

***In Vitro* Differentiation of Teratomas and the Distribution of Creatine Phosphokinase and Plasminogen Activator in Teratocarcinoma-derived Cells¹**

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Summary

Mouse teratocarcinoma cells from embryoid bodies were cultured *in vitro* to permit their differentiation into a number of cell types. Two enzyme activities, creatine phosphokinase (CPK) and the protease plasminogen activator, were studied to follow the developmental sequence of events in these embryoid body-derived cell cultures. CPK activity increased with time in culture, indicating the appearance of new cell types with brain- or muscle-specific enzyme activities. Plasminogen activator was detectable in extracts of embryoid bodies. This protease activity first increased and then decreased to a low level as the embryoid bodies in culture developed into differentiated cell types. These cell cultures also showed a decreased potential for tumor formation in syngeneic mice as a function of time in culture. This decrease in tumorigenic potential was correlated with the appearance of differentiated cells *in vitro*.

Simian virus 40 (SV40) was used to infect and transform cells derived from embryoid bodies in culture. This was done to permit the establishment of cloned teratocarcinoma-derived cell lines. Twenty-nine distinct cloned permanent cell lines (called SVTER) containing the SV40-specific tumor antigen were obtained. None of these cell lines was capable of producing tumors in syngeneic mice. An analysis of the levels of creatine phosphokinase and plasminogen activator in these SVTER cell lines indicated that: (a) some cell lines had high CPK activity and little or no plasminogen activator activity, (b) some cell lines contained high levels of plasminogen activator activity with little or no CPK activity, and (c) some cell lines contained neither of these enzyme activities. No example of a cell line with high levels of both enzyme activities was observed, indicating that these two enzymes may participate in mutually exclusive developmental pathways. The SVTER cell lines may therefore be useful in reconstructing these developmental pathways *in vitro*.

Introduction

Testicular teratomas in 129/Sv mice originate from male primordial germ cells produced during the 12th to 13th day

of fetal life (19, 25-27). The mature tumor is composed of 2 classes of cells: (a) tumorigenic embryonal carcinoma cells that, under the appropriate conditions, produce (b) a variety of differentiated, nontumorigenic tissue types. Some of these teratocarcinomas have been established as transplantable tumors in 129/Sv mice (17, 26). The embryonal carcinoma cells transplanted s.c. give rise to both malignant teratocarcinoma cells and benign differentiated cells (17, 26). Injection of these same embryonal carcinoma cells into the peritoneal cavity of a mouse, however, results in the appearance of spherical multicellular aggregates called embryoid bodies (18, 24). Simple embryoid bodies resemble 5- to 6-day-old mouse embryos with endodermal cells surrounding several layers of embryonal carcinoma cells (24, 28).

Over the past few years it has been possible to reproduce this developmental sequence of events in tissue culture. Starting with either cloned embryonal carcinoma cell lines (14, 15) or embryoid bodies (12, 28), the pluripotent teratocarcinoma cells have been shown to differentiate *in vitro* into a variety of new cell types. The criteria used to demonstrate differentiation *in vitro* have included: (a) morphological alterations (14, 15, 28); (b) the acquisition or loss of enzymatic activities (5, 12); (c) the presence or absence of cell surface antigens (1, 23); and (d) the tumorigenic potential of either embryonal carcinoma or the differentiated cells derived from them (7).

This communication studies the expression of 2 enzymatic activities, CPK³ and the protease, plasminogen activator, in embryoid bodies, embryonal carcinoma cells, and the cells derived from these pluripotent stem cells. CPK is not present in embryonal carcinoma cells but is found in extracts of adult mouse brain and muscle tissue (12). Plasminogen activator activity can be detected in embryoid bodies and is a component of the parietal endodermal cells (7, 22)⁴. This protease is also found in some differentiated cell types like macrophages, endothelial cells, and ovarian granulosa cells (2, 21, 29, 30). Normal fibroblasts in culture do not have detectable plasminogen activator, while viral-transformed fibroblasts appear to acquire this protease activity (16, 31). Hence, these 2 enzymatic activities are associated with distinctly different cell types and may therefore

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³ The abbreviation used is: CPK, creatine phosphokinase.

⁴ S. Strickland, E. Reich, and M. Sherman, manuscript in preparation.

participate in distinct sets of developmental pathways or stages of differentiation.

In order to examine the distribution of these enzymatic activities in individual cells derived from embryonal carcinoma stem cells, it was necessary to first permit the embryonal carcinoma cells to differentiate in culture and then to establish cloned cell lines of these differentiated cells. This has proven to be a difficult task because the teratocarcinoma-derived differentiated cells grow poorly, if at all, in culture. To obtain permanent teratocarcinoma-derived cell lines, the tumor virus SV40 was used because primary cultures of mouse cells transformed by this virus acquire the potential for infinite growth in culture. Following viral infection, 29 cloned SV40-transformed teratocarcinoma derived cell lines were established. An analysis of these 29 cloned cell lines revealed that a reciprocal relationship existed between the expression of CPK and the plasminogen activator protease activity. Cell lines that contained high levels of one of these enzymes invariably contained low levels of the other enzyme activity. While a number of these cell lines contained neither of these enzyme activities, no cell line was detected with high levels of both CPK and plasminogen activator. These results are consistent with the observation that CPK and plasminogen activator are associated with different cell types *in vivo*. The SV40-transformed, teratocarcinoma-derived cell lines may represent cells undergoing differentiation along a number of different developmental pathways and could therefore be useful for studying these processes of differentiation and tumorigenesis.

Materials and Methods

Teratocarcinoma Cells and Tumors. The transplantable tumor OTT605OA, a pluripotent teratoma, was a gift of Dr. L. Stevens. The ascites tumor, consisting of simple embryoid bodies, was transplanted at 3-week intervals in 129Sv/Sl male mice (28). Transplantable tumor 402A contains only embryonal carcinoma cells and was transplanted in 129/Sv mice by s.c. injection at monthly intervals (12, 28). PCC4 aza-1 is an embryonal carcinoma stem cell line in tissue culture isolated by Jakob *et al.* (9).

Culture Conditions. Embryoid bodies were obtained from the peritoneal cavity of 129Sv/Sl mice and were purified by unit gravity sedimentation (28). Approximately 0.1 ml of packed cells was then plated in five 100-mm plastic culture dishes containing 10 ml of Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 1% pen-strep (Grand Island Biological Co., Grand Island, N. Y.). The embryoid bodies attach to the surface of the culture dish and differentiate (12, 28).

SV40 Infection and Cloning Techniques. One-month-old cultures of embryoid bodies were infected with SV40 (approximately 10⁸ plaque-forming units per plate) or were mock infected. After a 2-hr adsorption period, the culture was incubated at 37° for 3 weeks. Cells were rough cloned from separate parts of the culture dish with the use of steel cloning cylinders. Colonies resulting from this cloning procedure were picked and cloned a second time by plating 0.3 cells/well in Linbro microwells.

SV40 Tumor Antigen Staining. Cells on a coverslip were fixed with formalin for 15 min and acetone dehydrated. The cells were then treated with hamster antibody (Flow) against SV40 T-antigen at 37° for 1 hr. The coverslips were then rinsed with phosphate-buffered saline and stained with a fluorescein-conjugated goat anti-hamster IgG. The coverslips were then rinsed with phosphate buffered saline, mounted, and examined on a Zeiss Photomicroscope II.

Tumorigenicity of Cell Lines. Cell lines were washed 3 times in Dulbecco's modified Eagle's medium without serum and scraped from the surface of a culture dish. Approximately 10⁶ cells were injected s.c. or i.p. into male or female 129Sv/Sl or 129Sv mice. The incidence of tumors was followed up to 7 to 8 months.

CPK Assay. CPK activities were determined by methods described by Levine *et al.* (12). Protein determinations were performed by the method of Lowry *et al.* (13).

Plasminogen Activator Assay. Assays for soluble plasminogen activator were performed as described by Pollack *et al.* (20). The plasminogen activator plaque assay was performed by the procedure of Goldberg (6), with either purified human plasminogen or acid-treated fetal calf serum (10).

Results

Characterization of Enzymatic Activities in Teratocarcinoma Cells Developing *in Vitro*. CPK and plasminogen activator were used as enzyme markers to follow changes in cellular gene expression during differentiation of teratocarcinoma cells *in vitro*. A high level of CPK was detected in adult mouse brain and skeletal muscle (12) (see Table 1). Mouse lung, kidney, liver, and spleen contain less than 1% of the enzyme activity present in muscle (12). In contrast, 402A, a transplantable tumor composed only of embryonal carcinoma cells, and PCC 4 aza-1, a cloned embryonal carcinoma stem cell line, do not contain detectable levels of CPK (Table 1). Likewise, embryoid bodies, composed of endodermal cells and embryonal carcinoma cells, do not contain CPK activity (Table 1).

Plasminogen activator is present in crude cell extracts of embryoid bodies (7) (Table 1) which are composed of endodermal cells and embryonal carcinoma cells. A cloned embryonal carcinoma stem cell line (PCC 4 aza-1) or trans-

Table 1
Specific activity of CPK and plasminogen activator in normal and tumor tissue extracts

The CPK and plasminogen activator assays were performed as described in "Materials and Methods."

Tissue or cell line	CPK (A ₅₂₀ /mg protein)	Plasminogen activator (units relative to EB ^a /μg protein)
Brain, 129 mouse	37	ND
Muscle, 129 mouse	59	ND
EB's	0-0.1	100
402A	0	10-20
PCC 4 aza-1	0	2

^a EB, embryoid body; ND, not detectable over background values.

plantable tumor 402A, composed only of embryonal carcinoma cells, contains much lower levels of plasminogen activator (2 to 20% of the activity found in embryoid bodies) (Table 1). These data suggest that the parietal endodermal cells of the embryoid bodies may contain higher levels of plasminogen activator than the embryonal carcinoma cells themselves. Sherman *et al.* (22) have independently corroborated this result.

The measurements of plasminogen activator in Table 1 are obtained from crude cell extracts that detect intracellular and cell-associated levels of plasminogen activator. This enzyme activity is also excreted from cells, and a plaque assay has been devised to detect colonies of cells with extracellular plasminogen activator activity (6). Fig. 1 presents photographs of the plaques produced by PCC4 aza-1 colonies. The agar overlays in these assays contain casein, giving the agar a cloudy appearance. The clear areas or plaques above each colony result from cleavage by plasminogen activator of plasminogen to plasmin which, in turn, degrades the casein. Fig. 1 also demonstrates that the appearance of plaques is totally dependent upon the presence of plasminogen (present in serum), indicating that this assay is specific for plasminogen activator and not some other extracellular protease. Similar results have been obtained with colonies of embryoid bodies (data not shown). Hence, the embryonal carcinoma stem cell lines or a cell derived from PCC-4 aza-1 in culture contains low but detectable levels of extracellular plasminogen activator activity.

When embryoid bodies are placed in cell culture and permitted to attach to the surface of a Petri dish, the cells either migrate out of these bodies or divide to cover an area of the culture dish (Fig. 2). Over the next 3 to 4 weeks, a large number of morphologically distinct cell types appear in these cultures (Fig. 2) (7, 28). The levels of CPK and plasminogen activator in these developing cell cultures have been determined in a number of experiments (7), and

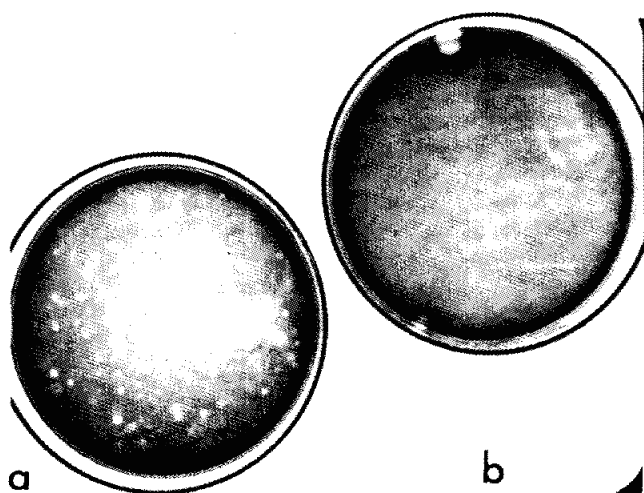


Fig. 1. Plasminogen activator plaque assay of embryonal carcinoma cell line. PCC 4 aza-1 cells were plated on plastic Petri dishes and grown until colonies formed. The colonies were then overlaid with casein-agar either with fetal calf serum (a) or without serum (b). The plaques were developed over 48 hr at 37°.

Table 2

Specific activity of CPK and plasminogen activator in EB^a-derived cells in culture

The CPK and plasminogen activator assays are described in "Materials and Methods."

EB-derived cells (days in culture)	CPK (A ₅₂₀ /mg protein)	Plasminogen activator (units relative to EB/ μ g protein)
1	0.05	100
5	0.2	205
10	1.6	80
20	4.4	20
30	6.4	20

^a EB, embryoid body.

these data are summarized in Table 2. The specific activity of CPK increases from a background level at Day 1 and continues to rise over the 30-day period, reaching a level, in this experiment, 130-fold greater than that observed on Day 1. The plasminogen activator activity first increases 2-fold in this experiment (Table 2) and then declines to a low level approximately one-fifth that observed in the starting embryoid body cultures.

Establishment of Cloned Teratocarcinoma-derived Cell Lines. To obtain a clearer understanding of the expression of CPK and plasminogen activator in developing teratocarcinoma cell cultures, it would be desirable to examine the levels of CPK and plasminogen activator in the individual cells (or clones of these cells) derived from the embryoid body cultures. Since differentiated cells derived from the embryonal carcinoma stem cells usually grow poorly in culture, it has not been possible to develop cloned teratocarcinoma-derived cell lines. In order to establish such cell lines, cultures of embryoid bodies (4 weeks old) were infected with tumor virus SV40. This virus has the capacity to transform primary mouse cells yielding cell lines capable of unlimited growth in culture. Three weeks after SV40 infection, the teratoma-derived cells were cloned. Twenty-nine distinct clones were isolated and designated SVTER followed by a clone number. Mock-infected teratocarcinoma-derived cells were also cloned, but these cells failed to sustain growth in culture, whereas greater than 95% of the SV40-infected clones produced permanent cell lines. Thus SV40 infection clearly permitted the establishment of cloned cell lines derived from embryoid body cultures.

All 29 cell lines (SVTER) were shown to contain the SV40-specific tumor antigen by an immunofluorescent assay. The growth properties of these cells are presently being characterized and will be reported elsewhere.⁵

CPK and Plasminogen Activator Levels in the SVTER Cell Lines. The establishment of the 29 distinct SVTER cell lines permitted an investigation of the levels of CPK and plasminogen activator in cell lines obtained from single teratocarcinoma-derived cells. Plasminogen activator and CPK levels were measured in each of these 29 SVTER cell lines. The plasminogen activator specific activity was expressed as a fraction of the level detected in a standard,

⁵ W. Topp, J. Hall, D. Rifkin, A. Levine, and R. Pollack. The Characterization of SV40-transformed Cell Lines Derived from Mouse Teratocarcinoma: The Relationship between the Malignant and Differentiated Cell Phenotype, manuscript in preparation.

high-activity cell line (4-71-1, isolated by Imada and Sueoka) designated 1.0 relative unit of activity per μg of protein. The CPK levels were expressed as a percentage of the specific activity (A_{520}/mg protein) found in adult mouse muscle. A graph of the levels of plasminogen activator and CPK in each of these 29 cell lines indicates that no SVTER cell line

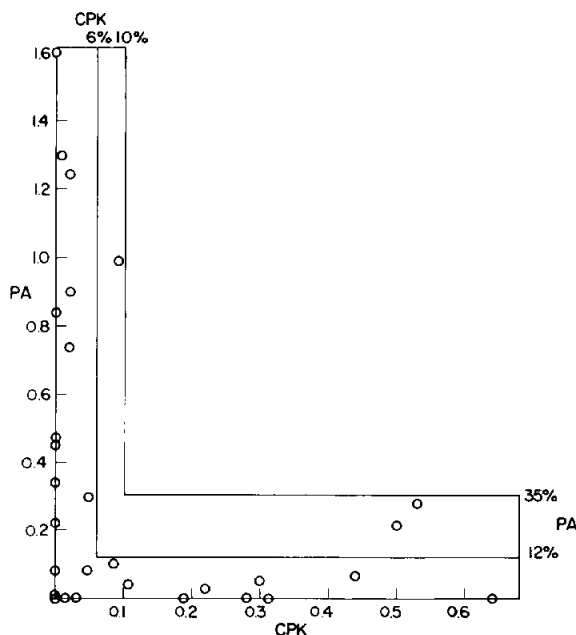


Chart 1. CPK and plasminogen activator levels in 29 different teratocarcinoma-derived cloned cell lines (SVTER). Differentiated embryoid body cell cultures were infected with SV40 and 29 permanent cell lines containing SV40 tumor antigens were cloned from this culture. Each cell line was assayed for the level of CPK and plasminogen activator (PA). Each point, a different cell line with a defined specific activity for (a) CPK, expressed as the fraction of the specific activity (A_{520}/mg protein) found in adult mouse muscle; and (b) plasminogen activator, expressed as the fraction of the specific activity (cpm of ^{125}I -labeled fibrin hydrolyzed per μg of protein) found in a standard high-activity cell-line (4-71-1) designated 1.0 relative unit of activity per μg of protein.

contained high levels of both enzyme activities (Chart 1). Nine of these teratocarcinoma-derived cell lines contained moderate to high quantities (20 to 64% of the adult mouse muscle level) of CPK and low levels of plasminogen activator (0 to 30% of cell line 4-71-1). Nine of these SVTER cell lines contained moderate to high levels of plasminogen activator (40 to 160% of cell line 4-71-1) and little or no CPK (0 to 9% of the muscle levels). Eleven additional cell lines had very low or no plasminogen activator activity (0 to 35% of the cell line 4-71-1) or CPK activity (0 to 11% of the muscle levels) (Chart 1). The total absence of teratocarcinoma-derived cells that contain high levels of both CPK and plasminogen activator (Chart 1) suggests that these enzyme activities may be involved in different pathways of development. The SVTER cell lines may thus be useful tools for characterizing these pathways.

Tumorigenic Potential of Teratocarcinoma-derived Cells in Culture. Embryonal carcinoma cells are highly tumorigenic. As few as 5×10^4 cells produced teratocarcinomas in 100% of the 129Sv/SI mice tested (Table 3). When embryoid bodies were permitted to differentiate in cell culture, the teratocarcinoma-derived cells no longer formed tumors in animals with the efficiency of embryonal carcinoma cells (Table 3) (7). After 60 to 90 days in culture, 5×10^4 differentiated teratocarcinoma-derived cells no longer produce tumors in 129Sv/SI mice (0%) and 5×10^5 cells injected in these mice produce tumors with a reduced (50 to 60%) efficiency, compared with the embryonal carcinoma cells or embryoid bodies. The loss of tumorigenic potential of these teratocarcinoma-derived cells correlates with the acquisition of differentiated functions in culture. A similar correlation has been made *in vivo* with transplantable murine teratomas (17). The teratocarcinoma-derived SVTER cell lines were also tested for their tumorigenic potential. None of these SVTER cell lines was able to produce a tumor in 129Sv/SI mice following injection of 10^6 cells either s.c. or i.p. (Table 3).

Table 3
Tumorigenic potential of teratocarcinoma-derived cells

Cell line	No. of cells injected/129 mouse	No. of animals with tumors/total no. of animals	% of animals with tumors
PCC 4 aza-1	5×10^4	5/5	100
EB's.	$\approx 5 \times 10^4$	5/5	100
EB ^a -derived cells in culture for:			
30 days	5×10^4	3/5	60
	5×10^5	10/10	100
60 days	5×10^4	0/5	0
	5×10^5	6/10	60
90 days	5×10^4	0/5	0
	5×10^5	5/10	50
SVTER ^b cloned EB-derived cells in culture:	5×10^6	0/2 or 0/4	0
(in all cases)			

^a Cultures and EB (embryoid bodies) were prepared by the method described by Teresky et al. (28).

^b Twenty-nine different SVTER lines were prepared as described in "Materials and Methods."

Discussion

When embryoid bodies (the ascites form of a transplantable teratocarcinoma) are placed in culture, they attach to the surface of the culture dish, proliferate, and, after prolonged incubation, acquire differentiated functions (7, 28). These teratocarcinoma-derived cells undergo a variety of morphological alterations (14, 28); they acquire new enzyme activities (5, 12), and the teratocarcinoma-derived cells lose their tumorigenic potential (7). The experiments presented here study the expression of 2 enzyme activities by these cells: CPK, a brain and muscle specific enzyme, and plasminogen activator, a component of parietal endoderm and some differentiated cell types (macrophages, endothelial cells) (2, 13, 21, 29, 30).

Plasminogen activator is present in at least some embryonal carcinoma cell lines (PCC 4 aza-1) and tumors (402A) but at only 2 to 20% of the level found in embryoid bodies. This observation suggests that plasminogen activator activity in embryoid bodies resides mainly in the endodermal cells (22).⁴ When embryoid bodies differentiate in culture, the plasminogen activator activity first increases, then declines during further incubation in cell culture. In the cloned teratocarcinoma-derived cells (SVTER cell lines), the levels of plasminogen activator vary from essentially 0 to 1.6 times the standard cell line (4-71-1). These results demonstrate that in embryoid body cultures, not all cells show a reduction in plasminogen activator activity during prolonged incubation, but rather a class of cells exists which retains high levels of plasminogen activator activity. These cells probably account for the low but detectable specific activity of plasminogen activator found in these embryoid body-derived cell cultures. Similarly, CPK activity in the cloned teratocarcinoma-derived cells (SVTER) is found in only a subset of these lines. This distribution of enzyme activities is expected if cells developing along different pathways are randomly transformed with SV40 and cloned. The absence of a class of SVTER cells with high levels of both plasminogen activator and CPK supports the notion that these enzymes are expressed during differentiation along mutually exclusive developmental pathways.

Several additional lines of evidence indicate that the SVTER clones represent teratocarcinoma-derived differentiated cells. Ten of these SVTER lines have been tested for the presence of H/2^b K gene product (31). Embryonal carcinoma cells do not contain detectable H-2 antigens, while differentiated cells derived from this stem cell population acquire this cell surface antigen (4, 23, 32). All 10 SVTER cell lines tested had the 129 H-2^b K antigen (private specificity, 33). In addition, the SVTER cell lines, like teratocarcinoma-derived differentiated cells and unlike embryonal carcinoma cells, did not form tumors in animals.

Embryonal carcinoma stem cells infected with SV40 are not capable of expressing the SV40-specific tumor antigen (11). When these cells differentiate in culture, transformation by SV40 and the expression of the viral-specific tumor antigen becomes possible (11). In this manner, SV40 was used to establish differentiating teratocarcinoma-derived (SVTER) cell lines that were able to grow indefinitely in culture. At the outset it was not clear what effect SV40 might

have upon the gene expression or developmental sequence of events in these transformed cells. The fact that some or all of the SVTER clones retain several differentiated characteristics, *i.e.*, CPK, plasminogen activator, H-2^b, and no tumorigenic potential, indicates that these cell lines may indeed be useful models of developing or differentiated cells. It should be acknowledged, however, that these cells could represent aberrant examples of development and unusual gene expressions. Several examples of viruses altering normal cellular gene functions are known (3, 8). Only further study of these cell lines will indicate whether they will be useful in elucidating the sequence of events occurring during differentiation.

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References

1. Artzt, K., Bennett, D., and Jacob, F. Primitive Teratocarcinoma Cells Express a Differentiation Antigen Specified by a Gene at the T-Locus in the Mouse. *Proc. Natl. Acad. Sci. U. S. A.*, 71: 811-814, 1974.
2. Beers, W. H., Strickland, S., and Reich, E. Ovarian Plasminogen Activator: Relationship to Ovulation and Hormonal Regulation. *Cell*, 6: 387-394, 1975.
3. Easton, T. G., and Reich, E. Muscle Differentiation in Cell Culture: Effects of Nucleoside Inhibitors and Rous Sarcoma Virus. *J. Biol. Chem.*, 247: 6420-6431, 1972.
4. Forman, J., and Vitella, E. S. Absence of H-2 Antigens Capable of Reacting with Cytotoxic T Cells on a Teratoma Line Expressing a T/t Locus Antigen. *Proc. Natl. Acad. Sci. U. S. A.*, 72: 3661-3665, 1975.
5. Gearhard, J. D., and Mintz, B. Creatine Kinase Myokinase, and Acetylcholinesterase of Mouse Teratocarcinoma Cells. *Cell*, 6: 61-66, 1975.
6. Goldberg, A. R. Increased Protease Levels in Transformed Cells: A Casein Overlay Assay for the Detection of Plasminogen Activator Production. *Cell*, 2: 95-102, 1974.
7. Hall, J. D., Marsden, M., Rifkin, D., Teresky, A. K., and Levine, A. J. The *In Vitro* Differentiation of Embryoid Bodies Produced by a Transplantable Teratoma of Mice. In: M. Sherman and D. Sotter (eds.), *Teratomas and Differentiation*, pp. 251-270. New York: Academic Press, Inc., 1975.
8. Holtzer, H., Biehl, J., Yeoh, G., Magauathan, R., and Kaji, A. Effect of Oncogenic Virus on Muscle Differentiation. *Proc. Natl. Acad. Sci. U. S. A.*, 72: 4051-4055, 1975.
9. Jakob, H., Boon, T., Gaillard, J., Nicolas, J-F., and Jacob, F. *Teratocarcinome de la Souris: Isolerment, Culture et Propriétés de Cellules a Potentialités Multiples*. *Ann. Microbiol.*, 124B: 269-282, 1973.
10. Jones, P., Benedict, W., Strickland, S., and Reich, E. Fibrin Overlay Methods for the Detection of Single Transformed Cells and Colonies of Transformed Cells. *Cell*, 5: 323-329, 1975.
11. Lehman, J. M., Klein, I. B., and Hackenberg, R. M. The Response of Murine Teratocarcinoma Cells to Infection with DNA and RNA Viruses. In: M. Sherman and D. Sotter (eds.), *Teratomas and Differentiation*, pp. 289-301. New York: Academic Press, Inc., 1975.
12. Levine, A. J., Torosian, M., Sarokhan, A. J., and Teresky, A. K. Biochemical Criteria for the *In Vitro* Differentiation of Embryoid Bodies Produced by a Transplantable Teratoma of Mice. The Production of Acetylcholine Esterase and Creatine Phosphokinase by Teratoma Cells. *J. Cellular Physiol.*, 84: 311-381, 1974.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. Protein Measurements with the Folin Phenol Reagent. *J. Biol. Chem.*, 193: 256-275, 1951.
14. Martin, G. R., and Evans, M. J. Multiple Differentiation of Clonal Teratocarcinoma Stem Cells following Embryoid Body Formation *In Vitro*. *Cell*, 6: 467-474, 1975.
15. Martin, G. R., and Evans, M. J. Differentiation of Clonal Lines of Teratocarcinoma Cells: Formation of Embryoid Bodies *In Vitro*. *Proc. Natl. Acad. Sci. U. S. A.*, 72: 1441-1445, 1975.
16. Ossowski, L., Unkeless, J. C., Tobia, A., Quigley, J. P., Rifkin, D. B., and Reich, E. An Enzymatic Function Associated with Transformation of Fibroblasts by Oncogenic Viruses. II. Mammalian Fibroblast Cultures

- Transformed by DNA and RNA Tumor Viruses. *J. Exptl. Med.*, *137*: 112-129, 1973.
17. Pierce, G. B. Teratocarcinoma: Model for a Developmental Concept of Cancer. *Current Topics Developmental Biol.*, Vol. 1, 233-246, 1967.
 18. Pierce, G. B., and Dixon, E. J. Testicular Teratomas. II. Teratocarcinoma as an Ascites Tumor. *Cancer*, *72*: 584-589, 1959.
 19. Pierce, G. B., Stevens, L. C., and Nakane, P. K. Ultrastructural Analysis of the Early Development of Teratocarcinomas. *J. Natl. Cancer Inst.*, *39*: 755-773, 1967.
 20. Pollack, R., Risser, R., Conlon, S., and Rifkin, D. Plasminogen Activator Production Accompanies Loss of Anchorage Regulation in Transformation of Primary Rat Embryo Cells by Simian Virus 40. *Proc. Natl. Acad. Sci. U. S.*, *71*: 4792-4796, 1974.
 21. Risberg, B., Peterson, H-I., and Zettergren, L. Fibrinolysis of the Lung: Experimental Study on the Localization of Plasminogen Activator in the Rat Lung. *Microvascular Res.*, *9*: 222-229, 1975.
 22. Sherman, M., Strickland, S., and Reich, E. Differentiation of Early Mouse Embryonic and Teratocarcinoma Cells *in Vitro*: Plasminogen Activator Production. *Cancer Res.*, *36*: 4208-4216, 1976.
 23. Stern, P. L., Martin, G. R., and Evans, M. J. Cell Surface Antigens of Clonal Teratocarcinoma Cells at Various Stages of Differentiation. *Cell*, *6*: 455-465, 1975.
 24. Stevens, L. C. Embryology of Testicular Teratomas in Strain 129 Mice. *J. Natl. Cancer Inst.*, *23*: 1249-1295, 1959.
 25. Stevens, L. C. Testicular Teratomas in Fetal Mice. *J. Natl. Cancer Inst.*, *28*: 247-268, 1962.
 26. Stevens, L. C. The Biology of Teratomas. *Advan. Morphogenesis*, *6*: 1-32, 1967.
 27. Stevens, L. C. Origin of Testicular Teratomas from Primordial Germ Cells in Mice. *J. Natl. Cancer Inst.*, *38*: 549-552, 1967.
 28. Teresky, A., Marsden, M., Kuff, E., and Levine, A. J. Morphological Criteria for the *in Vitro* Differentiation of Embryoid Bodies Produced by a Transplantable Teratomas of Mice. *J. Cellular Physiol.*, *84*: 319-332, 1974.
 29. Todd, A. S. The Histological Localization of Fibrinolysin Activator. *J. Pathol. Bacteriol.*, *78*: 281-289, 1959.
 30. Unkeless, J. C., Gordon, S., and Reich, E. Secretion of Plasminogen Activator by Stimulated Macrophages. *J. Exptl. Med.*, *139*: 834-850, 1974.
 31. Unkeless, J. C., Tobia, A., Ossowski, L., Quigley, J. P., Rifkin, D. B., and Reich, E. An Enzymatic Function Associated with Transformation of Fibroblasts by Oncogenic Viruses. I. Chick Embryo Fibroblast Cultures Transformed by Avian RNA Tumor Viruses. *J. Exptl. Med.*, *137*: 85-111, 1973.
 32. Vitetta, E. S., Artzt, K., Bennett, D., Boyse, E. A., and Jacob, F. Structural Similarities between a Product of the ^{T/t}-Locus Isolated from Sperm and Teratoma Cells, and H-2 Antigens Isolated from Spermocytes. *Proc. Natl. Acad. Sci. U. S.*, *72*: 3215-3219, 1975.

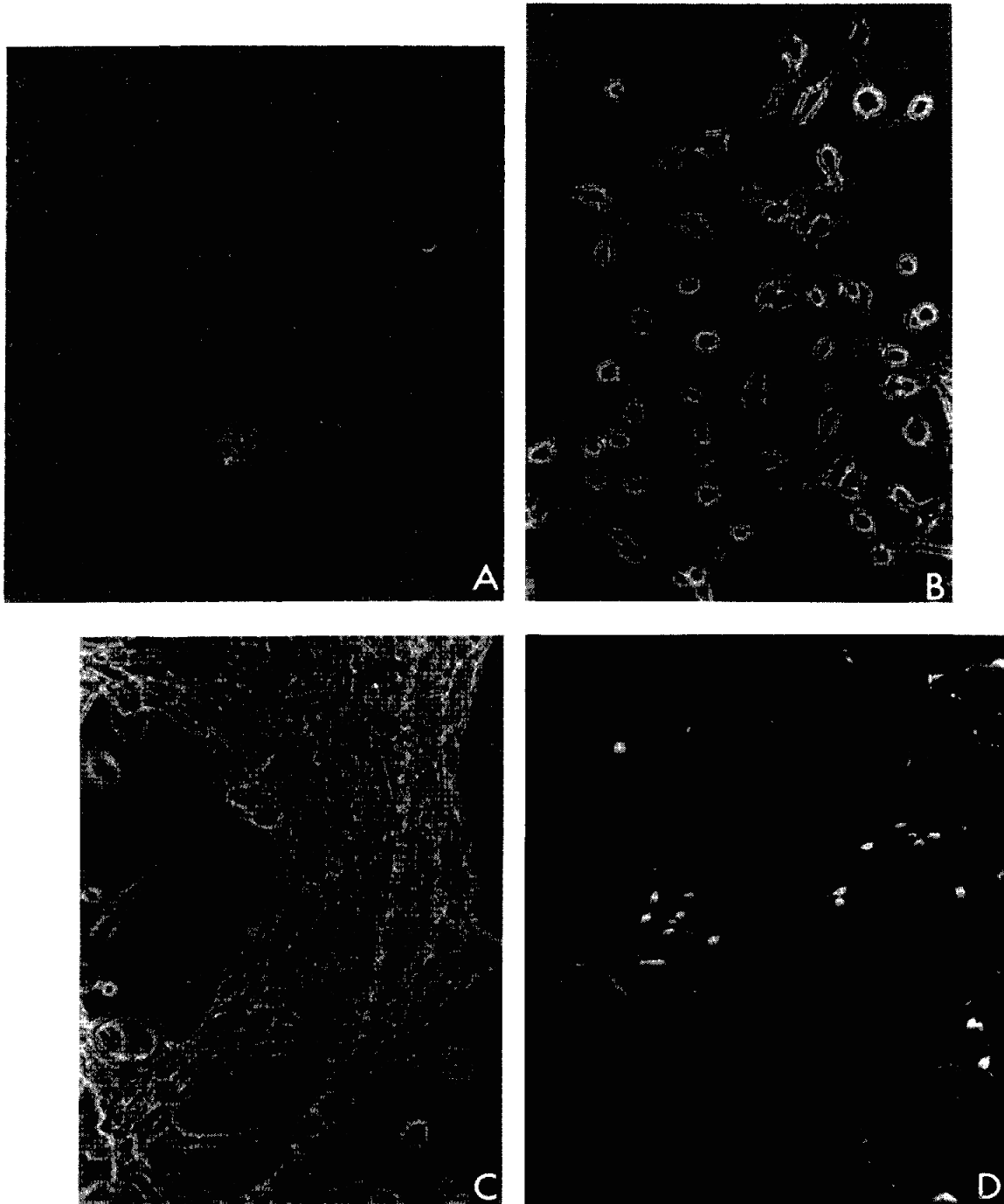


Fig. 2. Differentiated cells derived from embryoid body cultures. Embryoid bodies from the peritoneal cavity of a mouse were placed in culture and allowed to grow. *A*, scanning electron micrograph of embryoid bodies and surrounding cells (1 week in culture); *B* and *C*, phase contrast microscopy of differentiated cell types (3 weeks in culture); and *D*, scanning electron micrograph of differentiated cell types (1 to 2 weeks in culture).