

Expression and Function of Cell Surface Extracellular Matrix Receptors In Mouse Blastocyst Attachment and Outgrowth

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Abstract. Mouse-hatched blastocysts cultured *in vitro* will attach and form outgrowths of trophoblast cells on appropriate substrates, providing a model for implantation. Immediately after hatching, the surfaces of blastocysts are quiescent and are not adhesive. Over the period 24–36 h post-hatching, blastocysts cultured in serum-free medium become adhesive and attach and spread on the extracellular matrix components fibronectin, laminin, and collagen type IV in a ligand specific manner. Attachment and trophoblast outgrowth on these substrates can be inhibited by addition to the culture medium of an antibody, anti-ECMr (anti-extracellular matrix receptor), that recognizes a group of 140-kD glycoproteins similar to those of the 140-kD extracellular matrix receptor complex (integrin) recognized in avian cells by CSAT and JG22 monoclonal antibodies. Addition to the culture

medium of a synthetic peptide containing the Arg-Gly-Asp tripeptide cell recognition sequence of fibronectin inhibits trophoblast outgrowth on both laminin and fibronectin. However, the presence of the peptide does not affect attachment of the blastocysts to either ligand. Immunoprecipitation of ¹²⁵I surface-labeled embryos using anti-ECMr reveals that antigens recognized by this antibody are exposed on the surfaces of embryos at a time when they are spreading on the substrate, but are not detectable immediately after hatching. Immunofluorescence experiments show that both the ECMr antigens and the cytoskeletal proteins vinculin and talin are enriched on the cell processes and ventral surfaces of trophoblast cells in embryo outgrowths, in patterns similar to those seen in fibroblasts, and consistent with their role in adhesion of the trophoblast cells to the substratum.

ADHESIVE interactions, between cells, and between cells and their surrounding extracellular matrix, are important in many developmental events. Changes in cell-cell and cell-matrix interactions are required for the ingression of both sea urchin primary mesenchyme cells (Fink and McClay, 1985) and chick endocardial cells (Markwald et al., 1975, 1977), while cell-matrix interactions are involved in the morphogenesis of branching organs, such as salivary gland (Banerjee et al., 1977; Bernfield and Banerjee, 1982) and lung (Bluemink et al., 1976; Grant et al., 1983). In addition, cellular migration through an extracellular matrix (ECM)¹ is a fundamental part of the morphogenesis of many other tissues including the cornea (Toole and Trelstad, 1971; Bard et al., 1975), the endocardial cushions (Manasek, 1968; Markwald et al., 1975, 1977) and all the derivatives of the neural crest (Weston, 1983; Erickson, 1986).

The earliest detectable adhesive interactions between cells in the early mouse embryo occur at the eight-cell stage when the blastomeres of the mouse embryo become closely apposed in a process known as compaction (Lewis and Wright, 1935; Ducibella and Anderson, 1975). This morphogenetic event is dependent upon intercellular interactions: treatment

of embryos with antibodies to the cell-adhesion molecule cell-CAM 120/80 (also known as uvomorulin or E-cadherin), will prevent compaction as well as subsequent blastocoele formation (Damsky et al., 1983; Peyrieras et al., 1983; Shirayoshi et al., 1983). Treatment of mouse morulae with UDP-galactose (Shur et al., 1979; Bayna et al., 1986) can also inhibit blastocyst formation, suggesting a role for cell surface galactosyltransferases in blastomere adhesion.

During the peri-implantation period after hatching of the blastocyst from the zona pellucida, the outer epithelial layer of the embryo, known as the trophoblast, differentiates to form trophoblast (Hsu, 1971). These cells show considerable protrusive activity and become highly adhesive to the substrate; characteristics that will lead, *in vivo*, to implantation in the uterus, and *in vitro*, to formation of an embryo outgrowth, considered a model for implantation (Mintz, 1964; Cole and Paul, 1965; Gwatkin, 1966*a,b*; Enders et al., 1981).

The formation of outgrowths is dependent upon the presence of suitable culture conditions (Spindle and Pedersen, 1973; Hsu, 1971, 1973). FCS and amino acids are required in the medium to obtain trophoblast spreading with differentiation of ectoderm and endoderm, while further modifications are required to obtain development to subsequent stages (Hsu, 1978). An important requirement for trophoblast out-

1. *Abbreviations used in this paper:* Anti-Col IV, anti-collagen IV; anti-fn, anti-fibronectin; anti-Ln, antilaminin; ECM, extracellular matrix; Vnc, vinculin.

growth is the presence of a suitable substrate for trophoblast attachment and spreading; embryos cultured on agarose will not form outgrowths even in the presence of serum (Wilson and Jenkinson, 1974). Early studies showed that matrices of rat-tail collagen would support trophoblast spreading in the absence of serum, although no differentiation of inner-cell mass cells was seen under these conditions (Wilson and Jenkinson, 1974). More recently, it has been reported that substrates of the adhesive molecules fibronectin or laminin will also support trophoblast spreading in serum-free medium (Armant et al., 1986a).

The ability of blastocysts to attach and spread on these defined ligands suggests the presence of specific cell surface receptors. A complex of cell-matrix adhesion-related glycoproteins of M_r 140,000 has been described in mammalian (Knudsen et al., 1981; Damsky et al., 1981; Brown and Juliano, 1985, 1986) and avian (Neff et al., 1982; Greve and Gottlieb, 1982) cells, that has properties of both a fibronectin and a laminin receptor (Horwitz et al., 1985, 1986; Hasagawa et al., 1985; Akiyama et al., 1986; Hall et al., 1987; Tomaselli et al., 1987). This complex, recently named integrin in the avian system (Tamkun et al., 1986), has been shown to be a member of a broad family of adhesion receptor complexes interacting with their respective ligands via cell recognition sites containing the tripeptide Arg-Gly-Asp (Pierschbacher et al., 1984b). Among these are the fibronectin, vitronectin, and fibrinogen receptors (Pytela et al., 1985a, b; see reviews by Ruoslahti and Pierschbacher, 1986; Hynes, 1987).

The M_r 140,000 avian integrin complex is involved in the migration of neural crest cells (Bronner-Fraser, 1985, 1986) and in neurite outgrowth from sympathetic neurons (Hall et al., 1987). In addition, changes in its distribution correlate with the morphogenetic changes in the developing chick lung (Chen et al., 1986). A role for this receptor complex during implantation of the mouse embryo has also been postulated, since an antiserum, anti-GPI40, which recognizes this receptor complex in mammalian cells (Knudsen et al., 1981; Damsky et al., 1982) blocks attachment of hatched blastocysts to tissue culture plastic in the presence of serum (Richa et al., 1985). In addition, synthetic hexapeptides containing the Arg-Gly-Asp tripeptide will inhibit trophoblast outgrowth on fibronectin (Armant et al., 1986b). In this study, we have examined whether blastocysts express the 140-kD adhesion receptor family at a time and location consistent with its proposed function in implantation, and whether blastocysts specifically recognize defined substrates, such as fibronectin, laminin and collagen type IV, that they are likely to encounter during invasion of the uterine stroma.

Materials and Methods

Antisera and Peptides

Antibodies used included rabbit anti-fibronectin (anti-Fn; Cappel Laboratories, Cochranville, PA), rabbit anti-laminin (anti-Ln, gift of Dr. Deborah Hall, University of California, San Francisco), affinity purified rabbit anti-type IV collagen (anti-Col IV; purchased from Dr. Heinz Furthmayr, Yale University, New Haven, CT) and goat anti-ECMr (formerly called anti-GPI40; Knudsen et al., 1981). Anti-ECMr was produced in a goat by Dr. Karen Knudsen against a purified glycoprotein fraction of baby hamster kidney (BHK) cells, that consisted predominantly of a group of cell-substratum adhesion proteins of M_r 120,000–160,000 (Knudsen et al., 1981; Dam-

sky et al., 1982). All of the above antibody preparations were used as purified IgG fractions; anti-ECMr was also used as an antiserum in some experiments. Rabbit antiserum against the cytoskeleton associated protein talin (Burrige and Connell, 1983) purified from chicken gizzard, was a gift of Dr. Mary Beckerle, University of Utah [Salt Lake City, UT] and Dr. Keith Burrige, University of North Carolina [Chapel Hill, NC]. Monoclonal antibodies to vinculin (anti-Vnc) were purchased from Miles Scientific (Naperville, IL). Anti-fibronectin receptor (anti-FNR) antiserum was a gift of Drs. Erkki Ruoslahti and Michael Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA). Anti-entactin antiserum was a gift of Dr. Albert Chung (University of Pittsburgh, Pittsburgh, PA). To remove any contaminating anti-Ln antibodies from the anti-entactin, 100 μ l of a 1:10 dilution of anti-entactin serum was absorbed on one well of a 96-well plate that had been coated with 100 μ l of 25 μ g/ml Ln for 2 h and rinsed three times with PBS containing 0.2% BSA. The absorbed serum retained its activity for entactin, as assessed by immunoblot analysis, and was used in outgrowth experiments without further dilution. Purified laminin (Ln) was a gift of Dr. Deborah Hall, while purified fibronectin (Fn) and collagen type IV (Col IV) were purchased from Collaborative Research (Lexington, MA). Purified vitronectin was a gift of Drs. Erkki Ruoslahti and Michael Pierschbacher.

Two different types of synthetic hexapeptides were used in the experiments described below: (a) Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP). This hexapeptide contains the tripeptide cell-binding region of fibronectin, and is active in preventing the adhesion of cultured cells to fibronectin (Pierschbacher and Ruoslahti, 1984a; Yamada and Kennedy, 1984; Ruoslahti and Pierschbacher, 1986). (b) Gly-Arg-Gly-Glu-Ser-Pro (GRGESP). This hexapeptide contains one amino-acid substitution relative to the first hexapeptide, and does not prevent the adhesion of cultured cells to fibronectin (Pierschbacher and Ruoslahti, 1984b). The peptides used were obtained from two different sources, from Peninsula Laboratories, Belmont, California, and as gifts from Drs. Michael Pierschbacher and Erkki Ruoslahti (La Jolla Cancer Research Foundation). Peptides were used at 250 and 500 μ g/ml.

Embryo Culture

Female ICR mice (12-w-old, Harlin) were superovulated by injection with 5 IU of pregnant mare's serum gonadotropin (Teikoku Hormone Mfg. Co., Japan) followed after 48 h by an injection of 2.5 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, MO) and were then caged with ICR males. Embryos were collected at the 2-cell stage (48 h post-hCG injection) and cultured for 96 h to the hatched blastocyst stage (144 h post-hCG) in microdrops of embryo culture medium (Biggers et al., 1971) under mineral oil, at 37°C in a humid atmosphere of 5% CO₂ in air. Embryos were routinely cultured *in vitro* to the blastocyst stage instead of being flushed from the uterus at the blastocyst stage, since a much higher yield of embryos is obtained this way. The *in vitro* culture period does not affect the process of development.

Experimental Culture

Antibody and peptide dilutions were prepared either in Eagle's Basal Medium (BME) supplemented with 4 mg/ml BSA and 1% Nutridoma-HU (Boehringer Mannheim Biochemicals) (modified from Spindle, 1980); or in CMRL medium 1066 (Gibco, Grand Island, NY), supplemented with 0.5 mg/ml calcium lactate, 0.05 mg/ml sodium pyruvate, 4 mg/ml BSA (Sigma Chemical Co.), and 1% Nutridoma-HU (Armant et al., 1986b). All media (with or without antibody or peptide) were filter-sterilized using syringe-tip filters of 0.45 μ m pore size (Millipore, HV) as they were loaded into the previously prepared wells (see below).

Substrates for experimental culture were prepared essentially as described previously (Giancotti et al., 1985) in 96-well tissue culture dishes with flat bottom wells (Falcon, No. 3075). Coating solutions used were: Ln (25 μ g/ml), Fn (10 μ g/ml), Col IV (25 μ g/ml), vitronectin (20 μ g/ml), or BSA (25 μ g/ml). The coated wells were rinsed twice with sterile PBS, once with medium, then filled with the appropriate antibody or peptide dilution for the experiment and equilibrated at 37°C for 30–60 min.

Hatched blastocysts (144 h post-hCG) were collected and rinsed in medium in agarose-coated dishes, then placed into the previously prepared wells, at 10–30 embryos per well, and incubated for 48–72 h. Outgrowth was examined at various times during the culture period with a Nikon Diaphot inverted microscope equipped with Hoffman Modulation Contrast optics, and photographed using Kodak Tri-X film. Embryo attachment in the absence of spreading was quantified by gently blowing a small amount of

medium on each embryo, using a glass pipette pulled to a very fine bore. Those that did not move were considered to be attached.

The extent of spreading in peptide incubation experiments was obtained by photographing embryos at a magnification of 20 \times , printing each negative at the same size, and then measuring the area of each outgrowth using a Numonics digitizer. The final value for each embryo was calculated as the average of three tracings, and the final value for each treatment was calculated as the average of the values from at least 11 embryos.

Immunofluorescence

For immunofluorescence examination of hatched blastocysts (144 h post-hCG) in suspension, a group of 20–50 hatched blastocysts were rinsed in PBS and then fixed for 30 min in freshly prepared 2% paraformaldehyde in PBS (pH 7.3). Embryos were reacted with 0.15 M glycine in PBS for 15 min to block free aldehyde groups, followed by rinsing in PBS containing 0.5% BSA (PBS-BSA) to prevent nonspecific antibody binding. Embryos were then incubated for 60 min in primary antibody. After a series of rinses in PBS-BSA, the embryos were incubated for 30 min in affinity-purified fluorescein-conjugated secondary antibody, rinsed again in PBS-BSA, mounted on slides and viewed on a Zeiss Universal microscope outfitted for epifluorescence.

For immunofluorescence examination of embryo outgrowths, circular glass coverslips (Fisher, 12 mm) were coated, as described above for the 96-well plates, with Fn (10 μ g/ml). After coating, the coverslips were placed into 35-mm petri dishes (Falcon, No. 1008; five coverslips per dish), covered with medium, and 10–20 hatched blastocysts (144 h post-hCG) were placed on each coverslip. After 48–72 h culture, embryo outgrowths were rinsed, fixed, and reacted with 0.15 M glycine in PBS to block free aldehyde groups, as described above for the hatched blastocysts. They were then permeabilized with a 3-min treatment in acetone at -20°C , and subsequently incubated in PBS containing 0.5% BSA to prevent non-specific antibody binding. After several rinses in PBS, the outgrowths were incubated for 60 min in either anti-ECMr, anti-FNR, anti-Vnc, anti-talin, or nonimmune antibodies (or combinations of two of the above for co-localization experiments). The embryos were then rinsed five times in PBS and incubated in affinity-purified fluorescein-conjugated and/or rhodamine-conjugated secondary antibodies (Cappel Laboratories) for 60 min, then rinsed a further five times in PBS, and mounted on slides in mounting medium containing p-phenylene diamine to prevent quenching of the fluorescent signal. Embryos were examined with a Zeiss Universal microscope outfitted for epifluorescence, and photographed using Kodak Tri-X film.

Radiolabeling of Cells and Embryos

Cultured cells (mouse mammary tumor epithelial cells [MMTE], or human trophoblast cells [JAR]), hatched blastocysts (144 h post-hCG), and embryo outgrowths (216 h post-hCG) were surface labeled with ^{125}I using the lactoperoxidase-glucose oxidase catalyzed radioiodination method. Both the cultured cells and the hatched blastocysts were labeled in suspension. Cells were harvested using 2 mM EDTA, 0.5% BSA in PBS, rinsed three times with 20 mM glucose in PBS (PBS-Glucose), then resuspended in 1 ml of the same buffer. Lactoperoxidase (50 μ g; Boehringer-Mannheim Biochemicals), glucose oxidase (50 U; Sigma Chemical Co.), and Na^{125}I (400 μCi ; Amersham) were then added and the reaction was allowed to proceed, with mixing, for 10 min on ice. After labeling, cells were rinsed three times in DME (UCSF Cell Culture Facility) and resuspended in one ml of DME. For radiolabeling of hatched blastocysts, a group of 300 hatched blastocysts was suspended in 200 μl of PBS-Glucose, and lactoperoxidase, glucose oxidase, and Na^{125}I were added. The iodination reaction was allowed to proceed for 10 min on ice, and the embryos were then rinsed three times in DME and resuspended in 200 μl of DME. For radiolabeling of embryo outgrowths, a group of 200 72-h embryo outgrowths in one well of a 96-well plate (Costar) was rinsed six times with PBS-Glucose, then left in a final volume of 200 μl of the same buffer. Lactoperoxidase, glucose oxidase, and Na^{125}I were added and reacted with the embryos for 10 min on ice. The outgrowths were then rinsed 10 times with DME and left in a final volume of 200 μl .

Immunoprecipitation

For immunoprecipitation of radiolabeled proteins, either normal goat or anti-ECMr antibodies were added to the intact labeled cells, hatched blastocysts, and embryo outgrowths. In the case of outgrowths, 10 μg of cold Fn was added to the mixture to reduce non-specific precipitation of Fn substrate labeled during the iodination of the attached outgrowths. All samples

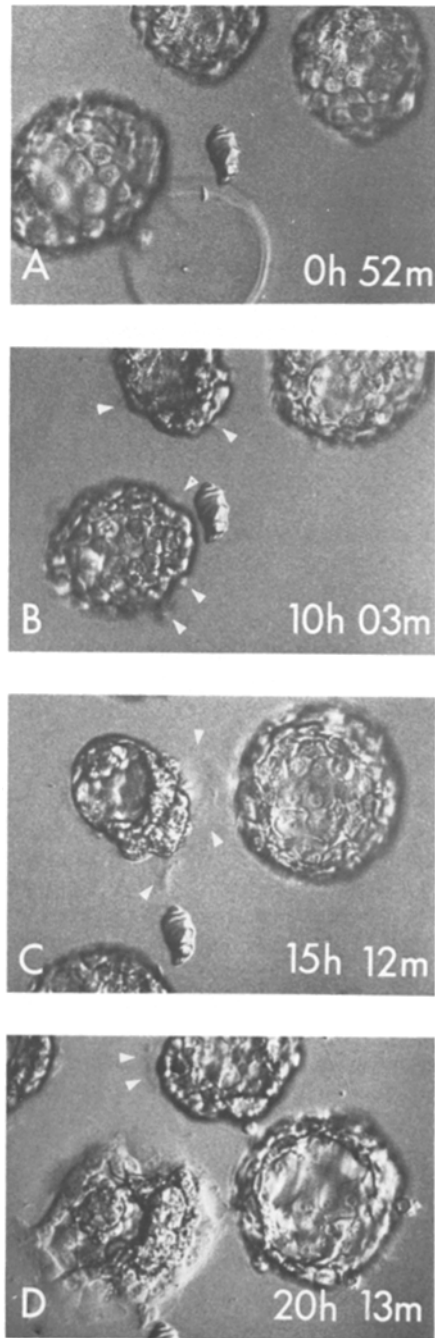


Figure 1. Cinemicrographic observations of mouse blastocyst outgrowth. Embryos were put into culture for filming at 147 h post-hCG. (A) Immediately post-hatching; none of the embryos are attached, and none show any protrusive activity. (B) 10 h post-hatching; protrusive activity is evident in two of the three embryos (arrows). (C) 15 h post-hatching; spreading is seen in one of the embryos (arrows). (D) 20 h post-hatching; trophoblast spreading has become prominent in one embryo, is just beginning in another, and has yet to begin in a third.

were then incubated on ice for 1 h, rinsed three times with PBS, and lysed for 30 min in lysis buffer (200 mM octylglucoside, 1 mM PMSF in PBS) with frequent agitation. Cell or embryo lysates were spun for 20 min in the cold at 12,000 rpm to pellet nuclei and other insoluble components, and the resulting supernatant was precleared twice on 100 μl pellets of unconjugated Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) for 30

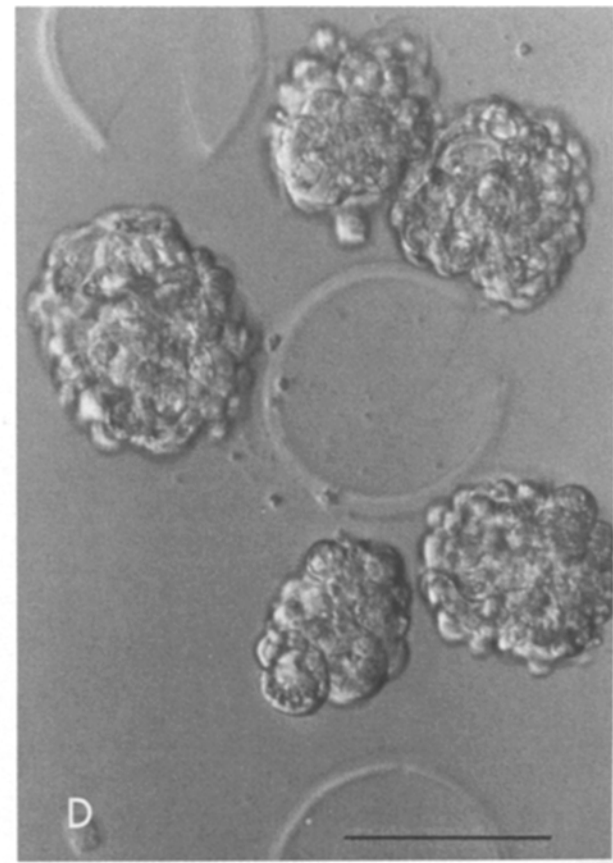
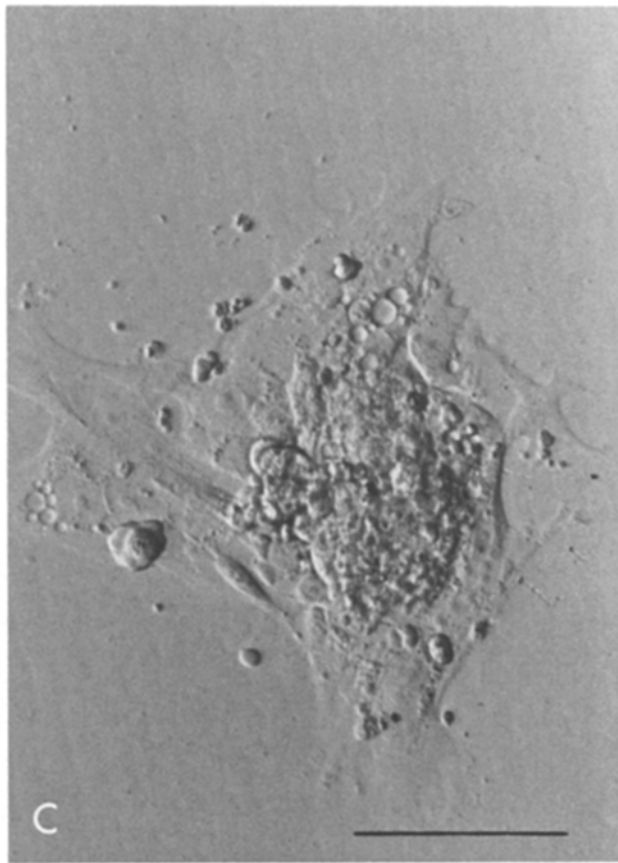
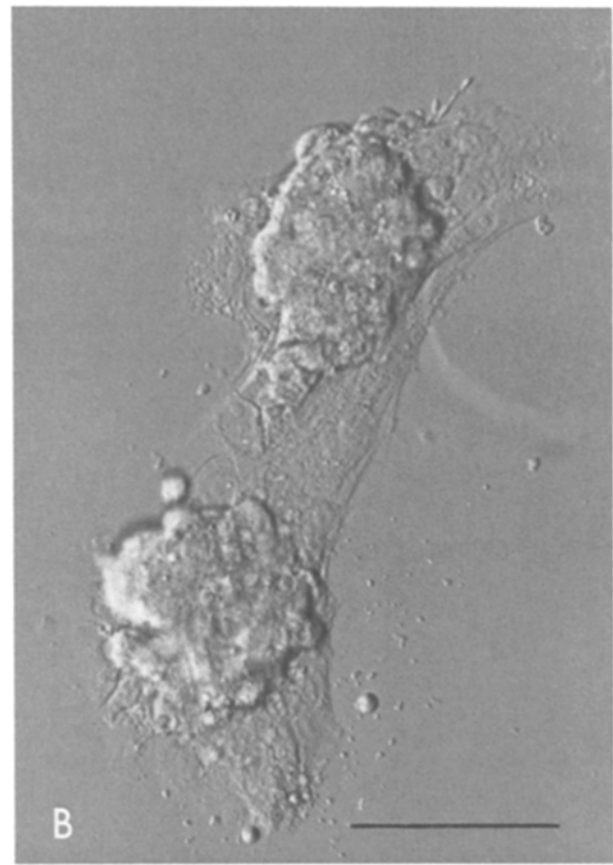
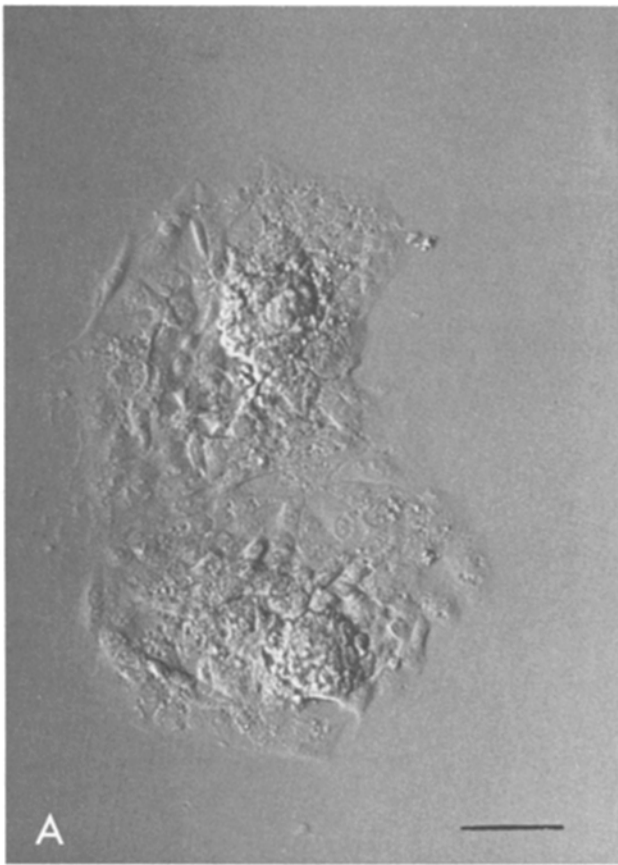


Figure 2. Embryo outgrowth on defined substrates in serum-free medium. (A) Fn substrate (10 $\mu\text{g/ml}$). (B) Ln substrate (25 $\mu\text{g/ml}$). (C) Col IV substrate (25 $\mu\text{g/ml}$). (D) BSA substrate (25 $\mu\text{g/ml}$). Bars, 100 μm .

min at 4°C with mixing. The precleared supernatant was then incubated on a 100 µl pellet of Protein A-Sepharose 4B beads (Pharmacia Fine Chemicals) for 1 h at 4°C with mixing. The beads were pelleted with a low-speed spin, and washed with a series of buffers: (a) Tris-NP40 buffer (0.05 M Tris, pH 8.0, 0.4 M NaCl, 0.005 M EDTA, 1% NP40) (TNB). (b) TNB with 0.1% SDS. (c) TNB with 1.0 M NaCl. After washing, the beads were pelleted and resuspended in electrophoresis sample buffer without 2-mercaptoethanol, and the immunoprecipitated proteins solubilized by heating the sample at 100°C for 3 min. In some experiments, the surface labeled embryos and cells were lysed and spun for 10 min in a microfuge before a 1-h incubation with the primary antibodies. After primary antibody incubation, the lysates were precleared with unconjugated Sepharose beads and immunoprecipitation was performed as described above.

Polyacrylamide Gel Electrophoresis

Immunoprecipitates were analyzed under non-reducing conditions after the method of Laemmli (1970) using 7.0% polyacrylamide slab gels containing SDS. Prestained molecular weight markers (BRL) were used, and consisted of myosin (200 kD), phosphorylase b (97.4 kD), and BSA (68 kD). The gels were stained with Coomassie Brilliant Blue, fixed, dried down, and exposed to X-ray film (Kodak XAR-5).

Cinematicography

Hatched blastocysts (144 h post-hCG) were placed in a microdrop of BME medium under oil in a Falcon 35-mm tissue culture dish which had been equilibrated at 37°C for 4 h. The cover of the dish was inverted and two holes bored into opposite sides: one a pinhole, the other large enough to accept a syringe tip. The cover was then sealed onto the dish using high vacuum grease (Dow Corning Corp., Midland, MI). A piece of tubing was inserted into the large hole in the cover, and attached at the other end to a tank containing 5% CO₂ in air. The flow of gas was adjusted to a level that did not disturb the surface of the oil.

The chamber was then placed into a stage heater (Steier, 1975) controlled by a proportional temperature controller (Yellow Springs, model No. 72) on the stage of a Nikon Diaphot inverted microscope outfitted with Hoffman Modulation Contrast optics. The embryos were filmed using a 10× objective, and their development recorded on Kodak Tri-X reversal film with a Bolex H-16 camera controlled by a Nikon CFX intervalometer. Frames were taken at 30-s intervals.

Results

Normal Outgrowth Formation by Hatched Blastocysts

Blastocyst outgrowth in culture is asynchronous among embryos and occurs after hatching of the embryo from its zona pellucida. Immediately after hatching, blastocysts are not adhesive and when viewed by time lapse cinematicography their surfaces are quiescent (Fig. 1 A). After 10–12 h in culture in serum-containing medium, the trophoblast cells of most embryos show signs of protrusive activity (Fig. 1 B). Spreading is first detectable in the faster-developing embryos after 15 h in culture (Fig. 1 C), and becomes extensive after 20–24 hours; other embryos have yet to begin spreading at this time (Fig. 1 D). By 36 h in culture, the slower-developing embryos have also begun to spread, and by 48 h all have formed extensive outgrowths. This sequence of events is similar when the embryos are grown in the absence of serum, however, the time span over which it occurs is more extended. Embryos cultured in the absence of serum have begun to spread after ~36 h, and have formed extensive outgrowths by 72 h in culture.

The timing of outgrowth formation was similar for embryos cultured in serum-free medium on either Fn or Ln, but seemed slightly slower on Col IV. Most embryos had attached and begun to spread on these substrates after 36 h, and reached maximal spreading after 48–60 h (Fig. 2, A, B,

and C). Identical results were obtained for all substrate coating concentrations used. Embryos cultured on a substrate of BSA in serum-free medium did not attach or spread (Fig. 2 D).

Specificity of Blastocyst Interactions with Defined Ligands

The interactions of embryos with Fn, Ln, and Col IV were ligand specific; embryos attached and spread on Fn in the presence of nonimmune antibodies or anti-Ln, but not in the presence of anti-Fn (Figs. 3 and 5). Outgrowth on Fn was not due to the presence of small amounts of vitronectin (a possible contaminant of Fn preparations and therefore of anti-Fn sera), since attachment and spreading on vitronectin substrates were unaffected by the presence of anti-Fn antibodies (results not shown). Embryos attached and spread on Ln in the presence of either non-immune antibodies or anti-Fn but not in the presence of anti-Ln (Figs. 3, 4, and 5). Embryo outgrowth on Ln was also unaffected by the presence of antibodies against Col IV (Figs. 4 and 5) and against en-tactin (not shown), two common contaminants of Ln preparations. Finally blastocyst adhesion and spreading on Col IV are inhibited by anti-Col IV antibodies, but not by non-immune antibodies or anti-Ln antibodies (Figs. 4 and 5).

Effects on Blastocyst-Ligand Interactions of Anti-ECMr Antibodies and Synthetic Peptides

Anti-ECMr, a polyclonal antibody made against 120–160-kD adhesion glycoproteins isolated from hamster fibroblasts, completely inhibited both attachment and outgrowth of embryos on Fn, Ln (Figs. 6 and 7) and Col IV (not shown). The effects of these antibodies were reversible; embryos removed from anti-ECMr-containing medium after 48 h culture, rinsed and put back into control medium began to spread within 4 h, and formed outgrowths within 12 h (Fig. 6).

Peptides from two different sources (see Materials and Methods) were used to examine the role of the Arg-Gly-Asp-containing cell-recognition site in the formation of embryo outgrowths. Results were similar for peptides from both sources. Neither the active (GRGDSP) nor the control (GRGESP) peptide at concentrations as high as 0.5 mg/ml prevented attachment of embryos to substrates coated with 10 µg/ml Fn. However, GRGDSP had a pronounced effect on trophoblast outgrowth; both the incidence and extent of outgrowth on Fn were greatly reduced (Figs. 8, 9, and 10). Embryo outgrowth on Fn in the presence of GRGESP was similar to that seen in the absence of peptide, both in incidence and extent (Figs. 8, 9, and 10).

For embryos on Ln substrates, a similar effect was seen. Again, neither peptide prevented attachment of blastocysts to Ln, but both the incidence and extent of trophoblast outgrowth were reduced in the presence of GRGDSP (Figs. 8, 9, and 10). In contrast to its lack of effect on outgrowth on Fn, the control peptide, GRGESP, also had some effect on embryo outgrowth on Ln, although to a lesser extent than did GRGDSP (Figs. 9 and 10).

Synthesis and Expression of Adhesion Receptors by Embryo Outgrowths

The goal of these experiments was to determine, by immunoprecipitation and immunofluorescence, whether mouse tro-

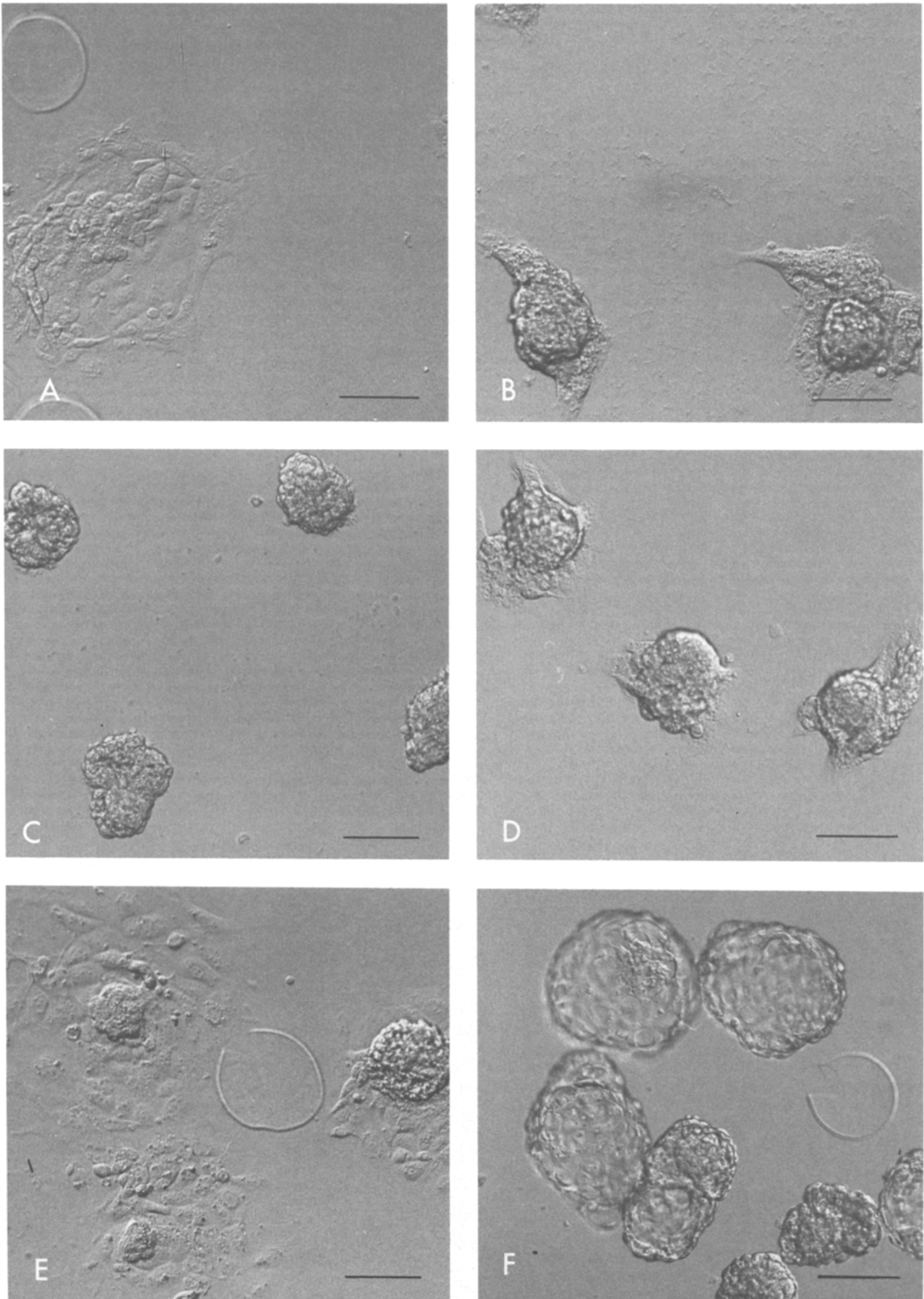


Figure 3. Blastocyst outgrowth on either Fn (10 $\mu\text{g/ml}$) and Ln (25 $\mu\text{g/ml}$) substrates in the presence of substrate-specific antibodies. (A, C, and E) Fn substrate. (B, D, and F) Ln substrate. Embryos were cultured in the presence of: preimmune rabbit antibodies (A and B); anti-Fn antibodies (C and D); or anti-Ln antibodies (E and F). Bars, 100 μm .

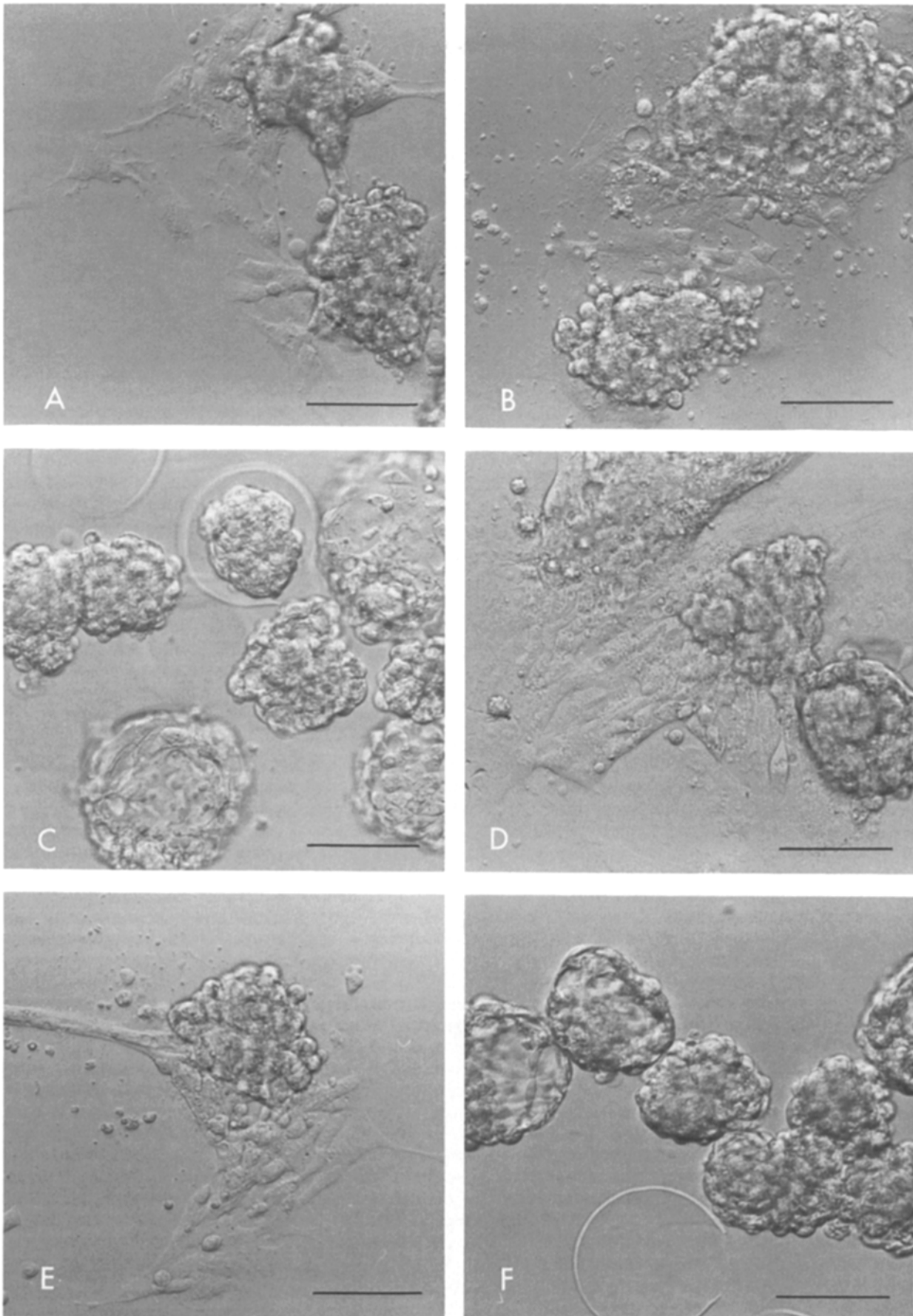


Figure 4. Blastocyst outgrowth on Ln (25 $\mu\text{g/ml}$) and Col IV (25 $\mu\text{g/ml}$) in the presence of substrate-specific antibodies. (A, C, and E) Ln substrate. (B, D, and F) Col IV substrate. Embryos were cultured in the presence of: preimmune rabbit antibodies (A and B); anti-Ln antibodies (C and D); or affinity purified anti-Col IV antibodies (E and F). Bars, 100 μm .

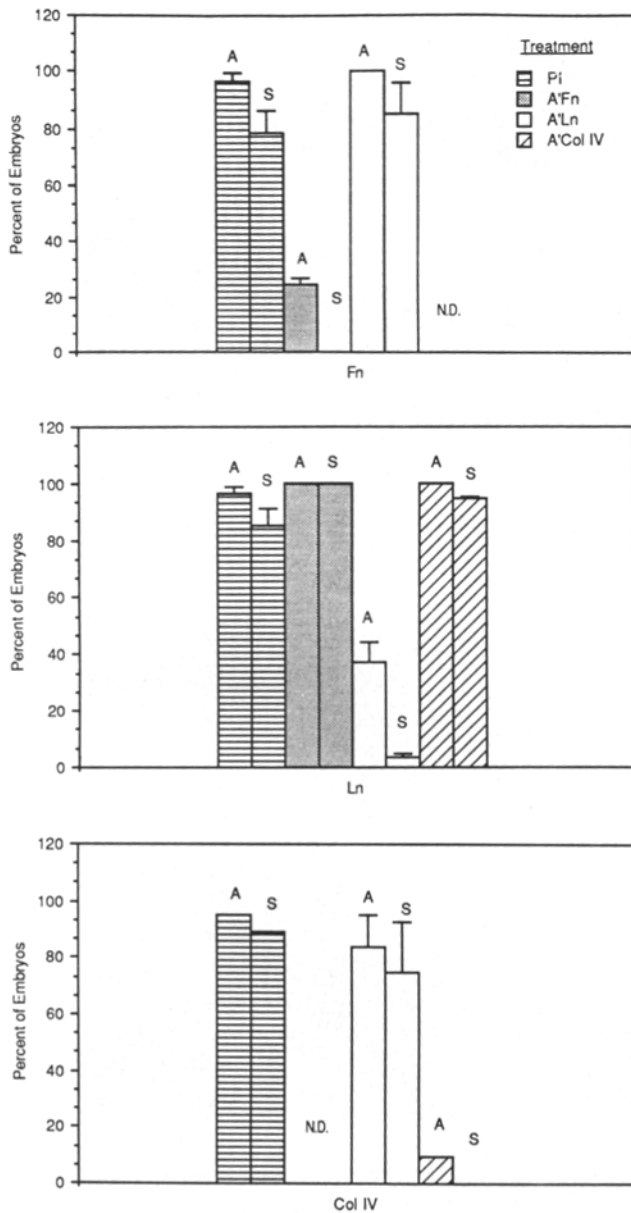


Figure 5. Graph of attachment and spreading of mouse blastocysts on Fn (10 $\mu\text{g/ml}$), Ln (25 $\mu\text{g/ml}$), and Col IV (25 $\mu\text{g/ml}$) in the presence of the different substrate-specific antibodies. Bars labeled *A* represent the percentage of embryos that attached to the substrate in each case, while bars labeled *S* represent the percentage that spread on the substrate. *N.D.*: not done.

phoblast cells produce 140-kD antigens similar to those synthesized by cultured cells, and whether these antigens are expressed at a time and location consistent with their proposed function in outgrowth formation. Two different cell lines were examined; mouse mammary tumor epithelial cells (MMTE), and human trophoblast cells (JAR). Two batches of embryos were also examined: (a) embryos surface-labeled with ^{125}I at the hatched blastocyst stage, and (b) embryos cultured 72 h from the hatched blastocyst stage on a Fn substrate, then surface-labeled with ^{125}I . Immunoprecipitation of labeled cell-surface proteins was then carried out, either

by adding the antibody to lysates of surface-labeled cells or embryos, or by incubating the intact cells or embryos in antibody before lysis. The immune complexes were then precipitated using protein A-Sepharose. Fig. 11 *A* shows the pattern precipitated when anti-ECMr was exposed to the surfaces of labeled intact embryos and cells. Two bands are recognized on the trophoblast surface of embryo outgrowths: one at ~ 144 kD, and the other more diffuse, at ~ 120 – 125 kD (lane *A1*). This pattern is similar to that recognized on intact MMTE, although the lower band is more diffuse and migrates slightly more slowly in the latter (125–135 kD; lane *A2*). When embryos that had been labeled with ^{125}I at times during the first 24 h after hatching were precipitated, no distinct bands in the 120–160 kD region of the gel were recognized above the background (lane *A3*), although a band at ~ 70 kD, and another faint band at 56 kD were detected.

When surface-labeled embryos and cells were lysed before exposure to anti-ECMr (Fig. 11 *B*), the patterns were somewhat more complex. In addition to the two bands recognized previously, bands at ~ 200 kD and 94 kD were precipitated by anti-ECMr from the embryo outgrowths (lane *B1*). A band at about 200 kD is also recognized on the MMTE cells under these conditions (not shown). On JAR cells, anti-ECMr recognizes bands at 120 and 150 kD (lane *B2*), which are likely to be analogous to the 120- and 140-kD bands in the embryo outgrowths. Anti-ECMr also recognizes a band at 200 kD in JAR cells, as well as an additional band at ~ 180 kD, suggesting that it has a more complex array of ECMr-related components than the mouse trophoblast.

These results indicate that mouse embryo outgrowths display ECMr related antigens at the cell surface when they are capable of interacting with the substrate. Furthermore, the components recognized on the outgrowths are similar to those recognized by anti-ECMr on cultured cells of the same species (MMTE). They are similar as well, to the components recognized on a human trophoblast cell line by anti-ECMr, although the pattern in the JAR cells contains an additional component at 180 kD.

To determine the distribution and organization of ECMr antigens in embryos, hatched blastocysts and embryo outgrowths were stained with anti-ECMr and an antibody against the human FN receptor (anti-FNR). Like anti-ECMr, the antiserum against the FNR recognizes the β chain of the β 1 subfamily of integrin. Previous studies on murine melanoma cells using anti-ECMr and anti-FNR showed that both antibodies immunoprecipitate components at 120 and 140 kD and immunoblot the 120-kD component (Kramer and Damsky, 1986; Kramer, R., K. McDonald, E. Crowley, D. Ramos, and C. H. Damsky, manuscript submitted for publication). The distribution patterns of these two antibodies were correlated with those of the cytoskeletal proteins talin (Burrige and Connell, 1983), and vinculin (Vnc; Geiger, 1979), which are components of cell-matrix contact sites (adhesion plaques) of cultured cells (Burrige and Feramisco, 1980; Geiger, 1979; Burrige and Connell, 1983). Indirect immunofluorescence was performed on several groups of embryos: hatched blastocysts, and hatched blastocysts cultured 48 and 72 h post-hatching on Fn substrates.

The embryo outgrowths are highly three dimensional with a rounded inner cell mass (ICM) and a surrounding flattened area of trophoblast outgrowth (Fig. 12 *A*). By focusing at different optical planes of this structure, the distribution pat-

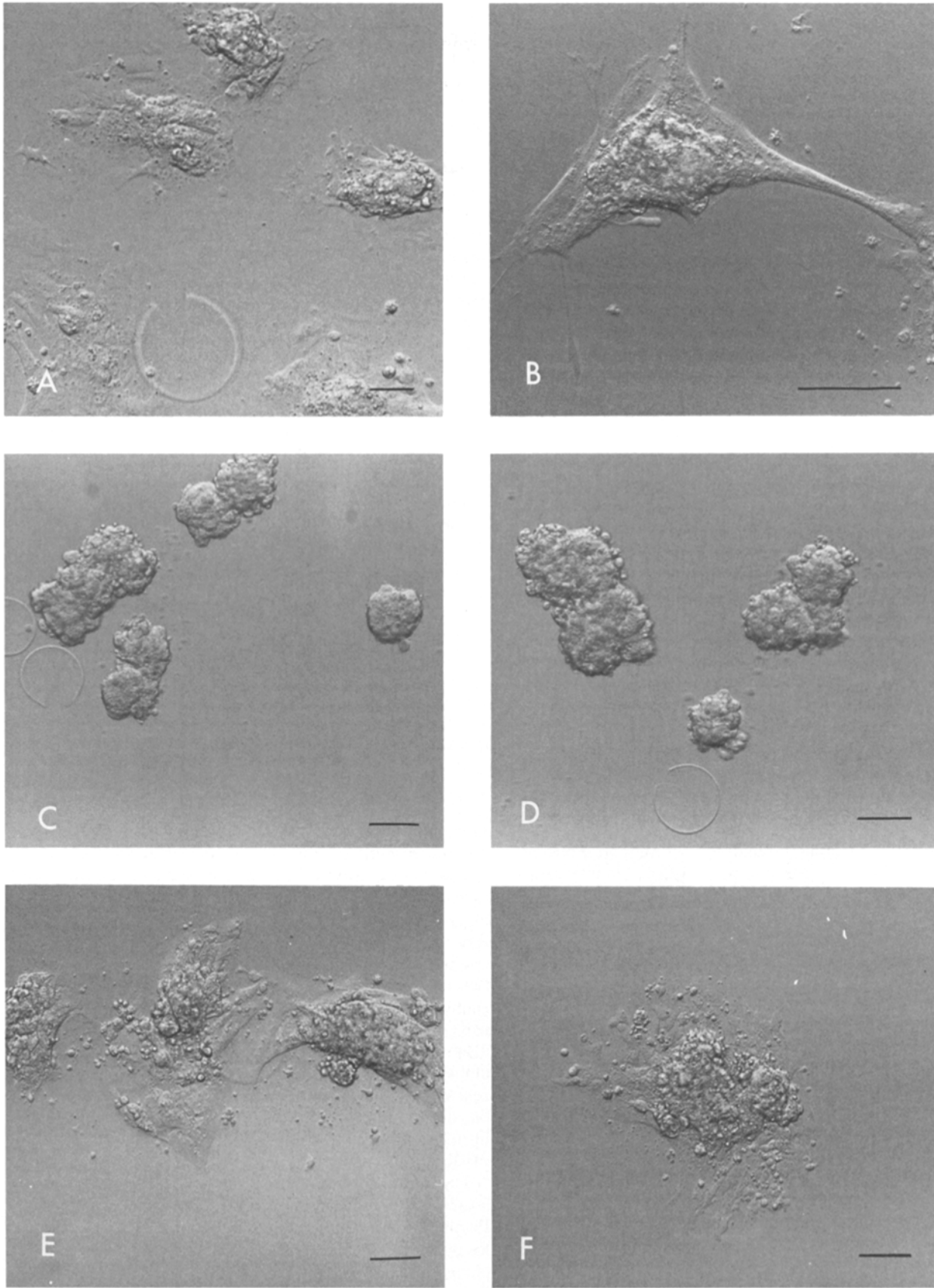


Figure 6. Blastocyst outgrowth on Fn (10 $\mu\text{g/ml}$) and Ln (25 $\mu\text{g/ml}$) in the presence of anti-ECMr antibodies: (A, C, and E) Fn substrate. (B, D, and F) Ln substrate. Embryos were cultured in the presence of: preimmune goat antibodies (A and B) or anti-ECMr antibodies (C and D). (E and F) embryos were cultured 48 h in the presence of anti-ECMr antibodies, then removed, rinsed, and cultured for a further 12 h in control medium. Bars, 100 μm .

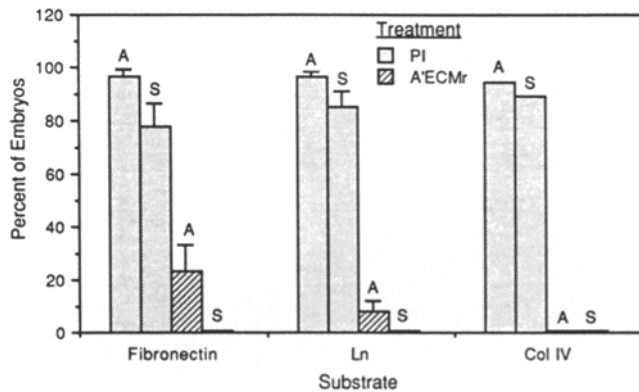


Figure 7. Graph of embryo outgrowth on Fn (10 $\mu\text{g/ml}$), Ln (25 $\mu\text{g/ml}$), and Col IV (25 $\mu\text{g/ml}$) in the presence of either preimmune antibodies (PI) or anti-ECMr antibodies (A'ECMr). Bars labeled A represent the percentage of embryos that attached to the substrate in each case, while bars labeled S represent the percentage that spread on the substrate.

tern of the adhesion receptor antigens and the cytoskeletal antigens Vnc and talin can be compared. In this study, the combination of antibodies that gave the best results in double indirect immunofluorescence experiments was anti-FNR and anti-Vnc. Results from single staining experiments using anti-ECMr alone gave similar staining patterns as anti-FNR (e.g. cf. Figs. 12 B and 13 A, and 12 G and 13 G). At both early (48 h post-hatching) and later (72 h post-hatching) periods, anti-ECMr and anti-FNR staining were strongly enriched at the surface of cells in the top part of the ICM (Fig. 12 B and 13 A). In contrast, staining for both Vnc (Fig. 13 B) and talin (not shown) in this region was diffuse and only slightly above the preimmune control. In the early outgrowth period, at the earliest time points that we could document, the pattern of cell-matrix contact sites was highly organized in the lamellopodial protrusions of the spreading trophoblast cells, as detected by staining with anti-Vnc (Fig. 12, C and D). Staining for anti-ECMr or anti-FNR in trophoblast cells was somewhat more diffuse. Double staining for FNR and Vnc showed extensive colocalization of discrete cell-matrix contact sites in these early outgrowths (Fig. 12, E and F). Examination of the cells at the periphery of later outgrowths shows that anti-ECMr continues to be enriched on filopodial protrusions. In areas where portions of the outgrowth have been torn away, anti-ECMr staining is seen on the substrate as a series of bright strips and dots (Fig. 12 G). Talin is also arrayed in streaks and spots in areas of trophoblast spreading (Fig. 12 H). In all of these samples the pattern of highly fluorescent patches is reminiscent of the staining patterns for avian integrin (the CSAT antigen complex) and for talin and Vnc on avian fibroblasts (Damsky et al., 1985a, b).

In an effort to examine more carefully the organization of adhesion receptors and the cytoskeleton, FNR and Vnc were co-localized in three areas of a 72-h outgrowth (Fig. 13). As indicated above, when focusing near the top of the rounded inner cell mass, anti-FNR (and anti-ECMr) staining is strongly enriched at the cell surface, while Vnc (and talin) staining is diffuse and only slightly above background (Fig. 13, A and B). A group of cells lying on the embryo surface in a region intermediate to the ICM and the trophoblast were

photographed at two planes of focus: one near the apical regions of these cells and the other more basally. This group of cells has smaller nuclei than those at the periphery of the outgrowth and may represent endoderm (Gonda and Hsu, 1980). Both anti-Vnc and anti-FNR staining are strongly enriched at the cell surface and are largely co-extensive. The organization of Vnc changes dramatically in the more basal regions of these cells, becoming highly discrete and punctate. The pattern of anti-FNR also changes. It is enriched in the general areas of strong Vnc staining (see arrows, Fig. 13), but is more diffuse. At the periphery of the outgrowth at the level of the substrate, a discrete pattern of contact sites is visible for both antigens, although the anti-FNR staining, like that of anti-ECMr, has additional diffuse staining.

In hatched blastocysts stained with either anti-ECMr or anti-FNR, no fluorescent signal above the control level was seen (not shown).

Discussion

The results described above make three major points. First, mouse blastocysts will specifically attach and spread on a variety of defined extracellular matrix glycoproteins including Fn, Ln, and Col IV (Figs. 2-5). Second, as described in Figs. 6 and 7, the family of cell surface ECM receptors, variously called, in avian cells, the 140-kD complex (Akiyama et al., 1986), the CSAT antigen (Neff et al., 1982) and, more recently, integrin (Tamkun et al., 1986) is involved in all these blastocyst-ligand interactions. Third, the immunoprecipitation and immunofluorescence data (Figs. 11, 12, and 13) show that blastocysts synthesize and express 140-kD integrin-like matrix receptors at a time and location consistent with their proposed role in blastocyst attachment and outgrowth.

Blastocyst Interactions with Defined ECM Ligands

Recognition of Fn, Ln, and Col IV by blastocysts is ligand specific and not due to cross contamination among the ligands, since antibodies against the individual ECM components interfere only with blastocyst attachment to that particular ligand (Fig. 2-5). These observations in part support those of Armant et al., (1986a), who reported that blastocysts could attach and spread on Fn and Ln. However, the lack of outgrowth on collagen types I and IV reported in those studies contrasts both with the data reported here (Figs. 4 and 5) and with previous observations that the presence of a substrate derived from rat tail collagen could also promote embryo outgrowth in the absence of serum (Wilson and Jenkinson, 1974). The reasons for this discrepancy between our data and that of Armant et al. are not clear, but may lie in differences in the source of Col IV, in the nature of the plastic substrate, or in the method used to coat the substrates.

The invading trophoblast would be expected to encounter a variety of ECM ligands during implantation. In fact, both Fn and Ln have been found to be prominent components of the uterine basement membrane and stroma at the time of implantation (Wewer et al., 1985; Grinnell et al., 1982). Ln may be particularly significant in this regard, as it has been shown that uterine decidual cells in the vicinity of the implanting conceptus synthesize and surround themselves with a Ln-rich basal lamina (Wewer et al., 1985). At the same

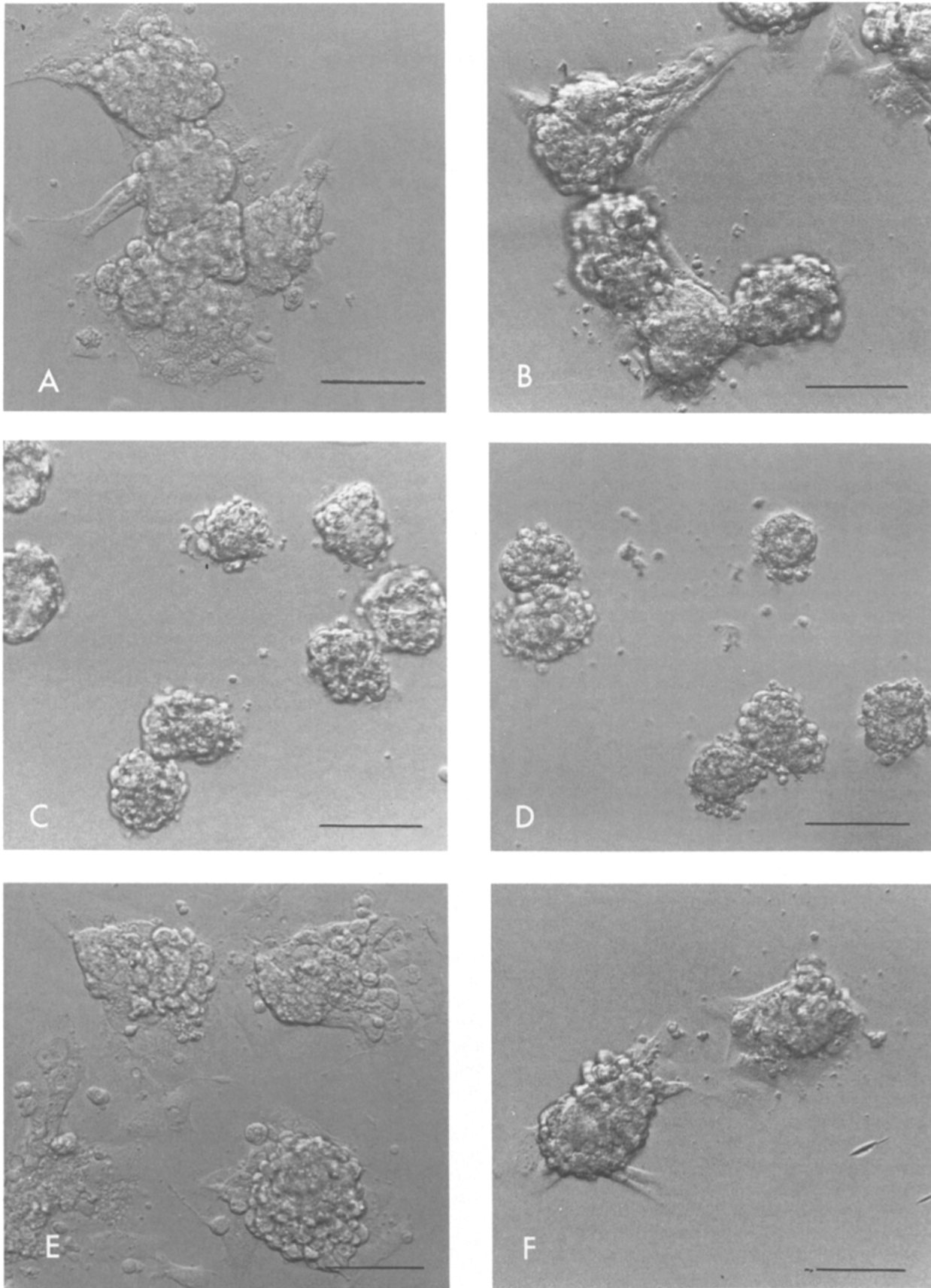


Figure 8. Blastocyst outgrowth on Fn (10 µg/ml) and Ln (25 µg/ml) in the presence of GRGDSP and GRGESP synthetic hexapeptides. (A, C, and E) Fn substrate. (B, D, and E) Ln substrate. Embryos were cultured in: control medium (A and B); medium containing 500 µg/ml GRGDSP (C and D); or medium containing 500 µg/ml GRGESP (E and F). Bars, 100 µm.

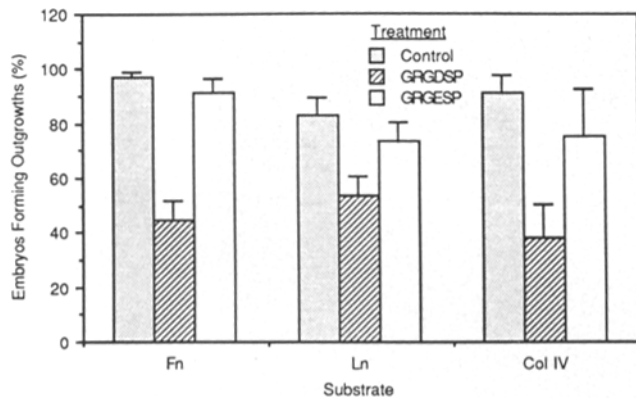


Figure 9. Graph showing the percent of embryos which spread on either Fn (10 $\mu\text{g/ml}$), Ln (25 $\mu\text{g/ml}$), or Col IV (25 $\mu\text{g/ml}$) in normal medium (Control), medium containing 500 $\mu\text{g/ml}$ of the active peptide (GRGDSP) or medium containing 500 $\mu\text{g/ml}$ of the control peptide (GRGESP).

time, the amount of Fn in the uterine stroma near the implantation site decreases considerably (Grinnell et al., 1982).

Both Fn and Ln, and perhaps collagen as well, have more than one mechanism by which they can interact with the cell surface. In addition to the RGD-containing cell-binding domain (Pierschbacher and Ruoslahti, 1984a), Fn has a heparin-binding domain (Hakomori et al., 1984) which may mediate cell attachment via heparin- or heparan sulfate-containing cell surface molecules (Saunders et al., 1986). Ln is reported to have at least two cell-binding regions, one near the intersection of the long and short arms (Liotta et al., 1987; Graf et al., 1987) and the other near the end of the long arm (Engvall, 1986).

Armant et al., (1986b) have reported that small peptides containing the cell-binding sequence of Fn (i.e., GRGDSP), at concentrations as low as 200 $\mu\text{g/ml}$, inhibit both blastocyst attachment and outgrowth on Fn, but not on Ln. In contrast to those results our experiments show that the GRGDSP peptide at 250–500 $\mu\text{g/ml}$ does not inhibit attachment to either Fn or Ln, but inhibits trophoblast outgrowth on both ligands. Recently Farach et al. (1987) have also reported that some outgrowth can occur on Fn in the presence of RGDS. We found that the GRGESP (control) peptide had no effect on blastocyst-Fn interactions, but had a slight effect on trophoblast outgrowth on Ln. The differences in the data on attachment from our study and that of Armant et al. (1986b) may be related to differences in the methods used to assess attachment in the absence of spreading. In the present study, a gentle stream of medium was blown against the embryos whereas Armant et al. (1986a, b) report shaking the dish. The reason that we find an effect of these peptides on outgrowth on Ln while Armant et al. do not is more difficult to ascertain, but may reflect differences in the sources of Ln, or in methods of Ln purification. In our hands, antibodies against Col IV, entactin, and Fn do not inhibit outgrowth on Ln, arguing that these molecules, possible contaminants of Ln preparations, are not responsible for the outgrowth of embryos on Ln.

There is precedent for an effect of RGD- and RGE-containing peptides on cell interactions with Ln. Madri et al. (1987) have reported effects of both RGD- and RGE-containing peptides on endothelial cell-Ln interactions, Grabel and

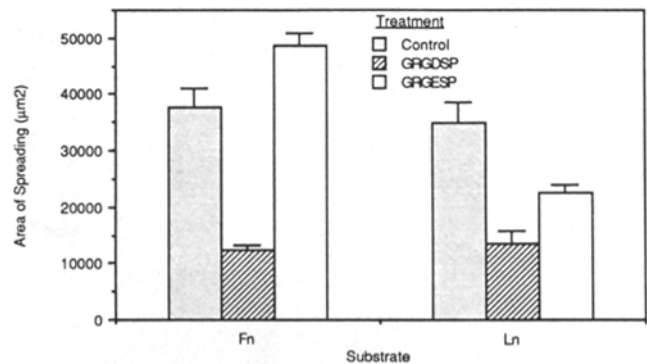


Figure 10. Graph showing the area of outgrowth (in μm^2) of embryos cultured on either Fn (10 $\mu\text{g/ml}$) or Ln (25 $\mu\text{g/ml}$) substrates in normal medium (Control), medium containing 500 $\mu\text{g/ml}$ active peptide (GRGDSP), or medium containing 500 $\mu\text{g/ml}$ control peptide (GRGESP). Each value represents the mean \pm SEM for at least 11 embryos.

Watts (1987) find that GRGDSP peptides inhibit teratocarcinoma cell migration on Ln as well as on Fn, and Thiery et al. (1987) have reported that GRGESP affects migratory behavior of neural crest cells on Fn. The inhibition of trophoblast outgrowth on both Fn and Ln by GRGDSP is also consistent with equilibrium gel filtration experiments showing

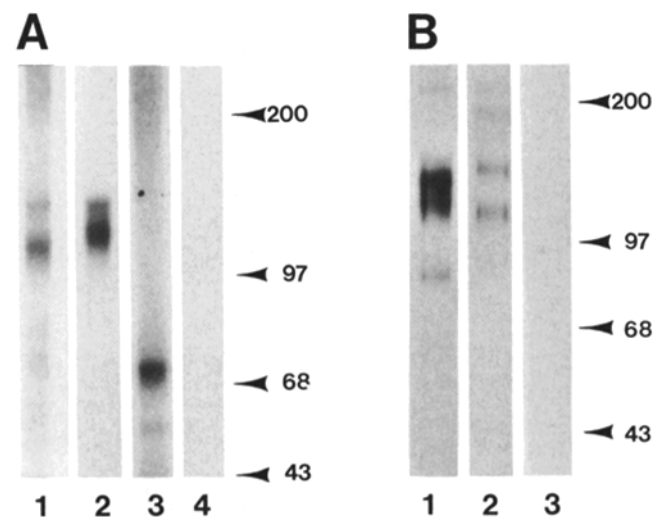


Figure 11. Immunoprecipitation of anti-ECM antigens from cells and embryos. (A) Immunoprecipitation by anti-ECM of ^{125}I -labeled cell surface proteins from 72-h mouse embryo outgrowths (lane 1), MMTE cells (lane 2), and mouse hatched blastocysts (lane 3). (Lane 4) Immunoprecipitation by normal goat serum of ^{125}I -labeled cells surface proteins from MMTE cells. Cells and embryos were surface-labeled with ^{125}I , and then incubated in medium containing either anti-ECM or preimmune antibodies prior to lysis (see Materials and Methods). (B) Immunoprecipitation of ^{125}I -labeled cell surface proteins from 72-h mouse embryo outgrowths (lane 1) and JAR cells (lane 2). (Lane 3) immunoprecipitation by normal goat serum of ^{125}I -labeled cell surface proteins from MMTE cells. Cells and embryos were surface-labeled with ^{125}I , then lysed and the labeled lysate subjected to immunoprecipitation with either anti-ECM or preimmune antibodies. Immunoprecipitation by normal goat serum of the above embryo samples were similar to those shown for MMTE cells.

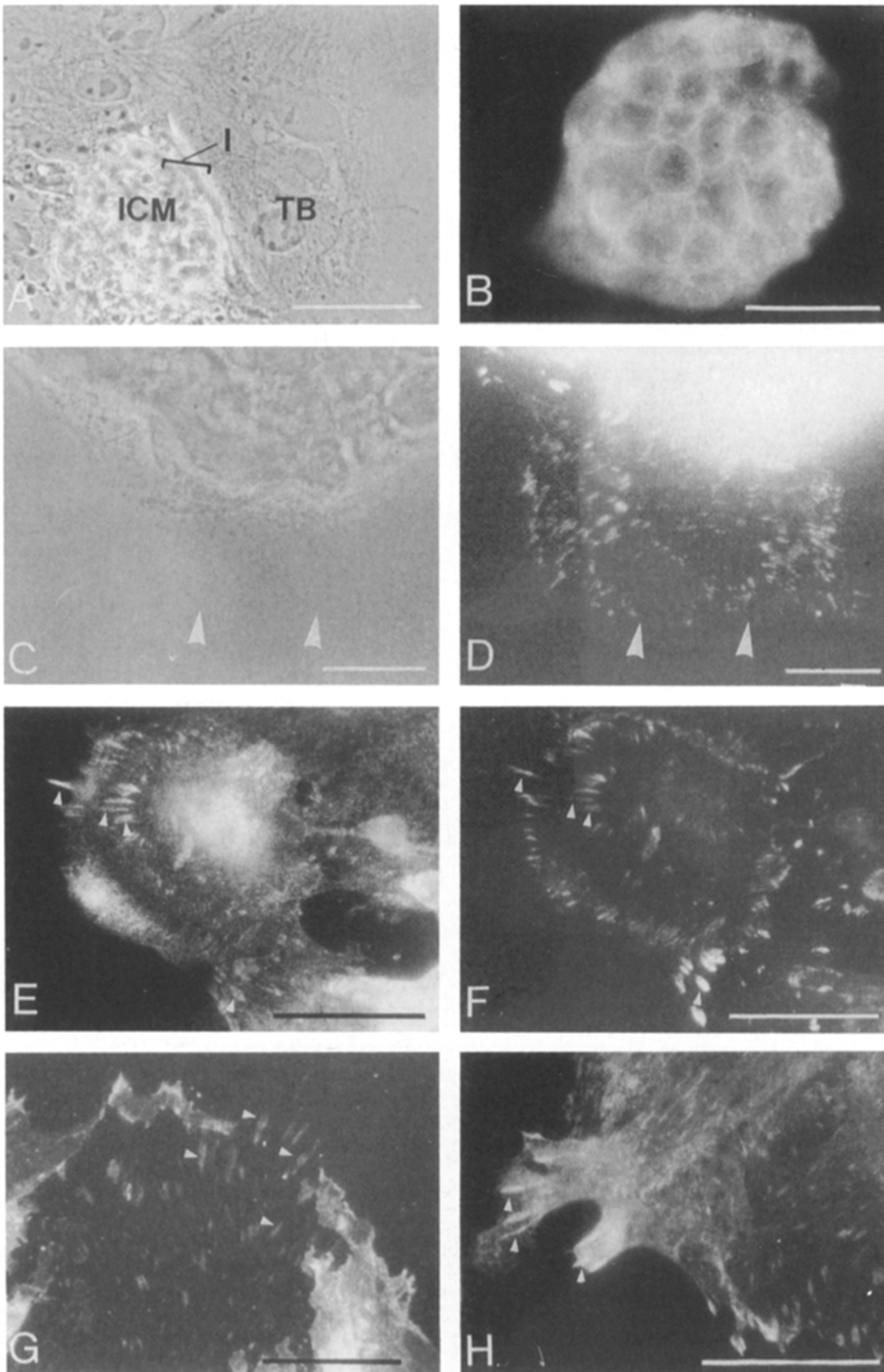


Figure 12. Immunofluorescent staining of antigens recognized by anti-ECMr, anti-FNR, anti-Vnc, and anti-talin antibodies on blastocyst outgrowths. (A) Phase photograph of an outgrowth, demonstrating the regions shown in the other photographs. *ICM*, inner cell mass. *TB*, trophoblast cells. *I*, intermediate region. (B) Anti-ECMr staining is concentrated at the cell surface in the ICM, outlining the cells. (C) Phase photograph of trophoblast cells beginning to spread in a 48-h outgrowth. (D) Anti-Vnc staining in the same early outgrowth, demonstrating organization of cell-matrix contacts even in early stages of outgrowth. (E and F) Anti-FNR (E) and anti-Vnc (F) double staining on a 48-h outgrowth. Adhesion receptors and Vnc colocalize in discrete cell-matrix contact sites (*arrowheads*). (G) In areas where the trophoblast has been torn away from the substrate, anti-ECMr staining is seen on the substrate in bright strips and dots (*arrowheads*). (H) Anti-talin fluorescence is seen in similar patterns in peripheral processes of trophoblast cells (*arrowheads*). Bars, 25 μ m.

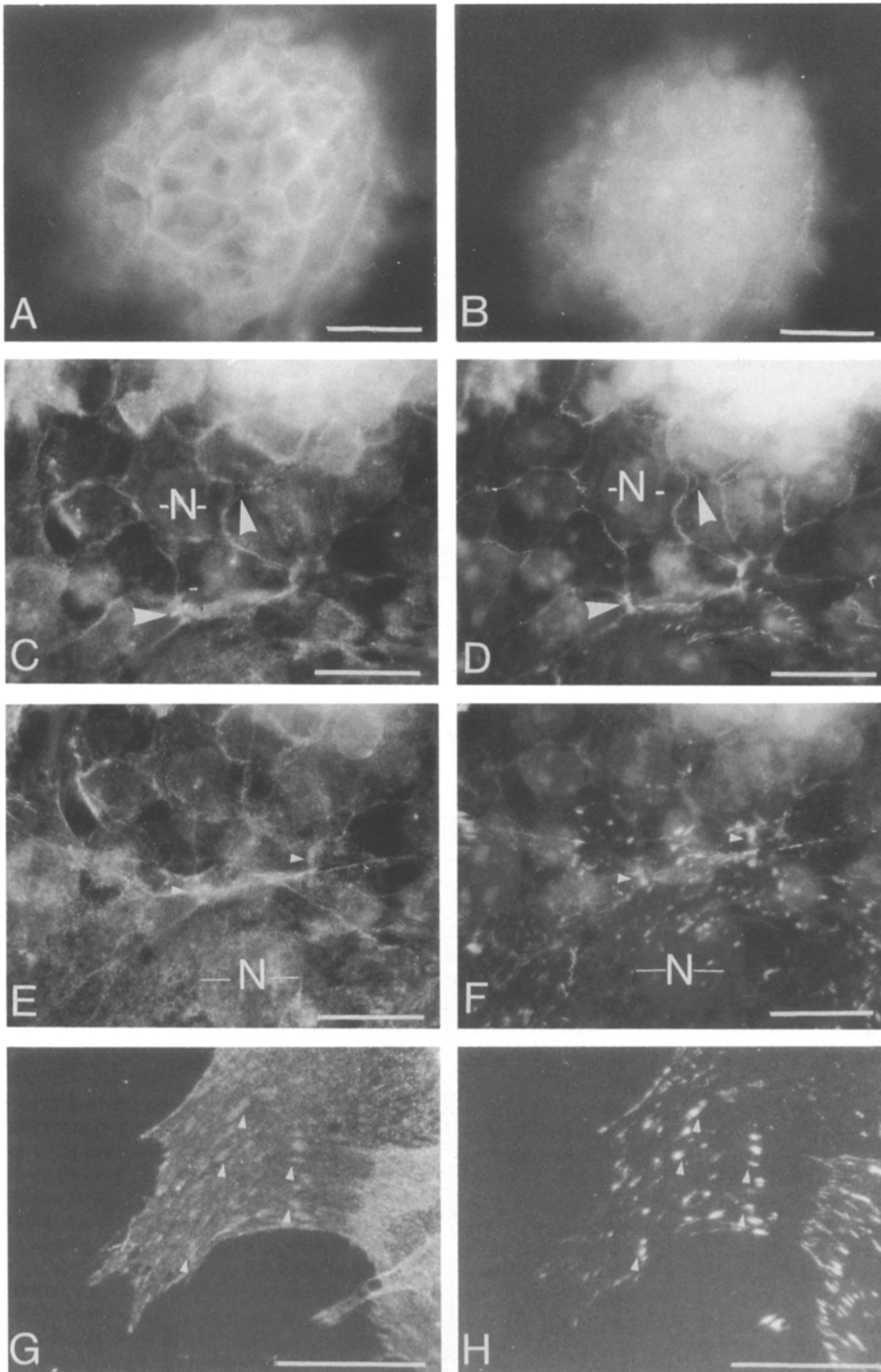


Figure 13. Double staining for anti-FNR and anti-Vnc on a 72-h outgrowth, examined in various regions of the outgrowth. (A, C, E, and G) anti-FNR staining. (B, D, F, and H) anti-Vnc staining. (A and B) Anti-FNR is localized at the surface of cells in the top of the ICM, while anti-Vnc staining is diffuse and only slightly above background. (C and D) When focussing on the apical ends of cells found in

that the interactions of purified avian integrin with either Fn or Ln can be disrupted by small RGD-containing peptides (Horwitz et al., 1985). Altogether, these observations suggest that small peptides related to the cell recognition sequence of Fn, can have diverse effects depending on the cell type, the substrate and the biological activity being monitored.

The fact that in our hands, the active peptide, RGDS, affects embryo outgrowth on, but not attachment to, both Fn and Ln suggests that trophoblast attachment is mediated by additional mechanisms. Farach et al., (1987) recently reported that soluble heparin inhibited mouse embryo attachment and outgrowth on both Fn and Ln to a greater extent than did RGDS, and that substrates of a heparin-binding protein, platelet factor 4, supported attachment but only limited outgrowth. These results, taken with those reported here, imply that outgrowth relies primarily on mechanisms that involve recognition of sequences related to the cell binding region of Fn, while attachment may be mediated through heparin- or heparan sulfate-containing moieties as well.

The Role of the Integrin Receptor Family in Blastocyst-ECM Interactions

In their interactions with Fn, Ln, and Col IV, blastocysts appear to use a 140-kD family of cell surface adhesion receptors, inasmuch as both trophoblast attachment and spreading on all three of these substrates are inhibited by anti-ECMr. The 140 kD receptor complex recognized in mouse blastocysts and other mammalian cells by anti-ECMr is very similar to the integrin complex identified by the CSAT and JG22 monoclonal antibodies in avian systems. Such similarity is demonstrated by several criteria: firstly, anti-ECMr, CSAT, and JG22 all affect cell attachment to a broad range of ECM ligands including Fn, Ln, and Col IV (Hall et al., 1987; Horwitz et al., 1985; Tomaselli et al., 1987); secondly, the constituent polypeptides of both the ECMr complex and avian integrin behave similarly on reduced and non-reduced gels (Knudsen et al., 1985; Tomaselli et al., 1987); thirdly, the anti-ECMr used in this study and an antibody against band 3 of the integrin complex (Buck et al., 1986) precipitate identical polypeptide patterns from labeled rat PC12 cells (Tomaselli et al., 1987); and finally, the anti-integrin band 3 antibody reacts in the immunoblot procedure with the lowest molecular weight bank (120 kD) immunoprecipitated from rat PC12 cells by anti-ECMr (Tomaselli et al., 1987). Gene cloning and protein sequencing data have shown in turn that the avian and mammalian adhesion receptor complexes are also closely related to the dimeric fibronectin receptor isolated using fibronectin affinity chromatography (Pytela et al., 1985a; Patel and Lodish, 1986). In addition, the anti-human FNR used in these studies and anti-ECMr recognize a similar pattern of polypeptides on murine melanoma cells (Kramer, R., K. McDonald, E. Crowley, and C. H. Damsky,

manuscript submitted for publication). These observations suggest that the mammalian adhesion receptors recognized by anti-ECMr in the mouse embryo are members of the large family of adhesion receptors that includes both the Fn receptor and the avian integrin complex. Embryo attachment to the broad range of substrates studied here may reflect the presence of several ligand-specific heterodimeric receptors. It is also possible, however, that the 120 and 140 kD components that comprise most of the anti-ECMr-reactive material constitutes, at least in part, a promiscuous receptor, capable of interacting with more than one ECM ligand. Such a receptor, in the beta 1 integrin family has been proposed by Wayner and Carter (1987). The platelet adhesion receptor GP IIb-IIIa, a member of the beta 3 subfamily of integrin has also been shown previously to act as a receptor for at least four ECM ligands, including fibronectin (Pytela et al., 1986).

Anti-ECMr inhibits attachment to a broad range of substrates, but does not inhibit all cell-matrix interactions: for example, it does not affect cell attachment to thrombospondin (Tuszynski et al., 1987) nor does it affect attachment of PC12 cells to polylysine or to the surfaces of muscle or neuronal cells (Tomaselli et al., 1987). Anti-ECMr inhibits both attachment and spreading of mouse blastocysts on defined substrates (Fn, Ln, and Col IV). Natural ECM is more complex, however, containing proteoglycans, glycosaminoglycans, and thrombospondin in addition to Fn, Ln, and collagens. Anti-ECMr does not inhibit blastocyst attachment to the complex ECM laid down by bovine corneal endothelial cells, but does inhibit trophoblast outgrowth on this matrix (Glass, R. H. and C. H. Damsky, unpublished experiments). These observations stress the importance of integrin-like adhesion receptors in trophoblast outgrowth while demonstrating that blastocysts are able to interact with their surrounding ECM by other mechanisms as well.

Expression and Distribution of Integrin on Blastocysts

Immunoprecipitation of ¹²⁵I-labeled surface proteins from intact embryo outgrowths with anti-ECMr produces a set of proteins that are very much like those found in MMTE cells. These bands in mouse embryos have molecular weights of ~140 and 120 kD, similar to those in MMTE cells, but closer in molecular weight to each other than those found in human trophoblast cells (Fig. 11). In addition, a band at ~200 kD and another at ~95 kD are precipitated from whole lysates of surface-labeled mouse embryo outgrowths. The 200 kD band is also precipitated from both the MMTE and JAR cells, and is of similar molecular weight in all three cell types. This band may be related to attachment to Ln or Col IV since it is not precipitated from JAR cells by a monoclonal antibody that inhibits JAR cell attachment to Fn only, whereas both the 120- and 150-kD bands are precipitated by that antibody (Damsky and Crowley, unpublished results). The 95-kD band was observed only in embryo outgrowths,

the region intermediate to the ICM and the trophoblast, both anti-FNR and anti-Vnc staining are observed to be localized at the cell surface (arrowheads). *N*, nucleus. (*E* and *F*) When focussing more basally in the same region pictured in *C* and *D*, anti-Vnc staining is seen in a discrete, punctate pattern. Anti-FNR staining is enriched in areas of discrete Vnc staining, but is much more diffuse. Arrowheads point to some areas of codistribution. *N*, nucleus. (*G* and *H*) In the highly spread trophoblast cells at the periphery of the outgrowth, both anti-FNR and anti-Vnc staining are organized into a series of discrete cell-matrix contacts. Arrowheads point to some areas of codistribution. Bars, 25 μm.

and its relationship to the higher molecular weight components is unknown. The fact that these two additional bands are precipitated from whole lysates but not from lysates of cells or embryos exposed to antibody while still intact probably means that the epitopes recognized by anti-ECMr on these proteins are not accessible to antibody while the cells are intact.

Immunoprecipitation of blastocysts that were ^{125}I surface-labeled at times during the first 24 h post-hatching showed no resolvable bands in the 120–160 kD area of the gel, suggesting that these ECMr antigens are either not expressed on the cell surface or are not detectable by our labeling procedures on these earlier stages (Fig. 11). These findings correlate with both the observation that embryos at this stage are not adherent and do not spread on the substrate during the first 24 h post-hatching, and immunofluorescence data demonstrating no specific staining above the level seen in controls.

Indirect immunofluorescence experiments using anti-ECMr show that ECMr antigens are present on both the ICM and trophoblast outgrowth portions of attached embryos. Observation of the flattened trophoblast cells shows that, although there is quite a bit of diffuse fluorescence, the antigen is enriched on cell processes and on the ventral surfaces of cells. In regions where the trophoblast has been ripped away, there is a characteristic pattern of substrate attached “footpads” like that seen in fibroblastic cells stained with CSAT monoclonal antibody (Damsky, et al., 1985a).

The distribution in trophoblast cells of the adhesion plaque protein Vnc was also examined. This 130-kD intracellular protein has been shown to be a component of adhesion plaques (Geiger et al., 1980; Burridge and Feramisco, 1980). In cell–substratum adhesion plaques, it codistributes with and binds to talin and is proposed to interact indirectly with actin microfilaments (reviewed in Burridge, 1986). In the trophoblast outgrowth of attached blastocysts, anti-Vnc is highly localized in streaks and patches very similar to those found in other cell types (Geiger, 1979; Burridge and Feramisco, 1980; Geiger et al., 1980; Damsky et al., 1985a). Likewise, the staining pattern seen for talin, which is known to bind to integrin (Horwitz et al., 1986) consists of the same kinds of linear patches and dots, especially at the margins of the trophoblast cells. The staining pattern generated by anti-Vnc colocalizes with that generated by anti-FNR (Figs. 12 and 13). These results strongly suggest that the integrin-like adhesion receptors are found in the adhesion plaques of mouse trophoblast cells, and are thus in an appropriate location to participate in trophoblast cell–substratum adhesion.

The staining pattern for cytoskeletal and adhesion receptors differs markedly in different parts of the embryo. In the uppermost region of the inner cell mass, ECMr and FNR are distributed uniformly but strongly at the cell surface, while Vnc and talin are distributed diffusely throughout the cytoplasm. This implies that the cells in this region are not associated with one another via well organized junctional complexes, and that adhesion receptors are not well integrated with the cytoskeleton. There is, however, intense cell surface laminin staining on the inner cell mass region of the embryo, that colocalizes with the adhesion receptor staining (not shown). In the apical regions of cells in the region intermediate to the ICM and the trophoblast, both Vnc staining and FNR or ECMr staining are enriched at the cell

surface. This may represent a zone that has plentiful adherens junctions. Vnc is a peripheral cytoplasmic component of these junctions, while adhesion receptors such as avian integrin, have been shown to extend to areas of cell–cell as well as cell–matrix contact in sections of embryonic tissue (Chen et al., 1986; Duband et al., 1986; Krotoski et al., 1986). In the basal region of cells of this intermediate zone and throughout the flat trophoblast area of the outgrowth, ECMr, FNR, and especially Vnc become localized more discretely. The most intense patches of adhesion receptor staining coincide with areas of closest cell–substratum contact as monitored by Vnc staining.

In summary, this study shows that mouse embryos express components of the integrin family of cell surface ECM receptors, as well as a known cytoskeletal ligand for this receptor family, at the time when they are competent to interact with ECM molecules *in vitro*, and with the uterine stroma during implantation. Our studies with defined ECM ligands, cell recognition peptides and anti-ECMr suggest that trophoblast cell outgrowth relies strongly on cell interactions with RGD-containing cell–recognition sites on ECM ligands. Our results agree with those in other systems showing that these interactions are involved in cell adhesion and migration during development (Boucaut et al., 1984; Bronner-Fraser, 1985, 1986; Duband et al., 1986; Grabel and Watts, 1987). The fact that anti-ECMr inhibits outgrowth on, but not attachment to, the complex bovine corneal endothelial cell-derived ECM (Glass and Damsky, unpublished observations), and that RGD peptides inhibit blastocyst outgrowth on but not attachment to Fn and Ln, suggest that blastocysts have a complex overall strategy for reacting with ECM components *in vitro*, and by analogy, with the uterine stroma during implantation.

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