

In vivo-like growth of human tumors *in vitro*

(collagen gels/tissue organization/tumor subpopulations)

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ABSTRACT We show that diverse human tumors obtained directly from surgery or biopsy can grow at high frequency *in vitro* for long periods of time and still maintain many of their *in vivo* properties. The *in vivo* properties maintained *in vitro* include three-dimensional growth; maintenance of tissue organization and structure, including changes associated with oncogenic transformation; retention of differentiated function; tumorigenicity; and the growth of multiple types of cells from a single tumor.

It has not been known whether human tumors can grow at high frequency for long periods of time *in vitro* and still maintain many of their critical *in vivo* properties. Tumor cells growing in monolayer culture or as clones in semisolid medium are, at best, only partly representative of the original tumor. Cells in monolayer culture have no chance to grow in three dimensions and to form organized tissues as would be the case *in vivo*. Clones of tumor cells in semisolid medium may represent only a minority of the cells of the tumor, with the rest incapable of growing under the particular *in vitro* conditions provided. Cloning eliminates cell-cell interactions among heterogeneous cell types in tumors, which may be critical for the expression of many of their properties (1, 2). Other workers (for example, refs. 3-6) have stressed the importance of three-dimensional *in vitro* growth of tumor cells in representing the *in vivo* situation. For a cultured tumor to be representative of actual cancer, it is essential that the tumor, as it proliferates *in vitro*, maintain its tissue organization and structure, its oncogenic properties, its differentiated functions, and any cellular heterogeneity that may have been present *in vivo* (7).

If human tumors growing *in vitro* can satisfy the above criteria and, in addition, can be grown at high frequency for long periods of time in culture, they should prove valuable for basic studies in cancer biology as well as for clinically relevant testing. This report addresses the important question whether human tumors can indeed satisfy the above criteria *in vitro*.

MATERIALS AND METHODS

Establishment of Human Tumor Explants in Culture. The specialized collagen gel (A.E.F., P. H. Gumerlock, and S. H. Hinrichs, unpublished data) is a commercial product of Health Design Industries (Rochester, NY) and is manufactured from pigskin. The material comes as dehydrated squares, which were removed from their sterile packages and placed in 60-mm plastic tissue-culture dishes containing Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (Irvine Scientific), 0.1 mM nonessential amino acids, and the antibiotics gentamicin (100 µg/ml) and cefotaxime (95 µg/ml). The gels were soaked in this medium with at least one fluid change before use.

Immediately after surgery or biopsy, tumor sections in MEM with Hanks' salts and 10% fetal bovine serum were brought to the laboratory. Necrotic tissue was cut away, and the remaining healthy tumor tissue was minced with scissors into ≈1-mm³ bits. Five to ten of these tumor bits were placed on the collagen surface, where they tended to stick or become embedded in loose fiber structure. Medium was added until the upper part of the gel was reached but not covered. The cultures were refed twice a week.

Fixation, Embedding, and Staining of Tumor Cells. Cells growing in the collagen gels were fixed in 10% formalin for at least 48 hr. The fixed material was washed for 1 hr in slowly running tap water to rinse out the formalin and then was processed through a series of changes of ethanol (70-100%), with each change lasting 1 hr. The material was then treated with xylene or chloroform and finally embedded in paraffin. After the paraffin hardened, the material was sectioned at 5 µm and then dried for 10-15 min on a slide warmer. The next day the slides were stained with Gill's hematoxylin no. 2 and eosin and then mounted.

autoradiography of Tumor Cells. Cells were labeled metabolically, in the above-described medium used for establishment of tumor explants, with [*methyl*-³H]thymidine (1 µCi/ml, 60-90 Ci/mmol, ICN; 1 Ci = 37 GBq) for 24 hr. After labeling, the medium was removed and the gels were washed three times for 5 min each with nonradioactive medium. Gels were then fixed, dehydrated, embedded in paraffin, and sectioned. The slides, after deparaffinization with xylene, were coated with Kodak NTB-2 emulsion (diluted 1:1 with water) at 40°C and stored in the dark at 4°C for 1 week. The slides were developed for 5 min in Kodak D-19, fixed for 5 min in Rapid Fix, washed well in water, and then dried and stained.

RESULTS

Proliferation of Human Tumors Growing in Primary Culture. To give an example of the possible extent of macroscopic growth of whole human tumor explants, Fig. 1A compares the melanoma of patient 174 after 1 month of *in vitro* growth to a typical start-up culture. Note that the tumor, which was initiated from five 1-mm³ fragments, fills up most of the gel. The macroscopic growth of this tumor is easily monitored because of the extent of melanin production.

It is also essential that individual cells within the tumor explant proliferate at high frequency *in vitro*. For an example of the measurement of proliferation of individual cells within human tumor *in vitro*, see Fig. 1B, which is an autoradiogram of a 6-day culture from the Wilms tumor of patient 181; it can be seen that a large percentage of the cells incorporated thymidine during the 24-hr labeling period. Another example is the 12-day *in vitro* culture of the squamous-cell lung tumor of patient 179 (Fig. 1C), where the majority of the proliferative cells are on the periphery of the tumor, while the interior

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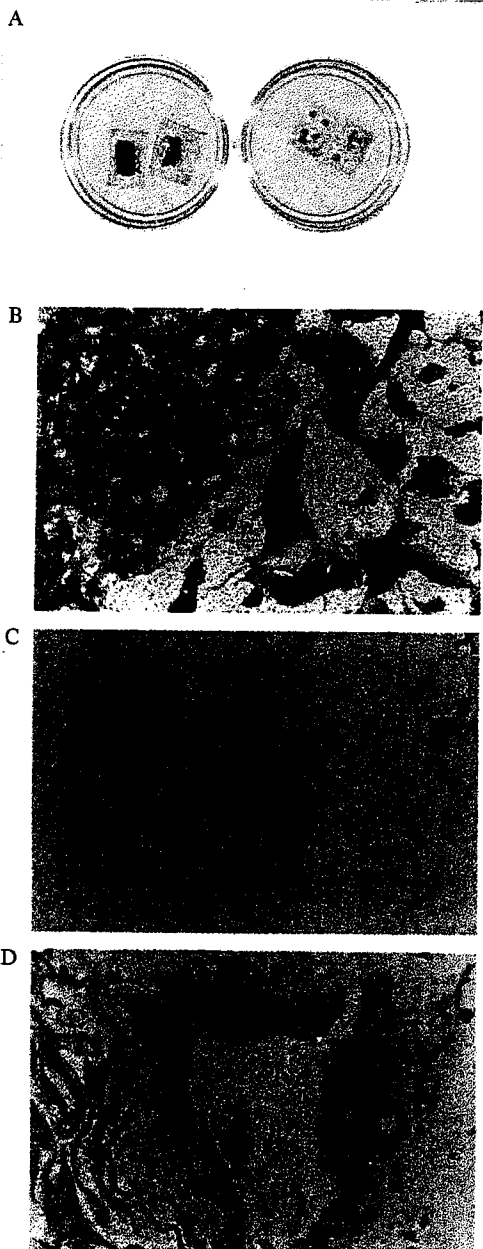


FIG. 1. (A) Human melanoma (patient 174) on collagen gels after 1 month in culture (Left), compared to a typical start-up culture (Right). Note that the melanoma has not only proliferated but continued to produce melanin, indicating maintenance of differentiation in culture. (B) *In vitro* incorporation of [³H]thymidine by Wilms tumor of the kidney (patient 181). After 6 days in culture, cells were labeled for 24 hr with [³H]thymidine at 1 μ Ci/ml. Note the proliferating cells in the explant itself as well as proliferating cells in the gel. (Autoradiogram, Giemsa counterstain; $\times 670$.) (C) Squamous-cell carcinoma of the lung (patient 179), labeled with [³H]thymidine after 12 days in culture. Note the high percentage of cells proliferating on the periphery of the tumor explants as well as cells proliferating in the gel. (Autoradiogram, hematoxylin/eosin counterstain; $\times 170$.) (D) Colon cancer metastasis to the liver (patient 245). Labeling with [³H]thymidine was done after 28 days in culture. Note proliferating, densely packed cells and large, darkly staining nuclei. (Autoradiogram, hematoxylin/eosin; $\times 670$.)

contains a lesser number of proliferating cells, a situation similar to that *in vivo*. In both cases, not only cells within the explant itself but also cells that had migrated into the gel were proliferative. Fig. 1D demonstrates cellular proliferation in the 28-day culture of a liver metastasis of a colon carcinoma. Note the relatively high percentage of cells labeled and the

large darkly staining nuclei in the densely packed cells, all indicating malignancy.

Three-Dimensional Organization of Tumor and Normal Tissues *in Vitro*. In order for a tumor growing *in vitro* to maintain the cell-cell interactions that occur *in vivo*, tissue structure and organization resembling that of the *in vivo* state must be maintained *in vitro*. Cellular interactions in the tumor are critical in determining drug sensitivity and other properties (6). An example of the maintenance of tissue structure and organization of human tumors *in vitro* is seen in Fig. 2 A and B, which show fixed and stained cultured normal and cancerous breast tissue, respectively, from patient 127. Both cultures contain duct-like structures and more than one cell type. However, the cellular organization is diminished in the cancerous tissue, as can be seen from the cells surrounding the duct, which are no longer in parallel alignment. Thus, not only is general tissue organization maintained *in vitro*, but specific oncogenic changes are also maintained. Fig. 2 C and

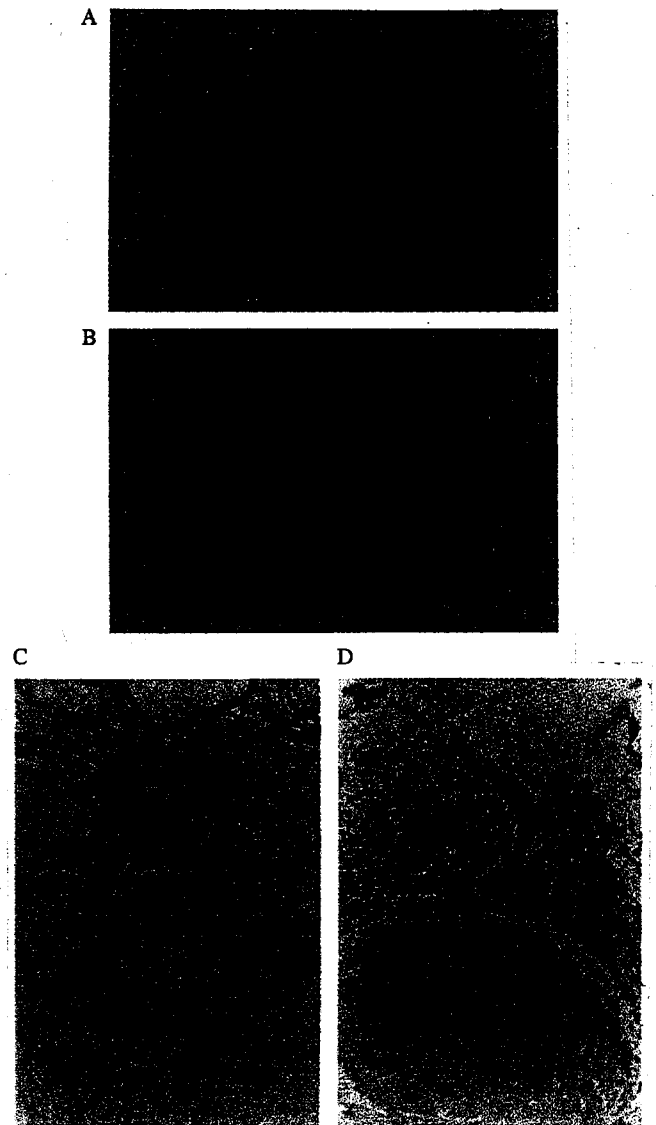


FIG. 2. (A) Normal breast tissue explant (patient 127) after 19 days in culture. Note maintenance of cellular organization, in particular in the cells lining the duct. ($\times 500$.) (B) Breast tumor tissue explant (patient 127) after 7 days in culture. Note decrease in cellular organization, in particular in the cells lining the duct. ($\times 500$.) (C) Normal stomach tissue (patient 123) after 36 days in culture. ($\times 340$.) (D) Cancerous stomach tissue (patient 123) after 36 days in culture. Note invasion of the tumor tissue into the collagen gel. ($\times 340$.)

D show fixed and stained cultured normal and cancerous stomach tissue, respectively, from patient 123. The cancerous tissue seems to have invaded the gel to a much greater degree than has the normal tissue. The nuclei of the cancerous tissue stain darker than the normal, which is frequently the case *in vivo*. In addition, the central areas of the explant seem to be necrotic.

Multiple Cell Types from Individual Tumors *in Vitro*. Tumors *in vivo* are frequently, if not always, composed of cell types with distinctly different properties. This phenomenon is termed tumor heterogeneity (6). We have observed that individual tumors growing *in vitro* also give rise to distinctly different cell types, even to the extent that cells show seemingly different degrees of anchorage requirements. For instance, of the cells that migrated from the explant of the lung tumor of patient 184, some grew in suspension in the medium (Fig. 3A), whereas others attached to the surface of the plastic culture dish (Fig. 3B). Fig. 3A suggests a highly malignant morphology of the lung-tumor cells that is consistent with the ability of the cells to grow in suspension. The cells growing attached to the plastic (Fig. 3B) may be of a less malignant cell type. In contrast, the plastic-surface-attached cells derived from the explant of the lung tumor of patient 237

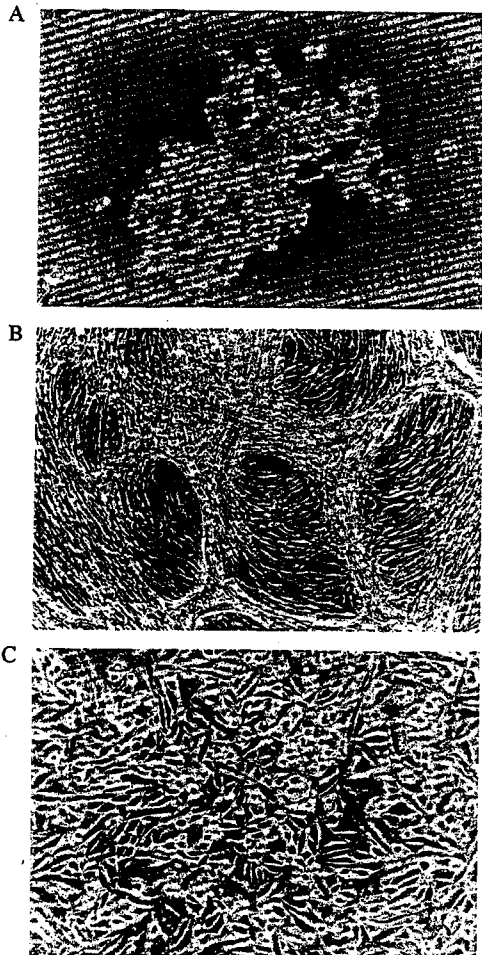


FIG. 3. Multiple cell types derived from lung carcinoma and growing in different phases. (A) Cells that have migrated from the original explant (patient 184) on the collagen gel to proliferate in suspension in the culture medium, after 21 days in culture. ($\times 250$.) (B) Cells that have migrated from the same original explant (patient 184) to grow attached to the plastic surface of the culture dish, after 21 days in culture. ($\times 70$.) (C) Cells from a different lung tumor explant (patient 237) that have migrated to grow attached to the plastic surface. ($\times 170$.)

(Fig. 3C) are much more malignant in appearance than the attached cells shown in Fig. 3B. Perhaps the type of surface-growing cells derived from a given tumor type depends on the individual patient. Thus far we have found that cells from nine different tumors (three colon, one melanoma, one lung, one parotid adenoma, two lymph node metastases of unknown origin, and a lymph node metastasis of a thyroid papillary carcinoma), explanted as described above, can proliferate on the plastic surface of the culture dish. A cell line has been established from the melanoma in this group.

Fig. 4 illustrates the proliferation kinetics of suspension-growing cells derived from collagen-gel-based explants of a solid breast tumor (patient 187) and of a lymph node metastasis of a rectal tumor (patient 130). It can be seen that these solid tumor explants gave rise to cells that can proliferate continuously in suspension in the culture medium. A cell line has been established from the lymph node metastasis.

Fig. 5A demonstrates the heterogeneity in cell size and pigment production of the melanoma of patient 174 growing within the collagen gel after migration from the original explant. Fig. 5B demonstrates the possibly different cell types, growing as an attached culture, derived from the explant of the omentum metastasis of the ovarian tumor of patient 227.

Differentiated Functions of Tumor Expressed in Primary Culture. Tumors *in vivo* frequently maintain certain differentiated functions typical of the tissues of origin or even express new ones ectopically. The *in vivo*-like growth of tumors *in vitro* should also permit the expression of differentiated functions. A graphic example of differentiated-function maintenance among our set of tumors growing *in vitro* is the production of melanin in the culture of melanoma from patient 174 (Figs. 1A and 5A).

Tumorigenicity of Primary Tumor Cells. Five million melanoma cells derived from patient 174 and grown *in vitro* for 9 months were inoculated subcutaneously into three nude mice, each of which developed very large tumors within 2.5 months (Fig. 6).

Summary of Results of Human Tumor Culture. Table 1 summarizes our results. We have explanted 89 human tumors and have obtained growth for 65 representing more than 17 types of human tumors. The only major factor limiting culture seems to be bacterial infections, especially in the colon tumors. The tumors growing *in vitro* include those of the lung, colon, breast, bone, cervix, small bowel, rectum, testis, prostate, kidney, stomach, ovary, thyroid, and skin. They can be maintained in culture for long periods, often for >100

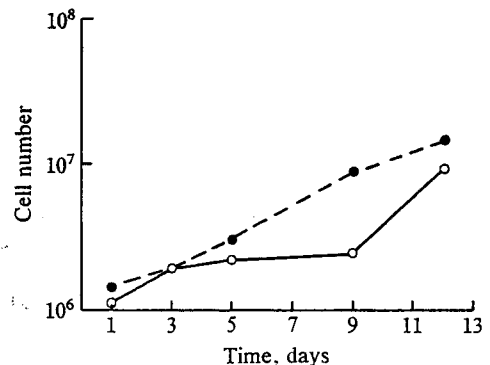


FIG. 4. Growth curves of cells derived from breast cancer explant (patient 187, ●) and from lymph node metastasis of anal carcinoma explant (patient 130, ○). Cells were grown in suspension in MEM containing 10% fetal bovine serum. Cells were enumerated in a Coulter counter. Both cell types originated from solid explants on collagen gels.

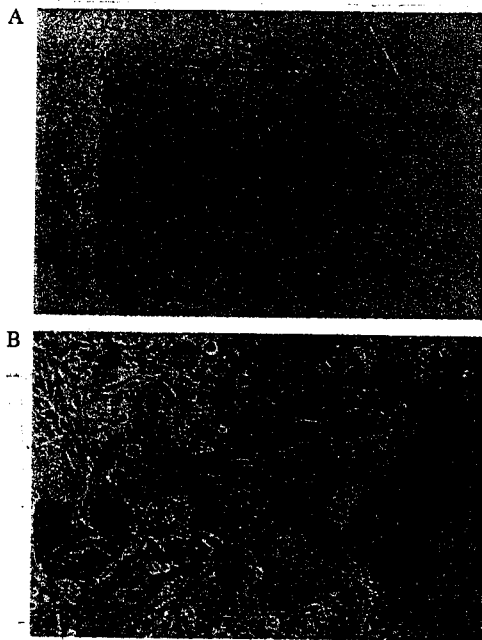


FIG. 5. (A) Melanoma cells (6 months in culture, from patient 174) growing in pigskin collagen gel after one transfer from original gel where tumor was explanted. Note the heterogeneity in cell size and pigment production. ($\times 170$.) (B) Cells (84 days in culture) migrating from explant on collagen gel of omentum metastasis of ovarian tumor (patient 227) to grow attached to the plastic surface of the cell culture dish. Note the apparent heterogeneity of the cells present. ($\times 170$.)

days. Most important of all, the tumors *in vitro* maintain critical properties of the *in vivo* state.

DISCUSSION

Our results show that diverse human tumors obtained directly from surgery and explanted on floating pigskin collagen gels can be cultured with high proliferative capacity for long periods of time, with a high frequency of success. The tumors can maintain properties of the *in vivo* state, including three-dimensional growth, tissue organization and structure, differentiated function, and the growth of multiple cell types from an individual tumor. Oncogenic changes seem to be maintained in the human tumors cultured *in vitro*, as seen, for example, in the altered tissue organization of breast cancer from patient 127 (Fig. 2B); the seemingly greater than normal

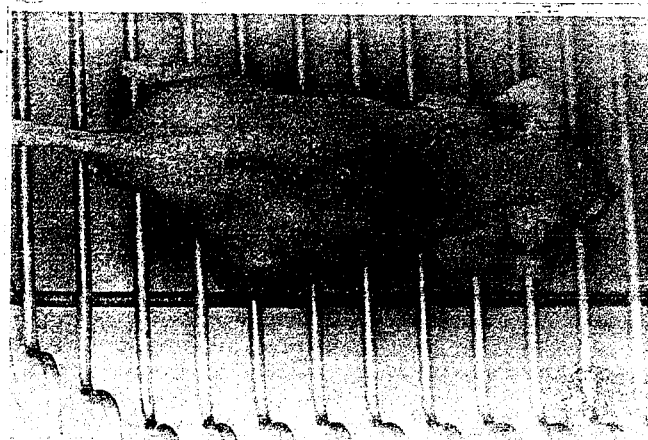


FIG. 6. Tumor formation in nude mice following inoculation of 5×10^6 melanoma cells of patient 174. The time from inoculation to the photograph was 85 days.

Table 1. Primary *in vitro* culture of human tumor tissue and cells

Tumor type	No. growing/no. explanted	Days in culture (mean \pm SD)
Lung	8/8 (100%)	103 \pm 50
Breast	14/17 (82%)	86 \pm 59
Colon	8/25 (32%)	95 \pm 64
Miscellaneous*	34/39 (87%)	95 \pm 67
Total	64/89 (72%)	94 \pm 63

Growth was indicated by any of the following: visible expansion of the explants, microscopic observation of tumor cells growing in the gel or cell-culture medium or attached to the cell-culture dish, or autoradiographic measurement of DNA synthesis within the explant. *Ovary, kidney, small bowel, rectum, stomach, parotid, lymphoma, melanoma, prostate, bone, cervix, thyroid, parathyroid, testis.

invasiveness of the stomach cancer of patient 123 (Fig. 2D); the suspension-growing cultures derived from the explant of the lung tumor of patient 184 (Fig. 3A); the plastic-surface-growing cells derived from the explant of the lung tumor of patient 237 (Fig. 3C); and the tumorigenicity of the melanoma cells of patient 174 (Fig. 6).

Collagen seems to be a good substrate for the culture of many types of normal and tumorous cells and tissues (4, 5, 8-42). Perhaps the flexibility of the collagen-gel substrate permits cells to assume specific shapes that in turn allow cellular growth and differentiation that would not be achievable on a rigid surface such as plastic (40). Indeed, it has been shown that morphology is a primary factor in determining the sensitivities of cells to mitogens (43, 44). With regard to the flexibility of the substrate and differentiation, it has been shown, for example, that mouse mammary tumor cells on floating collagen gels synthesize 3- to 10-fold more casein mRNA than cells on plastic or on attached collagen gels (9). It is also possible that the collagen gel acts as a basement-membrane substitute and thereby permits *in vivo*-type cellular proliferation and function (25).

With regard to attempts of primary human tumor culture by other investigators, the use of the extracellular matrix from smooth muscle has permitted primary culture of certain pediatric tumors (45, 46). Successful short-term culture has been achieved with 70% of human breast cancers on a plastic substrate in a very complex medium (47). Certain human tumors contain cells that will form clones in soft agar at very low frequency (48). None of these methods indicate significant maintenance *in vitro* of *in vivo* tissue structure and organization or cellular heterogeneity.

That the various cell types comprising most tumors *in vivo* should grow *in vitro* is a critical criterion for basic and clinical studies. Our data indicate in a number of ways that multiple cell types of the individual tumor can indeed grow *in vitro*. For example, one can observe the different types of cells in organized but abnormal tissue structure growing from the breast tumor of patient 127 (Fig. 2B); the varied appearance of the cells, growing in the gel, of the melanoma of patient 174 (Fig. 5A); the suspension and plastic-attached cells of lung tumor patient 184 (Fig. 3A and B, respectively); and the ovarian tumor cells of patient 227, growing while attached to plastic (Fig. 5B). We found that a "solid" tumor such as the breast tumor of patient 187 can produce cells that grow continuously in suspension (Fig. 4). Perhaps these are the cell types that form pleural effusions or ascites *in vivo*.

Melanoma cells from patient 174 produce large quantities of melanin *in vitro*, indicating long-term maintenance of differentiated function in primary culture (Figs. 1A and 5A). These cells are also tumorigenic and lethal in nude mice after primary culture (Fig. 6). Other workers have noted the exceptional lethality of human melanoma cells implanted in mice (49).

The data presented here therefore show that human tumors can grow at high frequency (64 of the 89 tumors explanted have grown) for prolonged periods *in vitro*. Further, these tumor explants maintain important *in vivo* properties, which include those that distinguish them from normal cells and tissues. The findings reported here should be important for basic biological studies as well as for clinical studies and applications.

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