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Plate-Induced Tumors of BALB/3T3 Cells Exhibiting Foci of Differentiation Into Pericytes, Chondrocytes, and Fibroblasts

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The BALB/3T3 clone A31 mouse embryo cell line has been used by many investigators as a model "normal" "fibroblast" line for a variety of in vitro studies. It has been shown, however, that these cells are not "normal" because they will produce tumors within 2–4 months if 3×10^4 cells are implanted subcutaneously in BALB/c mice attached to $0.2 \times 5 \times 10$-mm plastic plates. Previous studies also suggested that these cells were not fibroblasts because they gave rise to tumors with the characteristics of vascular endothelium not fibroblasts. We now report that BALB/3T3 (clone A31), BALB/3T3-T, a proadipocyte subclone of clone A31 cells, and six recent subclones of BALB/3T3-T cells show additional differentiation patterns when tumors derived by implantation of these cells attached to plastic plates are examined. Differentiation into pericytes, chondrocytes, and fibroblasts was observed. We conclude that the BALB/3T3 clone A31 cell line and related lines are multipotent mesenchymal cells which are capable of differentiation into a variety of cell types.

Key words: smooth surface tumorigenesis, cell differentiation, BALB/3T3, mesenchymal precursor cells

Numerous mouse embryo cell lines, especially BALB/3T3 cells, have been viewed by many investigators as a prototype "normal" "fibroblast" line suitable for a variety of in vitro studies on cell structure and function. BALB/3T3 cells have also been used as a model nontransformed cell line with which to compare tumorigenic sublines derived by in vitro transformation with viruses, chemicals, or other methods [1–3]. It has been shown, however, that BALB/3T3 and related mouse embryo cell lines are not "normal" because they will produce tumors if implanted subcutaneously to glass beads to small plastic plates [1–5]. The observation that plate-induced BALB/3T3 and C3H/10T½ tumors arising in different animals exhibited transplantation-rejection antigens that were unique for each individual tumor [2, 4] strongly suggests that the plate-induced tumors did not develop from tumorigenic cells already present in the cell monolayers at the time of subcutaneous implantation on plastic plates. This evidence favors the view that BALB/3T3 and C3H/10T½ and related mouse embryo cells are preneoplastic. That is, they are altered in the direction of neoplastic transformation by loss or gain of one or more characters, but they still retain anchorage dependence, which prevents them from forming a tumor when inoculated in fluid suspension.

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Not only has the hypothesis that BALB/3T3 represent "normal" cells been proved wrong, but the question as to whether they are fibroblasts has been raised. The histological appearance of plate-induced BALB/3T3 tumors described in previous studies suggested that they were derived from endothelial cells because they resembled malignant hemangioendothelioma and malignant fibrous histiocytoma [2]. We now report that BALB/3T3 (clone A31) and a variety of clonal cell lines derived therefrom have the capability to differentiate into many different cell types including capillary pericytes, chondrocytes, and fibroblasts. Based on these observations and additional reports in the literature, we suggest that BALB/3T3 and related lines are multipotential mesenchymal cells.

METHODS

Cell Lines

BALB/3T3 clone A31 cells (obtained from Dr. George Todaro) were cultured in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) containing 10% calf serum. BALB/3T3-T cells (obtained from Dr. Leila Diamond), which were cloned from BALB/3T3 clone A31 cells and possess the ability to differentiate into adipocytes, were grown in DMEM containing 10% fetal bovine serum. Subclones of BALB/3T3-T cells obtained by selected culture of microscopically verified single cells on small glass chips were also grown in DMEM containing 10% fetal bovine serum. These subclones were passaged for less than 3 months prior to use. All cell lines were shown to be free of mycoplasma contamination prior to use and all serum was purchased from KC Biologicals, Lenexa, Kansas.

Plates

Plates measuring 0.2 X 5 X 10 mm were cut from sheets of Lexan polycarbonate plastic, soaked overnight in distilled water, washed, and sterilized by autoclaving. The sterile plates were placed in 60-mm petri dishes to which approximately 10^6 cells in DMEM were added, and the cells incubated overnight. Approximately 3×10^4 cells attached per plate to form a confluent monolayer. Each plate was microscopically examined prior to implantation to verify that it contained a confluent monolayer of cells. Plates with attached cells were implanted subcutaneously in anesthetized mice by first making an incision with scissors over the dorsum and bluntly dissecting a tunnel in the subcutaneous tissue, then placing the plate in the tunnel, with cells facing the skin, and closing the incision with skin clips.

Histopathology

Tumors that developed were removed from the animals and at least five different portions of each tumor were fixed in 3.7% buffered formaldehyde overnight. Fixed tissue was then processed for histological examination by standard procedures. All sections were stained with hematoxylin and eosin or with other appropriate stains. Microscopic review of at least five sections of each tumor was performed and each section typically measured approximately 1 X 1 cm.

RESULTS AND DISCUSSION

BALB/3T3 clone A31 cells, BALB/3T3-T cells derived from clone A31 BALB/3T3 cells, and subclones of BALB/3T3-T cells were implanted subcutaneously attached to

plastic plates. Tumors typically arose after 12–24 weeks in 50% of the BALB/3T3 implanted animals and in 16% of the BALB/3T3-T-implanted animals. Greater variability in tumor incidence of BALB/3T3-T subclones was observed. The difference in tumor incidence between BALB/3T3 and BALB/3T3-T cells has been carefully analyzed in a separate study [6]. Grossly, all tumors measured 2–3 cm in average diameter, and consisted of a large central cyst filled with reddish-brown fluid. The cyst wall was 2–5 mm thick and the tumor mass surrounding the cyst was typically gray-pink in color (Fig. 1). Suspensions of 10^6 BALB/3T3 and BALB/3T3 cells were also injected subcutaneously into mice but such preparations failed to induce tumor development.

Microscopically the tumors that formed from the BALB/3T3 and BALB/3T3-T and subclones of BALB/3T3-T cells were similar. The predominant pattern was that of undifferentiated sarcoma (Fig. 2). However, in many tumors, numerous foci of differentiation were observed. Figure 3 illustrates differentiation to capillary pericytes. Differentiation into chondrocytes (Fig. 4) and fibroblasts (Fig. 5) was also seen. The incidence of foci of such differentiation was comparable in tumors derived from BALB/3T3 (clone A31) cells, from the BALB/3T3-T subclone of BALB/3T3 (clone A31) cells, and from subclones of BALB/3T3-T cells derived within 3 months prior to implantation (Table I). In addition, evidence of differentiation into tissue suggestive but not diagnostic of histiocytes, osteoblasts, and adipocytes was evident in occasional tumors induced by each cell type.

In view of these microscopic findings, which conflict somewhat with previous reports [1–3], histological sections of tumors from all previous experiments were reviewed [1–3]. No foci of cartilaginous differentiation was seen in this earlier material, and there was no evidence of differentiation into most other tissues described in this apper. However, the pattern of malignant hemangiopericytoma was consistently present in material from previous studies where BALB/3T3 clone A31 cells obtained from Dr. George Todaro were employed and cystic tumors resulted. In material from these studies, malignant cells were seen lining vascular channels in a pattern histologically diagnostic of malignant

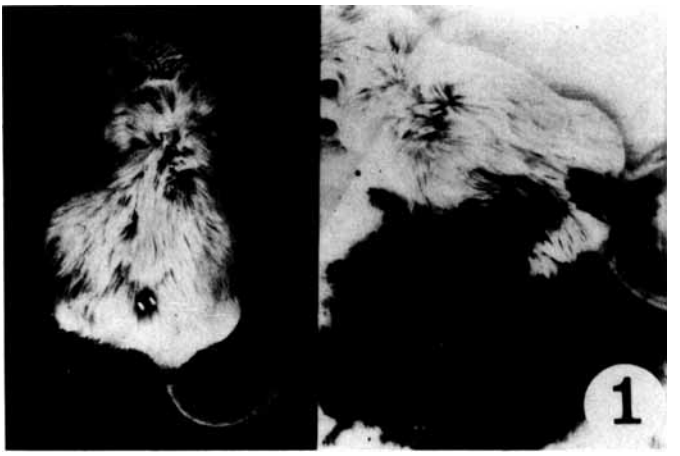

Fig. 1. Mouse bearing a tumor derived from BALB/3T3 cells implanted subcutaneously attached to a plastic plate. The tumors contained a large central cyst filled with bloody fluid. The photograph on the right was taken after the cyst was cut open and allowed to drain.

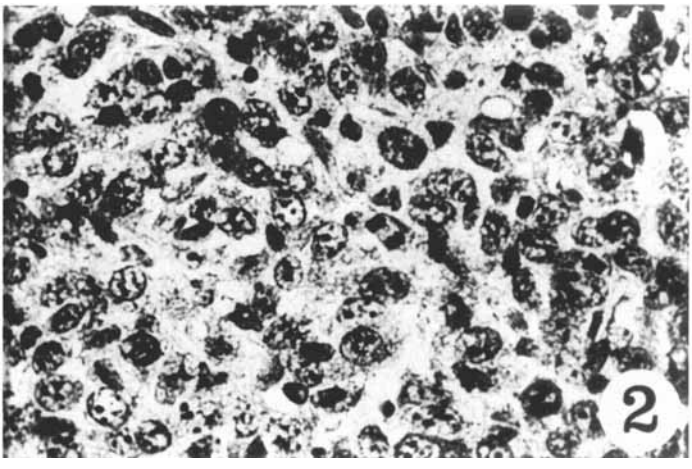

Fig. 2. Pattern of anaplastic sarcoma frequently seen in smooth surface-induced tumors derived from BALB/3T3 cells. H and E. ×400.

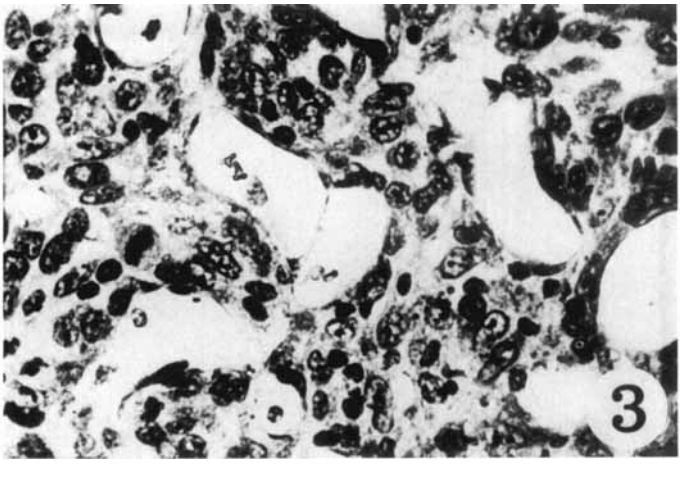

Fig. 3. Patern of malignant hemangiopericytoma observed in many smooth surface-induced tumors derived from BALB/3T3 cells. Multiple abnormally shaped blood channels surrounded by anaplastic sarcoma cells are present. Occasionally tumor cells could be seen directly lining the blood channels. H and E. ×400. H and E. ×400.

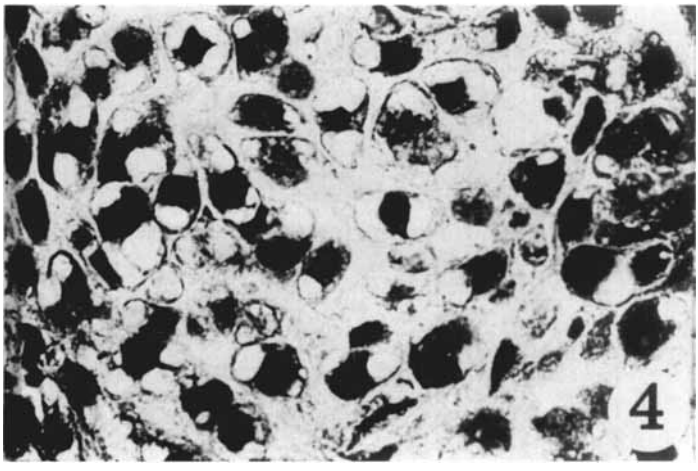

Fig. 4. Evidence of chondrocyte differentiation in smooth surface-induced tumors derived from BALB/3T3 cells. H and E. ×400.

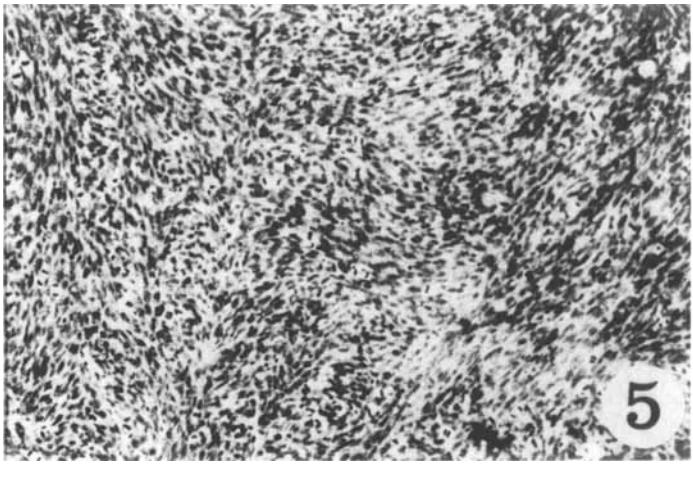

Fig. 5. Pattern of fibroblast differentiation characteristic of fibrosarcoma seen in smooth surface-induced tumors derived from BALB/3T3 cells. H and E. × 100.

TABLE I. Differentiation Characteristics of Plate-Induced Tumors Derived From BALB/3T3 Mouse Embryo Cells

	Differentiation characteristics[a]		
	Pericyte/vascular endothelium	Fibroblast	Chondroblast
3T3	18/89	4/89	11/89
3T3-T	3/7	1/7	1/7
3T3-T clone T1	1/2	0/2	0/2
clone T2	0/1	0/1	1/1
clone T3	1/1	1/1	1/1
clone T4	6/7	4/7	2/7
clone T6	1/1	1/1	0/1
clone T8	3/3	2/3	1/3

[a]Data expressed as the number of tumors with a specific differentiated phenotype over the total number of tumors examined. Many additional patterns of differentiation suggestive of osteocytes, histiocytes, and adipocytes were observed in selected tumors, but their phenotype could not be unequivocally established.

hemangioendothelioma. In other previous studies, BALB/3T3 A31 clone cells obtained from the American Type Culture Collection (ATCC) were used. These cells, by contrast, produced solid rather than cystic tumors, and they also failed to show differentiation into cartilage, pericytes, or other endothelial elements. Rather, tumors derived from ATCC BALB/3T3 clone A31 cells had a histology characteristic of the class of tumors designated fibrous histiocytomas.

These observations now make it obvious that the initial report describing tumors produced from BALB/3T3 cells implanted subcutaneously attached to glass beads [3] as cystic and blood-filled employed BALB/3T3 clone A31 cells obtained from Dr. George Todaro. By contrast, in previous studies where only solid tumors developed, the BALB/3T3 A31 clone cells obtained from the ATCC were used [1, 2].

The question remains, however, as to why different cultures of the presumed same cell line respond in distinct patterns when placed in animals on smooth-surfaced plates. The original BALB/3T3 clone A31 cell line that was developed many years ago could have represented a single cell type that underwent either genetic or epigenetic modification during prolonged culture. This could have resulted in changes in the differentiational capacity of various cultures even though they retained the same name, ie, BALB/3T3 clone A31 cells.

Alternatively, BALB/3T3 clone A31 and other related mouse embryo-derived cell lines could be multipotential mesenchymal cells, which always had the capacity to differentiate into different tissues under different microenvironmental influences or under different culture conditions.

We favor the latter possibility because of our results and those reported by other investigators. We observed that tumors derived from the original BALB/3T3 clone A31 cells show comparable differentiational characteristics to BALB/3T3-T cells derived from BALB/3T3 clone A31 cells years later. More convincing support for the possibility that BALB/3T3 and related cell lines are multipotential mesenchymal cells is that tumors derived from very recent subclones of BALB/3T3-T cells also show evidence of pericyte, fibroblast, and chondrocyte differentiation (Table I).

The differentiation of Swiss 3T3 and C3H/10T½ cells into contractile striated muscle, chondrocytes, and adipocytes following in vitro exposure to 5-azacytidine or 5-

aza-2′-deoxycytidine observed by Taylor and Jones [7] also supports the view that such mouse embryo cell lines are derived from multipotential mesenchymal cells. Although our studies on BALB/3T3 clone A31 cells using 5-azacytidine did not result in a change in their differentiational potential, such treatment did significantly affect the tumorigenicity of BALB/3T3 clone A31 cells (Table II). We have observed, however, that modification of the differentiation potential of BALB/3T3 cells can be induced in vitro under specific cultural conditions. We found that BALB/3T3 and a variety of clonal cell lines derived therefrom can be induced to differentiate in vitro into cells with the morphological, enzymatic, and antigenic characteristics of macrophages simply by exposure of the cells to human plasma clot [Krawisz and Scott, manuscript submitted for publication].

The available evidence, therefore, strongly supports the conclusion that BALB/3T3 and related mouse embryo-derived cell lines represent multipotential mesenchymal cells that are capable of differentiation into many cell types previously proposed to be derived from such precursor cells (Fig. 6) [8]. These include macrophages, endothelial cells, capillary pericytes, chondrocytes, striated muscle cells, fibroblasts, and adipocytes. It is now our goal to determine the mechanisms that control the expression of these differentiated phenotypes.

TABLE II. Effect of 5-Azacytidine on Smooth Surface Tumorigenesis of BALB/3T3 Cells

Cell line and treatment	Tumor latent period (weeks)	Tumor incidence (%)
BALB/3T3	16.4 ± 4.6	50 (15/31)
BALB/3T3 + 10^{-6} azacytidine	12.2 ± 2.6[a]	90 (9/10)[c]
BALB/3T3 + 10^{-5} azacytidine	9.6 ± 2.3[b]	80 (8/10)[d]

Statistical analysis:
[a]Student's t-test, $P < 0.025$.
[b]Student's t-test, $P < 0.005$.
[c]Chi-square analysis, $P < 0.02$.
[d]Chi-square analysis, $P < 0.05$.

Treatment of BALB/3T3 cells with azacytidine was for 24 h at 37° C. Thereafter the cells were washed in phosphate-buffered saline, trypsinized, and passaged on to smooth surface plates as described in the text.

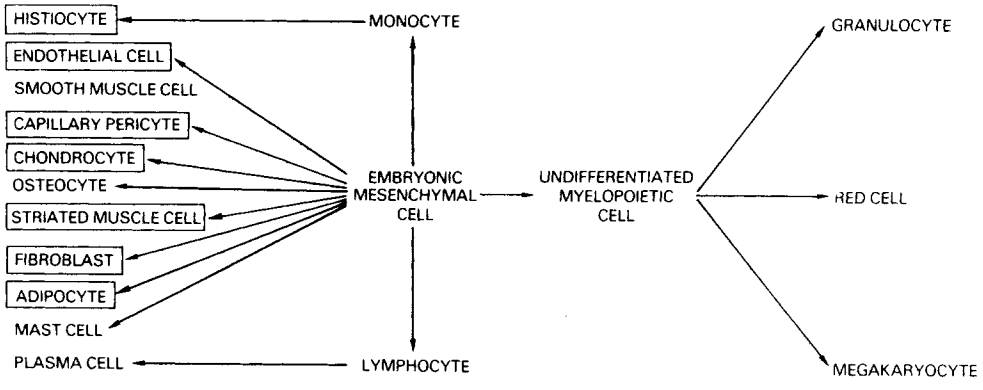

Fig. 6. Spectrum of differentiation pathways shown by embryonic mesenchymal cells. Boxed entries depict differentiated phenotypes demonstrated by BALB/3T3 cells or related cell lines in this article or in other studies [7, 9]

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