

*GROWTH CONTROL IN CULTURED CELLS: SELECTION OF
SUBLINES WITH INCREASED SENSITIVITY TO CONTACT
INHIBITION AND DECREASED TUMOR-PRODUCING ABILITY**

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As growing cultures of mammalian cells reach a high density, their growth rate decreases and in some cases a substantially nondividing population may result. This arrest of growth requires cell contact,^{1, 2} though soluble substances are also involved.³⁻⁵ The phenomenon has usually been called contact inhibition of division.

Transformants and tumors are relatively insensitive to growth inhibition, but the present experiments have shown that, even in such populations, it is possible to select for sensitive cells by exposure to 5-fluoro-2-deoxyuridine (FUdR) and to obtain stable variants greatly altered in their culture behavior and in tumor-producing ability.

Materials and Methods.—All cultures were maintained at 36.5°C in 20-cm² plastic Petri dishes in Dulbecco and Vogt's modification of Eagle's medium⁶ supplemented with 10% calf serum. The medium was changed twice weekly.

The cells used in these experiments were established fibroblast lines originating in this laboratory. 3T3-4 and 3T6-7 are established lines of mouse embryo origin.⁷ Py11 and SV101 are transformants of 3T3 produced by polyoma virus and SV40, respectively.⁸ PyH 75 is a hamster established line originating from a polyoma virus-induced tumor.⁹ The lines were cloned twice before they were used in selection experiments. All variants selected are designated as F1 (flat) with superscripts indicating the number of FUdR treatments preceding their isolation.

FUdR¹⁰ was obtained from Hoffmann-LaRoche, Nutley, New Jersey. Concentrated stock solutions were kept frozen until used. To remove FUdR before transferring surviving cells, cultures were incubated in fresh medium for 15-30 min before washing and trypsinization. In order to prevent any fluorouracil, produced by breakdown of the deoxyriboside, from interfering with RNA synthesis, a 10-fold greater quantity of uridine was added with the FUdR.¹¹

The capacity of a cell line to produce tumors was tested in 21-day-old Syrian hamsters. Known numbers of cells were injected subcutaneously over the back, and the animals examined for tumors 3 times weekly. A nodule exceeding 1 cm in diameter was considered positive. All tumors grew progressively thereafter.

Results.—*Sensitivity of different lines to killing by FUdR:* Antimetabolites that interfere with DNA synthesis affect the viability of cells only if they are in the S period of the division cycle, i.e., synthesizing DNA. For example, non-growing cells are unaffected by concentrations of iododeoxyuridine lethal to growing cells.¹²

The susceptibility of a number of lines to FUdR was tested by exposing exponentially growing cultures to FUdR for 48 hours, removing the FUdR, transferring the cultures at suitable dilutions, and counting colonies produced by the survivors 10-14 days later. Figure 1 shows the results obtained with PyH75, SV101, Py11, and 3T3-4. In all cases, the number of surviving cells could be reduced to 10⁻⁵ or less. This was accomplished at a concentration of 0.1

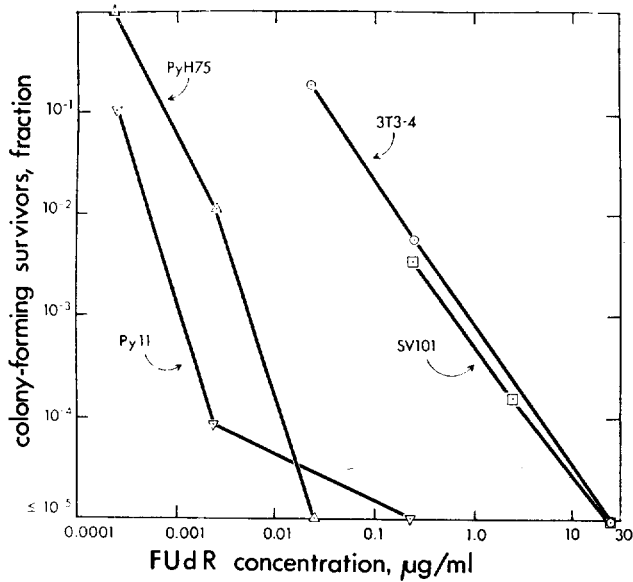


FIG. 1.—Fraction of cells forming colonies after 48-hr exposure to FUDR at low cell density.

$\mu\text{g/ml}$ for two polyoma virus-transformed fibroblast lines (PyH75 and Py11) and $25 \mu\text{g/ml}$ for SV101 and 3T3-4. Other lines examined were about as sensitive as SV101, so that the polyoma lines may be unusually sensitive to FUDR.

Survival of cells treated with FUDR at different cell densities and relation to contact sensitivity: Lines 3T3-4 and 3T6-7 differ greatly in their saturation densities.⁷ Cultures of each line were inoculated at low densities with the cells distributed as evenly as possible (Table 1). At intervals, a plate was trypsinized, and the cells were counted and plated to determine the number of colony-formers. FUDR was then added to a sister culture and allowed to remain there for two days. The cells were then trypsinized, counted, and the number of colony-forming survivors was determined. No increase in total cell number occurred in the presence of FUDR.

In Figure 2A, the number of colony-forming survivors expressed as a fraction of the control is plotted on a logarithmic scale against cell number per unit area at the time the FUDR was added. At a sufficiently low density, fewer than 10^{-4} cells survived. As the density increased, an increasing proportion of the cells survived, and the number of survivors always rose much faster than the cell density. For example, the number of 3T3 survivors rose from 10^{-3} to 1.0, whereas the cell density rose from 0.4 to $4 \times 10^4/\text{cm}^2$. At the same density, only 1.8×10^{-3} of the 3T6-7 population survived FUDR, and only at a density of $40 \times 10^4/\text{cm}^2$ (its saturation density) did the 3T6-7 survivor fraction reach 1.0. At a low density, the killing curves converge and might be expected to meet at a density sufficiently low that no cell contact occurs.

Properties of FUDR survivors: Colonies were isolated from cultures treated with FUDR at densities at which most of the cells were killed. These colonies

grew as rapidly as the parent line in sparse culture, but many were found to have lower saturation densities. They thus appear to be variants having greater contact sensitivity. Several of these variants were examined for sensitivity to FUdR while growing at very low cell densities and were found to leave fewer than 10^{-4} survivors. They did not therefore have increased FUdR resistance while synthesizing DNA. However, at sufficiently high cell densities their greater sensitivity to contact inhibition made them more resistant to FUdR than the parent population.

TABLE 1. *Saturation density and plating efficiency on 3T3 monolayers of various lines and their flat derivatives.*

	Saturation density (cells/cm ² × 10 ⁻⁴)	RPE*
3T6-7	40.0	1.0
Fl ¹ -1 3T6-7	7.0	0.003
3T3-4	4.0	0
SV101	46.0	0.6
Fl ² -1 SV101	7.0	0.06
Py11	37.5	0.1
Fl ¹ -1 Py11	7.0	0.01
PyH75	>75	1.1
Fl ² -1 PyH75	50	0.9
Fl ² -21 PyH75	45	0.2†

* RPE: relative plating efficiency = $\frac{\text{colonies on 3T3 monolayer}}{\text{colonies on bare Petri dish}}$.

† This value does not include a small number of microcolonies formed on 3T3. These colonies are visible several days after plating but do not continue to grow.

One variant colony of 3T6-7, Fl¹-3T6-7, was isolated, grown, and recloned. It was found to reach a saturation density of 7×10^4 /cm². When cultures at different cell densities were again treated with FUdR, a survival curve was obtained (Fig. 2A) that was very much displaced from that of the parent line and very similar to that of 3T3-4.

In contrast, the same selection procedure applied to 3T3-4 did not yield variants with appreciably lower saturation densities nor with displaced FUdR killing curves. Presumably 3T3 is already close to the maximum sensitivity to growth inhibition.

Selection of flat variants from virus-transformed cell lines: The FUdR killing curve of SV101, a cloned SV40 transformant of 3T3 that saturates at 47×10^4 /cm², is shown in Figure 2B. It can be seen that the number of survivors is several orders of magnitude below that of 3T3-4 treated at the same density. Only at densities higher than the saturation density of 3T3-4 are survivors detectable. The surviving fraction never reaches 0.1, even at the highest cell density. This probably means that even at the nominal saturation density the greater part of the culture is not resting but contains dividing and dying cells. Consistent with this, cells in mitosis can usually be seen even in very thick cultures.

From the surviving colonies of this first FUdR selection, a relatively flat colony was isolated and grown to mass culture. It had a saturation density of 15-20 ×

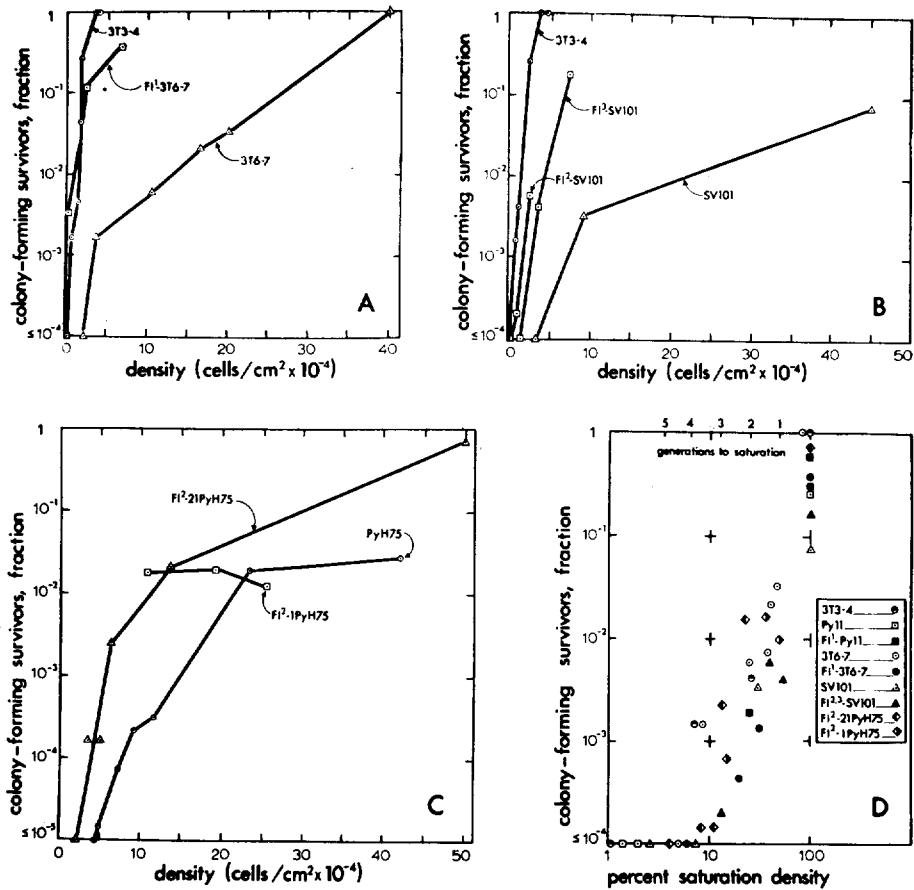


FIG. 2.—Colony-forming survivors following treatment with FUdR at different cell densities. (A) Cell lines of high and low saturation densities. (B) SV40-transformed line. (C) Polyoma tumor line. (D) Log-log plot of fraction of colony-forming survivors against cell density normalized to per cent saturation density. Pooled data for nine cell lines for which saturation densities are given in Table 1.

10⁴/cm². This line (FI¹-SV101) was carried through a second FUdR cycle, and the surviving colonies were more generally flat. One clone (FI²-SV101) was found to reach a saturation density of about 7 × 10⁴/cm². A cycle of FUdR treatment on this clone (Fig. 2B) showed marked displacement of the killing curve. It may be seen that at a density of 5 × 10⁴/cm² fewer than 10⁻⁴ of SV101 survived, whereas survivors of FI²-SV101 reached 10⁻². This behavior is still clearly distinguishable from that of 3T3-4, which was completely protected at this density. A further FUdR selection on a line originating from a surviving colony did not result in variants with improved survival in FUdR (Fig. 2B, FI³-SV101). Similar results were obtained with polyoma virus-transformant

Pyll. In this case, a single cycle of FUdR was sufficient to produce colonies of cells that had saturation density not much higher than 3T3-4 (Table 1).

Selection of flat variants from a virus-induced hamster tumor line: PyH75 is a tumor line produced by the injection of polyoma virus into newborn hamsters. Following its culture evolution into an established line, PyH75 retained its ability to produce tumors.⁹ The clone isolated from this line and used here grows in a very loose net characteristic of polyoma transformants.¹³ The cells are fusiform, and since they attach to the dish over a relatively narrow surface, they have a refractile appearance. As the cell density increases, nodules of more densely packed cells develop, perhaps as a result of retraction, and these break out of the plane of the cell layer, forming balls of largely unattached cell masses. As a result, no stable saturation density is attained though attached cell densities of $75 \times 10^4/\text{cm}^2$ have been measured.

When PyH75 was treated with FUdR, fewer than 10^{-5} of the cells survived when the density at the time of exposure was less than $4.5 \times 10^4/\text{cm}^2$ (Fig. 2C). At a fivefold higher density, the surviving fraction was about 10^{-2} . A culture grown up from a mixture of several surviving colonies was taken through a second cycle of FUdR. Of the colonies surviving the second cycle, some grew very slowly even at low densities and failed to do well on transfer. These colonies were discarded. Others grew at the usual rate, but the cells were obviously more adherent to the culture vessel than the parent line and were packed more tightly together. Two such colonies, Fl²-1 PyH75 and Fl²-21 PyH75, were grown up and subjected to third cycles. Figure 2C shows that at each density the survival was 10-100 times better than that of the parent. Both lines had quite high saturation densities (Table 1), but the cell layers were stable and, at least in the case of Fl²-21, the FUdR killing curve indicates that there was not much cell turnover at the saturation density.

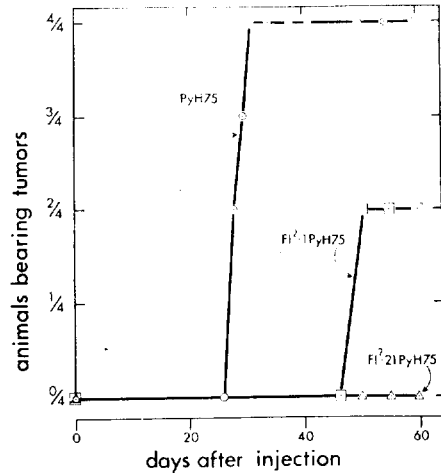
When the data for the nine lines with different stable saturation densities (Table 1) were pooled in a log-log plot (Fig. 2D) in which cell density was normalized to per cent of saturation density, all points clustered about a single curve. Thus all lines displayed a common response to increasing density. During the last 3.5 cell generations preceding saturation, the surviving fraction increased roughly as the fourth power of the density.

Colony-forming ability on a monolayer of 3T3: The ability of one cell type to inhibit the growth of another has been studied in various systems.¹⁴⁻¹⁷ Viral transformants are often able to form colonies very well on a confluent lawn of the parent cell type. For example, SV40 transformants of 3T3 or of human fibroblasts will grow very well when inoculated on a resting monolayer of either 3T3 or human diploid cells.

The ability of the flat variant lines to grow on a 3T3 monolayer was compared to their ability to grow on the bare surface of the Petri dish (Table 1). All flat derivatives showed a marked drop in colony-forming ability on 3T3 when compared with the parent line.

Tumor-producing ability of FUdR-selected variants: In general, a relation has been thought to exist between insensitivity to contact inhibition in culture and ability to produce tumors in animals. However, it has not been possible up to

FIG. 3.—Tumor incidence following injection of PyH75 and its flat variants into 21-day hamsters (5×10^4 cells/animal). With 5×10^5 cells/animal, one of four hamsters injected with Fl²-21 PyH75 cells developed a tumor 35 days after injection; the other three animals remained tumor-free.



now to select at will for sensitivity to contact inhibition of growth and to systematically correlate tumor-producing power with culture behavior. The PyH75 variants have made it possible to do this, and the results are shown in Figure 3.

Weanling hamsters were injected with 5×10^4 cells of PyH75, and two Fl² sublines, Fl²-1 PyH75 and Fl²-21 PyH75, obtained from the second cycle of FUDR treatment described above. PyH75 cells produced tumors of 1-cm diameter in all the injected animals within 30 days. Fl²-1 PyH75 cells produced tumors in only half the injected animals within 50 days. The same number of Fl²-21 PyH75 cells produced no tumors during the observation period of 60 days. In a further experiment, when a group of four animals was injected with cells of this line in ten times higher number (5×10^5 cells), one animal developed a tumor 35 days after injection. It is therefore clear, even on the basis of this small experiment, that FUDR-selected variants of a polyoma virus-induced tumor have reduced tumor-producing power.

Significance of flat variants for the study of transformation: When tumors are serially transplanted in animal hosts, the selective conditions favor those cells with the highest degree of neoplastic character; transplanted tumors evolve in this direction.¹³ Similarly, when established cell lines are serially cultivated, the culture conditions select in favor of variants with the ability to grow under conditions in which the growth of the rest of the population is arrested. This is probably why many long-term established lines do not reach any stable saturation density and have neoplastic character. Culture behavior and tumor-producing power for cells of similar origin have also been related by the observation that multilayer, but not monolayer, clones of polyoma virus-transformed cells are tumorigenic in hamsters.¹³

The present experiments show that the variation also occurs in the opposite direction and that, with FUDR, it is possible to select in culture for cells with increased sensitivity to growth inhibition. The cells so selected are stably different from the original population since their descendants show: (1) altered

morphology including greater flattening and adherence to the culture vessel; (2) lower saturation density; (3) reduced ability to form colonies on a 3T3 monolayer; (4) altered FUdR survival curve; and (5) reduced tumor-producing capacity. Since all of the original lines were cloned twice before the selection experiments were begun, the variants selected must have developed during the period of growth of the cloned lines preceding the selection experiment (20–40 cell generations). A fluctuation experiment^{19, 20} has confirmed the pre-existence of flat variants in an SV40-transformed 3T3 population at a frequency of approximately one in 10^5 .

The type of variation described here also occurred in lines that have never been infected with viruses. It could therefore be a cellular property independent of the virus. However, it seemed possible that since the virus transformants in general have low sensitivity to contact inhibition of growth, a powerful selection in favor of sensitive cells might disclose the presence of "revertants" that have lost the viral genome. This possibility was tested on flat variants of SV101 and PyH75.

The two tested variant clones that arose from SV101, like the original line, contained the SV40 T-antigen, and the two clones derived from the polyoma-transformed hamster line PyH75 still contained the polyoma T-antigen.²¹ Tests for the SV40 viral genome by cocultivation with monkey kidney cells in the presence of irradiated Sendai virus²² yielded SV40 from both SV101 and Fl²-SV101. This type of variation therefore occurs in cells expressing viral functions. However, examination of a larger number of flat variants may disclose whether or not revertants do occur, as in the case of Rous virus hamster transformants.²³

The relation between contact inhibition in culture and tumor-producing capacity has been clouded by the ambiguity of the criteria used and especially by the fact that some cell lines, though quite sensitive to contact inhibition of movement, are not sensitive to contact inhibition of growth.^{3, 24, 25} It should therefore be emphasized that the present studies are concerned only with effects on growth and that comparisons have been made between lines and their proximate clonal descendants.

Summary.—When cloned populations of cultured mammalian cells were treated with FUdR at different cell densities, the growing cells were killed, but the non-growing cells were unaffected and their progeny could be obtained selectively. Among these progeny were variants stably different from the original population in that they were more sensitive to contact inhibition of division and for this reason were protected from FUdR killing. Such variants could be isolated from established lines and from transformants produced by oncogenic viruses. They had a number of common properties in culture and greatly reduced tumor-producing ability *in vivo*.

The results give strong support to the hypothesis that contact inhibition of division in culture is related to growth control *in vivo*. Variation in the direction of greater sensitivity to both evidently occurs spontaneously even in viral transformants and in tumors in which some viral functions continue to be present.

The FUDR selection method may be of general applicability for the detection and isolation of cells with increased sensitivity to any form of growth control.

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