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Tumorigenicity of virus-transformed cells in *nude* mice is correlated specifically with anchorage independent growth *in vitro*

(malignant transformation/oncogenic virus/athymic *nude* mice/anchorage requirement)

SEUNG-IL SHIN*, VICTORIA H. FREEDMAN*, REX RISSER†‡, AND ROBERT POLLACK†§

* Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461; and † Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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ABSTRACT **Clonal isolates of mouse 3T3 cells and primary rat embryo cells, recovered nonselectively after infection by simian virus 40 (SV40), have been tested for tumorigenicity in the immune-deficient *nude* mice in order to determine the cellular growth properties *in vitro* specifically correlated with neoplastic growth *in vivo*. In addition, mouse 3T3 cells transformed by murine sarcoma virus (MuSV, Kirsten strain), and revertants isolated from cells fully transformed by either SV40 or MuSV were also studied. Results suggest that the single cellular property consistently associated with tumorigenicity in *nude* mice is the acquisition by virus-transformed cells of the ability to proliferate *in vitro* in the absence of anchorage. Other cellular parameters of virus-induced transformation, such as lack of sensitivity to high cell density and the capacity to grow in low serum concentration, are dissociable from cellular tumorigenicity. This conclusion is supported further by the demonstration that specific selection *in vivo* for tumorigenic cells from anchorage-dependent cells results in the isolation of anchorage-independent cells. Conversely, a single-step selection *in vitro* for anchorage-independent cells from nontumorigenic cells results in a simultaneous selection of highly tumorigenic subclones.**

Infection of susceptible animal cells *in vitro* by tumor viruses usually results in a spectrum of stable alterations in cellular growth properties, as well as in the appearance of virus-specific antigens in the transformed cells (1). In particular, division in populations of untransformed cells is inhibited by any of the following three environmental constraints: extensive cell–cell contact (2), reduction of serum concentration (3, 4), or deprivation of a solid substrate for cell anchorage (5, 6).

Recent results have demonstrated that cellular responses to the experimental parameters which differentiate the normal cell from its transformed counterpart are not coordinately controlled (7, 8). Each constraint is the source of a selective assay that yields a different class of transformed cell line. Nonselective transformations of 3T3 mouse cells and of primary rat embryo cells by simian virus 40 (SV40) yielded lines displaying many different transformed phenotypes. While some lines were fully insensitive to each of the three constraints, most transformed lines lost only one or two of these constraints and remained normal for the others. Negative selection of revertant cell lines from a fully transformed 3T3 cell also dissociated these three parameters of growth control (9, 10).

These observations suggested to us that not all of the altered cellular growth properties commonly associated with the transformed state would be required for neoplastic growth *in vivo*, and that it should be possible to test this prediction experimentally in the immune-deficient mouse mutant *nude*. Because of a recessive autosomal mutation, *nude* mice fail to develop the thymus, and are functionally devoid of T cell (thymus-derived cell)-dependent immunity (11, 12). These mice are therefore unable to reject transplanted tumor cells of allogeneic or xenogeneic origin (13, 14), and thus provide a convenient, immunologically neutral test system for cellular tumorigenicity (15).

In a recent series of experiments involving a large number of animal cells in culture, we have shown that there exists a high degree of correlation between cellular tumorigenicity in the *nude* mouse and anchorage-independent growth *in vitro* (15). Cells which require anchorage for growth uniformly failed to give rise to tumors in these mice. In another series of experiments, two independent sets of clonal isolates have been recovered from mouse 3T3 cells and primary rat embryo cultures after infection with SV40, and characterized extensively to determine the degree of coordinate expression of virus-induced transformed phenotypes (7, 8).

By combining these two approaches, we have attempted to identify the virus-induced cellular phenotype(s) *in vitro* specifically correlated with neoplastic growth potential *in vivo*. The results are described in this paper. A preliminary account of this work has been presented (16).

MATERIALS AND METHODS

Cell Culture Conditions. Mouse 3T3 cells (2), SV40-transformed 3T3-derivative SV101 (17), various revertant cell lines isolated from SV101, and other virus-transformed cell strains were maintained in Dulbecco-Vogt-modified Eagle's medium (Gibco, H21) with 10% calf serum in a humidified CO_2 incubator. Primary diploid cell strains and rat embryo cell clones were cultured in the same growth medium but with 10% fetal calf serum.

Cell Strains Used. Recovery and characterization of nonselectively isolated 3T3 cells transformed with SV40 have been described previously (7). In addition, the following revertants isolated from SV101 were also used: F1-SV, a density-sensitive "flat" revertant (9); aγ-SV and Ls-SV, two partial revertants, selected for sensitivity to agamma depleted medium and to low serum, respectively (18). KA31 is a nonproducer clone of Balb/3T3, transformed with the RNA-containing murine sarcoma virus, Kirsten strain (MuSV) (19), and M22 is a revertant selected from KA31 for inability to grow in methyl cellulose (20).

The preparation of rat embryo (RE) cells, and isolation of RE clones after infection with SV40 (SVRE clones), have also been described (8). Primary mouse embryo (ME) cultures and rabbit kidney cells (RK-1) were prepared similarly

Abbreviations: SV40, simian virus 40; MuSV, murine sarcoma virus, Kirsten strain; RE, rat embryo; ME, mouse embryo.

‡ Present address: National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014.

§ Present address: Microbiology Department, State University of New York, Stony Brook, N.Y. 11794.

from 13- to 15-day whole mouse embryos and an adult female New Zealand rabbit, respectively. For tests of tumorigenicity, ME cells were used within a few days after preparation without further passage.

Determination of Transformed Cellular Phenotypes *In Vitro*. Serum sensitivity, cell saturation density, anchorage requirement and viral T-antigen expression of each cell population were determined as described (7). In brief, serum sensitivity and saturation density were determined by measuring the average population doubling time and maximal cell density per unit surface area, at 1% and 10% serum concentrations. Anchorage requirement was determined by plating cells in culture medium containing 1.2% methyl cellulose (Methocel, 4000 cps, Dow Chemical Co.), over a layer of 0.9% agar (Difco Bacto Agar) in the same culture medium. In this assay, anchorage-dependent cells have a negligible efficiency of plating, but cells transformed with regard to anchorage have a relative efficiency of plating in methyl cellulose comparable to that in a liquid medium in which cell anchorage is permitted (6).

Breeding and Maintenance of *Nude* Mice. The *nude* mouse colony was originally initiated with a *+/nu* stock on BALB/c background, and was maintained by breeding *nu/nu* males and *+/nu* females. Under the semiaseptic conditions that have been instituted in our laboratory (15), the average life span of *nu/nu* has now increased to over 15 months.

Determination of Tumorigenicity in *Nude* Mice. Cells were harvested by trypsinization, and 2×10^6 washed cells suspended in 0.2 ml of phosphate-buffered saline were injected at a single subcutaneous site into each *nude* mouse at 6–8 weeks of age. All mice were examined regularly for development of tumors. Cells were scored as tumorigenic if a palpable nodule appeared at the site of injection within 10 weeks and increased in size thereafter. Otherwise, the injected mice were kept for at least 5 months after injection before scoring as nontumorigenic. With a few notable exceptions (see below), all tumorigenic cells regularly produced tumors within the first 8 weeks, always at the site of injection.

When further characterization of the tumor-derived cells was desired, tumors were removed aseptically from the mice, minced with dissecting scissors and sieved through a stainless-steel mesh to disaggregate tissues into single cells. In some instances, trypsinization of minced tumor fragments for 15 min at 37° was necessary to release a sufficient number of single cells. The single cell suspensions were then used to initiate new cultures, or reinjected into mice to test for serial transplantability.

RESULTS

Tumorigenicity of virus-transformed cells in *nude* mice

To first determine whether an overall association exists between tumor formation in *nude* mice and virus-induced transformed phenotypes *in vitro*, we compared the tumorigenicity of a group of diploid cell cultures and mouse 3T3 cell lines to that of the same cells transformed with either SV40 or MuSV. In addition, two partial revertant lines derived from the transformed cells were also tested. The results, given in Table 1, show clearly that an association does exist between virus transformation *in vitro* and tumorigenicity in the *nude* mice. Significantly, both of the revertants, F1-SV (a partial revertant of SV101) and M22 (a complete revertant of KA31), were no longer tumorigenic. F1-SV was selected from SV101 specifically on the basis of its reversion to sensitivity to contact inhibition, and still expresses the SV40 T-antigen (9). These data therefore suggest also that the loss of a specific transformed property *in vitro* may result in the loss of cellular tumorigenicity *in vivo*.

Table 1. Cellular tumorigenicity in *nude* mice: Association with virus-induced transformed phenotypes

Cell	Source of cell	Tumorigenicity*
Primary diploid cultures		
ME-CBA	Mouse embryo, CBA	0/2
ME-Balb	Mouse embryo, BALB/c†	0/7
RE-F	Rat embryo, Fisher	0/6
RK-1	Rabbit kidney, adult	0/2
Established cell lines		
3T3 (Swiss)	Mouse 3T3, Swiss	0/4
Balb/3T3	Mouse 3T3, BALB/c	0/2
CV-1	Monkey, adult kidney	0/2
Virus-transformed cell lines		
SV101	Mouse 3T3 (Swiss), transformed by SV40	5/6
KA31	Mouse Balb/3T3, transformed by murine sarcoma virus (MuSV)	2/2
RKT-TG1	Rabbit RK-1, transformed by SV40	2/2
Revertant cell lines		
F1-SV‡	Partial revertant of SV101	0/2
M22§	Complete revertant of KA31	0/2

* No. of mice which developed tumors within 10 weeks after injection/no. of mice injected.
† Pooled data, representing +/+, +/*nu*, and *nu/nu* genotypes.
‡ A revertant clone of SV101, which is density-sensitive, anchorage-dependent, serum-insensitive but T-antigen-positive (ref. 9).
§ Subclone derived from KA31, which retains none of the virus-induced transformed properties (ref. 20).

Tumorigenicity of mouse 3T3 cells transformed by SV40

A series of random clones isolated non-selectively from SV40-infected mouse 3T3 cells has been shown to express a spectrum of transformed phenotypes, which segregate among the clones in a non-coordinate fashion (7). A selected subset of these clones, representing each class of the transformed cell recovered, were injected into *nude* mice to test for tumorigenicity. Results of this experiment and the appropriate controls are presented in Table 2. The data indicate that the single *in vitro* correlate of tumorigenicity in *nude* mice is anchorage-independent growth, as measured by colony-forming ability in methyl cellulose. In particular, tumorigenicity of SV40-transformed cells is not correlated with either the loss of serum sensitivity (e.g., F1-SV, SVR 82), or insensitivity to contact inhibition (e.g., SVR 13).

Even though a comparable non-selective clonal analysis of MuSV-transformed 3T3 cells has not been carried out as yet, the data given in Table 1 with respect to KA31 and its revertant M22 are also consistent with this interpretation.

Table 2. Tumorigenicity of mouse 3T3 cells in *nude* mice: Association with SV40-induced anchorage independence

Cell	Transformed phenotype	Viral T-antigen	Sensitivity of cell growth to:* Cell density	Serum concentration	Anchorage	EOP in† methyl cellulose	Tumorigenicity‡
Untransformed control							
3T3 (Swiss)	Normal	—	+	+	+	<0.001	0/4
Fully transformed clone							
SV101	Transformed	+	—	—	—	27.0	5/6
Partial revertants of SV101							
F1-SV	Density revertant	+	+	—	+	0.01	0/2
Ls-SV	Serum revertant	+	+	+	+	0.04	0/2
Aγ-SV	Serum revertant	+	+	+	—	11.0	2/4§
SV40-infected 3T3 clones							
SVR 13	Intermediate	±¶	—	—	+	0.2	0/2
SVR 63	Intermediate	±	—	—	+	<0.001	0/2
SVR 82	Minimal	—	+	—	+	<0.001	0/2
SVR 86	Complete	+	—	—	—	58	3/4
SVR 95	Minimal	—	+	—	+	<0.001	0/4

* +, sensitive; −, insensitive. Data from ref. 7, 9, and 18.
† Efficiency of plating in percent.
‡ No. of mice which developed tumors/no. of mice injected.
§ Both tumors developed within 4 weeks after injection, but subsequently regressed completely.
¶ Intermediate (±) clones showed 10–20% bright positive nuclei, 50–80% weakly fluorescent nuclei, and 10–30% dark nonfluorescent nuclei. In contrast, control transformants (+) stained uniformly positive, whereas negative (−) clones did not show nuclear fluorescence.

Tumorigenicity of primary rat embryo cells transformed with SV40

An analogous study with a set of rat embryo cell clones transformed with SV40 and recovered as isolated colonies is summarized in Table 3. These results are essentially identical to those obtained from the mouse 3T3 clones (Table 2). Once again, tumorigenicity is specifically correlated with anchorage independence *in vitro*, but dissociable from cellular sensitivity to high cell density or low serum concentration.

Generality of the correlation between anchorage independent growth and tumorigenicity

If the specific correlation observed so far between anchorage independent growth *in vitro* and tumorigenicity in the immune-deficient animal is a truly general phenomenon, then a single-step selection *in vivo* for tumor-forming cells from normally anchorage-dependent cells should result in the selective rescue of anchorage-independent cells. Conversely, selection *in vitro* for cells that form colonies in methyl cellulose from normally nontumorigenic cells should result in highly tumorigenic derivatives.

These expectations were shown to be valid by the following experiments. Mouse embryo cultures representing four different parental genotypes (see Table 1), mouse 3T3 cells, and two independent SV40-transformed derivatives of 3T3 that had been shown previously to be anchorage-dependent for growth (i.e., Ls-SV and SVR 95) (see Table 2), were each injected into a group of *nude* mice and examined periodically for evidence of tumor development during the next 5 months. None of the mice receiving diploid embryo cells developed tumors. However, one mouse from each of the three groups of *nude* mice that had been injected with Balb/3T3, Ls-SV and SVR 95 eventually developed a slow-growing tumor (Table 4). These tumors were first detected more than 12 weeks after injection, whereas the usual lag time for tumorigenic cell lines was 4–8 weeks.

When these tumors were removed from the mice and introduced into culture, each gave rise to stable, dense monolayer cultures which morphologically resembled SV101. The nontumorigenic parental cells were all characterized by the

Table 3. Tumorigenicity of SV40-transformed rat embryo cell clones

Cell	Viral T-antigen	Sensitivity of cell growth to:* Cell density	Serum concentration	Anchorage	EOP in methyl cellulose (%)	Tumorigenicity
Untransformed control						
RE-F	—	+	+	+	<0.001	0/6
SV40-transformed clones						
SVRE 3	+	—	—	+	0.15	0/4
SVRE 5	+	—	—	—	2.58	2/2
SVRE 9	+	—	—	—	1.26	6/8
SVRE 12	+	—	—	+	0.01	0/8

EOP, efficiency of plating.
See footnotes to Table 2.
* Data from ref. 8.

Table 4. Selection *in vivo* of anchorage-independent cells from nontumorigenic mouse cells

Clone	Viral T-antigen	EOP in methyl cellulose (%)	No. of cells injected per mouse ($\times 10^{-6}$)	Development of tumors in *nude* mice*	
				After 8 weeks	After 16 weeks
Parental cells before selection in vivo					
3T3 (Swiss)	—	<0.001	10	0/4	0/4
Balb-3T3	—	<0.001	10	0/2	1/2
Ls-SV	+	0.04	2	0/2	1/2
SVR 95	—	<0.001	2	0/4	1/4
Tumor-selected cells					
B3T3-Nul	—	17	2	2/2	2/2
LSV-Nul	+	20	2	2/2	2/2
SVR 95T	—	N.D.†	2	4/4	4/4

EOP, efficiency of plating.
* No. of mice with tumors/no. of mice injected.
† Not done.

3T3-type morphology. Although each of the tumors had originated from anchorage-dependent cells, the tumor-derived cell lines were no longer anchorage-dependent, as shown by the high efficiency of plating in methyl cellulose. The major phenotypic parameters of these *in vivo* selected cells are presented in Table 4. As predicted, all of these lines were now highly tumorigenic when reinjected into *nude* mice.

In the second experiment, the above procedure was reversed. SVR 13 is an incomplete transformant of SV40 isolated from 3T3 (Swiss); it shows an intermediate level of T-antigen expression, and is density insensitive, anchorage-dependent, and nontumorigenic (see Table 2). From SVR 13, several anchorage independent subclonal derivatives were eventually isolated following plating in methyl cellulose (7). These subclones, when injected into *nude* mice, were now highly tumorigenic. Data for one such clone, 13MC2, are given in Table 5. It is apparent that selection *in vitro* for the anchorage-independent cells from an originally nontumorigenic clone leads to a simultaneous selection for the tumorigenic phenotype.

Selection *in vivo* of SV40-transformed cells in *nude* mice

The above results suggested that it may be possible to select for transformed cells from newly infected primary diploid cells by injection into *nude* mice. Positive confirmation of this possibility would be useful, since such a procedure would allow the isolation of transformed descendants from animal cells that are difficult to maintain in culture for a prolonged period of time. For example, the prospect for establishing a highly-differentiated human cell type into a cell line would be greatly enhanced by such a method (21).

Primary rat embryo (RE) cells were infected at subconfluence with SV40 at a multiplicity of infection of 400. After overnight incubation, the cells were washed with buffer and returned to normal culture conditions for 1 day. Cells were then harvested by trypsinization, and 12×10^6 cells were injected into each *nude* mouse. In controls, replicate RE cells were treated with saline instead of SV40, and injected after identical manipulations.

None of the *nude* mice with the mock-infected RE cells developed tumors, but both of the *nude* mice injected with SV40-infected cells eventually developed tumors at the site of injection. Cell culture lines derived from each of these tumors, VRE-Nul and VRE-Nu2, showed characteristic transformed morphology, expressed SV40 T-antigen, formed colonies in methyl cellulose, and were readily transplantable in *nude* mice. The genetic origin of VRE-Nu cells was confirmed as rat by the fact that their isozyme pattern for lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27.) was identical to that of control RE cells, but clearly different from SV101 and control ME cells. This evidence thus rules out the possibility that the SV40-infected RE cells caused tumors in *nude* mice by simply inducing the transformation of host cells with RE-bound SV40 particles.

Conclusion

As mentioned earlier, at least four independently controlled, virus-induced cellular phenotypes segregate among clonal isolates of SV40-transformed cells. These are: the expression of the SV40-specific nuclear (T) antigen, and insensitivity of cell growth to high cell density, low serum concentration, or absence of anchorage. Data presented in this paper show that the single cellular property *in vitro* correlated specifically with neoplastic growth *in vivo* as determined in *nude* mice is the virus-induced acquisition of anchorage independence.

DISCUSSION

Earlier studies have indicated that the increased capacity of virus-transformed cells to form colonies in a semi-solid growth medium is in general associated with transplantability in immunologically compatible hosts (2, 5, 9, 22, 23). Our

Table 5. Selection *in vitro* of tumorigenic cells from anchorage-dependent cells

Cell	Viral T-antigen	EOP in methyl cellulose (%)	Tumorigenicity† in *nude* mice
Nontumorigenic parent			
SVR 13*	±	0.2	0/2
Anchorage independent subclone			
13MC2	+	66.5	3/4

EOP, efficiency of plating.
* Data from Table 2.
† No. of mice with tumors/no. of mice injected.

results presented in this paper demonstrate that specific transformed cellular phenotypes other than anchorage independence are *not* necessarily associated with malignant growth *in vivo*. Thus, the genetic analysis of malignant transformation by tumor viruses may now be directed more fruitfully to the analysis of the molecular mechanism which mediates the loss of anchorage requirement in the transformed cells.

Whether the observed correlation between the anchorage regulation of cell growth *in vitro* and tumorigenicity in the *nude* mice holds true also in other cell types that are transformed by different oncogenic viruses remains to be established.

Recent work with the cell lines used in this study has shown, for instance, that the loss of anchorage dependence is accompanied by, and possibly causally related to, the production of a tumor-associated activator of serum plasminogen (24, 8). Upon anchorage-transformation, the internal structure of cells is also altered in a specific way: thick actin-containing bundles of microfilaments are replaced by a diffuse matrix (25). Understanding of the molecular changes associated with these phenomena may provide new information about the mechanism of oncogenic transformation.

Unambiguous determination of malignant growth potential *in vivo* of transformed cells has hitherto been complicated because cell lines widely used in tumor biology, notably mouse 3T3 (Swiss) and hamster BHK-21, often originated in noninbred strains. Even with cell lines derived from highly inbred animals, the possibility exists that the appearance of new cellular antigens, by *de novo* mutation in culture or as a result of viral gene activity, may provoke immunological rejection in the test animals. The use of *nude* mice obviates this potential problem. Furthermore, the availability of *nude* mice as an immunologically inert test system for tumorigenicity should make it possible to carry out this type of analysis with human cells transformed *in vitro* with carcinogens, mutagens or tumor viruses.

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