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High Frequency of Tumour Cell Reversion to Non-tumorigenic Phenotype

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Nine spontaneously transformed cell lines were isolated from embryo fibroblasts of mice and rats with different genotypes. In six cell lines highly tumorigenic cell variants were selected. At the start of culture all cell lines were of low or zero tumorigenicity. The same cells in a confluent monolayer *in vitro* had high contact inhibition of growth and proliferated in response to stimulation by growth factors. Tumour progression of the established lines was accompanied by significant changes of these properties. Clonal analysis of the six most malignant cell lines revealed their capacity to revert simultaneously to the non-tumorigenic state and to their initial growth characteristics. Frequencies of reversion to the non-tumorigenic phenotype were much higher than re-reversion to the tumorigenic phenotype. The reversions occurred in several sequential passages of transformed clones, with some variations in individual clones. These observations suppose that frequencies of tumour reversions are a constant genetic characteristic of every cell line.
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INTRODUCTION

THE DISCOVERY of polypeptide growth factors appears to link logically the regulation of proliferation of normal somatic cells and the most serious pathology of these processes, tumorigenesis [1–3]. There are many steps that can be defective in tumour cells, from perception of the growth signal by the cell membrane to realisation of the signal in DNA. The main defects, common to all tumour cells, are hard to detect because of their great variability. There may be no adequate models to describe repeatedly and independently neoplastic transformation compared with spontaneous reversals in cell culture. We propose one such model. Nine independent cell lines of fibroblasts with various genotypes were obtained from mice and rats. The cell lines transformed spontaneously *in vitro*. In six of them we succeeded to select highly tumorigenic variants. All nine lines at the beginning of transformation were found to be not tumorigenic at all or very slightly tumorigenic. At the same time they demonstrated high ability to the density-dependent inhibition of cell growth and possessed sensitivity to some serum growth factors. During the tumour progression both properties changed significantly. Besides that, selected highly tumorigenic cells were shown to be able to reverse to a non-tumorigenic or slightly tumorigenic state. At the same time cells reverse to the initial state regarding to both above-mentioned properties.

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MATERIALS AND METHODS

Cell lines

Inbred lines of mice, C3H/He, CBA, BALB/c, C57Bl/6, and rats, Wa, from our institute were used. Primary cultures of mouse FC3HO, FCBAO, FBALBO, FBIO and rat FWaO embryo fibroblasts were obtained by trypsinisation of embryos without head and inner organs. Established cell lines were maintained in culture for more than 1.5 years, and every 2–4 months cells were tested for tumorigenicity by inoculation under the dorsal skin of syngeneic animals (10^6 cells per animal). The observation period was 4 months. At the same time cells were frozen in liquid nitrogen. Six cell lines were selected for high tumorigenicity by passing them *in vivo*. They then were maintained *in vitro*: FC3H3v7, FC3H4v8, FCBA2v10, FBALB1v12, FB12v12, FWa3v6 (the figure after "v" is the number of passages *in vivo*). Moreover, highly metastatic cell lines, FC3H3m and FCBA2m, were selected from cell lines FC3H3 and FCBA2v40.

Primary cultures and established cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The cells were cultured in plastic dishes (Flow) and were trypsinised with 0.025% trypsin (Serva). The cells were passaged three times per week. All cell cultures were found to be mycoplasma-free [4]. Cell cloning was done by

limiting dilutions by seeding 0.5–1 cells per well in 96-well plates. Colonies were counted and estimated after 10–14 days. There were three types of clones, according to their phenotype: (1) transformed = semicircular, high cell saturation density; (2) non-transformed = flat, low density; and (3) intermediate = flat, higher density than non-transformed cells.

Tumorigenicity

The criteria used to identify the degree of tumorigenicity of cells in syngeneic animals were: (1) frequency (% of animals with tumour); (2) rate of tumour growth (weight of tumour on day 30); (3) tumorigenicity index, calculated as $\ln(A \times K1 \times K2)$. A is the average weight of tumours in grams on day 30 (without any tumours this weight was presumed to be minimal, 0.01 g), $K1$ is a coefficient varying from 2 to 1, depending on the proportion of animals that died before day 30. If the proportion was 100%, then $K1 = 2$. If none of the animals had died by day 30, then $K1 = 1$. $K2$ is a coefficient varying from 1 to 0.1, depending on the frequency of tumour bearing animals at day 30. If the frequency was 100%, then $K2 = 1$. If none of the animals had tumour at day 30, then $K2 = 0.1$.

Immunological characteristics

The cell sensitivity to natural killer (NK) cell and macrophage lysis was studied with a cytotoxicity assay in 96-well plates [5]. ^{3}H release from prelabelled targets in response to NK cells and macrophages was measured. The degree of density-dependent inhibition was estimated in comparison with the degree of cell proliferation on the monolayer of density-inhibited pseudonormal culture FC3H8 as described [6]. In brief, a confluent monolayer of FC3H8 was treated with mitomycin 1 μg/ml (Serva) for 5 h. Then the monolayer was washed three times. The $4–5 \times 10^3$ tested cells were added to the monolayer and to the plastic surface. All the cells attached. After 12–20 h. [^{3}H]thymidine was added for 2–3 h. The amount of radioactivity incorporated in tested cells harvested onto paper filters was measured by liquid scintillation. [^{3}H]thymidine incorporation of cells plated onto the plastic surface was taken as 100%. Inhibition of cell proliferation was estimated as inhibition of [^{3}H]thymidine incorporation by tested cells grown on a monolayer of fibroblasts compared with cells grown on the plastic surface, with the following equation: $[1-(A-B)/C] \times 100$, where A = radioactivity incorporated into tested cells and monolayer cells, B = radioactivity incorporated in monolayer cells only and C = radioactivity incorporated into tested cells growing on plastic. Moreover, density-dependent inhibition of cell growth was determined with respect to cell saturation density. The cells were seeded in the same culture dishes. They were allowed to become confluent and then the culture medium was changed every day for 5 days. After that the cells were trypsinised. Cell numbers in suspension were counted microscopically.

Response to growth factors

For measurements of cell reactions to polypeptide growth factors, such as epidermal growth factor (EGF) (Serva), endothelial cell growth factor (ECGF) (Sigma), platelet-derived growth factor (PDGF), partly purified by methods described by Raines and Ross [7], and fibroblast growth factor (FGF) (Serva), cells were seeded at 1.2×10^5/cm^2 in 96-well plates in MEM plus 10% CS. The next day the cell monolayers were washed twice with serum-free medium and fresh medium supplemented with 5% platelet-poor plasma [8] plus one growth factor. The concentrations of growth factors in culture medium were: EGF, 12.0 ng/ml; ECGF, 10.0 μg/ml; PDGF, 20.0 ng/ml, 1625 units of specific activity per mg; and FGF, 0.1 μg/ml. Medium plus 5% plasma only was added to control wells. 18–22 h later [^{3}H]thymidine was added for 2–3 h. The radioactivity of tested cells was measured on paper filters. The radioactivity of tested control cells was taken as one unit and the increase of [^{3}H]thymidine incorporation in response to growth factor was estimated.

RESULTS

Cell lines and tumorigenicity

Mammalian fibroblasts cultured *in vitro* can overcome Hayflick's limit and become immortalised, during which cells often gain a transformed phenotype and become able to form tumours in syngeneic animals [9,10]. Nine cell lines passed over a long period were obtained from initial cultures of embryo fibroblasts from mice and rats of five genotypes. Seven of these cultures, passed *in vitro*, demonstrated a gradual increase of tumorigenicity, gaining moderate permanent tumour potential over 1–2 years. Such cells led to the onset of slowly growing tumours, that arose usually only after a prolonged latent period (1–2 months). After repeated *in vivo* passage of such cells, six highly tumorigenic cell lines were obtained. They could be propagated *in vivo* in all syngeneic recipients and their rate of growth is shown in Fig. 1. The tumorigenicity index is shown in Fig. 2.

Inhibition of growth and sensitivity to growth factors

Figure 2 shows that cells of all lines which are progressing during early stages of passing *in vitro* demonstrated a high degree of density-dependent inhibition of proliferation. This made these cells unable to grow on a monolayer of pseudonormal fibroblasts FC3H8. Density-dependent inhibition was diminished with passages *in vitro* and became minimal in highly tumorigenic cells selected *in vivo* (Fig. 2). Normal embryo fibroblasts of mice, non-tumorigenic lines and non-tumorigenic clones of tumourigenic lines also possessed minimal capacity to proliferate on a monolayer of pseudonormal cells (Table 1). The degree of density-dependent inhibition of growth correlated with the sensitivity to serum polypeptide growth factors. Indeed, all the studied non-tumorigenic cell lines and clones responded to a certain degree to the addition of one of four growth factors. Under the same conditions, highly tumorigenic cells either did not respond to growth factors or showed decreased [^{3}H]thymidine-uptake (Table 1).

Reversion to non-tumorigenic phenotype

Highly tumorigenic cell lines, on being cloned, also formed some less dense colonies and monolayered colonies, which is typical of cells with strong density-dependent inhibition of growth. Four out of six highly tumorigenic cultures demonstrated such reversion (Fig. 3). Cells of rat culture FWa3v6 and of highly metastasing mouse line FC3H3m did not reverse, or the frequency of such events was below 1%. Experiments were done to demonstrate that this was a reversion and not a contamination of initial tumour culture with normal cells. In these experiments the high frequency of reversion of transformed clones to the non-transformed phenotype remained the same in the third and even in the sixth series of recloning (Figs 4 and 5). It is noteworthy that cells of phenotypically non-transformed colonies, on being repeatedly cloned, gave almost only colonies of a non-transformed phenotype (Figs 4 and 5).

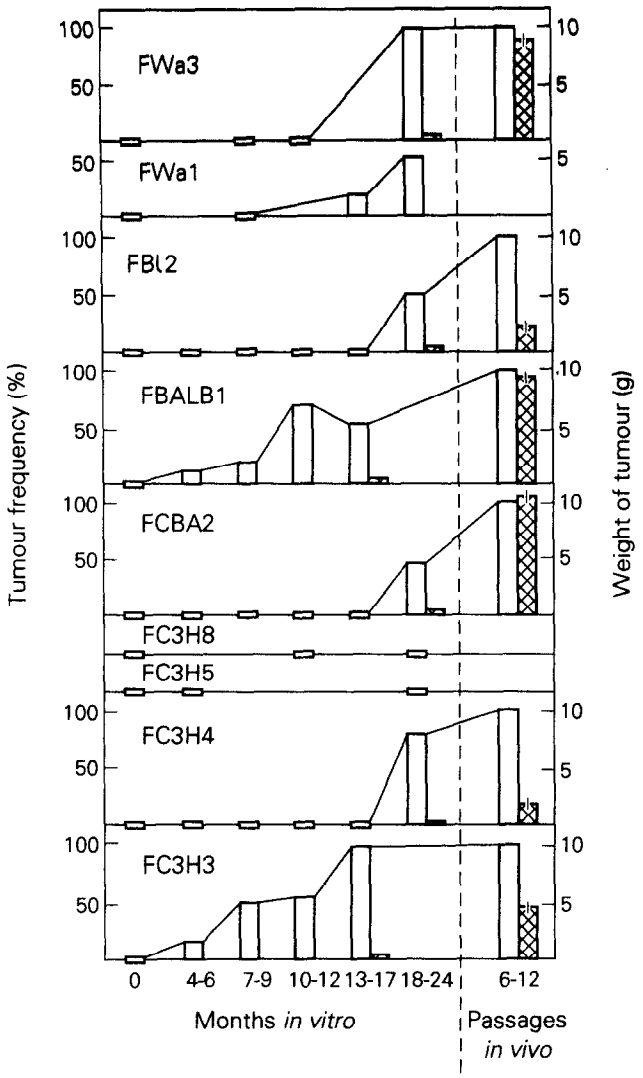

Fig. 1. Alteration of tumorigenicity in nine mice and rat cell lines during continued passaging *in vitro* and *in vivo*. Open column = % of animals with tumour, hatched column = weight of tumour on day 30.

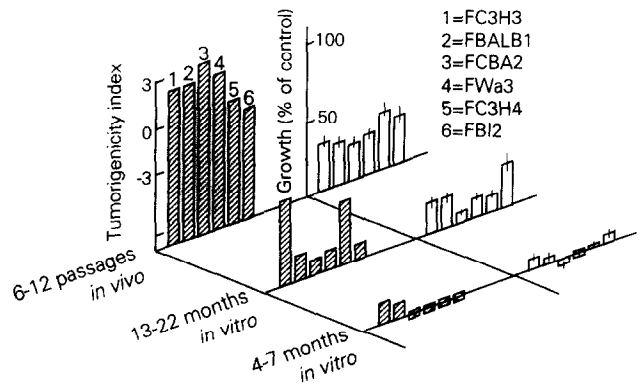

Fig. 2. Tumorigenicity (shaded columns) and degree of density-dependent inhibition of proliferation (open columns) of six cell lines at various stages of tumour progression. Degree of density-dependent inhibition of proliferation was compared with degree of cell proliferation on a monolayer of FC3H8.

Table 1. Inhibition of non-tumorigenic and tumorigenic cell proliferation on pseudonormal cell monolayer and sensitivity to growth factors

Cell type	% Inhibition of [^{3}H]thymidine incorporation	Fold increase of [^{3}H]thymidine incorporation in response to: EGF	ECGF	PDGF
Non-tumorigenic				
FCBAO	95.6 (2.1)*	1.6 (0.02)	1.2 (0.1)	1.1 (0.4)
FC3H5	99.0 (2.0)	6.2 (0.6)	1.3 (0.2)	1.7 (0.1)
FC3H8	89.6 (2.6)	3.3 (0.8)	3.4 (0.3)	1.7 (0.01)
FC3H31 (k42)	89.2 (1.5)	2.2 (0.2)	2.6 (0.9)	1.9 (0.3)
FCBA25 (k22)	91.8 (1.3)	4.8 (0.8)	1.9 (0.1)	1.6 (0.2)
NIH3T3	91.7 (3.3)	4.2 (1.0)	3.3 (1.3)	1.8 (0.6)
Highly tumorigenic				
FC3H3v7	68.0 (2.2)	0.8 (0.2)	1.0 (0.1)	1.0 (0.1)
FBALB1v12	71.8 (2.5)	0.8 (0.1)	0.8 (0.1)	1.0 (0.2)
FC3H4v8	60.0 (4.2)	1.2 (0.2)	0.9 (0.02)	1.0 (0.1)
FWa3v6	74.1 (3.2)	0.9 (0.1)	0.9 (0.03)	1.0 (0.03)

*Mean (S.E.) of three independent experiments.
k = designation of particular clone.

Tumorigenicity, immunological and growth properties of cells taken from colonies of contrasting phenotypes

Data that estimate the degree of tumourigenicity of 10 clones from culture FCBA2v10 are shown in Fig. 6. Cells were taken from colonies corresponding to transformed, intermediate and nontransformed phenotypes. These data show that the degree of density-dependent proliferation varied strongly in parallel to tumourigenicity. Immunological variables that can influence tumorigenicity varied only slightly. Additional investigations of several randomly chosen colonies with a transformed phenotype of two other cell cultures (Table 2) demonstrate their capacity

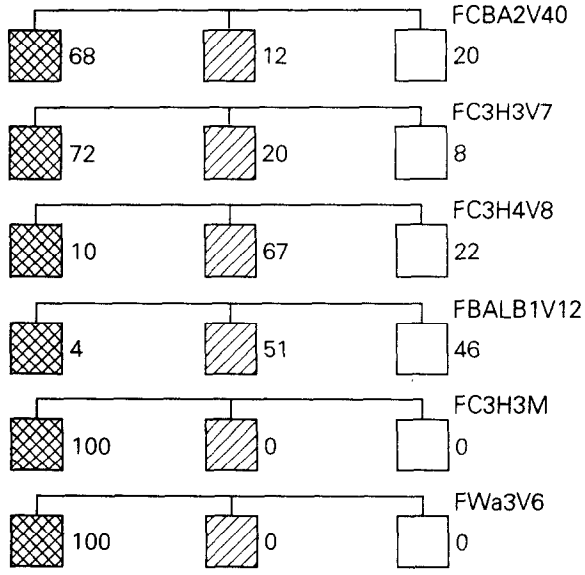

Fig. 3. Reversion to non-tumorigenic phenotype of six highly tumourigenic cell lines after cloning. hatched = transformed type; shaded = intermediate type; open = non-transformed type. Figures = % of each type of colonies. About 50 colonies were examined.

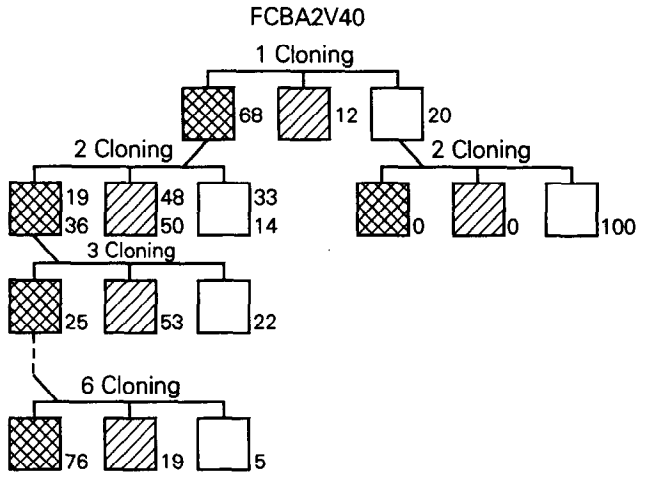

Fig. 4. Recloning of highly tumorigenic culture FCBA2v40. Symbols as for Fig. 3. Separate series of figures correspond to results from independently taken clones.

to form quickly growing tumours (latencies 7–10 days), while cells of all monolayer colonies could not form tumours at all or only formed them after a long latency (1–3 months). In addition, the cells of monolayer colonies showed high density-dependent inhibition of proliferation and were sensitive to the growth factors. Confluent density-inhibited monolayers of such cells, when individual growth factors or fetal calf serum were added to the medium, responded with an increase in DNA synthesis (Table 2).

DISCUSSION

Our data concern the high frequency of reversion of highly tumourigenic cells to the non-tumorigenic state. The frequency of such reversions should be a constant genetic characteristic of every cell line, because they repeated in a sequence of cell generations with some variations in individual clones (Figs 4 and 5). It is interesting that the frequencies of reversions were

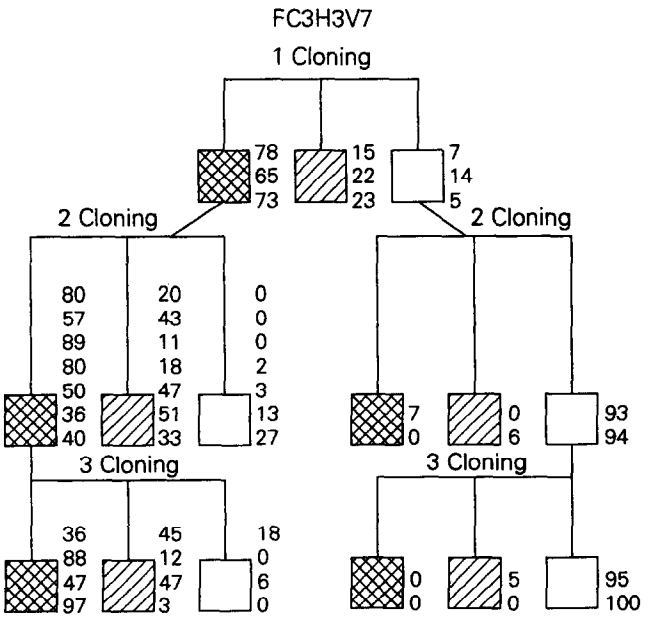

Fig. 5. Recloning of highly tumorigenic culture FC3H3v7. See legend to Fig. 4.

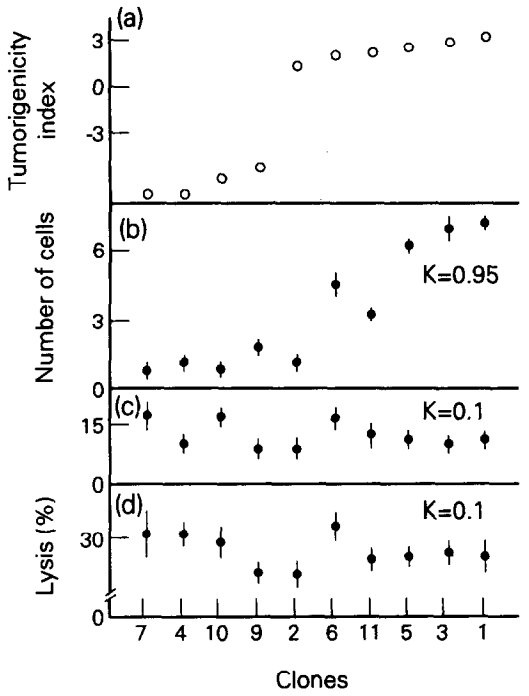

Fig. 6. Tumorigenicity (a), growth (b) and immunological (c, d) characteristics of clones of highly tumorigenic culture FCBA2v10. (a) Cells inoculated under dorsal skin at 10^6 cells per animal. (b) Number of cells × $10^5/cm^2$. (c) Degree of cell lysis by macrophages from peritoneal cavity of syngeneic mice (effector-to-target cell ratio, 30:1; time of reaction 48 h). (d) Degree of cell lysis by NK cells (from spleen) of syngeneic mice (300:1; 24 h). K = coefficient of correlation between tumorigenicity and the characteristic. Mean (S.E.) shown.

much higher than back reversions, although these did occur (Fig. 5). Cells of many non-transformed colonies can form tumours 2–4 months after inoculation (data not shown). This proves the possibility of rare back reversions. Reasons for the high phenotypical variability of tumour cells are still unclear [11–14]. Cases of sporadic reversion of tumorigenic cells to the non-tumorigenic phenotype have been reported. The cause of such reversions often appeared to be related to changes in the immunological characteristics of cells [15–18]. Our spontaneously transformed cells did not show much variability in immunological variables, but they did demonstrate a high and constant frequency of forward and back reversions in tumorigenicity. Similar phenotype reversion has been described for cell lines C3H10T1/2 and NIH 3T3 [19, 20]. Our data support the hypothesis [19, 20] that cell transformation behaves as an adaptive alteration rather than a mutation because of reversibility of the phenomenon. However, our findings are not consistent with the reports in which the changes of the phenotype occur in the entire cell population. Possibly these disagreements are due to technical differences, including differences in the type and quantity of serum and cell saturation density. In addition, we have shown that the phenotypic reversions of definitive share of tumorigenic cells correlate with the changes in cell sensitivity to exogenous growth factors.

Although the mechanisms for the loss of sensitivity of tumour cell to signals regulating proliferation are unknown, some hypotheses have been discussed [21–23]. A fuller investigation of the putative mechanism of carcinogenesis is needed for understanding of its genetics or epigenetics, or some combination of the two. If the changes underlying the mutual cell

Table 2. Tumorigenicity, inhibition of proliferation on pseudonormal cell monolayer and sensitivity to growth factors

Cell from:	Tumour frequency*	% Inhibition of [^{3}H]thymidine incorporation	Fold increase of [^{3}H]thymidine incorporation in response to: EGF	ECGF	PDGF	FGF
Non-transformed colonies						
FBALB1v12 (k46)	2/6	89.0 (6.1)	2.6 (1.1)	1.7 (0.3)	ND	1.3 (0.1)
FCBA2v10 (k23)	0/6	102.0 (2.6)	5.6 (1.5)	6.3 (0.4)	2.1 (0.3)	2.5 (0.2)
FCBA2v40 (k11)	0/7	97.8 (1.9)	1.8 (0.1)	2.9 (0.2)	2.0 (0.3)	1.8 (0.1)
FC3H3v7 (k20)	1/6	101.0 (11.9)	ND	ND	ND	ND
FC3H3v7 (k18)	0/4	104.0 (2.5)	6.2 (1.7)	3.3 (0.9)	ND	1.6 (0.1)
FC3H3v7 (k31)	0/6	100.0 (2.8)	4.6 (1.2)	5.1 (1.3)	ND	2.5 (1.1)
Transformed colonies						
FBALB1v12 (k11)	5/5 [6.2 (0.6)]	67.3 (12.2)	ND	ND	ND	ND
FCBA2v10 (k1)	6/6 [8.4 (1.4)]	76.4 (3.1)	1.0 (0.1)	1.1 (0.1)	1.2 (0.1)	1.1 (0.1)
FCBA2m	6/6 [6.9 (0.8)]	58.4 (4.7)	0.8 (0.03)	0.6 (0.1)	1.0 (0.1)	0.8 (0.1)
FC3H3v7 (k29)	4/4 [4.5 (1.3)]	65.4 (8.0)	1.1 (0.03)	1.1 (0.1)	ND	1.1 (0.1)
FC3H3v7 (k33)	4/4 [4.3 (1.2)]	67.4 (8.5)	1.1 (0.01)	1.1 (0.1)	ND	1.0 (0.1)

*In square brackets, tumour weight (g).
ND = not done.

transmutation have a common base in all tested cell cultures, then these cultures are a convenient model for studying the mechanism of uncontrolled growth of tumour cells, and for clarifying the reasons for phenotypical and genotypical instability of transformed cells.

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