

Role of Laminin in Epithelium Formation by F9 Aggregates

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ABSTRACT The formation and maturation of the outer epithelial layer is essential for maximal alphafetoprotein (AFP) production during differentiation of F9 embryoid bodies in the presence of 5×10^{-8} M retinoic acid (Grover et al., 1983. *J. Cell Biol.* 96:1690–1696). The critical phase is between the third and the fourth day when the components of the extracellular matrix organize into a basement membrane. The role of some of these components in the process of epithelium formation and maturation is analyzed in this paper.

The role of laminin was investigated by testing the effect of exogenous laminin and antilaminin in cultures of differentiating F9 aggregates. Tests included growth rates, morphological changes, AFP production, determination of AFP mRNA levels, and fluorescent staining for basement membrane components and for epithelial markers. At concentrations $> 5 \mu\text{g}/\text{ml}$, exogenous laminin inhibited the production of AFP and prevented AFP gene transcription. On the basis of immunofluorescence tests, exogenous laminin appeared to act by preventing the accumulation of a basement membrane and by disrupting the organization of the outer layer into an epithelium. No such effects were produced by fibronectin or collagens type I or IV.

Aggregates cultured in the presence of antilaminin also failed to organize an epithelium and did not produce AFP, whereas those in normal rabbit serum differentiated normally. Therefore, endogenous laminin plays a key role not only as a basement membrane structural component but also in organizing the epithelial layer of endoderm cells and hence (indirectly) in gene expression.

F9 embryonal carcinoma (EC) cells, when induced with 5×10^{-8} M retinoic acid in suspension cultures, differentiate into embryoid bodies consisting of an outer layer of epithelial cells surrounding a central core of undifferentiated or partially differentiated cells (1). The cells of the outer layer synthesize and secrete alphafetoprotein (AFP) and resemble the cells of visceral endoderm in the mouse embryo (1). In our previous paper, we studied the process of differentiation of embryoid bodies (2). We showed that epithelial layer formation and basement membrane organization are essential for the full expression of visceral endoderm function (characterized by AFP production). AFP is produced at high levels only after the outer layer is properly aligned on a basement membrane. We have also shown that the appearance of AFP in the medium is affected by the density of the starting EC cells in the culture, indicating that the early appearance of AFP in the medium might be related to the conditioning of the medium by high densities of cells, although other explanations

are possible. We hypothesized that laminin (3, 4), fibronectin (5), or type IV collagen (6), components of extracellular matrices, might be involved at several stages in the differentiation process in addition to their accumulation as a basement membrane.

The involvement of laminin has been examined in detail by adding purified laminin or rabbit antilaminin serum to the culture medium in various doses and at various times during the 8-d process of embryoid body formation. Evidence is presented in this paper that low concentrations of exogenous or endogenous laminin are stimulatory or necessary, respectively, for embryoid body formation. However, high concentrations of exogenous laminin inhibit the process. Several roles of laminin are discussed.

MATERIALS AND METHODS

Cell Culture: A subclone of F9 EC cells, B1 (2), was used in all experiments. It is referred to as F9. The maintenance of the cells and the

conditions affecting the formation of F9 embryoid bodies have previously been described (2).

Differentiation of aggregates of F9 cells was induced by adding 5×10^{-8} M retinoic acid to the culture medium at the time of seeding (1). Cultures for testing AFP production were grown in 96-well untreated plastic plates as described (2). For detection of mRNA the aggregates were grown in 100-mm untreated plastic dishes starting with 5×10^6 F9 cells/10 ml for 1-d cultures, 3×10^6 cells/10 ml for 2- and 3-d cultures, 1×10^6 cells/10 ml for 4-d cultures, or more. For immunofluorescence tests, aggregates obtained from one 60-mm dish seeded with 5×10^5 F9 cells/5 ml were used.

Additions to Cultures: Rat laminin purified from tumors of a rat yolk sac tumor cell line (L2) (7) and human and bovine fibronectin purified from plasma (8) were kindly provided by Drs. E. Engvall and E. Ruoslahti of this Institute. Laminin (freshly prepared) at 1–2 mg/ml in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, was stored at -20°C and thawed only once before use. Fibronectin (2 mg/ml in 4 M urea) was brought into phosphate-buffered saline (PBS) by gel filtration through a column of Sephadex G-25. It was stored at 4°C and used within a week. Collagen type I was purified from rat tail tendon (9) and collagen type IV from bovine anterior lens capsules after limited pepsin digestion (10). These materials were taken up in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and sterile filtered (pore size, 0.45 μm) to give stock solutions with concentrations between 20 and 100 $\mu\text{g}/\text{ml}$. In one experiment, laminin was labeled with ^{125}I by the chloramine T method (11) and added at 10^6 cpm/ml to cultures over 8 d together with excess of cold laminin to a final concentration of 7 $\mu\text{g}/\text{ml}$. The aggregates were collected, washed with PBS, fixed in a mixture of ethanol and acetic acid, embedded in 1% agar (2), and processed for autoradiography (12).

Rabbit antisera to rat laminin (13) and to mouse fibronectin (14) were provided by Dr. E. Engvall. The antilaminin cross-reacts with laminin from mouse and other species. The antifibronectin cross-reacts with bovine serum fibronectin. These antisera and normal rabbit serum were heat-inactivated at 56°C for 30 min, dialyzed against PBS, sterile-filtered, and added at the start of F9 aggregate cultures at concentrations up to 5% (vol/vol). In all the above-described cultures, the medium was replaced on days 2, 4, 6, and 7, and, at day 8, AFP in the medium was assayed.

Measurement of AFP and Growth Rates: AFP was determined by sandwich enzyme-linked immunosorbent assay (ELISA), as described (15, 16). The growth of the embryoid bodies was measured by disrupting them in 1 M glycine, 2 mM EDTA for 3 h at 37°C , and determining the cell numbers as described (2).

Immunofluorescence: The embryoid bodies were washed, fixed in a mixture of ethanol and acetic acid, embedded in 1% agar, sectioned, and stained for detection of extracellular matrix components (laminin, fibronectin, and type IV collagen), cytoskeletal intermediate filament proteins, Endo A and B (17), and blood group antigens, I and i (18, 19). The antisera used were rabbit antibodies: antilaminin, antifibronectin, antitype IV collagen, anti-Endo A, and anti-Endo B; and human antibodies: anti-I and anti-i. The controls were normal rabbit serum and normal human serum. Details are described by Grover et al. (2).

Light Microscopy: The embryoid bodies were fixed in 2.5% glutaraldehyde buffered with 0.06 M sodium cacodylate at pH 7.3 for 4 h, rinsed in three changes of cacodylate buffer, and postfixed in 1% osmium tetroxide buffered with veronal acetate at pH 7.3. Dehydration was carried out in a series of graded ethanols at pH 7.3 before embedding in Araldite medium. Thick sections (1 μm) were mounted on glass slides and stained with 1% Toluidine Blue in 1% borax solution (20).

Dot Blots to Detect and Quantitate mRNA for AFP: To quantitate AFP mRNA production during the time course, F9 aggregates and monolayers were collected from large-scale cultures after 1–21 d. In another series, cultures contained various concentrations of extracellular matrix components, and these were cultured for 4 d (with one medium replacement at 2 d) before harvesting.

Total RNA was extracted from each sample of F9 aggregates and, for a positive control, from mouse embryo visceral yolk sacs, as described (21). Total RNA (5 μg in 15 μl of $3\times$ standard saline citrate: 0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.4 [SSC], 5 mM methyl mercury hydroxide) was dotted onto nitrocellulose membranes according to the method of Thomas (22). 10-fold serial dilutions of RNA were prepared in a solution of 1 mg/ml tRNA, 5 mM methyl mercury hydroxide, and $3\times$ SSC, and 5- μl aliquots were dotted.

Nitrocellulose filters were processed as described (23) and RNA was hybridized for 15 h at 65°C with 10–20 ng/ml heat-denatured probe ($1-3 \times 10^6$ cpm $^{32}\text{P}/\text{ml}$). The probe consisted of the plasmid pBR322 with a 1,700-base-pair cDNA insert containing the AFP-encoding sequence (24). Autoradiographs of the blots were exposed for 1–6 d and were densitometrically scanned and integrated to quantitate the data.

RESULTS

Morphological Effects of Exogenous Laminin

We have previously shown (2) that F9 aggregates cultured in the presence of 5×10^{-8} M retinoic acid develop a distinct outer layer of cells and that, subsequently, internal cysts appear as embryoid bodies form. Control aggregates (in the absence of retinoic acid) did not develop an outer layer of cells; the aggregates were dark in the center and did not become cystic.

When laminin was added to F9 aggregate cultures, the following observations were made. In the presence of low concentrations of laminin ($<5 \mu\text{g}/\text{ml}$) for 8 d, the cultures morphologically resembled the embryoid bodies produced in the absence of added laminin, that is, an epithelial layer appeared. However, at concentrations of exogenous laminin $>5 \mu\text{g}/\text{ml}$, the aggregates did not develop an outer layer of cells at any time. They appeared slightly smaller compared with those cultured without laminin, but the cell numbers were similar (Table I), indicating that laminin was not toxic to cell metabolism or growth. Furthermore, aggregates that formed in the presence of high laminin concentrations ($>5 \mu\text{g}/\text{ml}$) remained solid and spherical, did not become cystic, and, unlike the control aggregates, they were not dark in the center. The morphological effect was specific for laminin and was not observed with human or bovine fibronectin, bovine collagen type IV, and rat collagen type I at similar concentrations. The addition of these materials did not alter the production or time of appearance of an outer layer of epithelium.

Microscopic Appearance of Aggregates Cultured with Laminin

Sections of F9 cell aggregates cultured in the presence of retinoic acid for 8 d showed a well-defined endoderm cell layer forming an epithelium resting on a basement membrane and with several layers of less differentiated cells within (Fig. 1a). The other obvious feature was the cystic center containing some cellular debris. Most of the cells of outer layer resembled visceral endoderm of the postimplantation mouse embryo (20) and had numerous microvilli and pinocytotic and lysosomal vacuoles. The presence of numerous fat droplets gave a lacy appearance to the outer cell layer. In the presence of exogenous laminin ($>5 \mu\text{g}/\text{ml}$), the cells resembled the cells of aggregates cultured without retinoic acid in that no central cyst formed and there was no epithelial layer and no basement membrane. The outer layer of cells did not develop into visceral endoderm-type cells. The cells did not

TABLE I
Effect of Laminin on Differentiation of F9 Embryoid Bodies

Days of treatment	AFP	Cell number
	% control	% control
0–8	3 ± 2	117 ± 24
0–3	54 ± 15	116 ± 9
3–8	18 ± 6	109 ± 32
6–8	73 ± 17	117 ± 20

Rat laminin (10 $\mu\text{g}/\text{ml}$) was added to the medium for the indicated time periods during the 8-day culture. Laminin was included for all 8 d (line 1), or was added from the start of the culture for only 3 d (line 2), or was added on day 3 of the culture for the last 5 d (line 3), or added for the last 2 d (line 4). AFP was measured as nanograms per milliliter on day 8 when the total cell numbers were counted (see Materials and Methods). There were three determinations.

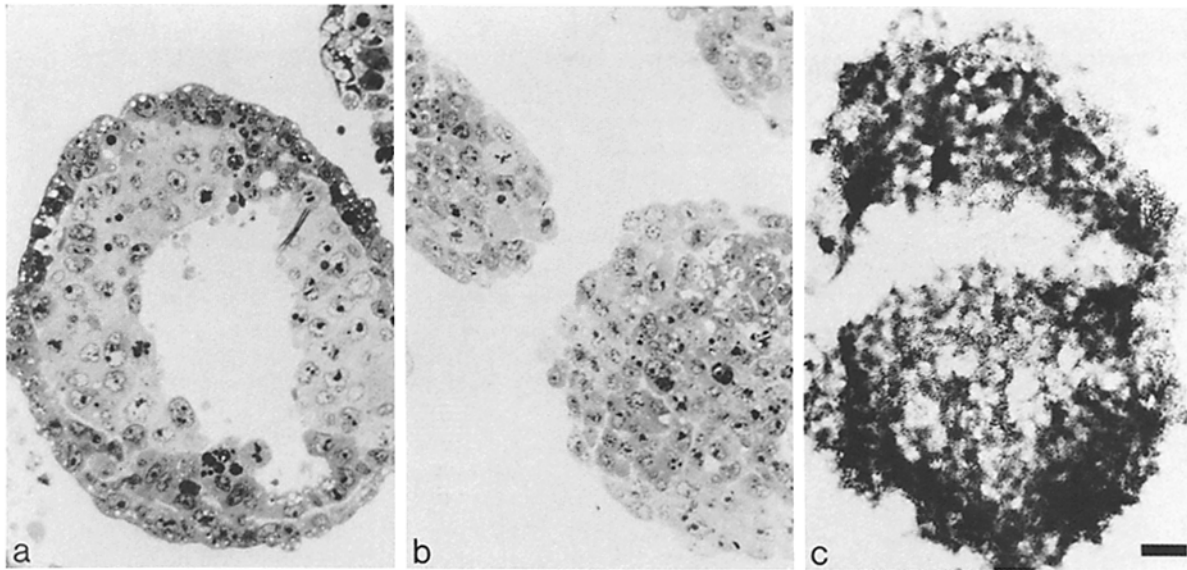


FIGURE 1 Photomicrographs of sections of F9 aggregates. (a) Control cultures of embryoid bodies at 8 d. (b) Aggregates cultured for 8 d in the presence of 10 $\mu\text{g/ml}$ rat laminin. a and b are 1- μm sections stained with Toluidine Blue. (c) Autoradiograph of sections of F9 aggregates cultured with 7 $\mu\text{g/ml}$ laminin and tracer levels of ^{125}I -laminin. Bar, 20 μm . Original magnification, \times 400.

appear pinocytotic, had no microvilli, and no distinct vacuoles: All cells appeared similar (Fig. 1 b). In contrast, the outer endoderm layer of normal embryoid bodies stained more intensely with Toluidine Blue, and this together with the basement membrane served to make the epithelial layer distinct from the rest of the more rounded, paler-staining cells.

We also cultured aggregates with 7 $\mu\text{g/ml}$ laminin together with radio-iodinated laminin at tracer level. These aggregates were fixed and processed for autoradiography as described in Materials and Methods. The resulting sections of the cell aggregates showed that exogenous laminin had indeed penetrated and was found around most cells (Fig. 1 c). The greatest label intensity was found adhering to the outer edge of the aggregates, and this fact was taken into account when considering the possible modes of action of laminin.

Immunofluorescence Tests for Epithelium Formation

The results of immunofluorescence staining of sections of embryoid bodies cultured for 8 d in the presence of 10 $\mu\text{g/ml}$ bovine fibronectin (Fig. 2, column C) were similar to those of bodies cultured in its absence (Fig. 2, column A). There was an outer layer of cells underlain by a distinct basement membrane that stained for laminin, fibronectin, and type IV collagen. In contrast, sections of aggregates cultured with laminin (10 $\mu\text{g/ml}$) for 8 d did not show a basement membrane. Instead, laminin, fibronectin, and type IV collagen were uniformly distributed over nearly all the cells (Fig. 2, column B).

In immunofluorescence staining for epithelial markers, an abnormal pattern was observed in cultures containing laminin. The cytoskeletal protein, Endo B, was present in low amounts and in a scattered pattern (Fig. 3, compare column A and B). Staining for i blood group antigen was positive on most cells in the aggregate instead of being restricted to the very outermost margin of the outer layer as it is in embryoid bodies formed in the absence of laminin. I antigen similarly showed a wider distribution on the outermost layers of cells

(Fig. 3). The latter observations were important because they indicated that the markers were not correctly distributed as would be expected for an epithelium, and also that the cells of the aggregate were differentiated to some extent. This was confirmed by lack of stage-specific embryonic antigen (SSEA-1; [25]) staining (not shown), which was earlier shown to be expressed on the undifferentiated cells (2).

Effect of Exogenous Laminin on AFP Production

We have previously shown (2) that maximal production of AFP takes place when the epithelial layer is fully organized on a basement membrane, that is, at 8–10 d of culture. We analyzed the effect of laminin on endoderm expression by adding it at a range of concentrations and at various stages of the process. The AFP secreted into the medium was tested at 8 d in all cases. Fig. 4 shows the effect of various concentrations of rat laminin on AFP production. At concentrations of laminin >5 $\mu\text{g/ml}$, AFP production was greatly inhibited at all starting cell densities of F9 EC cells. We observed total inhibition of AFP production at 10 $\mu\text{g/ml}$ of laminin in the medium.

The effect of lower laminin concentrations differed according to the density of the cultures. Within a range of 0.1 to 1 $\mu\text{g/ml}$, less inhibition of AFP production occurred in cultures of lower cell density (e.g., 600 cells) compared with the cultures of higher cell density (e.g., 2,400 cells). In fact, at the lowest cell density (300 cells), we observed a slight increase in AFP production (Fig. 4). Since we did not know the rate of cell proliferation in this study, increased AFP production could have been caused either by increased numbers of endoderm cells or by increased AFP production per cell. We concluded that under specific conditions low concentrations of exogenous laminin may stimulate growth or differentiation, but that the predominant effect at average or high cell densities is that laminin disrupts or inhibits the formation of visceral endoderm, as measured by AFP production.

When 10 $\mu\text{g/ml}$ laminin was added to cultures for all 8 d, maximal inhibition of AFP production was observed (Table

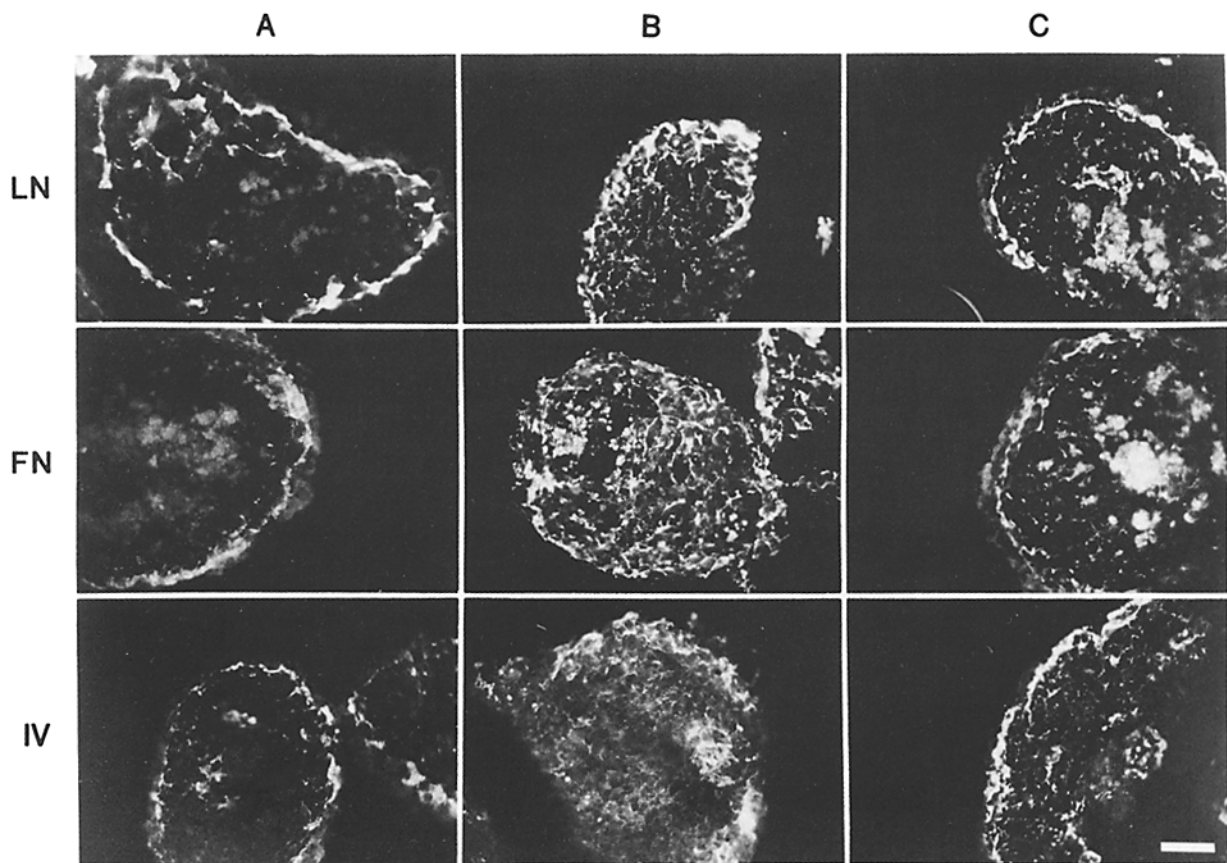


FIGURE 2 Immunofluorescent staining of sections of F9 aggregates for basement membrane components. Column A, 8-d control cultures (embryoid bodies); column B, 8-d aggregates cultured with 10 $\mu\text{g}/\text{ml}$ rat laminin; column C, 8-d cultures with 10 $\mu\text{g}/\text{ml}$ bovine fibronectin. LN, stained for laminin; FN, stained for fibronectin; IV, stained for type IV collagen. Bar, 50 μm . Original magnification, $\times 250$.

I). Very little inhibition was observed when laminin was included in the culture medium for the first 3 d only (17%), showing that the major deleterious effect of laminin was on the later phases of the process. In fact, laminin added from days 3 to 8 had almost as much effect as that added from days 0 to 8, whereas laminin present from days 6 to 8 had no effect and therefore the process of secretion itself was not affected. We concluded that laminin inhibits embryoid body formation by affecting a process that occurs between the third and the sixth day. We have shown previously that this period is important for the organization of the basement membrane and the appearance of the visceral endoderm phenotype (2).

Other components associated with the basement membrane were tested similarly. Fibronectin and type IV collagen added at concentrations up to 10 $\mu\text{g}/\text{ml}$ had little or no effect on AFP production. Fibronectin, type IV collagen, and bovine serum albumin at 20 $\mu\text{g}/\text{ml}$ or higher slightly inhibited AFP levels produced by densely-seeded cultures, although the degree was variable and inconsistent. The effect of laminin therefore appears to be specific.

Effect of Exogenous Laminin on AFP mRNA Production

Since AFP was not found in cultures of F9 aggregates containing laminin, we examined the cultures for AFP mRNA production. Fig. 5a shows that extracts of total RNA from normal cultures hybridized to labeled cDNA for AFP in a pattern and in amounts reflecting the amount of mRNA for

AFP in the extract. The first visible increase in the amount of probe hybridized was on the third day (shown at highest sensitivity in Fig. 5b) but the largest increase of 20-fold occurred between days 3 and 4. Thereafter, a steady rise of mRNA occurred to a maximum between day 8 and 15 (Fig. 5).

In the next series of experiments, extracts of RNA were made from F9 aggregates cultured in the presence of a range of extracellular matrix components at various concentrations. To conserve our purified matrix components, we stopped the cultures after 4 d when the maximum increase of AFP mRNA should have occurred. The results in Fig. 5 show that the culture containing laminin contained little or no mRNA for AFP since the dots reacted at background level. Some cultures containing matrix components contained somewhat less mRNA for AFP, but in general the levels were within the experimental variation of control. Therefore, we concluded that laminin specifically modulated differentiation of F9 embryoid bodies by inhibiting AFP production at the level of transcription.

F9 Aggregates Cultured in Antilaminin

We observed earlier that low concentrations of laminin were stimulatory to AFP production when added to cultures containing very few cells (75–300 cells, Fig. 4). We have observed that laminin is synthesized by F9 cells throughout the time course of embryoid body formation, with a sharp increase on day 2 (2). Therefore, we attempted to remove this

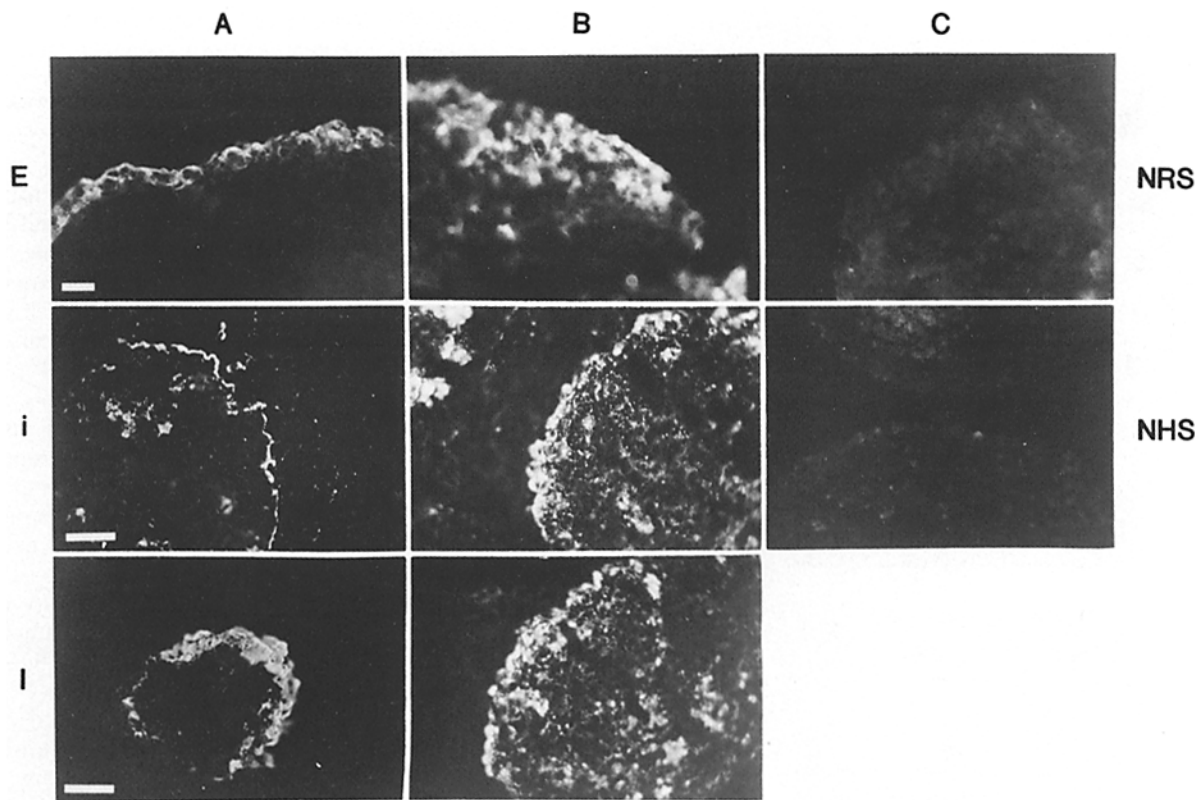


FIGURE 3 Immunofluorescent staining of sections of F9 aggregates for cytoskeletal and cell surface antigens. Column A, 8-d control cultures (embryoid bodies); column B, 8-d aggregates cultured with 10 $\mu\text{g}/\text{ml}$ rat laminin; column C, sections stained with preimmune normal rabbit or human sera (NRS, NHS). E, stained for Endo B, bar = 20 μm ; i, stained for i antigen; bar = 50 μm ; I, stained for I antigen, Bar = 50 μm . Original magnifications, $\times 400$ (E); $\times 250$ (i and I).

laminin or to neutralize its effectiveness in any stimulatory process by adding rabbit antilaminin antiserum to cultures. The result was a dose-dependent inhibition of AFP production measured on the eighth day (Table II). For example, AFP production was inhibited 66% in cultures containing antilaminin antiserum (1:40 dilution) compared with those containing an unrelated antiserum (not shown) or normal rabbit serum. Interestingly, rabbit antibovine fibronectin also inhibited AFP production at similar dilutions.

Since both antilaminin and antifibronectin inhibited AFP production by F9 aggregates (Table II), it was of interest to see whether they interfered with the formation of the epithelial layer. This was tested by studying the distribution of basement membrane components (laminin, fibronectin, and type IV collagen) and cell surface markers (blood group antigens I and i) in 8-d cultures. Figs. 6 and 7 show the immunofluorescence staining of sections of F9 aggregates cultured in antilaminin and antifibronectin. Aggregates cultured in antilaminin (1:20) and stained for laminin, fibronectin, and type IV collagen lacked a basement membrane. Instead, laminin, fibronectin, and type IV collagen were uniformly distributed (Fig. 6). Similarly, there was uniform distribution of I antigen on outer layers of sections, whereas i antigen failed to appear at all (Fig. 7).

On the other hand, sections of aggregates cultured in antifibronectin (1:20) and stained for laminin, fibronectin, and type IV collagen showed a normal distribution of these markers in the basement membrane (Fig. 6). In addition, both I and i antigens were expressed normally on the outer cell surface (Fig. 7). Similar staining patterns were observed with

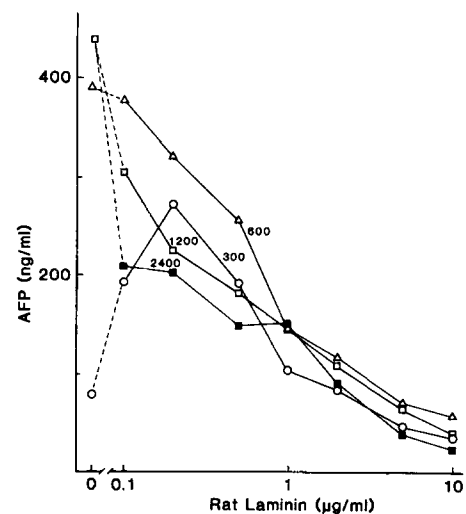


FIGURE 4 Effect of exogenous laminin on AFP production. Differentiating F9 aggregates were cultured for 8 d in the absence or presence of rat laminin at various concentrations. The numbers indicate the number of F9 cells seeded per well in a 96-well microtiter plate. Δ , 600; \circ , 300; \square , 1,200; \blacksquare , 2,400. AFP was assayed on day 8. The AFP measurements of cultures without added laminin are connected to those with laminin by dotted lines.

bodies cultured in normal rabbit serum (1:20, not shown).

In summary, we found that endogenous laminin was necessary for forming a basement membrane as well as for organizing the outer epithelial layer of visceral endoderm

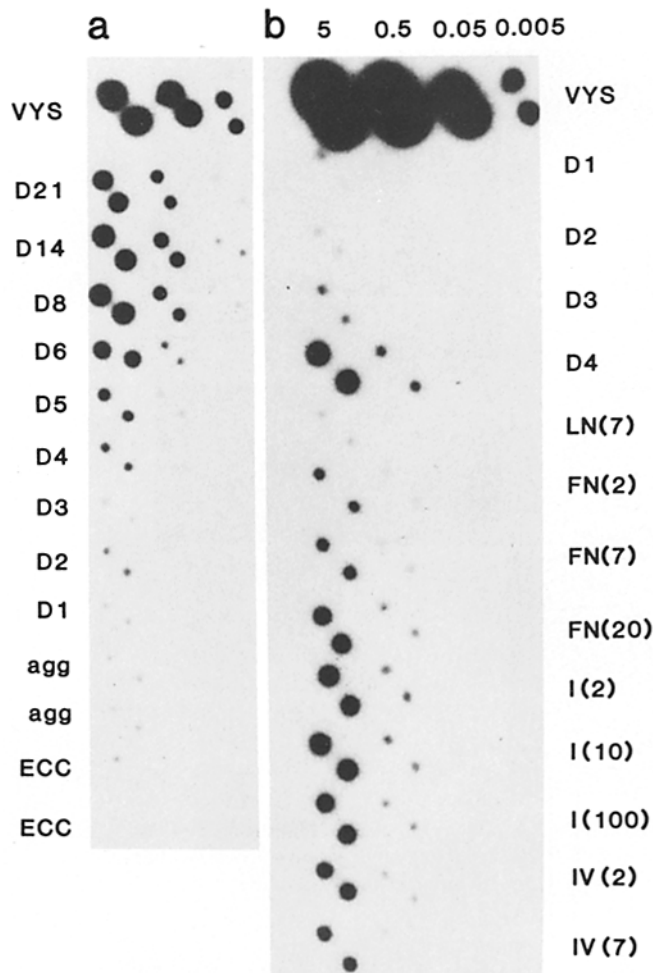


FIGURE 5 Detection of AFP mRNA by dot hybridization. (a) Duplicate dots of 5, 0.5, and 0.05 μg total RNA extracted from cultures over a time course were dried onto nitrocellulose membranes. Radioactive cDNA for AFP was applied and the filters were processed as described in Materials and Methods. RNA extracted from mid-gestation mouse embryo visceral yolk sacs (VYS) was added for a comparison. The largest proportion of AFP mRNA was found between day 8 and 14 of F9 embryo body cultures. The largest single day increase was between days 3 and 4 (RNA was inadvertently lost from the third day sample and different extracts are shown again at a more sensitive level in the upper portion of *b*). *ECC*, F9 embryonal carcinoma cell monolayers; *agg*, F9 aggregates cultured for 2 and 3 d in the absence of retinoic acid; D1 through 21, day of culture of embryo bodies from 1 to 21. (b) RNA extracted from F9 aggregates cultured with extracellular matrix components for 4 d was dotted and hybridized as described in *a*. *LN*, laminin; *FN*, fibronectin; *I*, type I collagen; *IV*, type IV collagen. The number in parentheses indicates the concentration in $\mu\text{g}/\text{ml}$.

cells. This is consistent with the inability of aggregates to produce AFP when cultured in antilaminin. F9 aggregates cultured in antifibronectin, on the other hand, were not affected in their ability to form an epithelium. However, this layer of cells was unable to produce AFP, and therefore fibronectin appears to play a distinctly different role in embryo body formation.

DISCUSSION

We showed earlier (2) that the organization of an epithelium of visceral endoderm cells as an outer layer on aggregates of

differentiated F9 cells involves many overlapping steps: aggregation, proliferation, differentiation, basement membrane formation and organization, and maturation and secretion. The secretion of large amounts of AFP (up to $4 \mu\text{g}/10^6$ cells/day) occurs at a late stage (day 8–10) and seems to correlate with the formation of a mature, polarized epithelial layer, properly aligned on a thin basement membrane. The organization of the epithelium starts at a critical stage during the differentiative process, that is, the fourth day. Before this time, there is no indication of asymmetric accumulations of extracellular matrix materials, although new gene activity is seen prior to the fourth day. Increased synthesis of laminin occurs on the second day. Radiolabeled Endo A and B and AFP polypeptides are first detected intracellularly on day 3. It is only on the fourth day that basement membrane components, laminin, fibronectin, and type IV collagen, start to accumulate in the outer layer of cells. These deposits change to a thin layer under the newly-forming epithelium. On the sixth day AFP can be detected in the medium, and this rises extremely rapidly up to the tenth day. The evidence presented in this paper shows that the increased production of mRNA for AFP (Fig. 5) starts on the third day and, since small amounts of AFP are detected on day 3 by immunoprecipitation (2), it appears that the message is translated immediately. However, the AFP is either retained within the cell or is diluted so much in the medium that it is not detectable by ELISA (lower limit 2 ng AFP/ml) until day 6.

When laminin (above $5 \mu\text{g}/\text{ml}$) was added to cultures of differentiating F9 aggregates, the events leading to the formation of an epithelium did not occur. No outer differentiated layer of visceral endoderm cells was seen (Fig. 1*b*), no basement membrane was formed (Fig. 2), no AFP was produced (Fig. 4), and no AFP mRNA was detected (Fig. 5). However, laminin did not prevent differentiation in general since SSEA-1 disappeared (not shown), and I and i antigens were expressed, although abnormally (Fig. 3). The normal embryo body displays I antigen throughout the outer epithelial layer and i is normally on the outermost margin of that layer. In cultures containing laminin, the cell-surface antigens and also Endo B cytoskeletal protein were found throughout the 3–5 outer cell layers of the aggregates. The overall appearance was lack of the normal asymmetry and therefore, we concluded that laminin disrupted the “organization” required for epithelium formation. Since exogenous laminin was seen predomi-

TABLE II
Effect of Antilaminin and Antifibronectin on AFP Production by F9 Embryo Bodies

Antiserum	Dilution	AFP % control
Antilaminin	1:40	34
	1:80	50
	1:160	57
	1:320	80
	1:640	112
Antifibronectin	1:40	42
	1:80	37
	1:160	42
	1:320	78
	1:640	118

Antilaminin, antifibronectin, or normal rabbit serum (control) was included for all 8 d of culture. AFP was measured on day 8 and the cells were counted. Percentages were calculated as micrograms AFP produced per 10^6 cells per day. Results shown are from one of two similar experiments.

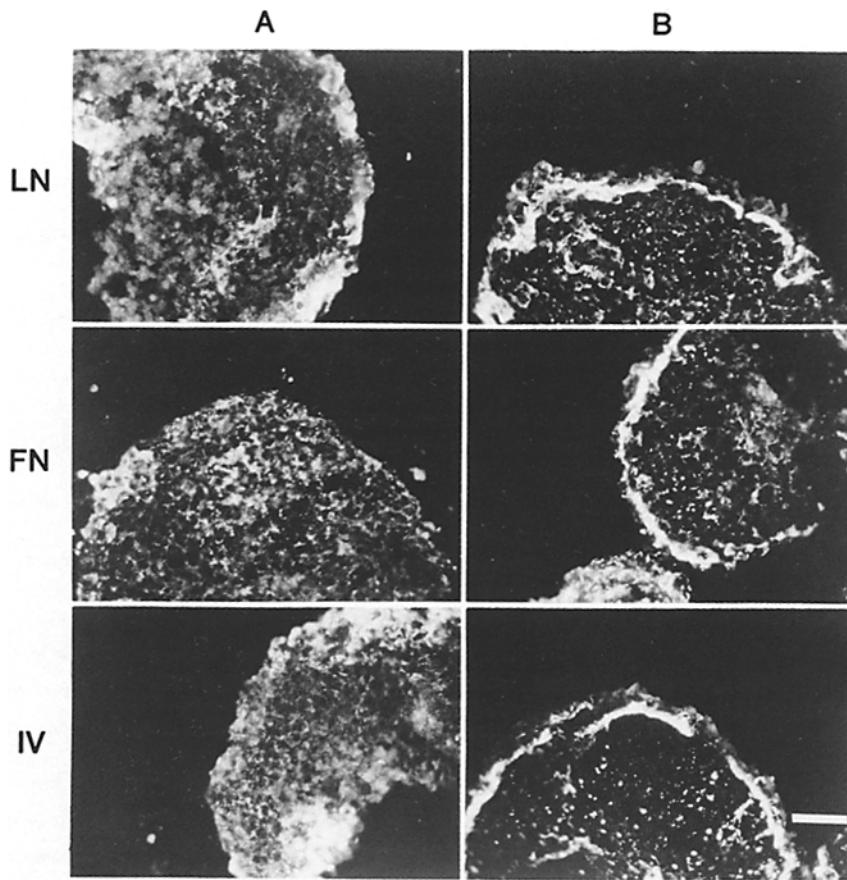


FIGURE 6. Immunofluorescent staining of sections of F9 aggregates cultured with antisera for 8 d; staining for basement membrane components. Column A, cultured in 1:20 dilution of antilaminin; column B, in 1:20 dilution of antifibronectin; LN, stained for laminin; FN, stained for fibronectin; IV, stained for type IV collagen. Bar, 50 μ m. Original magnification, \times 250.

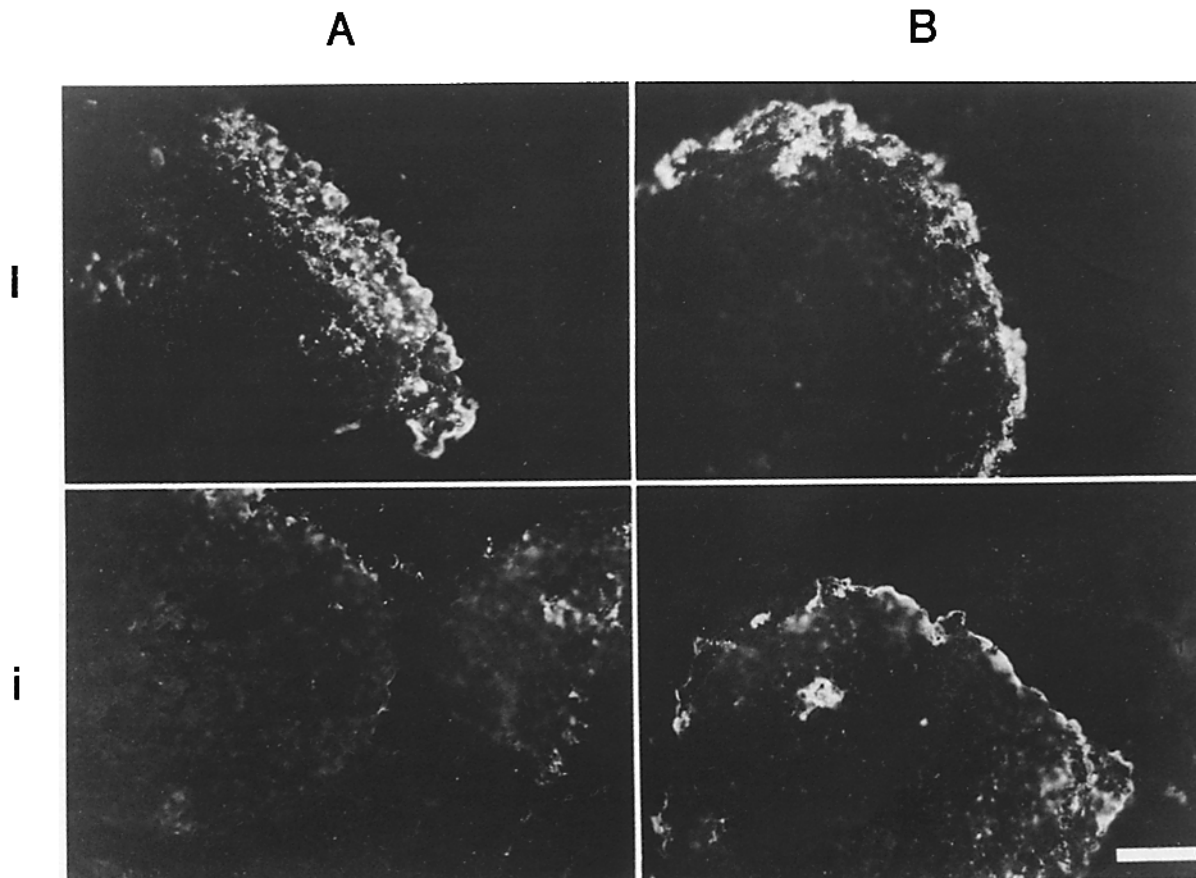


FIGURE 7. Immunofluorescent staining of sections of F9 aggregates cultured with antisera for 8 d; staining for cell surface markers. Column A, cultured with antilaminin (1:20); column B, cultured with antifibronectin (1:20); I, stained for I antigen; i, stained for i antigen. Bar, 50 μ m. Original magnification, \times 250.

nantly on the outside of aggregates (Fig. 1c), we hypothesize that the disruption was caused by laminin being on the wrong side of the outer cell layer such that asymmetric arrangements could never form. This hypothesis is supported by the results obtained when laminin is added at different stages of the process (Table 1). If the basement membrane does not form, it appears that an epithelial layer cannot align properly and "mature" with correctly organized cytoskeletal elements, microvilli, and desmosomes, etc.

We observed that mRNA for AFP was extremely low in 4-d cultures in the presence of laminin (Fig. 5), although it was detected readily in control cultures at 3 d. Since other differentiated products were observed (Figs. 2 and 3), it must be concluded that the expression of new genes is modulated differentially by laminin. It is possible that AFP mRNA appears in older cultures but this is unlikely because no AFP is detected as late as the 8-d stage. We can speculate that the AFP gene is expressed only when all the other visceral endoderm characteristics are present and functioning correctly: for example, when a cytoskeleton of intermediate filaments is organized, possibly to maintain the asymmetry and alignment on the basement membrane, or to maintain the functional integrity of the tight apical junctions and desmosomes that characterize the epithelium.

Another significant finding relevant to the role of laminin is that dense cultures differentiate earlier and synthesize greater amounts of AFP per cell (2). Since we know that laminin is a product whose synthesis increases early (2 d), we hypothesize that endogenous laminin in the medium may form a part of the stimulatory mechanism. This is supported by the finding that low concentrations of exogenous laminin stimulate AFP production in low density cultures but not high density cultures (Fig. 4). Likewise, it has been reported that laminin supports the growth and differentiation of F9 monolayers (26). Further evidence is the reduction in AFP production when aggregates are cultured in antiserum to laminin (Table II). Of course, there is an obvious need for both laminin and fibronectin, since they appear to form part of the basement membrane (Fig. 2A). Cultures in either antilaminin or antifibronectin fail to produce AFP, but the effect of antilaminin is clearly different and more fundamental since it causes the failure of epithelium formation. The effect of antifibronectin appears to occur at a later stage, and this is being examined further.

In summary, endogenous laminin is essential for forming and organizing the epithelial layer on F9 embryoid bodies. Exogenous laminin supplied in low amounts stimulates AFP production, possibly by stimulating the formation and organization of the epithelial layer. Excess exogenous laminin, however, is deleterious to epithelium formation, possibly because asymmetry cannot be established and therefore a coherent basement membrane cannot partition off the outer layer and form the basis of its polarity.

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REFERENCES

- Hogan, B. L. M., A. Taylor, and E. D. Adamson. 1981. Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature (Lond.)* 291:235-237.
- Grover, A., R. G. Oshima, and E. D. Adamson. 1983. Epithelial layer formation in differentiating aggregates of F9 embryonal carcinoma cells. *J. Cell Biol.* 96:1690-1696.
- Chung, A. E., I. L. Freeman, and J. E. Braginiski. 1977. A novel extracellular membrane elaborated by a mouse embryonal carcinoma derived cell line. *Biochem. Biophys. Res. Commun.* 79:859-868.
- Timpl, R., H. Rohde, P. G. Robey, S. I. Rennard, J.-M. Foidart, and G. R. Martin. 1979. Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933-9937.
- Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369-377.
- Kefalides, N. A., and B. Denduchis. 1969. Structural components of epithelial and endothelial basement membranes. *Biochemistry* 8:4613-4621.
- Wewer, U., R. Albrechtsen, and E. Ruoslahti. 1981. Laminin, a non-collagenous component of epithelial basement membranes synthesized by a rat yolk sac tumor. *Cancer Res.* 41:1518-1524.
- Ruoslahti, E., E. Engvall, and E. G. Hayman. 1981. Fibronectin—current concepts of its structure and functions. *Collagen Research* 1:95-128.
- Linsenymer, T. F., E. Gibney, B. P. Toole, and J. Gross. 1978. Cellular adhesion to collagen. *Exp. Cell Res.* 116:470-474.
- Adamson, E. D., and S. E. Ayers. 1979. The localization and synthesis of some collagen types in developing mouse embryos. *Cell* 16:953-965.
- Hunter, W. M., and F. C. Greenwood. 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.)* 194:495-496.
- Adamson, E. D., M. J. Deller, and J. B. Warshaw. 1981. Functional EGF receptors are present on mouse embryo tissues. *Nature (Lond.)* 291:656-659.
- Albrechtsen, R., M. Nielsen, U. Wewer, E. Engvall, and E. Ruoslahti. 1981. Basement membrane changes in breast cancer detected by immunohistochemical staining for laminin. *Cancer Res.* 41:5076-5081.
- Ruoslahti, E., M. Vuento, and E. Engvall. 1978. Interaction of fibronectin with antibodies and collagen in radioimmunoassay. *Biochem. Biophys. Acta.* 534:210-218.
- Engvall, E. 1980. Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* 70:419-439.
- Freeman, A. E., E. Engvall, K. Hirata, Y. Yoshida, R. H. Kottel, V. Hilborn, and E. Ruoslahti. 1981. Differentiation of fetal liver cells *in vitro*. *Proc. Natl. Acad. Sci. USA.* 78:3659-3663.
- Oshima, R. G. 1981. Identification and immunoprecipitation of cytoskeletal proteins from extra-embryonic endodermal cells. *J. Biol. Chem.* 256:8124-8133.
- Marsh, W. L. 1961. Anti-i: cold antibody defining the I: relationship in human red cells. *Br. J. Haematol.* 7:200-209.
- Fukuda, M. N., M. Fukuda, and S. Hakomori. 1979. Cell surface modification by endo- β -galactosidase. *J. Biol. Chem.* 254:5458-5465.
- Hogan, B. L. M., and R. Tilly. 1981. Cell interactions and endoderm differentiation in cultured mouse embryos. *J. Embryol. Exp. Morphol.* 62:379-394.
- Andrews, G. K., and C. S. Teng. 1979. Studies on sex-organ development. *Biochem. J.* 182:271-286.
- Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201-5205.
- Andrews, G. K., M. Dziadek, and T. Tamaoki. 1982. Expression and methylation of the mouse α -fetoprotein gene in embryonic, adult and neoplastic tissues. *J. Biol. Chem.* 256:5148-5153.
- Law, S., T. Tamaoki, F. Kreuzalar, and A. Dugaiczky. 1980. Molecular cloning of DNA complementary to a mouse α -fetoprotein mRNA sequence. *Gene (Amst.)* 10:53-61.
- 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.
- Rizzino, A., V. Terranova, D. Rohrbach, C. Crowley, and H. Rizzino. 1980. The effects of laminin on the growth and differentiation of embryonal carcinoma cells in defined media. *J. Supramol. Struct.* 13:243-253.