mM sodium acetate, pH 7, 1 mM EDTA) buffer, then blotted to Duralon membrane in $20\times$ SSC overnight. The RNA was UV cross-linked to the membrane in a Stratelinker (Stratagene), prehybridized for 30 min at 65°C in Quikhyb and hybridized overnight in 2×10^6 cpm of probe per ml of hybridization buffer. Blots were then washed in $1\times$ SSC, 0.1% SDS at room temperature followed by washes in $0.2\times$ SSC, 0.1% SDS at 60°C. Blots were exposed to Kodak X-O-mat XAR film (Eastman Kodak Co., Rochester, NY) for 24–48 h. To confirm that equal amounts of RNA were loaded per lane, blots were stripped and reprobed with GapDH.

Probe Synthesis

α FP (gift of S. Tilgman, Princeton University, Princeton, NJ), Endo B (gift of R. Oshima, La Jolla Cancer Foundation, La Jolla, CA) and GapDH probes were all generated by random priming of cDNA.

Results

Inactivation of β 1 Integrin by Gene Targeting

To prevent expression of β 1 integrin in F9 cells, two vectors carrying either the neomycin-resistance (*neo*^r) or hygromycin-resistance (*hyg*^r) gene were constructed (Fig. 1) (Mansour et al., 1988). Each vector contained a 6-kb EcoRI/BamHI fragment of the β 1 integrin gene spanning the first three coding exons. We made use of the positive/negative selection scheme (Mansour et al., 1988) using neomycin and hygromycin for positive selections and the HSV *tk* gene for negative selections. Furthermore, we took advantage of the fact that the β 1 integrin subunit is expressed strongly in F9 cells, by using promoterless *neo*^r and *hyg*^r genes to select further against random integrations. Recombinant PCR (see Materials and Methods) was employed to insert the promoterless selective markers in frame with and immediately downstream of the endogenous β 1 AUG start site. This approach avoided inserting the selective marker genes significantly downstream of the β 1 start site which would have resulted in β 1/*neo*^r or β 1/*hyg*^r fusion proteins with potentially compromised abilities to confer resistance.

F9 cells were transfected with pBS β 1/*neo*/*tk* and, by Southern blotting, four out of 80 clones showed the appearance of the diagnostic 1.2-kb PvuII restriction fragment, in addition to the wild-type 3.5-kb fragment (Fig. 2 a, lane 2). A neomycin probe hybridized exclusively to this 1.2-kb fragment in all four β 1 single knock-out (SKO) clones, indicating single insertion events of the targeting vector in each of these clones (data not shown).

Initial characterization of these four clones showed no significant differences from the parental F9 cells in adhesiveness or ability to form morphologically normal parietal and visceral endoderm (not shown). One clone, β 1 SKO-59, was used for inactivation of the second β 1 integrin allele using the pBS β 1/*hyg*/*tk* targeting vector. Cells were selected in both G418 (to retain the *neo*-targeted allele) and hygromycin

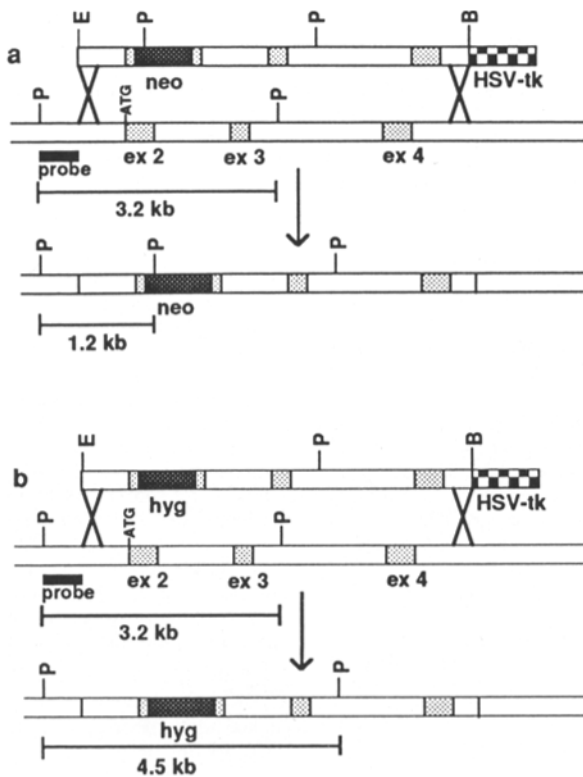


Figure 1. $\beta 1$ integrin targeting vectors used for homologous recombination. A 6-kb EcoRI/BamHI fragment spanning the first three coding exons (ex 2–4) of the $\beta 1$ integrin gene received either the neomycin-resistance gene (a) or the hygromycin-resistance gene (b), inserted into the first coding exon to generate the pBS $\beta 1$ /neo/tk vector (a) and the pBS $\beta 1$ /hyg/tk vector (b). Integration of the construct at the homologous site creates the novel locus characterized by a novel 1.2-kb PvuII site in the case of the pBS $\beta 1$ /neo/tk vector, or a novel 4.5-kb PvuII site in the case of the pBS $\beta 1$ /hyg/tk. E, EcoRI; B, BamHI; P, PvuII.

B. Out of 142 clones screened, four $\beta 1$ double knock-out (DKO) colonies were identified by the presence of both the 1.2-kb (neo) and a 4.5-kb (hyg) PvuII restriction fragments on Southern blots (Fig. 2 a, lane 3). However, all four clones still carried at least one additional, nontargeted copy of the $\beta 1$ integrin gene, as indicated by the presence of the normal, 3.5-kb PvuII fragment. A third round of selection was carried out by culturing clone $\beta 1$ DKO 84 in highly elevated levels of G418 (3.5 mg/ml). This procedure selected for cells which had undergone a spontaneous gene conversion event (Mortensen et al., 1992), replacing a normal copy of the $\beta 1$ integrin with the previously targeted copy carrying the neo insertion. Of 36 clones screened, six $\beta 1$ integrin triple knock-out ($\beta 1$ TKO) clones were identified by the disappearance of the normal 3.5-kb PvuII band and an apparent enrichment of the 1.2-kb neo band on Southern blots (Fig. 2 a, lane 4). Successful inactivation of all three $\beta 1$ integrin genes was further confirmed by immunoprecipitation of biotinylated cell surface proteins with anti- $\beta 1$ antibodies (Fig. 2 b). Each sequential inactivation of the $\beta 1$ integrin genes resulted in correspondingly lower levels of $\beta 1$ integrin protein, indicating that all three copies of the $\beta 1$ integrin genes were active in the parental F9 cells (Fig. 2 b, lane 1) and that all three copies had been inactivated in the TKO clones (Fig. 2 b, lane 4).

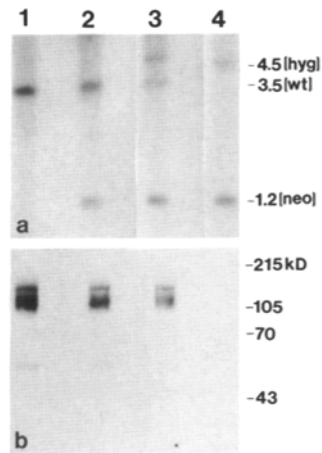


Figure 2. Southern blot (a) and immunoprecipitation (b) showing successful inactivation of all three $\beta 1$ integrin alleles. (a) PvuII-digested genomic DNA from F9 (lane 1), SKO (lane 2), DKO (lane 3), and TKO (lane 4) cells was hybridized with a $\beta 1$ cDNA probe shown in Fig. 1. The 3.5-kb band represents the untargeted $\beta 1$ allele, while the 1.2 and 4.5 kb bands are obtained after successful targeting with the pBS $\beta 1$ /neo/tk and pBS $\beta 1$ /hyg/tk constructs, respectively. The TKO cells (lane 4) have lost the normal

3.5-kb band, indicative of a complete knock-out. (b) Biotinylated cell surface proteins were immunoprecipitated with an antibody against the cytoplasmic domain of $\beta 1$ integrin. The $\beta 1$ subunit runs as a broad band between 105–120 kD. Each round of transfection and selection resulted in a decrease of $\beta 1$ integrins until no $\beta 1$ integrins could be detected in the TKO lines (lane 4).

Inactivation of $\beta 1$ Integrins Alters F9 Cell Morphology

All six $\beta 1$ TKO clones attached to plastic and gelatin in the presence of serum, but spread poorly in comparison to F9 or SKO cells, as indicated by the highly rounded morphology of TKO colonies (Fig. 3 d) compared to the flattened, triangular phenotype characteristic of F9 (Fig. 3 a), SKO (Fig. 3 b), and DKO lines (Fig. 3 c). $\beta 1$ TKO cells maintained very close cell-cell contacts within colonies such that cell borders were often indistinguishable at the level of the light microscope. This phenotype appears stable since several TKO clones have maintained this morphology in culture after more than 80 cell doublings.

$\beta 1$ TKO Cells Do Not Express α Subunits That Interact Exclusively With $\beta 1$, But Continue to Express Integrin Complexes Containing Other β Subunits

To determine how the loss of $\beta 1$ integrins affected expression of other α and β integrin subunits, biotinylated cell surface proteins from parental F9 cells and two separate TKO clones were immunoprecipitated with a variety of antibodies against α and β subunits. As expected, neither the $\alpha 3$ or $\alpha 5$ subunits, both of which form heterodimers only with $\beta 1$, were detected on the surface of $\beta 1$ TKO cells (Fig. 4, a and b, lanes 2 and 3), while both subunits were present at high levels on the parental F9 cells (Fig. 4, a and b, lane 1). In contrast, a small amount of $\alpha 6$, which can associate with $\beta 4$ as well as $\beta 1$, was immunoprecipitated from the surface of $\beta 1$ -deficient cells (Fig. 4 c, lanes 2 and 3) and was associated with a band that co-migrated with $\beta 4$ from mouse mammary epithelial cells (lane 4). In contrast, parental F9 cells contained high levels of $\alpha 6$, associated predominantly with $\beta 1$ (Fig. 4 c, lane 1).

Immunoprecipitation with antibodies against other β subunits was carried out to determine whether loss of the $\beta 1$ subunit resulted in any compensatory up-regulation of other β subunits. As shown in Fig. 4, d and e, parental F9 cells (lane 1) and two separate $\beta 1$ TKO clones (lanes 2 and 3) expressed at least two alternative β subunits, $\beta 3$ and $\beta 5$, which

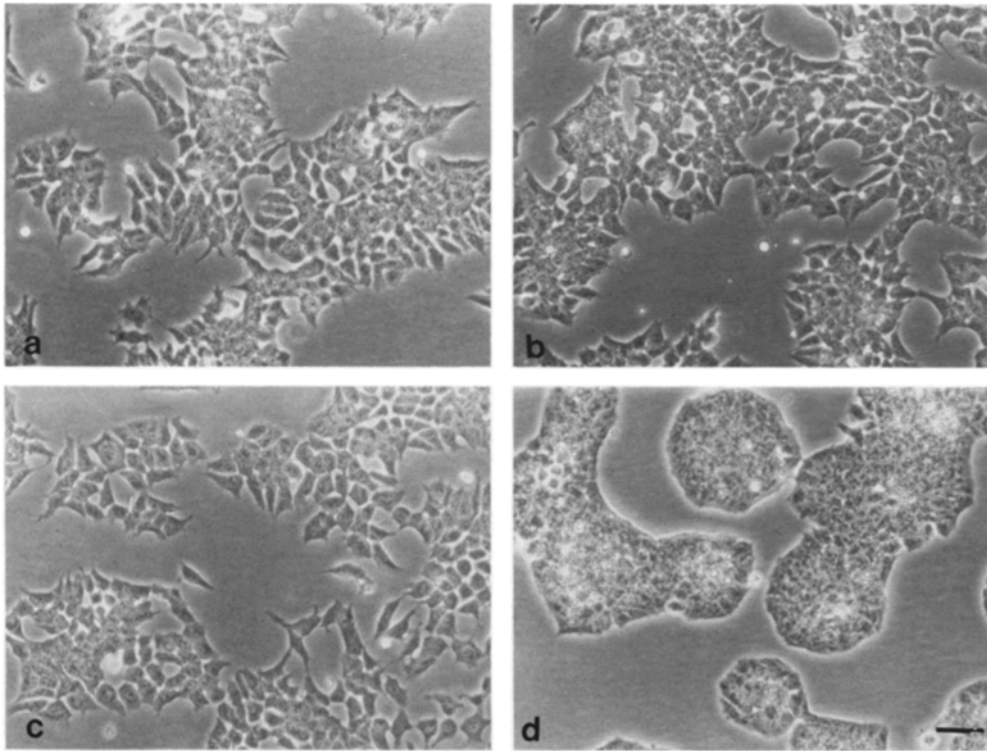


Figure 3. Photomicrograph of parental F9 cells (a), $\beta 1$ SKO (b), $\beta 1$ DKO (c), and $\beta 1$ TKO cells (d) plated on tissue culture plastic in the presence of serum. Cells in F9, SKO and DKO colonies are well spread and flattened, while cells in $\beta 1$ TKO colonies are more tightly packed and poorly spread. Bars, 25 μm .

are known to associate with αv . Immunoprecipitations were carried out in the presence of excess antibody so that all of the cognate integrin present in the cell lysate was bound in the first round of precipitation. This permitted a quantitative comparison of the relative amounts of each integrin expressed on the surface of F9 and TKO cells by performing densitometric analysis (Table II). In three separate experiments using two different TKO cell lines, the $\beta 3$ subunit was present in slightly reduced quantities, and although this difference was of marginal significance (p values 0.04 and 0.16), it was consistently observed. In the case of $\beta 5$, both TKO lines showed a consistent but not statistically significant increase in the levels of this subunit (p values 0.11 and 0.10).

Inactivation of $\beta 1$ Integrins Results in Decreased Adhesion to Fibronectin, Laminin, and Collagen IV, but Increased Adhesion to Vitronectin

One of the major functions of integrins is to mediate cell-substrate attachment. Therefore, we tested how the loss of $\beta 1$ integrins altered adhesion to specific substrates. Adhesion of two separate $\beta 1$ TKO lines was compared to parental F9

cells on purified substrates in the absence of serum. As illustrated in Fig. 5 A, attachment of $\beta 1$ TKO cells to laminin, collagen IV and fibronectin were all reduced to background levels (nonspecific binding to BSA). Surprisingly, attachment to vitronectin was greatly enhanced in both $\beta 1$ TKO clones, reaching seven times that exhibited by the parental line.

$\beta 3$ and $\beta 5$ integrins are the principal vitronectin receptors in the integrin family (Pytela, 1985; Cheresh et al., 1989). Therefore, to determine whether the enhanced adhesion to vitronectin was integrin mediated, it would have been ideal to incorporate function-perturbing antibodies against the $\beta 3$ and $\beta 5$ integrins into the adhesion assays. However, such reagents are not available for mouse cells. Therefore, RGD-containing peptides (GpenRGDSPCA or RGDSP) which interfere with adhesion to vitronectin (Pierschbacher and Ruoslahti, 1987), were incorporated into the adhesion assays. Fig. 5 b shows that the adhesion of F9 and both TKO lines to vitronectin was almost completely inhibited by the cyclic peptide, and similar results were obtained with the linear GRGDSP (not shown). A control RGE peptide (GRGESp) had no effect on adhesion. These results sug-

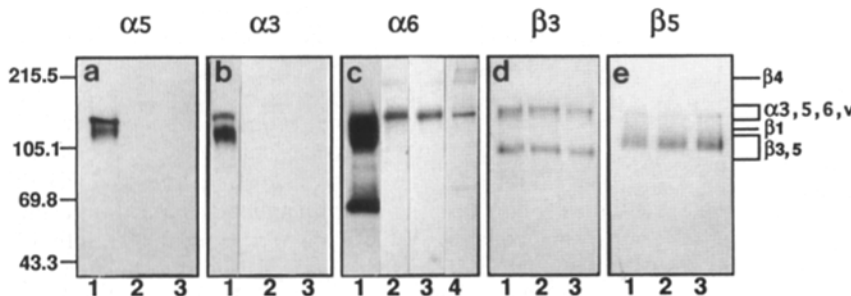


Figure 4. Immunoprecipitations of biotinylated cell surface proteins from F9 and TKO cells with antibodies against $\alpha 5$ (a), $\alpha 3$ (b), $\alpha 6$ (c), $\beta 3$ (d), and $\beta 5$ (e) integrin subunits. Equal amounts of protein from F9 cells (lanes 1) and two separate TKO cell lines (lanes 2 and 3) were immunoprecipitated as described in Materials and Methods. A positive control cell line, mouse mammary tumor epithelium (MMTE), was used in immunoprecipitation with anti- $\alpha 6$ antibody (c, lane 4) to indicate the position of the $\beta 4$ subunit.

Table II. Levels of $\beta 3$ and $\beta 5$ Protein Measured by Densitometric Scanning of Immunoprecipitated Cell Lysates

	$\beta 3$			$\beta 5$		
	F9	TKO-1	TKO-2	F9	TKO-1	TKO-2
	%	%	%	%	%	%
Mean ($n = 3$)	100	68	82	100	118	122
Standard Error	—	9.6	13.3	—	10.7	12.2
<i>P</i> value	—	0.04	0.16	—	0.11	0.10

Values obtained by densitometric analysis (see Materials and Methods) of three separate experiments were averaged and expressed as a percentage of F9 controls, which were arbitrarily set at 100%. *P* values were derived by the Student's *t* test.

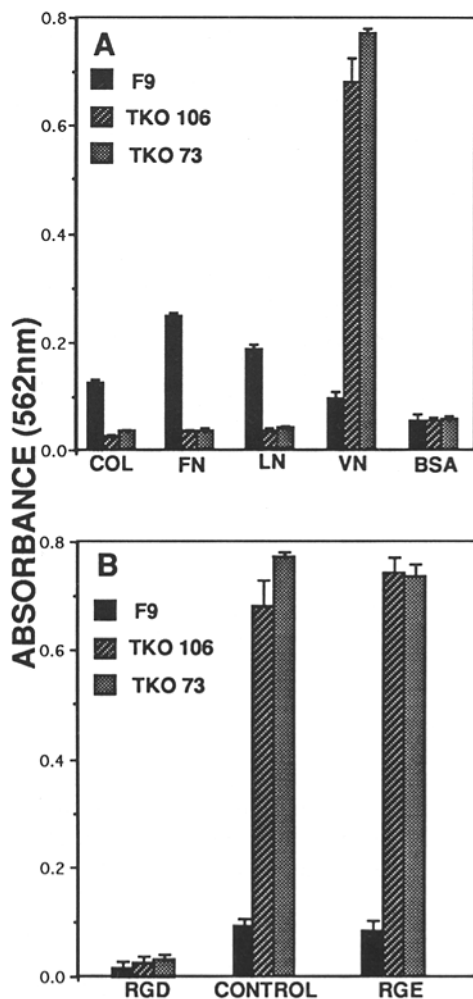


Figure 5. Adhesion assays performed with F9 and two TKO cell lines on purified substrates in the absence of serum. (A) F9 and TKO cells were plated in 96 well plates coated with 5 $\mu\text{g}/\text{ml}$ collagen IV, 5 $\mu\text{g}/\text{ml}$ fibronectin, 5 $\mu\text{g}/\text{ml}$ laminin, and 10 $\mu\text{g}/\text{ml}$ vitronectin. Cells were in contact with collagen IV, laminin, and vitronectin for 3 h and with fibronectin for 15 min. (B) Adhesion of F9 and TKO cells to plates coated with 10 $\mu\text{g}/\text{ml}$ vitronectin in the presence of 0.1 mg/ml GpenRGDSPCA or GRGESP peptide, or no peptide. Adhesion assay was performed 3 h after plating.

gested that the enhanced binding to vitronectin was integrin mediated. Thus, while changes in the levels of $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ were very modest, the increase in adhesion to vitronectin was dramatic, suggesting that the actual activity of these receptors was modulated due to the absence of the $\beta 1$ subunit.

$\beta 1$ Integrin Deficient Cells Differentiate, But Fail to Undergo Normal Morphogenesis

As mentioned previously, F9 cells are capable of differentiating into two types of extra-embryonic endoderm: parietal (PE) and visceral (VE) endoderm. In both cases, it has been suggested that extracellular matrix molecules may provide signals to these differentiating cells (Grover and Adamson, 1985; Grabel and Watts, 1987), and these signals may be transduced via integrin receptors. To assess directly the role of the $\beta 1$ integrin family in PE and VE differentiation, $\beta 1$ TKO cells were induced to differentiate and were examined for their morphological properties as well as for the expression of VE- and PE-specific biochemical markers.

PE was induced by culturing aggregates of normal F9 or TKO cells in suspension for 3 d and then plating these "bods" on fibronectin or vitronectin in the presence of RA and dbcAMP as described in Materials and Methods. After 4 d of outgrowth on vitronectin, cells from the normal F9 bods (Fig. 6 a) had migrated away from the central aggregate as single cells and differentiated into highly refractile cells with extensive cellular processes. In contrast, cells from $\beta 1$ TKO bods (Fig. 6 b) progressed only a short distance from the original aggregate, forming a nearly continuous ring of rounded cells around the bod. The same results were seen using fibronectin as a substrate (data not shown). These observations were quantitated by measuring how far the cells had migrated out from the edge of the bods. The ring of TKO cells extended out only one-third the distance traveled by F9 cells on fibronectin and traveled one-fifth the distance on vitronectin (Fig. 6 c). Thus the loss of $\beta 1$ integrins resulted in significantly reduced migration of TKO-derived PE cells.

We then examined whether $\beta 1$ TKO cells would express the tissue specific pattern of genes typical of PE, despite their poor migratory ability. F9 or TKO cells were plated on glass coverslips in serum and induced to differentiate to PE for 4 d in the presence or absence of RA and dbcAMP. In the absence of inducing agents, colonies of F9 cells showed bright cell surface staining throughout the colony for the stem cell marker SSEA-1 (Fig. 7 a). After induction to PE, cells within the growing colonies differentiated and migrated away as single cells. Those cells which had migrated away from the colonies were well spread, contained numerous cell processes, and were SSEA-1 negative (Fig. 7, c and d). Similarly, all cells in colonies of untreated $\beta 1$ TKO also stained strongly for SSEA-1 (Fig. 7 b). In colonies of TKO cells treated with inducing agents (TKO PE), most cells were SSEA-1 negative, even though they were unable to migrate extensively (Fig. 7, e and f) and retained a rounded morphology. As with the F9 PE cells, occasional clusters of positively stained undifferentiated cells remained in the $\beta 1$ TKO PE cultures (Fig. 7 c). Staining with antibodies to laminin (Fig. 7, g-j) and collagen IV (not shown) revealed that both F9 PE (Fig. 7 i) and $\beta 1$ TKO PE (Fig. 7 j) cells accumulated high levels of these matrix proteins while untreated cultures

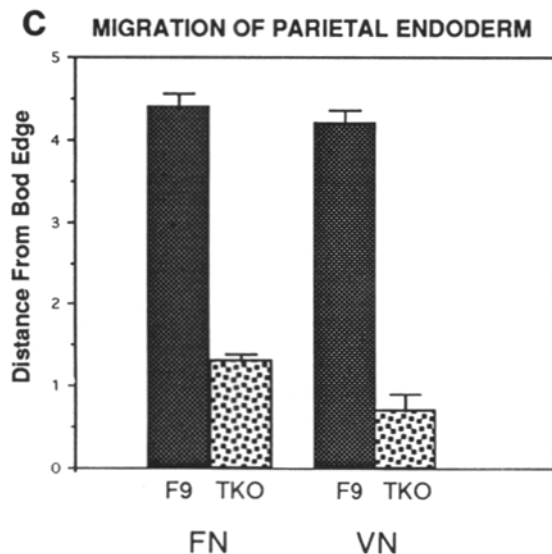
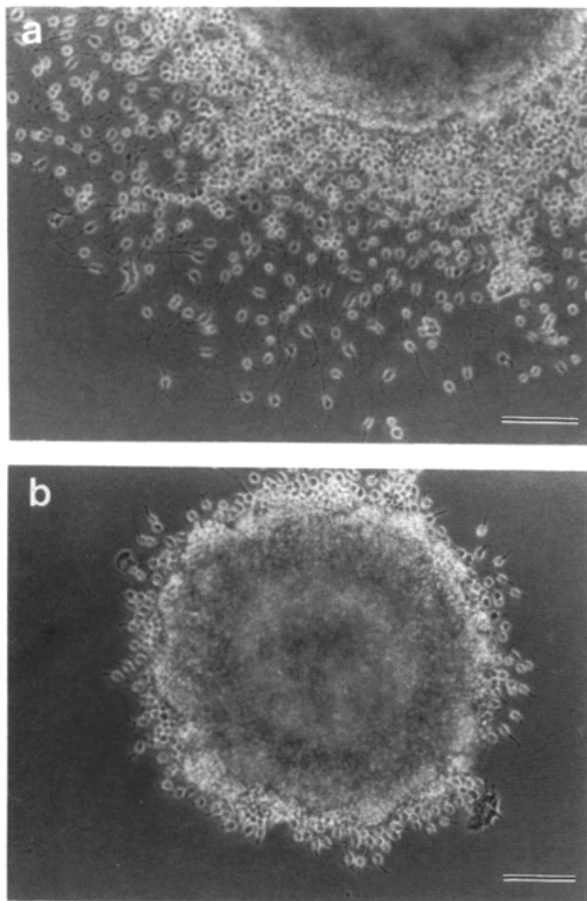


Figure 6. Phase micrographs of outgrowth of F9 and $\beta 1$ TKO PE bods plated on fibronectin and vitronectin-coated substrates. Three day old untreated F9 or TKO bods were plated on vitronectin (*a* and *b*) or fibronectin (not shown) for 4 d in serum-free medium containing RA and dbcAMP. F9 PE cells migrated extensively away from the bods as single cells (*a*). TKO PE formed a solid halo of cells around the bod (*b*). The distance to the edge of the F9 and TKO PE outgrowths on fibronectin and vitronectin was determined as described in Materials and Methods (*c*). Bars, 25 μ m.

showed only low levels of diffuse or punctate staining for these markers (Fig. 7, *g* and *h*).

To further establish that the RA/dbcAMP-induced $\beta 1$ TKO cells had in fact differentiated to PE, levels of tPA activity were examined on casein substrate gels. Two separate untreated TKO lines (Fig. 8 *a*, lanes 1 and 2) and untreated F9 (Fig. 8 *a*, lane 3) had little or no detectable tPA activity, while the corresponding RA/dbcAMP-treated cultures showed distinct lysis zones indicating the presence of the enzyme in all cases (Fig. 8 *b*, lanes 1–3). These results, in combination with the immunofluorescence data, suggest that $\beta 1$ TKO cells can differentiate to PE in response to inducing agents in the absence of $\beta 1$ and in spite of their altered migratory characteristics.

To determine the ability of $\beta 1$ TKO cells to differentiate to VE, F9 and $\beta 1$ TKO cells were aggregated and cultured in suspension in the presence of 5×10^{-8} M RA for 7–10 d. The epithelial structure of these VE-containing bods was examined by embedding the bods in paraffin, sectioning, and staining the material with antibodies to the basement membrane protein laminin and to the cell–cell adhesion molecule E-cadherin. As shown in Fig. 9 *a*, parental F9 cells formed an epithelial monolayer of VE with an underlying laminin-rich basement membrane surrounding an undifferentiated core of stem cells. This epithelium was generally well organized and laminin was confined to a continuous layer of basement membrane at the basal surface of the epithelium (VE). Very little laminin could be found along the lateral and apical surfaces of the epithelial cells (Fig. 9 *b*). In contrast, the $\beta 1$ TKO cells (Fig. 9 *c*) showed areas of dense laminin deposition and irregular epithelium formation interrupted by patches of loose cell aggregates. These loosely adherent aggregates were often lost from the cell surface, leaving the underlying matrix exposed. Laminin deposition was disorganized and patchy, extending throughout multilayered clusters of differentiated cells. Furthermore, laminin was often uniformly distributed around the circumference of a cell or group of cells (Fig. 9 *d*), reflecting an absence of the basal enrichment of laminin associated with normal epithelialization. A monolayer of epithelium was present in some areas, but in general the cells at the surface appeared as clumps of rounded, non-polarized cells.

Staining with E-cadherin also revealed a lack of epithelial organization and polarization. In normal, polarized epithelial cells, E-cadherin has a baso-lateral distribution (reviewed by Takeichi, 1988). This pattern of expression is seen in the F9 VE bods (Fig. 9 *e*) where E-cadherin is localized to areas of cell–cell contact throughout the bod, but becomes restricted to the baso-lateral surfaces in the outer layer of visceral endoderm. In contrast, E-cadherin in the TKO VE bods was generally not localized to any definitive baso-lateral surfaces, and its expression was highly erratic in the cells and clumps of cells at the periphery of the bods. Some cells showed cadherin staining around their entire surfaces, while others showed partial staining, or even none at all, particularly in the cells at the outer edge of the clumps (Fig. 9 *f*). These results, together with the laminin localization, indicate that cells lacking $\beta 1$ integrins do not organize a normal epithelial monolayer and fail to establish the typical apical-basal polarity of the cells within that layer.

The TKO VE bods were examined for the expression of biochemical markers by staining with anti- α FP and anti-

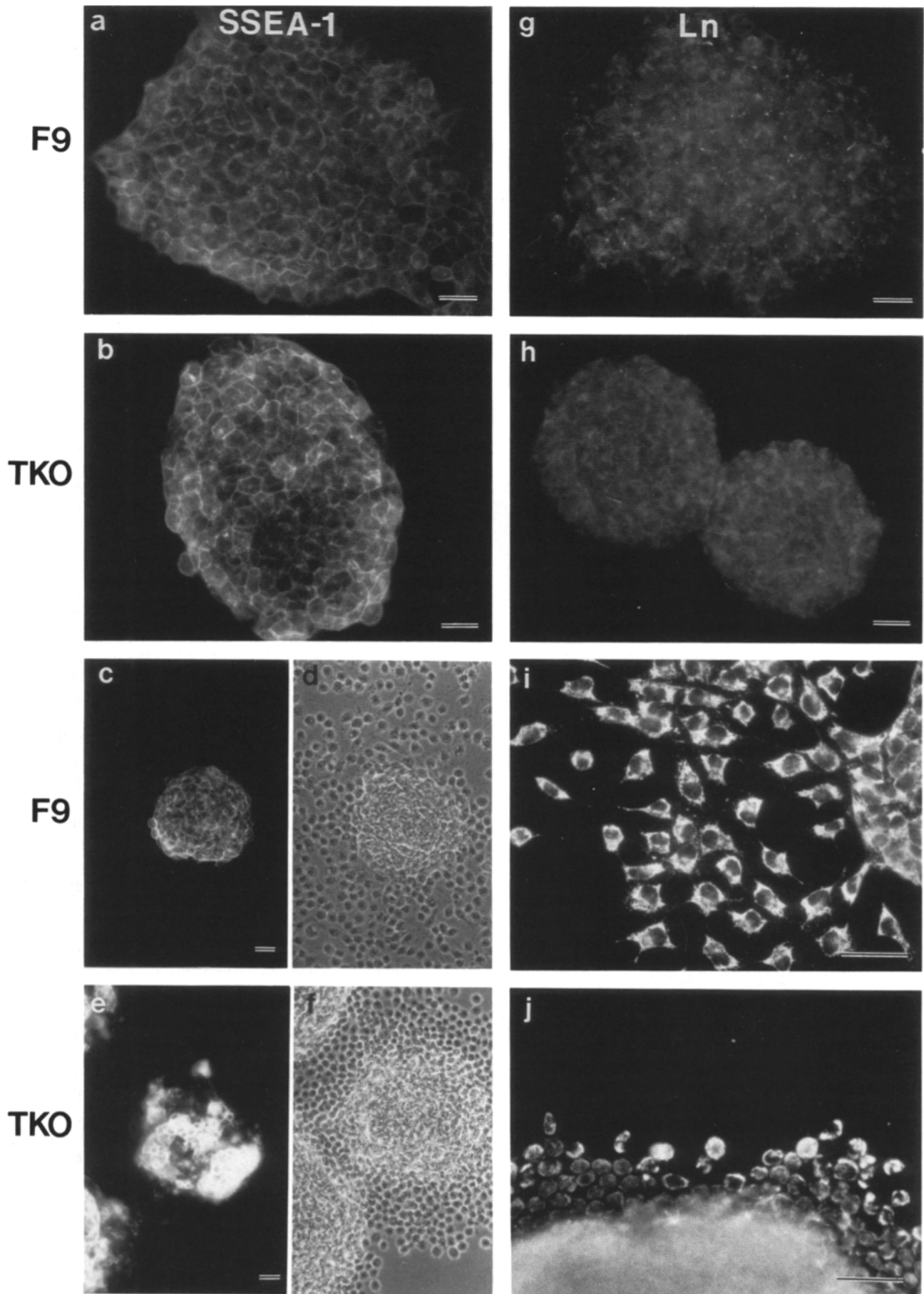


Figure 7. F9 and TKO cells were stained with anti-SSEA-1 (a-f) or anti-laminin (g-j) antibodies following culture on glass coverslips in the presence or absence of RA and dbcAMP. Uninduced F9 stained with SSEA-1 (a) or laminin (g), uninduced TKO cells stained with SSEA-1 (b) or laminin (h), drug-induced F9 PE cells stained with SSEA-1 (c and d) or laminin (i), drug-induced TKO PE stained with SSEA-1 (e and f) or laminin (j). d and f are phase-contrast images while a, b, c, e, and g-j are fluorescence images. Bars, 25 μm.

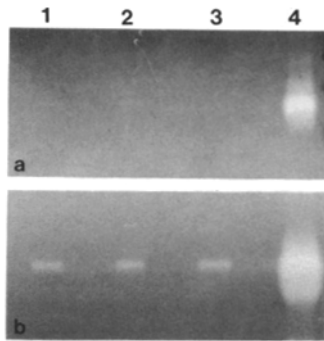


Figure 8. tPA activity in the conditioned medium of untreated and RA/dbcAMP-treated F9 and TKO cells. Samples of conditioned medium from monolayer cultures grown in serum-free medium for 5 d were run on casein-containing substrate gels. (a) Conditioned media from two uninduced TKO cell lines (lanes 1 and 2) and from uninduced F9 cells (lane 3). (b) Conditioned media from RA/dbcAMP treated cells: TKO lines (lanes 1 and 2), F9 (lane 3). Lane 4, tPA standard.

SSEA-1 antibodies. As expected, SSEA-1 was no longer expressed in the VE layer of F9 bods (Fig. 10, *a* and *b*) and it was also lost from the clusters of cells at the periphery of $\beta 1$ TKO VE bods (Fig. 10, *c* and *d*). In both cases, SSEA-1 continued to be expressed in the core cells. Staining with α FP, a VE-specific marker, showed patchy but positive staining in the peripheral cells in both the F9 VE bods (Fig. 10 *e*) and the $\beta 1$ TKO VE bods (Fig. 10 *f*). This expression of α FP and the loss of SSEA-1 in peripheral cells of $\beta 1$ TKO VE bods demonstrates clearly that $\beta 1$ TKO cells synthesize markers of VE differentiation in spite of their abnormal morphological differentiation, their compromised ability to polarize, and their lack of $\beta 1$ integrins.

The ability of $\beta 1$ TKO cells to differentiate to VE was further confirmed by northern blot analysis (Fig. 11). RNA isolated from RA-treated F9, SKO, and TKO bods was hybridized with probes for both α FP, which is VE-specific, and the cytokeratin Endo B, which is expressed in differentiated endoderm (Oshima, 1981). Uninduced F9 (Fig. 11, lane 1) expressed very low levels of these markers, while the induced cultures of F9 VE (Fig. 11, lane 2), SKO VE (Fig. 11, lane 3), and TKO VE (Fig. 11, lane 4) accumulated very high levels of α FP and Endo B. Glyceraldehyde phosphate dehydrogenase (GapDH) was also used as a probe to confirm that similar amounts of RNA were loaded in each lane. As in the case of PE, VE-specific gene expression was induced in the $\beta 1$ TKO cells in spite of their abnormal morphological differentiation.

Discussion

We have eliminated $\beta 1$ integrin expression in F9 embryonal carcinoma cells through targeted mutagenesis of the $\beta 1$ integrin genes. Four major conclusions can be derived from this study regarding the role of $\beta 1$ integrins in adhesion, morphology and differentiation in the F9 system: (a) $\beta 1$ integrins are required for maintaining the morphological characteristics of F9 cells in monolayers and for regulating the adhesive behavior of these cells on a variety of substrates. (b) This receptor family is critical for normal morphological differentiation to both PE and VE. (c) The expression of tissue-specific genes in response to differentiation-promoting factors is not necessarily dependent upon a signal received from or transmitted by $\beta 1$ integrins, since both PE and VE-spe-

cific gene products are expressed in the RA- or RA/dbcAMP-treated TKO cells. (d) The expression of PE- and VE-specific gene products is not dependent on normal morphological differentiation, suggesting that morphological differentiation and tissue-specific gene expression are not inter-dependent in this system.

$\beta 1$ Integrins Regulate F9 Cell Morphology, Adhesion, and Migration

$\beta 1$ TKO cells grew in rounded, tightly packed colonies in serum-containing monolayer culture and were easily distinguished from colonies of parental F9, SKO and DKO cells. $\beta 1$ TKO cells exhibited greatly reduced attachment to $\beta 1$ integrin substrates, including fibronectin, laminin and collagen IV, but showed both a strongly elevated, RGD-dependent adherence to vitronectin (7–8-fold) and reduced migration (fivefold). These behavioral changes were not accompanied by substantial changes in the surface expression of the $\alpha v \beta 3$ or $\alpha v \beta 5$ receptors in either the control $\beta 1$ TKO cells (Fig. 4, *d* and *e*) or in the $\beta 1$ TKO cells which had been induced to differentiate to PE (not shown). However, a slight alteration in the ratio of $\alpha v \beta 3$ to $\alpha v \beta 5$ might be significant. This possibility is consistent with previous studies demonstrating that the relative abundance of $\beta 3$ and $\beta 5$ subunits can influence cellular responses to ECM (Leavesley et al., 1992). In that work, human pancreatic carcinoma cells (FG cells), which express $\alpha v \beta 5$ but lack $\alpha v \beta 3$, attach but do not spread on or migrate in response to vitronectin. When transfected with the $\beta 3$ subunit, FG cells acquire the ability to spread on and migrate in response to vitronectin. Therefore, in those cells, attachment to vitronectin is mediated by $\alpha v \beta 5$, while $\alpha v \beta 3$ also promotes spreading and migration. If this observation holds true for F9 cells, then an increase in the $\beta 5$ relative to the $\beta 3$ subunit might disrupt the normal balance of these receptors, resulting in the observed changes: enhanced attachment of undifferentiated $\beta 1$ TKO cells and reduced migration of $\beta 1$ TKO PE on vitronectin. However, the changes we observed in levels of vitronectin receptors were very small when compared with the enhanced adhesion to vitronectin, suggesting that these changes in TKO cell behavior may result more directly from the loss of the $\beta 1$ subunit. For example, previous studies have suggested that $\beta 1$ -containing complexes influence the function of complexes containing other β integrin subunits. In those studies, a CHO cell line expressing very low levels of the $\alpha 5 \beta 1$ fibronectin receptor migrated poorly on vitronectin, as well as on fibronectin, despite the presence of vitronectin receptors (Bauer et al., 1992). How this influence is exerted is not known, although several hypotheses have been discussed (see Bauer, 1992). For example, the cytoplasmic domains of integrin β subunits interact with cytoskeletal and other components in response to interaction with ligand. In control F9 cells, some binding sites might be recognized by more than one β subunit cytoplasmic domain (e.g., $\beta 1$ and $\beta 3$). If such sites were present in limiting amounts, there would be competition between $\beta 1$ and other β subunits for binding. In the $\beta 1$ TKO cells, more of these sites would be available for the alternative β subunits, at least two of which promote adhesion to vitronectin. This may lead, in turn, to stronger adhesion and reduced migration. Clearly, more studies are needed to determine the underlying mechanisms responsible

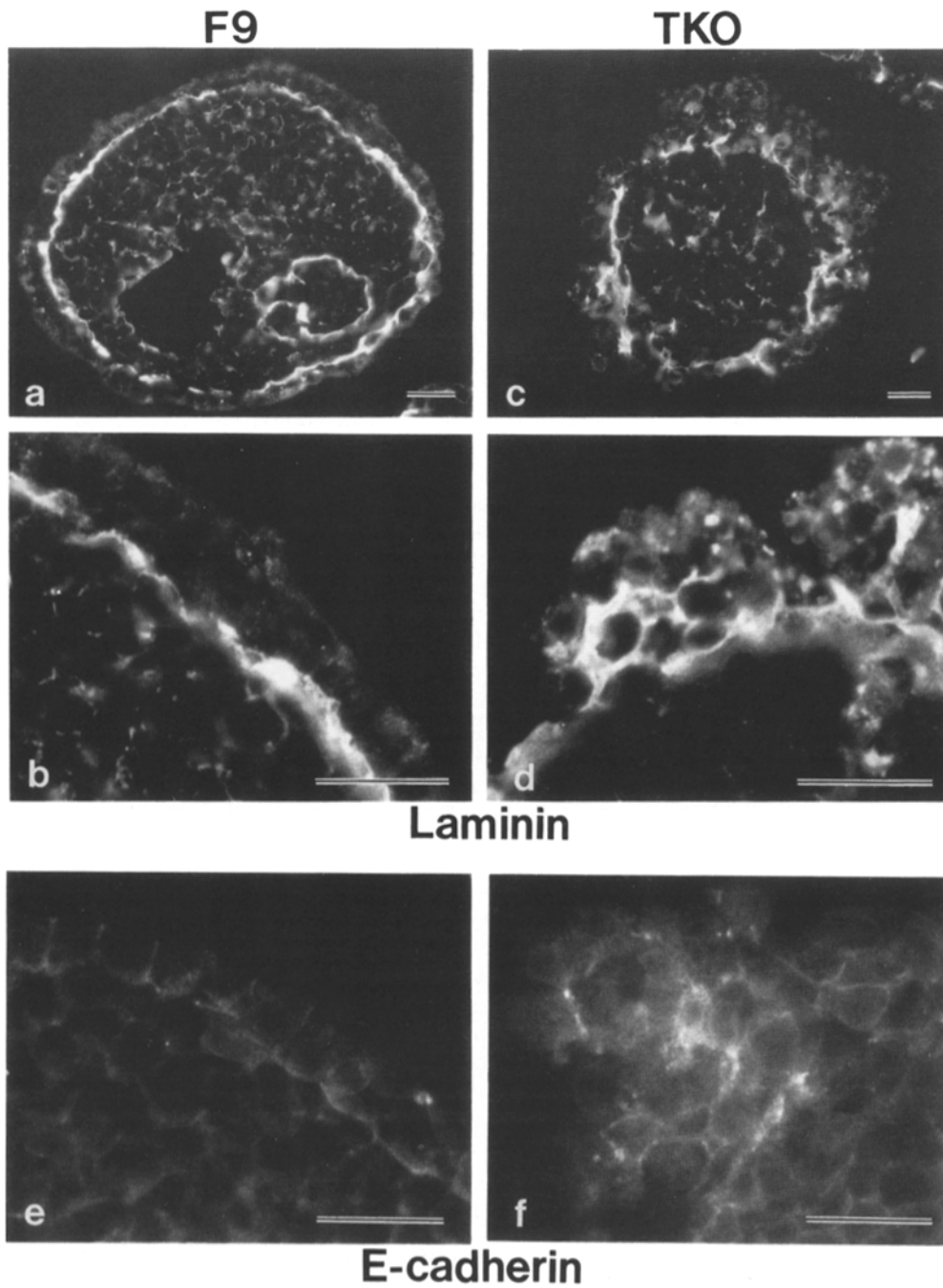


Figure 9. Anti-E-cadherin and anti-laminin staining of 7 day VE-containing bods. F9 and TKO bods were cultured for 7 d in the presence of RA, and fixed, embedded in paraffin and sectioned as described in Materials and Methods. F9 VE sections (a, b, and e) and TKO VE sections (c, d, and f) were stained with laminin (a-d) and E-cadherin (e and f). Bars, 25 μ m.

for the observed increase in adhesion of $\beta 1$ TKO cells to vitronectin and for their compromised ability to migrate on that substrate.

$\beta 1$ Integrins Are Critical for Normal VE Epithelial Formation

When compacted aggregates (bods) of $\beta 1$ TKO were treated with RA, formation of an organized epithelial monolayer at the periphery of the bod did not occur, as it did in RA-treated parental F9 and SKO cultures. Although differentiation to VE was restricted to the periphery of the TKO bods, the differentiating cells formed clumps of rounded, loosely adherent cells surrounded by matrix, suggesting that the cells were not polarized and could not distinguish basal from apical sur-

faces. That the cells in these clumps were in fact VE-like, rather than PE or stem cell-like, was indicated by their expression of α FP and Endo B and loss of SSEA-1.

The relationship between $\beta 1$ integrins, epithelialization and cell polarity has been suggested previously by Sorokin et al. (1990) in their studies on developing kidney tubules. They demonstrated the co-appearance of the $\alpha 6$ subunit (which, when associated with $\beta 1$, functions as a laminin receptor) and the laminin A-chain in regions of embryonic kidney where nonpolarized mesenchyme is converting to polarized epithelium. Furthermore, organ culture experiments in which either anti-laminin-A chain or anti- $\alpha 6$ antibodies were present prevented cell polarization and kidney tubule formation. Their results provide strong evidence that

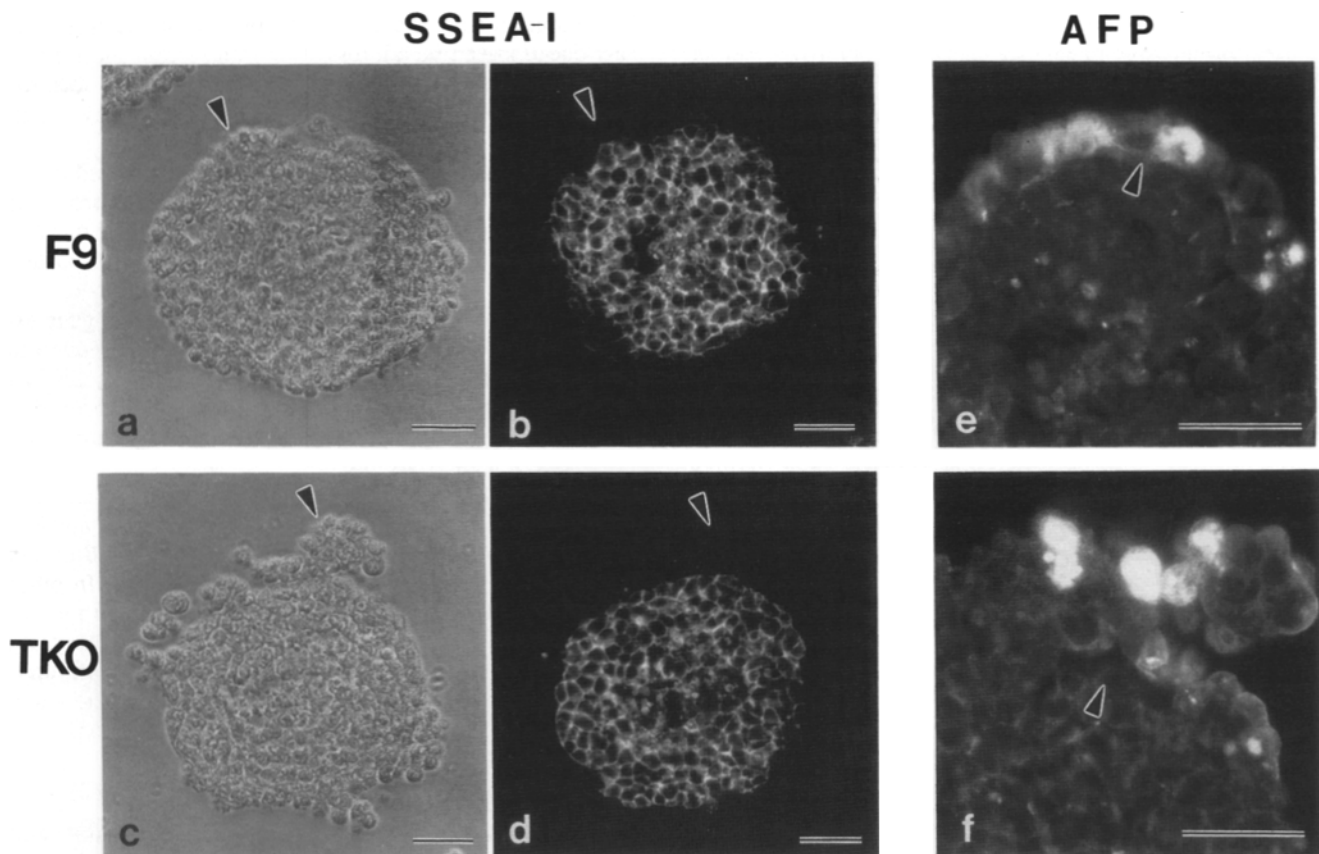


Figure 10. Anti-SSEA-1 and anti- α FP staining of 7-d VE-containing bods. F9 and TKO bods were cultured for 7 d in the presence of RA, and fixed, embedded in paraffin and sectioned as described in Materials and Methods. F9 VE sections (a, b, and e) and TKO VE sections (c, d, and f) were stained for SSEA-1 (a and c) or α FP (e and f). Arrows in a–d indicate regions where SSEA-1 staining is negative. Arrows in e and f indicate the boundary between undifferentiated “stem” cells and differentiated cells. Bars, 50 μ m.

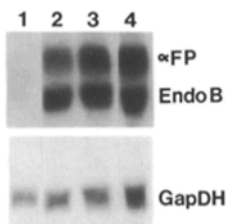
laminin promotes initial polarization of kidney tubules through the $\alpha 6 \beta 1$ integrin receptor. Similar studies on F9 cells demonstrated a role for laminin in epithelial organization during VE differentiation (Grover et al., 1983a). In that work, the investigators added either laminin or anti-laminin antibodies to cultures of differentiating VE bods and showed that both reagents interfered with the formation of a polarized epithelial monolayer. Those authors suggested that an asymmetric localization of laminin in the nascent basement membrane is essential for normal polarization to occur. While that study did not specifically address the role of integrins in polarization, their results in combination with our data support the hypothesis that a signal provided by the

asymmetric distribution of ECM is mediated through $\beta 1$ integrin receptors to promote the normal morphological differentiation (epithelialization) of visceral endoderm in F9 cells.

$\beta 1$ Integrins Are Not Required for Tissue-specific Gene Expression of VE or PE Cells in Response to Exogenous Differentiation Agents

The expression of a variety of markers for both PE and VE in the induced TKO cells demonstrates that $\beta 1$ integrins are not required for expression of tissue-specific genes in response to differentiation-promoting agents such as RA and dbcAMP. These results are surprising in light of previous studies suggesting roles for the integrin ligands fibronectin and laminin in terminal differentiation (Grabel and Watts, 1987; Grover et al., 1983a). For example, when VE bods made from PSA-1 embryonal carcinoma cells were plated in the presence of fibronectin, cells migrated out in an integrin-dependent manner (Grabel and Watts, 1987). They were PE-like based on their morphology, absence of SSEA-1, loss of α FP expression and elevated expression of laminin. In contrast, very little outgrowth from the VE bods occurred in the absence of fibronectin, or in the presence of laminin or collagen IV. Those cells which did grow out from the VE bod in the absence of fibronectin stained positively for SSEA-1, indicating that they had either reverted to a stem cell pheno-

Figure 11. Northern blot of RNA from 7-d control and VE bods probed with α FP, Endo B and GapDH. RNA was isolated from untreated F9 bods and from F9 VE, SKO VE and TKO VE bods cultured for 7 d in RA. Northern analysis was carried out as described in Materials and Methods. Uninduced F9 (lane 1), and induced cultures of F9 VE bods (lane 2), SKO VE bods (lane 3), and TKO VE bods (lane 4). α FP is a 2.2-kb transcript, Endo B is 1.6-kb and GapDH is 1.9 kb.



type or originated from the undifferentiated core of cells. Those authors concluded that interaction with fibronectin through its integrin receptor(s) initiated the differentiation as well as the migration of PE. Our data do not support this interpretation since we observed expression of PE markers in RA dbcAMP treated TKO cells in the complete absence of $\beta 1$ integrins. However, there are two important differences between these two experiments. First, Grabel and colleagues used PSA-1 EC cells which are normally maintained on feeder layers, a condition which prevents differentiation. Thus, PSA-1 cells require release from a negative signal, while F9 cells require stimulation from a positive signal (e.g., RA, dbcAMP) for differentiation. Second, the experiments with PSA-1 involved outgrowth of PE from fully differentiated VE bods, while our experiments with F9 cells involved outgrowth from undifferentiated bods. Thus, the initial differentiation of either VE or PE from a common stem cell may not require $\beta 1$ integrins, while "transdifferentiation" from VE to PE may be more dependent on a substrate-initiated signal conveyed by integrins. This may in fact have greater relevance to the situation *in vivo*, where migratory PE continue to be generated at the periphery of the stationary VE layer in the peri-implantation mouse embryo.

In the case of initial differentiation to VE, our data are more in agreement with previous cell biological studies by Grover and colleagues (Grover et al., 1983a). As discussed earlier, addition of excess laminin, or anti-laminin antibodies, to differentiating F9 VE bods interfered with the formation of a normal epithelium. The authors also observed a decrease in the accumulation of α FP mRNA as compared with untreated controls, suggesting that either excess laminin or laminin antibodies can interfere with the organization of the basement membrane and exert effects on differentiation as well as morphogenesis. However, treatment with high levels of exogenous laminin did not interfere with modulation of other differentiation markers, such as the upregulation of blood group antigens I and i, Endo B and the loss of SSEA-1. Thus, those studies, as well as our present data, indicate that substantial biochemical differentiation of the VE lineage can occur in the absence of functional $\beta 1$ integrin-ligand interactions and despite abnormal morphogenesis.

Our experiments suggest that $\beta 1$ integrins are not required for tissue-specific differentiation in the F9 system. This observation is supported by studies of the β integrin null mutation in *Drosophila*, *lethal-1-myospheroid* (*l-1-mys*), in which muscle-specific gene expression appears normal in these β integrin-deficient flies in spite of abnormal morphogenesis of muscle attachment sites (Leptin et al., 1989). However, there are numerous studies using anti-integrin antibodies which suggest that these receptors are important in regulating tissue-specific terminal differentiation in mammary epithelium, keratinocytes, and chick myoblasts (Streuli et al., 1991; Adams and Watt, 1990; Menko and Boettiger, 1987). This apparent conflict in conclusions regarding $\beta 1$ integrin function may actually reflect the differences in experimental approach. Homologous recombination and null mutations completely eliminate the $\beta 1$ family from the cell surface, while antibodies affect the function of pre-existing $\beta 1$ integrins in the membrane. The antibodies may stabilize a particular functional state of the integrins that either blocks or constitutively permits a signal that is normally subject to regulation. This may have a qualitatively different effect on

differentiation than the complete absence of the integrins. This question can be addressed by transfecting mutated $\beta 1$ subunits into the TKO cells. Good candidates for such experiments include the cDNAs encoding the naturally occurring $\beta 1$ variants (Altruda et al., 1990; Balzac et al., 1993), which have different cytoplasmic domains and would therefore be expected to transduce signals differently from the dominant form of the $\beta 1$ subunit, and a $\beta 1$ cDNA that has been mutated at a location shown to be required for normal ligand binding (Ginsberg et al., 1992).

An alternative explanation of why tissue-specific gene expression does not require $\beta 1$ integrin-ligand interaction in this system relates to the existence of two potentially distinct effects of matrix on tissue-specific gene expression operating in different cell types. A positive signal requiring matrix contact appears to be required for terminal differentiation in some tissues, including mammary epithelium. In that case, both contact with basement membrane via integrins, and lactogenic hormones are required for expression of β casein (Streuli et al., 1991; Schmidhauser et al., 1992). In other systems, including keratinocytes and chondrogenic mesenchyme (Adams and Watt, 1990; for review see Adams and Watt, 1993), contact with matrix, fibronectin in particular, suppresses differentiation while release from matrix, along with other signals, promotes terminal differentiation.

F9 Cells Carry Three Copies of the $\beta 1$ Integrin Gene

It is of interest to note that F9 cells carry three separate and transcriptionally active copies of the $\beta 1$ integrin gene. Why the $\beta 1$ integrin gene is present in triplicate is not known, however, it is presumably due to a chromosomal abnormality of F9 cells. Karyotype analyses performed in our lab (unpublished results) show that F9 cells have some chromosomal abnormalities: they carry 39–41 chromosomes, with one small satellite chromosome and at least one large, fused chromosome. It is quite possible, therefore, that these cells are trisomic for the chromosome or part of the chromosome on which $\beta 1$ integrin resides. Furthermore, it would appear that all three copies are active and functional, since sequential inactivation of each copy resulted in correspondingly lower levels of $\beta 1$ integrin protein. Nonetheless, these results clearly establish that multiple copies of a gene or genes can be successfully targeted in cultured cells by homologous recombination.

Summary

In summary, our data show that $\beta 1$ integrins are required for normal adhesion, migration and the execution of normal tissue-specific morphological differentiation in the F9 endoderm differentiation model. Of particular significance is the observation that the lack of $\beta 1$ integrins inhibits migration of TKO-derived PE cells on substrates for which they still have other integrin receptors. We also conclude that morphological differentiation and tissue-specific gene expression can be uncoupled in this system, since the absence of $\beta 1$ integrins profoundly affects morphogenesis in both lineages, but does not prevent expression of VE- and PE-specific markers in response to differentiation factors.

In addition to the immediate insights derived from this study, the F9 TKO cells present several advantages for future studies. To date, no known cell lines exist which fail to ex-

press $\beta 1$ integrins. Therefore, a system lacking this major receptor will be extremely amenable to transfection of altered $\beta 1$ subunits, allowing a greater understanding of the functional complexity inherent in this molecule. In addition, these $\beta 1$ -deficient cells will also lend themselves to the study of other integrins whose functions are normally difficult to evaluate in the presence of the $\beta 1$ complexes. Furthermore, insights gained from these studies will provide additional clues for analyzing $\beta 1$ -deficient mouse embryos derived from ongoing $\beta 1$ knock-out experiments in embryonic stem (ES) cells. It is anticipated that a combined approach using both F9 cells for in vitro studies, as well as ES cells for in vivo studies, will advance our understanding of the function of $\beta 1$ integrins in basic cell biological processes, such as cell motility and signaling, as well as in embryonic morphogenesis and differentiation.

L. E. Stephens dedicates this work to the memory of Kelly E. Stephens, 1955–1993.

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References

- Adams, J. C., and F. M. Watt. 1990. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha 5 \beta 1$ integrin loss from the cell surface. *Cell*. 63:425–435.
- Adams, J. C., and F. M. Watt. 1993. Regulation of development and differentiation by the extracellular matrix. *Development (Camb.)*. 117:1183–1198.
- Altruda, F., P. Cervella, G. Tarone, C. Botta, F. Balzac, G. Stefanuto, and L. Silengo. 1990. A human integrin beta 1 subunit with a unique cytoplasmic domain generated by alternative mRNA splicing. *Gene (Amst.)* 95:261–266.
- Balzac, F., A. M. Belkin, V. E. Kotliansky, Y. V. Balabanov, F. Altruda, L. Silengo, and G. Tarone. 1993. Expression and functional analysis of a cytoplasmic domain variant of the beta 1 integrin subunit. *J. Cell Biol.* 121:171–178.
- Bauer, J. S., C. L. Shreiner, F. G. Gaincotti, E. Ruoslahti, and R. L. Juliano. 1992. Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. *J. Cell Biol.* 116:477–487.
- Bogaert, T., N. Brown, and M. Wilcox. 1987. The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell*. 51:929–940.
- Boucaut, J. C., T. Darribere, T. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development; a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J. Cell Biol.* 99:1822–1830.
- Bronner-Fraser, M. 1986. An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Dev. Biol.* 117:528–536.
- Casanova, J. E., and L. B. Grabel. 1988. The role of cell interactions in the differentiation of teratocarcinoma-derived parietal and visceral endoderm. *Dev. Biol.* 129:124–139.
- Cheresh, D. A., J. W. Smith, H. M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin ($\alpha v \beta 3$) is responsible for distinct adhesive properties of carcinoma cells. *Cell*. 57:59–69.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
- Damsky, C. H., and Z. Werb. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772–781.
- Dziadek, M., and E. Adamson. 1978. Localization and synthesis of alpha-fetoprotein in post-implantation mouse embryos. *J. Embryol. Exp. Morphol.* 43:289–313.
- Ginsberg, M. H., X. Du, and E. F. Plow. 1992. Inside-out integrin signalling. *Curr. Opin. Cell Biol.* 4:766–771.
- Grabel, L. B., and T. D. Watts. 1987. The role of extracellular matrix in the migration and differentiation of parietal endoderm from teratocarcinoma embryoid bodies. *J. Cell Biol.* 105:441–448.
- Grover, A., and E. D. Adamson. 1985. Roles of extracellular matrix components in differentiating teratocarcinoma cells. *J. Cell Biol.* 260:12252–12258.
- Grover, A., G. Andrews, and E. D. Adamson. 1983a. Role of laminin in epithelium formation by F9 aggregates. *J. Cell Biol.* 97:137–144.
- Grover, A., R. G. Oshima, and E. D. Adamson. 1983b. Epithelial layer formation in differentiating aggregates of F9 embryonal carcinoma cells. *J. Cell Biol.* 96:1690–1696.
- Higuchi, R. 1990. Recombinant PCR. In PCR protocols: A Guide to Methods and Applications. M. A. Innes, D. H. Gelfand, J. J. Sninsky and T. J. White, editors. Academic Press, Inc. San Diego, CA. 177 pp.
- Hogan, B. L. M., and A. Taylor. 1981. Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature (Lond.)*. 291:235–237.
- Hogan, B. L. M., D. P. Barlow, and R. Tilly. 1983. F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surv.* 2:115–140.
- Hynes, R. O. 1992. Integrins: Versatility, Modulation, and Signaling in Cell Adhesion. *Cell*. 69:11–25.
- Imada, S., H. Yamaguchi, and M. Imada. 1990. Differential expression of fetomodulin and tissue plasminogen activator to characterize parietal endoderm differentiation of F9 embryonal carcinoma cells. *Dev. Biol.* 141:426–430.
- Leavesley, D. I., G. D. Ferguson, E. A. Weyer, and D. A. Cheresh. 1992. Requirement of the integrin $\beta 3$ subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J. Cell Biol.* 117:1101–1107.
- Leptin, M., T. Bogaert, R. Lehmann, and M. Wilcox. 1989. The function of PS integrins during *Drosophila* embryogenesis. *Cell*. 56:401–408.
- Mansour, S. L., K. R. Thomas, and M. R. Capecchi. 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells; a general strategy for targeting mutations to non-selectable genes. *Nature (Lond.)*. 336:348–352.
- Marotti, K. R., D. Belin, and S. Strickland. 1982. The production of distinct forms of plasminogen activator by mouse embryonic cells. *Dev. Biol.* 90:154–159.
- Menko, A. S., and D. Boettiger. 1987. Occupation of the extracellular matrix receptor, Integrin, is a control point for myogenic differentiation. *Cell*. 5:51–57.
- Mortensen, R. M., D. A. Conner, S. Chao, A. A. T. Geisterfer-Lowrance, and J. G. Seidman. 1992. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell Biol.* 12:2391–2395.
- Oshima, R. G. 1981. Identification and immunoprecipitation of cytoskeletal proteins from extra-embryonic endodermal cells. *J. Biol. Chem.* 256:8124–8133.
- Pierschbacher, M. D., and E. Ruoslahti. 1987. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J. Biol. Chem.* 262:17294–17298.
- Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. A 125/115 kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc. Natl. Acad. Sci. USA*. 82:5766–5770.
- Schmidhauser, C., G. F. Caspersen, C. A. Myers, K. T. Sanzo, S. Bolten, and M. J. Bissell. 1992. A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of beta-casein gene expression. *Mol. Biol. Cell*. 3:699–709.
- Solter, D., and B. B. Knowles. 1978. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA*. 75:5565–5569.
- Sorokin, L., A. Sonnenberg, M. Aumailley, R. Timpl, and P. Ekblom. 1990. Recognition of the laminin E8 cell-binding site by an integrin possessing the $\alpha 6$ subunit is essential for epithelial polarization in developing kidney tubules. *J. Cell Biol.* 111:1265–1273.
- Streuli, C. H., N. Bailey, and M. J. Bissell. 1991. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *J. Cell Biol.* 115:1383–1395.
- Strickland, S., K. K. Smith, and K. R. Marotti. 1980. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyl cAMP. *Cell*. 21:347–355.
- Sutherland, A. E., P. G. Calarco, and C. H. Damsky. 1993. Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development (Camb.)*. In press.
- Takeichi, M. 1988. The cadherin cell-cell adhesion molecules controlling animal morphogenesis. *Development (Camb.)*. 102:639–655.
- Tomaselli, K., C. H. Damsky, and L. Reichardt. 1987. Interactions of a neuronal cell line (PC12) with the extracellular matrix proteins laminin, collagen IV and fibronectin: identification of cell surface glycoproteins involved in attachment and outgrowth. *J. Cell Biol.* 105:2347–2358.
- Tominaga, S. 1988. Murine mRNA for the $\beta 1$ -subunit of integrin is increased in BALB/c-3T3 cells entering the G₁ phase from the G₀ state. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 238:315–319.