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Correlation of Cellular Aggregation of Transformed Cells with Their Growth in Soft Agar and Tumorigenic Potential[1] (39836)

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Various morphological and physiological changes occur during or after the transformation of normal cells to the neoplastic state. Newly acquired properties such as colony formation in semisolid medium (1), enhanced fibrinolytic activity (2), growth in medium deficient in serum growth factor (3), and increased thymidine uptake (4) are often used as *in vitro* indicators of transformation. However, no single *in vitro* assay is consistently accurate in the prediction of tumorigenicity, the ultimate criterion for cellular transformation.

Using an agitation or rotation system, the formation of large aggregates by tumor cells has previously been reported (5-8). Namba and Sato (9) found that chemical-transformed rat liver cells and recultured tumor cells formed aggregates in rotation culture which were twice as large as those formed by untreated cells. They proposed this difference in aggregate size as an indicator of the malignant transformation in culture of cells treated with chemical carcinogens.

A cellular aggregation assay (static system) has recently been reported for the evaluation of *in vitro* viral transformation of rat and hamster embryonic cells (10). Transformed cells formed larger aggregates than counterpart normal cells when suspended in liquid medium above an agar base. Using a human osteosarcoma (HOS) cell line and its viral and chemical transformants, transformed cell aggregates were further characterized by an increase in cell growth and viability over untreated control cells (11). In keeping with these observations, this study reports the correlation between the cell aggregation assay and tumorigenicity using cell lines selected to represent spontaneous transformants as well as DNA tumor virus, RNA tumor virus, and chemical-induced transformed cells.

Materials and methods. The history of the cell lines used in this study is summarized in Table I. Cells were grown and maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 2 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin per milliliter.

Formation of cellular aggregates by normal and transformed cells was tested in triplicate using the method described by Steuer *et al* (11). Freshly trypsinized viable cells (2×10^5) were seeded into 35-mm plastic dishes containing a 2-ml agar base layer of 0.9% Bacto agar (Difco, Detroit, Michigan) in growth medium containing 20% FBS. The dishes were incubated undisturbed at 37° under 5% CO_2 in air. Viable cell counts were usually performed on day 4.

For the soft agar assay, cells were suspended in 0.36% agar medium (EMEM containing 20% FBS and antibiotics). This suspension was layered onto a 0.9% agar base layer at concentrations of 10^2 to 10^5 cells per 60-mm dish. Dishes were incubated in a humidified atmosphere (5% CO_2) at 37°. After 2 weeks, colonies 0.125 mm in diameter and larger were counted. Results are expressed as percentage plating efficiency (% PE = number of colonies × 100/ number of cells plated).

Transformed and untransformed rat, mouse, and hamster cell lines were tested for their ability to produce tumors in syngeneic animals after sc inoculation of $0.5-1.0 \times 10^6$ cells. Tumorigenicity studies of human cells were done in NIH athymic "nude" mice by Dr. Paul Arnstein, National Cancer

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TABLE I. DESCRIPTION AND HISTORY OF CELL LINES.

Cell Line	Description	Comments or literature references
Rat cells		
F-111	Fisher rat embryo	(12)
PyTRE/F-111	Polyoma-transformed F-111	(13)
3MC/F-111	3MC[a]-transformed F-111	Rhim *et al.*, unpublished data
Ki-MSV/F-111	Ki-MSV-transformed F-111	F-111 was infected with rat grown Ki-MSV and tests performed on early passage levels
RL #33	W/Fu rat liver #33	Provided by Dr. T. Okigaki
RL #34	W/Fu rat liver #34	Provided by Dr. T. Okigaki
RLC #5	Spontaneous transformed rat liver, Clone #5	Provided by Dr. T. Okigaki
Mouse cells		
Cl #36	NIH-Swiss mouse embryo, Clone #36	Rhim *et al.*, unpublished data
3MC/Cl #36	3MC-transformed Cl #36	Rhim *et al.*, unpublished data
MT-2	3MC/Cl #36 tumor cells	Recultured 3MC/Cl #36 tumor
NIH-MEC	NIH-Swiss mouse embryo	Obtained from Microbiological Associates
TME	Spontaneous transformed NIH-Swiss MEC	Obtained after prolonged *in vitro* culture
SMT	Spontaneous mouse tumor cells	Recultured TME tumor
BALB/3T3	BALB/c mouse embryo	(14)
Ki-MSV/3T3/NP	Ki-MSV-transformed 3T3 nonproducer cells	(14)
Hamster cells		
HEC	Syrian hamster embryo	Prepared from embryos removed from pregnant Syrian Golden hamster
BP/HEC	BP[b]-transformed hamster embryo	Provided by Dr. J. G. Vidrine
HT #3	Polyoma-transformed hamster embryo	Rhim *et al.*, unpublished data
HTC	HT #3 tumor cells	Recultured HT #3 tumor
Human cells		
HOS	Human osteosarcoma TE-85 clone F-5	(15)
Ki-MSV/HOS	Ki-MSV-transformed HOS	(16)
KHOS	Ki-MSV-transformed nonproducer HOS	(16)
240S	Revertant of KHOS	(17)
312H	Revertant of KHOS	(17)
MNNG-HOS	MNNG[c] (0.01 and 0.1 μg/ml)-transformed HOS clonal lines	(18)

[a] 3MC, 3-Methylcholanthrene.
[b] BP, Benzo(*a*)pyrene.
[c] MNNG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

Institute, National Institutes of Health, at the Viral and Rickettsial Disease Laboratory, California State Department of Health, Berkeley, California. Animals were observed for tumor development for at least 3 months following challenge.

Results. Single cell suspensions formed cell aggregates within 24 hr after seeding onto an agar base. Viable cell counts on trypsinized aggregates were performed on 5 consecutive days for rat embryo (F-111) cells and transformants (Fig. 1). Normal rat embryo cell aggregates were characterized by a rapid decline in cell viability while transformed cell aggregates showed a progressive increase in viable cell counts over this time period.

Cells suspended above an agar base (static system) formed aggregates with a morphology distinct for each cell line (Fig. 2). Aggregates varied in appearance from compact spheroids to irregular, diffuse forms. No single morphology was associated with the transformation event. Aggregate sizes were highly variable within experiments, with normal cells ranging from 0.01 to 0.10 mm in diameter and transformed cells ranging from 0.01 to 0.30 mm. On day 4, aggregates were classified according to their mean diameter as small (<0.04 mm), moderate (0.04–0.08 mm), or large (>0.08 mm). Characteristic morphology and size

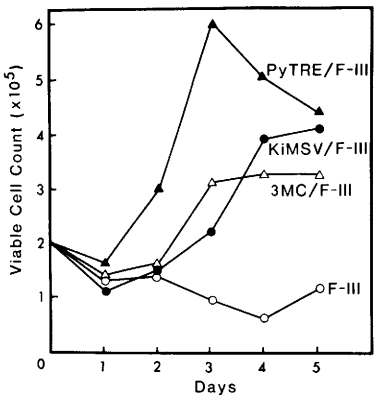


FIG. 1. Short-term growth curves of rat embryo cell lines. Viable cell counts were performed on trypsinized aggregates (initial cell concentration was 2×10^5 cells/dish). ○——○, F-111 (normal Fisher rat embryo); △——△, 3MC-treated F-111; ●——●, Ki-MSV-transformed F-111; and ▲——▲ polyoma virus-transformed F-111.

distribution were consistently reproducible between experiments.

Viability and proliferation of aggregates were compared to growth in soft agar and tumorigenicity of rat cells (Table II). Normal rat embryo and rat liver cells were unable to grow in soft agar or proliferate in the

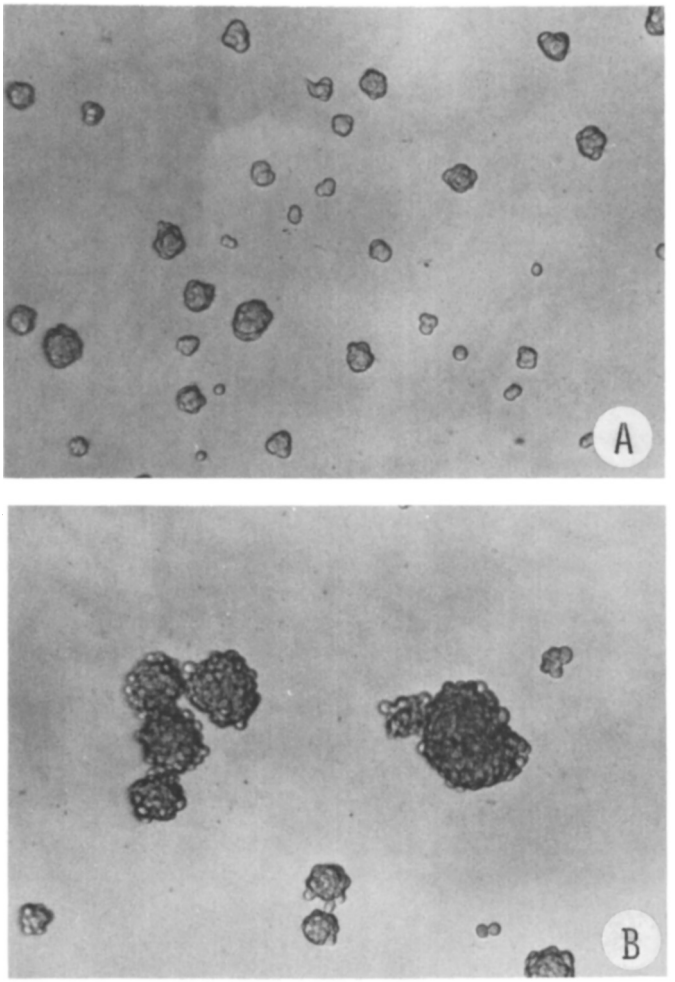
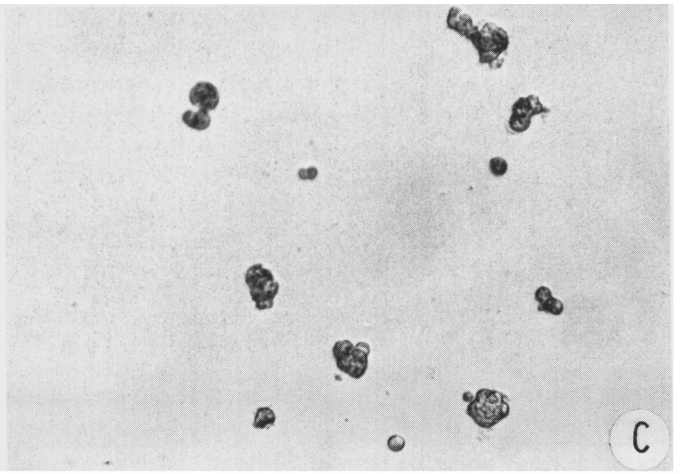
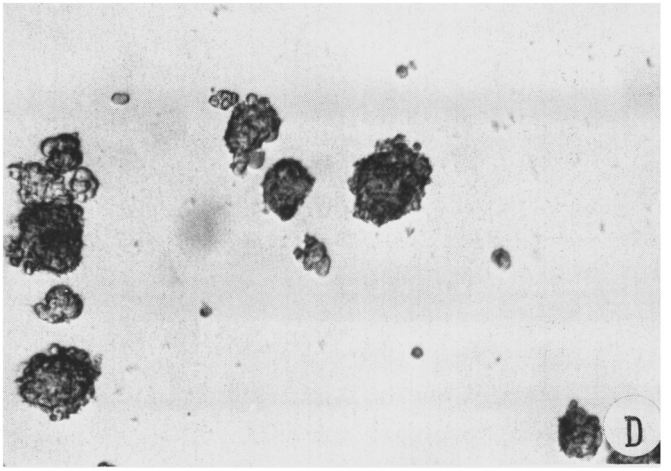


FIG. 2. Aggregates formed after 4 days using a static agar system. Cells were allowed to aggregate undisturbed in liquid growth medium above an agar base layer. 70 ×. (a) Aggregates of NIH Swiss MEC, clone #36; (b) aggregates of 3MC-treated clone #36; (c) aggregates of hamster embryonic cells; (d) aggregates of BP-treated hamster embryonic cells; (e) aggregates of Fisher rat embryo cell line F-111; (f) aggregates of 3MC-treated F-111; (g) aggregates of Ki-MSV-transformed F-111; and (h) aggregates of polyoma virus-transformed F-111.





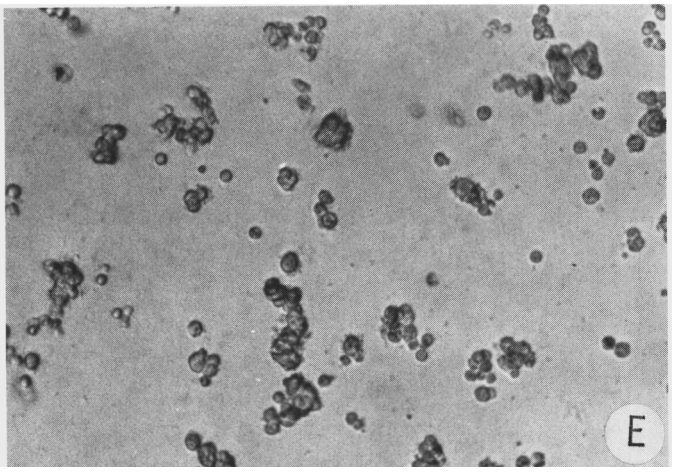
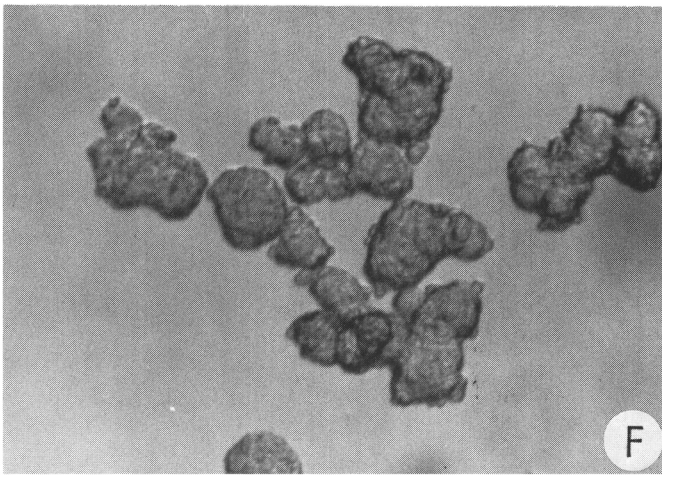
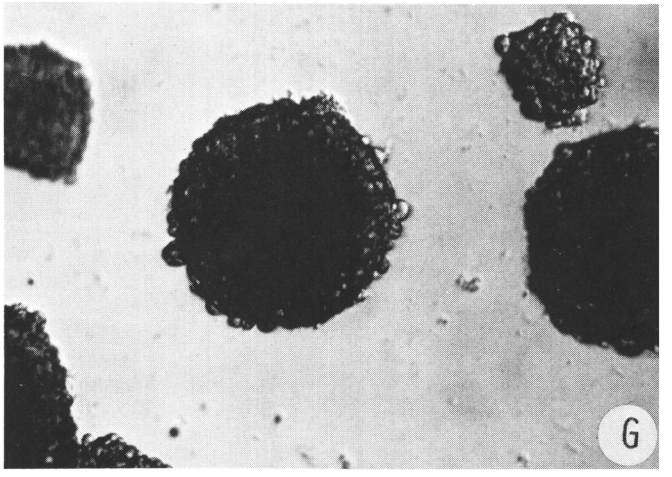


FIG. 2 C-E





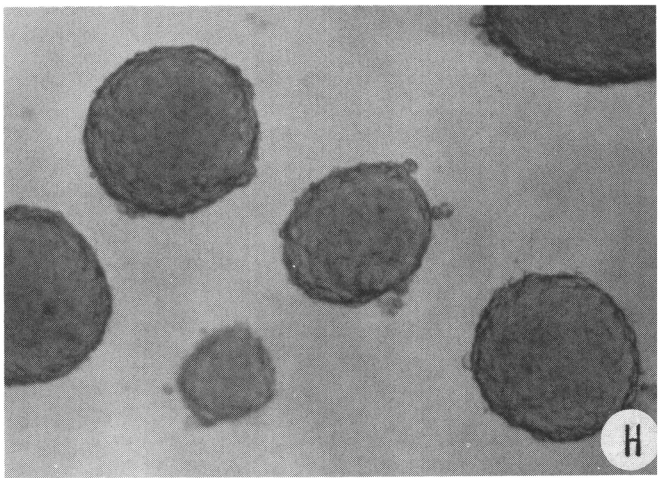


FIG. 2 F–H

aggregate form. Tumorigenic rat cells, regardless of the method of induction (viral, chemical, or spontaneous), grew in soft agar as well as in the aggregate form.

Normal mouse embryo cells did not form colonies in soft agar nor proliferate in the aggregate form (Table II). Ki-MSV and 3-methylcholanthrene(3MC) transformed mouse cells were tumorigenic and grew in soft agar and in aggregates. Unlike other transformed mouse cells, spontaneous transformed Swiss mouse embryo cells (TME) were unable to proliferate in the aggregate form. TME was, however, able to maintain viability in the aggregate form when compared to its normal counterpart cell line (NIH-ME). Recultured tumor cells (SMT) grew well in the aggregate form.

Normal hamster embryo cells failed to grow in either soft agar or in the aggregate form (Table II). Polyoma-transformed hamster embryo (HT #3) grew in soft agar and maintained viability in the aggregate form while its counterpart transplanted tumor

TABLE II. VIABILITY OF CELL AGGREGATES, GROWTH IN SOFT AGAR, AND TUMORIGENICITY OF NORMAL AND TRANSFORMED RAT, MOUSE, AND HAMSTER CELLS.

Cell line	Cell aggregates: Size	Cell aggregates: Cell count[a] ($\times 10^5$)	Plating efficiency in soft agar[b] (%)	Incidence of tumors in syngeneic host[c]
Rat cells				
Normal				
F-111	Small	0.6	0.3	0/5
RL #33	Small	1.2	<0.01	0/5
RL #34	Small	0.5	<0.01	0/5
RNA virus-transformed				
Ki-MSV/F-111	Large	3.9	4.4	NT[d]
Ki-MSV/RL #33	Large	4.6	18.6	5/5
Ki-MSV/RL #34	Large	3.6	9.0	5/5
DNA virus-transformed				
PyTRE/F-111	Large	5.0	47.0	5/5
Chemical-transformed				
3MC/F-111	Large	3.2	67.0	5/5
Spontaneous-transformed				
RLC	Large	8.8	48.0	5/5
Mouse cells				
Normal				
BALB/3T3	Small	1.2	0.02	NT
Cl #36	Small	1.3	0.03	0/4
NIH-MEC	Small	1.0	0.01	0/4
RNA virus-transformed				
Ki-MSV/BALB/3T3	Large	4.3	25.5	NT
Chemical-transformed				
3MC/Cl #36	Large	4.0	4.2	4/4
MT-2	Large	3.4	6.0	4/4
Spontaneous-transformed				
TME	Moderate	1.8	6.7	4/4
SMT	Large	4.0	42.0	4/4
Hamster cells				
Normal				
HEC	Small	0.5	0.01	0/5
DNA virus-transformed				
HT #3	Large	2.0	2.7	5/5
HTC	Large	2.4	1.8	5/5
Chemical-treated				
BP/HEC	Large	2.1	6.5	5/5

[a] Viable cell counts were determined by trypan blue dye exclusion on day 4.
[b] Percentage was calculated as number of colonies formed × 100/number of cells plated.
[c] Number of animals that developed tumors/number of animals challenged.
[d] NT, not tested.

cells (HTC) and benzo(*a*)pyrene(BP) transformed hamster embryo cells grew in soft agar and in aggregates.

Viability and proliferation of virus and chemical-transformed human osteosarcoma cells were compared to growth in soft agar and tumorigenicity in nude mice (Table III). The control HOS, dimethylsulfoxide (DMSO)-treated HOS clone 5, and KHOS revertants 240S and 312H formed colonies in soft agar but failed to proliferate in the aggregate form. All were nontumorigenic. Ki-MSV-transformed producer and nonproducer HOS and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-transformed HOS cells grew in the aggregate form, formed colonies in soft agar, and were tumorigenic in nude mice.

Discussion. Viability and proliferation in the aggregate form were compared to growth in soft agar, as indicators of the tumorigenic potential of cells in culture. Transformed rat, mouse, hamster, and human cells were found to form aggregates twice as large as those formed by counterpart nontransformed cells. Similar observations have been reported for chemical-transformed rat liver cells (9) and viral transformed rat and hamster embryo cells (10). Transformed cells either maintained viability or proliferated in the aggregate form while normal cells were characterized by a progressive decline in cell viability. Because of the varied size distribution of normal and transformed cell aggregates, proliferation in the aggregate form was considered to be more indicative of tumorigenic potential than aggregate size differences alone.

Earlier studies have indicated that an increased capacity of transformed cells to form colonies in soft agar was associated with tumorigenicity in immunologically compatible hosts (1) or in nude mice (19). DMSO-treated and untreated HOS cells and KHOS revertants were able to form colonies in soft agar. Since these cells were unable to produce tumors in ATS-treated hamsters (20), ATS-treated mice (21), as well as nude mice (17, 18), colony formation in soft agar was not a reliable indicator of tumorigenicity. The virus and chemical-transformed HOS cells did grow in the aggregate form while the control HOS cells and KHOS revertants did not; thus the proliferation of cellular aggregates was a reliable indicator of tumorigenic potential.

Growth in soft agar is a transformed characteristic associated with loss of anchorage dependence. Cells in the aggregation system initially adhere to one another providing a

TABLE III. RELATIONSHIP OF VIABILITY OF CELL AGGREGATES AND GROWTH IN SOFT AGAR TO TUMORIGENICITY OF HUMAN CELL LINES.

Cell line	Cell aggregates			
	Size	Cell count[a] ($\times 10^5$)	Plating efficiency in soft agar[b] (%)	Incidence of tumors in nude mice[c]
HOS, Human osteosarcoma TE85, clone F-5	Small	1.2	1.0	0/5
Ki-MSV/HOS, Ki-MSV-transformed HOS, producer	Large	3.3	10.7	5/5
KHOS, Ki-MSV-transformed nonproducer HOS	Large	3.2	7.0	5/5
Revertants of KHOS				
240S	Small	0.5	1.1	0/2
312H	Small	0.4	1.1	0/2
DMSO-Treated HOS				
Clone 5	Small	1.0	2.0	0/5
Clone 6	Small	1.3	0.5	0/5
MNNG-Treated HOS (0.1 μg/ml)				
Clone 2	Large	2.3	2.0	3/5
MNNG-Treated HOS (0.01 μg/ml)				
Clone 3	Large	3.3	2.1	5/5
Clone 5	Large	3.2	5.4	5/5

[a] Viable cell counts were determined by trypan blue dye exclusion on day 4.
[b] Percentage was calculated as number of colonies formed × 100/number of cells plated.
[c] Number of nude mice that developed tumors/number of nude mice injected.

cellular substratum which may favor cell growth. Significant aggregate size differences occur after 3–4 days. This suggests that the variations in size were due to different survival and proliferative capabilities of normal and transformed cells. The mechanisms for these survival differences are not known, but may be related to cellular nutritional requirements. It could be that transformed cells survive better than normal cells under conditions of nutrient deprivation and waste buildups which exists in the central regions of cell aggregates. Three-dimensional stresses on cells in the aggregate form could affect cell shape and thus influence membrane transport of nutrients. Whatever the mechanisms of survival may be, the aggregation assay resembles the three-dimensional situation found in the early stages of *in vivo* tumor formation. In fact, Sutherland *et al.* (8) have proposed the growth of multicell spheroids as an *in vitro* model for nodular carcinomas.

Evidence is presented here to show that the aggregation assay may be used to monitor the tumorigenic potential of spontaneous-, virus-, or chemical-transformed cells of various animal species. This assay could provide a fast, accurate, and reproducible means of evaluating *in vitro* transformation.

Summary. Formation of cellular aggregates by rat, mouse, hamster, and human transformed cells was compared to aggregate formation by untransformed control cells. Viability and proliferation of cells in the aggregate form were found to correlate well with tumorigenic potential regardless of the method of transformation (spontaneous, chemical, or virus-induced). This assay has the potential for a fast and accurate means of evaluating *in vitro* transformation.

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