

## BALB/c 3T3 cell transformation assays for the assessment of chemical carcinogenicity

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### Abstract

*In vitro* cell transformation assays have been proposed for predicting the carcinogenic potential of chemicals. Morphological transformation can be induced by exposing mammalian cells to carcinogens. The cellular and molecular processes involved in cell transformation in the cultures are assumed to be similar to those of *in vivo* carcinogenesis. BALB/c 3T3 is an established cell line transformable with genotoxic and nongenotoxic carcinogens and produce foci in the cultures which lose the property of contact-inhibition of growth. We can perform a two-stage cell transformation assay which mimics a two-stage carcinogenicity test in experimental animals. In the two-stage cell transformation assay, a tumor promoter is identified if a test chemical enhances cell transformation by exposure of cells in the promotion stage following a known carcinogen (a tumor initiator) in the initiation stage; initiating activity of a chemical is tested by post-treatment of cells with a known tumor promoter in the promotion stage. The BALB/c 3T3 cell transformation assay has been modified by several research groups to improve its performance including sensitivity, reliability, assay period and cost. Recently a short-term cell transformation assay has been developed using the v-Ha-*ras*-transfected cell line, Bhas 42.

**Keywords:** cell transformation, BALB/c 3T3 cells, Bhas 42 cells, nongenotoxic carcinogen, tumor promoter

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### Introduction

Genotoxicity assays *in vitro* have been routinely used to predict the carcinogenicity of chemicals and contributed to eliminating or reducing carcinogens in human environments. However, it is an issue that a considerable number of chemicals are negative in genotoxicity assays but carcinogenic in experimental animals. The battery of screening tests for carcinogens is required to include an assay of a category different from the genotoxicity assays in order to increase the accuracy of the chemical safety assessment. An *in vitro* cell transformation assay is a unique system that offers potential benefit in such screening (Kakunaga and Yamasaki, 1985; Montesano et al., 1986).

### Cell transformation assays

Cell transformation is defined as the acquisition of certain malignant characteristics in morphology, behavior, growth control or function, and induced by chemical carcinogens *in vitro*. Transformation occurs as a result of comprehensive cellular responses to direct and indirect damage to DNA, genes and cellular machinery, including altered gene expression and signal transduction. A cell transformation assay is an *in vitro* assay measuring the phenotypic

conversion from normal to malignant characteristics in mammalian cells exposed to test chemicals, and capable of detecting non-genotoxic as well as genotoxic carcinogens (Sakai et al., 2002). There exist several types of cell transformation assay. Focus assays are carried out using BALB/c 3T3 cells or C3H/10T1/2 cells, and their endpoint is loss of contact inhibition of cell growth. Soft agar-colony assays are performed with BHK21 cells and JB6 cells, and their endpoint is loss of anchorage independence. A colony assay utilizes Syrian hamster embryo (SHE) cells and the morphological change of colonies as the endpoint. BALB/c 3T3, C3H/10T1/2, BHK21 and JB6 cells are established cell lines, but SHE cells are primary cultures.

### BALB/c 3T3 cell transformation assay

BALB/c 3T3 cells originated from BALB/c mouse whole embryo cultures (Aaronson & Todaro, 1968). They possess the ability to divide indefinitely, but are highly sensitive to the post-confluence inhibition of cell division. The cells grow in a monolayer and never pile up, ceasing division when they cover the dish surface. However, if the cells are treated with a carcinogen at the beginning of cultivation,

there appears a population of cells which do not stop proliferation at confluence but grow over contact-inhibited normal cells to produce foci of aberrant cell morphology. These transformed cells are spindle-shaped, multilayered, dense and with basophilic staining, and show random orientation at the focus edge. The most transformed BALB/c 3T3 cells are tumorigenic in nude mice. At present, the clone A31-1-1 is used for the BALB/c 3T3 cell transformation assays, unless otherwise specified. This clone is sensitive to chemical transformation and is stable for spontaneous transformation (Kakunaga and Crow, 1980).

### 1) Standard cell transformation assay

The BALB/c 3T3 cell transformation assay is designed to allow the expression of transformed foci (IARC/NCI/EPA Working Group, 1985). In the standard assay, the cells are seeded at  $1 \times 10^4$  cells/60-mm dish in at least 12 replicates and exposed to a test chemical in the culture medium for 72 h from 24 h after inoculation. On termination of exposure, the treatment medium is replaced with normal medium and the cultures are maintained for a further 4 to 6 weeks receiving medium change twice a week. In these conditions, the cells grow to sub-confluence in a week and attain confluence in 8 days. During this growing phase, carcinogen-treated cells can divide several times and some DNA damage caused by the carcinogen can be fixed as mutations. After confluence the normal cells move to a stationary phase but the transformed cells continue multiplying to form foci.

### 2) Two-stage cell transformation assay

Cell transformation mimics some stages of *in vivo* multistage carcinogenesis. A tumor promoter, which is a typical non-genotoxic carcinogen, produces no foci by itself in BALB/c 3T3 cultures. However, when the cells are treated with a sub-threshold dose of a carcinogen (a tumor initiator) at the beginning of cultivation and then with a tumor promoter, transformed foci are produced. Thus we can perform two-stage cell transformation assays similar to *in vivo* two-stage carcinogenesis tests (Sasaki et al., 1986; Sakai and Fujiki, 1991). The starting time of promoter treatment seems to be critical to design a sensitive two-stage transformation assay. We researched the optimum starting time of promoter treatment, using a sub-threshold dose of 3-methylcholanthrene (MCA) as the initiator and 12-*O*-tetradecanoylphorbol 13 acetate (TPA) as the promoter (Sakai and Sato, 1989). Maximum focus formation was obtained when the promoter treatment was started at 1-2 days subconfluence of the cultures (Fig. 1). Thus, in the two-stage cell transformation assay, the cells seeded at  $1 \times 10^4$  cells/60-mm dish are treated with an tumor

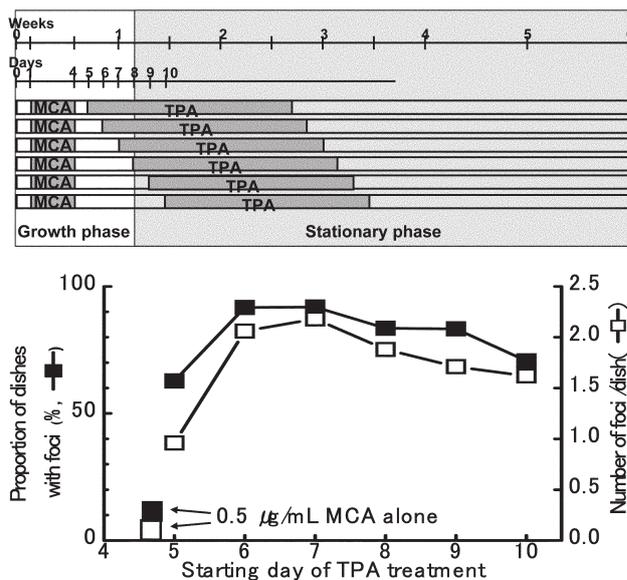


Fig. 1. Changes in the sensitivity of the two-stage BALB/c 3T3 cell transformation assay by the starting time of promoter treatment.

BALB/c 3T3 cells were seeded at a density of  $4 \times 10^4$  cells/60-mm dish and treated with  $0.5 \mu\text{g/mL}$  of 3-methylcholanthrene for 3 days from 24 h after inoculation. The promoter treatment was started at different days and continued for 2 weeks as indicated in the figure. After cultivation for 6 weeks in total, cells were fixed and stained with Giemsa solution and transformed foci were counted.

initiator for 3 days from 24 h after inoculation, cultured in normal medium for 3 days, then exposed to a tumor promoter for 2 weeks, and thereafter maintained in normal medium a further 3 weeks. The assay duration in total is usually 6 weeks.

The two-stage protocol is utilized for measuring tumor promoting activities and also for enhancing the sensitivity of cells to the weak initiators the standard assay is unable to detect (Sakai and Sato, 1989).

### Performance of the cell transformation assays

Results of BALB/c 3T3 cell transformation assays – including standard and modified methods, and two-stage assays – have been reported on about 180 chemicals. We evaluated the performance of the BALB/c 3T3 cell transformation assay to predict chemical carcinogenicity, using the assay results reported for 143 chemicals whose carcinogenicity data in human or rodents were available. The concordance, sensitivity and specificity were 69%, 76% and 52%, respectively. For the C3H/10T1/2 cell transformation assay, results of about 140 substances have been reported and its concordance, sensitivity and specificity were 72%, 71% and 78%, respectively, as evaluated for 98 compounds. Tumor promoters were excluded from these evaluations. The performance level of cell transformation assays was similar to that of genotoxicity assays. However, the concordance and sensitivity of Ames assay, the

Table 1. Correlation of the two-stage transformation assays with *in vivo* assays for tumor promoters

Chemical	Transformation Focus assay		<i>In vivo</i>		Reference of two-stage cell transformation
	BALB/c 3T3	C3H/10T1/2	Mouse	Rat	
TPA	+	+	Skin		Mondal & Heidelberger, 1976 Sakai & Sato, 1989 and others
Phorbol 12,13- didecanoate	+		Skin		Enomoto & Yamasaki, 1985 Katoh et al., 1990 and others
Mezerein	+	+	Skin		Boreiko et al., 1986 Sasaki, unpublished data
Okadaic acid	+		Skin		Katoh et al., 1990 Sakai & Fujiki, 1991
Dinophysistoxin-1	+		Skin		Sakai & Fujiki, 1991
Dihydrotelectocidin B	+		Skin		Hirakawa et al., 1982
2,3,7,8-Tetrachloro- dibenzodioxin (TCDD)	+	+	Skin, Lung, Liver		Abernethy et al., 1985 Fang, et al., 2001
Phenobarbital	+			Liver	Fang, et al., 2001
Sodium saccharin	-	+		Bladder	Mondal et al., 1978 Sakai, unpublished data
Catechol	+			Forestomach	Atchison et al., 1982
Chenodeoxycholate		+		Colon	Kaibara et al., 1984
Cholate	±	+		Colon	Kaibara et al., 1984 Umeda et al., 1989
Lithocholate	+	+		Colon	Kaibara et al., 1984 Umeda et al., 1989

most common genotoxicity assay, was somewhat low (52% and 40%, respectively), when the performances of the Ames assay was calculated for the set of chemicals evaluated for their transformation potency. This may indicate that more Ames negative carcinogens have been tested by cell transformation assays than Ames positive carcinogens. Even taking this into account, the above fact suggests that cell transformation assays are sensitive to a proportion of carcinogens not detected by Ames assay. Therefore, the cell transformation assay is recognized as a complementary *in vitro* partner of genotoxicity assays in the battery of tests to predict carcinogenicity of chemicals.

#### Correlation of the two-stage cell transformation assays with *in vivo* assays for tumor promoters

A considerable number of substances have been reported for promotional activity in the two-stage cell transformation assays (Sakai et al., 2002). Among them tumor promoters verified in rodents are listed in Table 1. All *in vivo* tumor promoters enhance the transformation of BALB/c 3T3 and/or C3H/101/2

cells except for saccharin in BALB/c 3T3 cells. They include mouse skin-, rat liver- and rat colon-tumor promoters. Thus two-stage transformation assays appear to be effective methods to screen tumor promoters.

There are conflicting though unpublished data for phenobarbital; promoting activity was not detected in one test in the two-stage BALB/c 3T3 cell transformation assay (Hayashi et al., personal communications).

#### A short-term transformation assay using a sensitive cell line, Bhas 42

The BALB/c 3T3 cell transformation assay is rapid and cost-efficient compared to *in vivo* assays, but is still time-consuming and expensive when compared with *in vitro* genotoxicity assays. Recently, a short-term cell transformation assay has been developed, using a sensitive cell line, Bhas 42. Bhas 42 was established by Sasaki et al from BALB/c 3T3 cells transfected with *v-Ha-ras* gene and is a morphologically non-transformed but TPA-sensitive clone (Sasaki et al., 1988). Bhas 42 cells are regarded

as being initiated (Sasaki et al., 1990).

The Bhas 42 cell transformation assay was proposed by Omori et al. to examine tumor-promoting activity of chemicals (Omori et al., 2004). The assay period has been shortened to 3 weeks in total thus half the 6 weeks of the BALB/c 3T3 assay. The dish size has been diminished from 60-mm dishes of the BALB/c 3T3 assay to 6-well dishes. Accordingly the volume of medium per dish has been reduced from 4 mL to 2 mL. The number of dishes for one dose group has been reduced from 10 dishes to 6 wells, and fetal bovine serum has been decreased from 10 % to 5 % of medium. Thus the Bhas assay is markedly scaled down from the BALB/c 3T3 assay and is more cost efficient.

Asada et al. reported that Bhas 42 cells are capable of detecting both tumor initiators and promoters as transformation initiators and promoters by a slight modification of the assay protocol (Asada et al., 2005). Moreover the modified Bhas assay can distinguish between tumor initiators and promoters by changing the inoculum cell number and the time of chemical treatment. In the initiation assay, the cells are inoculated at a low density, and treated with a test chemical for 3 days from 24 h after inoculation to detect initiating activity of the chemical. For the promotion assay, the cells are seeded more densely so that the cultures become sub-confluent at day 4. The chemical to be tested for promoting activity is added to the sub-confluent cultures and the chemical treatment continues for 10 days. The total culture duration is 21 days in either assay. A representative assay result is shown in Fig. 2. The typical carcinogen MCA induced many transformed foci in the initiation assay, but not in the promotion assay, and the tumor

promoter TPA produced a large number of foci in the promotion assay, but not in the initiation assay.

As the cell transformation assay has been scaled down by the use of Bhas 42 cells, the required amounts of test substances have become smaller. We have been testing various mycotoxins which are expensive natural products produced by fungi. Fumonisin B<sub>1</sub> and T-2 toxin showed promoting activities in the Bhas 42 cell transformation assay (Sakai et al., 2007).

### Gene expressions altered by tumor promoters and a plan of new assay system

Tumor promoters are known to induce altered expressions of various genes in animal organs and cultured cells. However, the changes causally related to the enhancement of tumorigenesis and cell transformation remain to be defined. It was postulated that the common alterations in the gene expressions induced by different tumor promoters are possibly highly involved in the enhancement of cell transformation and carcinogenesis (Sakai et al., 2003). The identification of such genes must aid to clarify the mechanisms of action for tumor promoters. We could develop a mechanism-based short term assay to predict tumor-promoting activity of chemicals by utilizing those genes as marker genes. We can estimate mRNA levels of the marker genes in the cells exposed to a test chemical with RT-PCR. Moreover we could view expressions of the marker genes under a microscope by constructing luminescent reporter cells which glow in response to tumor promoters.

Among the chemicals tested for promoting activity in the two-stage BALB/c 3T3 cell transformation assay in our laboratory, TPA, okadaic acid,

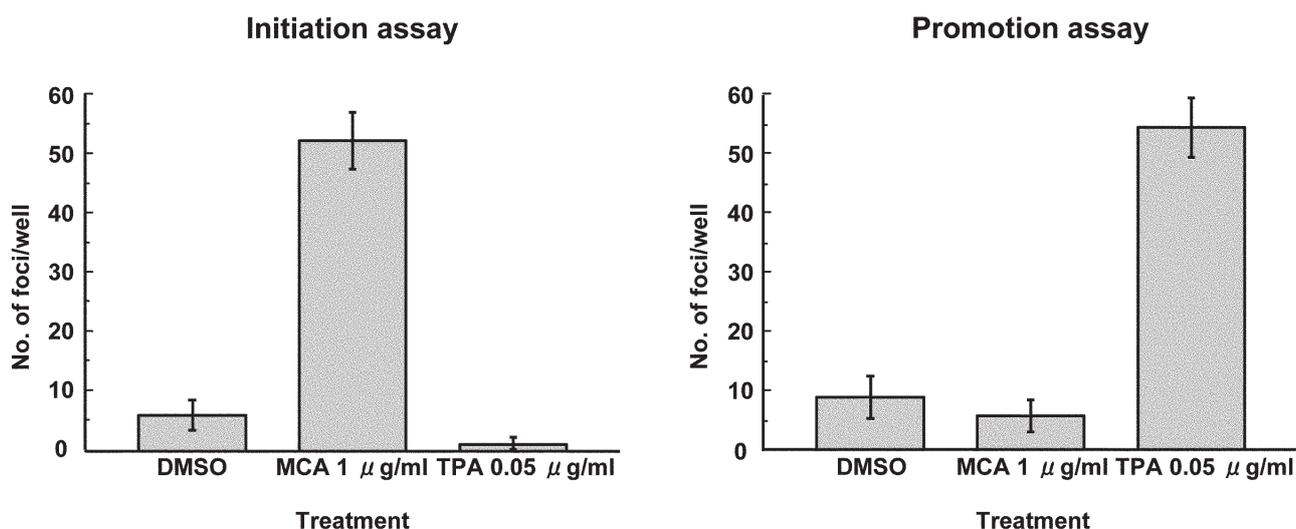


Fig. 2. Discrimination between a tumor initiator and a promoter in the Bhas 42 cell transformation assays. In the initiation assay, Bhas 42 cells were seeded onto 6-well microplates at a density of  $4 \times 10^3$  cells/well (Day 0), treated with MCA, TPA or the solvent for 3 days from 24 h after seeding and thereafter cultured with normal medium until Day 21. In the promotion assay, the cells were seeded at a density of  $2 \times 10^4$  cells/well, grown in normal medium for 4 days to achieve subconfluence, treated with medium containing MCA, TPA or the solvent from Day 4 to Day 14, and cultivated in normal medium till Day 21.

orthovanadate and *p*-nonylphenol were potent (Sakai et al., 2002). A preliminary study to search gene expression alteration induced by these transformation enhancers was carried out in BALB/c 3T3 cells under the condition of two-stage cell transformation

assay, using mRNA differential display analysis. The altered gene expressions were confirmed by RT-PCR. Elevated expressions of 7 genes and decreased expressions of 7 genes were found (Table 2). TPA and okadaic acid caused common changes in the

Table 2. Features of mRNAs increased and decreased in BALB/3T3 cells treated with tumor promoters<sup>a,b</sup>

Gene symbol	Promoter <sup>c</sup> : Time <sup>d</sup>	Gene name	NCBI RefSeq	Product	Human homolog
Increased					
Ass1	T: 7 hr O: 24 hr & 7 days	argininosuccinate synthetase 1	NM_007494	argininosuccinate synthetase NP_031520	ASS
Lgals3bp	O: 24 hr & 7 days	lectin, galactoside-binding, soluble, 3 binding protein	NM_011150	lectin, galactoside-binding, soluble, 3 binding protein NP_035280	LGALS3BP
Ly6e	T: 7 hr O: 24 hr & 7 days	lymphocyte antigen 6 complex, locus E	NM_008529	lymphocyte antigen 6 complex, locus E NP_032555	LY6E
Nudt9	T: 7 hr O: 24 hr & 7 days	nudix (nucleoside diphosphate linked moiety X)-type motif 9	NM_028794	nudix (nucleoside diphosphate linked moiety X)-type motif 9 NP_083070	NUDT9
Plat	O: 24 hr	plasminogen activator, tissue	NM_008872	plasminogen activator, tissue NP_032898	PLAT
Ssb	N: 7 hr	Sjogren syndrome antigen B	NM_009278	autoantigen La NP_033304	SSB
Sned1	N: 7 hr, 24 hr & 7 days	secreted protein SST3	NM_172463	Secreted nidogen domain protein, NP_766051	FLJ00133
Decreased					
AI428795	T: 7 hr	expressed sequence AI428795	NM_024477	expressed sequence AI428795 NP_077797	
ND1	N: 24 hr	NADH dehydrogenase subunit 1		NADH dehydrogenase subunit 1 NP_904328	MT ND1
Rpl3	O: 24 hr & 7 days	ribosomal protein L3	NM_013762	ribosomal protein L3 NP_038790	RPL3
Sparc	T: 7 hr & 7 days	secreted acidic cysteine rich glycoprotein	NM_009242	secreted acidic cysteine rich glycoprotein NP_033268	SPARC
Thbs1	T: 7 hr & 7 days O: 24 hr	thrombospondin 1	NM_011580	thrombospondin 1 NP_035710	THBS1
Vim	O: 24 hr & 7 days N: 24 hr	vimentin	NM_011701	Vimentin NP_035831	VIM
EST (BY594155)	T: 7 hr O: 7 days		BY594155 AA168524		

<sup>a</sup> Messenger RNAs whose expressions were increased or decreased beyond double or half of those in the corresponding controls are listed.

<sup>b</sup> This table was completed by summarizing the information offered by the National Center for Biotechnology Information in the USA at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

<sup>c</sup> T, TPA; O, Okadaic acid; N, Nonylphenol

<sup>d</sup> The time when up- or down-regulated expression of the mRNA was observed.

expressions of 5 genes, though the time courses of the changes were different between TPA and okadaic acid. The common alterations in gene expressions may suggest the existence of common actions on the cells. It is reported that TPA and okadaic acid upregulate protein phosphorylation through the acceleration of phosphorylation and inhibition of dephosphorylation, respectively, at the serine/threonine residues. No common gene has yet been identified among genes that were regulated by these four non-genotoxic transformation promoters. We have started a new project to construct luminescent reporter cells which are used for detection of carcinogens including tumor promoters. The project uses a DNA chip analysis, which is much more exhaustive, to detect marker genes.

### Conclusions

1. Cell transformation assays can complement genotoxicity assays in screening chemicals for carcinogenic activity.

2. We can conduct a two-stage cell transformation assay *in vitro* similar to two-stage carcinogenicity assays *in vivo*. In the two-stage transformation assay using BALB/c 3T3 cells, we can detect tumor promoters and also weak initiators we are unable to detect in the standard assay.

3. A short-term cell transformation assay has been developed using a sensitive cell line, Bhas 42.

4. The analysis of gene expression involved in cell transformation by carcinogens should be used for development of a high-throughput test to predict chemical carcinogenicity.

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