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Neoplastic transformation of mouse mammary epithelial cells by *in vitro* exposure to *N*-methyl-*N*-nitrosourea

(chemical carcinogenesis/mammary tumorigenesis/preneoplasia/collagen culture/serum-free)

SHIGEKI MIYAMOTO*, RAPHAEL C. GUZMAN, REBECCA C. OSBORN, AND SATYABRATA NANDI

Cancer Research Laboratory and Department of Zoology, University of California, Berkeley, CA 94720

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ABSTRACT **High-efficiency neoplastic transformation of mouse mammary epithelial cells in primary collagen gel culture was induced by *N*-methyl-*N*-nitrosourea (MNU). Mammary epithelial cells, isolated from virgin BALB/c mice, were embedded within collagen gels and grown in a serum-free medium containing prolactin, progesterone, and linoleic acid. The cells were then treated with MNU on day 3 of culture and subsequently at weekly intervals for up to 4 weeks. Eleven to 14 days after the final carcinogen treatment, the cells were removed from the collagen gels and injected into the cleared mammary fat pads of syngeneic hosts to assay for transformed cell populations. A single exposure or multiple exposures of these cells to MNU was effective in inducing tumorigenic cells that produced palpable tumors as early as 6 weeks after transplantation. Two treatments with MNU (100 μg/ml) were optimal for neoplastic transformation and produced tumors in 79% of the injected fat pads. All the tumors originated at the site of injection and had extensive central necroses. Histological examination indicated that the tumors were mammary carcinomas. Secondary transplantation of tumor pieces into intact mammary glands produced palpable carcinomas of the same histology within 1–8 weeks. Control cells cultured for the same periods of time as MNU-treated cells produced only ductal outgrowths that were morphologically similar to those found in the mammary glands of adult virgin hosts. This system provides a distinct means to study the mechanism of mammary neoplastic transformation at cellular and molecular levels.**

Mammary neoplasias induced by chemical carcinogens have been studied extensively in animal model systems. These *in vivo* studies have provided invaluable information about effective carcinogens, susceptible strains, the sensitive age groups, hormonal and dietary influences, and the presence of preneoplastic stages in mammary tumor development (1–3). It has been difficult, however, to directly study the mechanisms underlying the neoplastic transformation of mammary cells because of inherent complexities present *in vivo*.

In vitro transformation systems provide a distinct opportunity to directly study the mechanisms of transformation under well-controlled conditions because (*i*) mammary epithelial cells with or without their accompanying stroma can be treated with carcinogens, (*ii*) the direct effects of different hormones and growth factors on transformation can be studied, (*iii*) the molecular effects of carcinogens can be examined, and (*iv*) the cellular origin of various mammary tumors observed *in vivo* can be elucidated. Many attempts have been made to develop a suitable system to transform mammary cells in cell and organ cultures (4–10). In previous studies transformed mammary cells were identified by enhanced growth potential (4–6) or anchorage independence (7) in cell culture, induction of alveolar lesions (8, 9) in organ culture, and induction of preneoplastic lesions or carcinomas (9, 10) in animal hosts. Unfortunately, these systems have not afforded a significant advantage in understanding the mechanism of mammary cell transformation because of their low transformation efficiencies or prolonged latencies required for tumor induction.

We have described (11) a primary mouse mammary epithelial cell culture system in which carcinogen-treated cells could produce preneoplasias at high efficiencies. In this system, mammary epithelial cells were grown inside collagen gels (12, 13) in serum-free medium (14, 15). Transformation was assayed by transplanting cultured cells into cleared mammary fat pads of syngeneic female mice (16). Cells not exposed to carcinogens formed characteristic mammary ductal outgrowths. When the cells were grown in the serum-free medium and then treated with *N*-methyl-*N*-nitrosourea (MNU) or 7,12-dimethylbenz(a)anthracene, on the other hand, high incidences (up to 83%) of preneoplasias were observed. Some of these preneoplasias, upon secondary transplantation, produced mammary carcinomas after long latencies. Although these results are of great significance, no mammary carcinomatous transformation was directly induced in culture.

In the present study, we have used our earlier most effective transforming conditions, the serum-free growth medium containing prolactin, progesterone, and linoleate with the direct-acting chemical carcinogen MNU, which resulted in a high incidence of preneoplastic lesions. Utilizing these optimal conditions, we have made several significant changes in our experimental protocol in attempts to induce carcinomatous transformation of mouse mammary epithelial cells in primary culture. We describe here the finding that mammary carcinomas of extremely short latencies can be induced reproducibly in serum-free medium by *in vitro* exposure of normal mouse mammary cells to MNU.

MATERIALS AND METHODS

Animals. All mice were from the BALB/cCrgl strain maintained at the Cancer Research Laboratory, University of California, Berkeley. The BALB/cCrgl strain does not express murine mammary tumor virus and has a low incidence of spontaneous mammary tumors as well as of mammary dysplasias (17). Three- to four-month-old virgin female mice were sacrificed to obtain mammary tissue for cell culture, and 3-week-old female mice were utilized as transplantation hosts.

Tissue Dissociation. The procedure of mammary epithelial cell isolation was modified in the following way from the previously reported technique (11, 13, 15). Briefly, all 10 mammary glands were removed from mice, minced finely,

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Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; HANs, hyperplastic alveolar nodules; DHs, ductal hyperplasias; TPA, phorbol 12-tetradecanoate 13-acetate; Me_2SO, dimethylsulfoxide.

*To whom reprint requests should be addressed.

and then digested in a 150-ml flask containing 0.1% collagenase (CLSII, Worthington) for 2–3 hr until the mammary epithelial cell clumps appeared free of stromal components when examined under a microscope. The digested material was centrifuged at 200 × *g* for 5 min, and the cell pellet was washed once with medium 199 (GIBCO). The suspension was centrifuged as above, and the cell pellet was resuspended in a small volume (≈5 ml) of medium 199 and then mixed at "FAST" setting for 5 sec with a Super-Mixer (Lab-Line Instruments, Melrose Park, IL) in order to break up the larger cell clumps. This mechanical disruption of larger clumps was found to be less harmful to cells than the standard Pronase digestion, generating more uniform and hormone-responsive mammary cell populations. Mammary epithelial cells were then purified by centrifugation through a gradient of Percoll (Pharmacia), and 1×10^7 cells were mixed with isosmotic neutralized rat tail collagen (6 ml). This mixture was then placed over a bottom solidified collagen layer (4 ml) in a 75-cm^2 Corning tissue culture flask.

Medium. The basal medium, 1:1 (vol/vol) Hepes-buffered Dulbecco's modified Eagle's medium/Ham's F-12 medium (GIBCO), was supplemented with insulin (Sigma) at 10 μg/ml, ovine prolactin (a gift from the National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD) at 1 μg/ml, progesterone (Sigma) at 0.05 μg/ml, linoleic acid (Sigma) at 10 μg/ml, and bovine serum albumin fraction V (Sigma) at 5 mg/ml. Linoleic acid was conjugated to fatty acid-free bovine serum albumin (Armour, Kankakee, IL) as described (15). This medium will be referred to as "serum-free medium." Cultures were fed every 2 days.

Carcinogen Treatment. MNU (Sigma) was freshly dissolved in dimethylsulfoxide (Me_2SO) at 10 mg/ml and added to medium 199 at pH 6.0, and cultures were treated within 5 min of preparation instead of within 10 min as was done earlier (11). The final concentration of Me_2SO was between 0.67% and 6.7% of the medium. Me_2SO concentration was higher than that used previously (11) in order to rapidly solubilize MNU at different doses. The cultures were incubated in this medium for 1 hr at 37°C, and then the medium was replaced by the serum-free medium. MNU doses were determined by the cytotoxicity curve as reported (11). The first MNU treatment was on day 3 of culture, and treatments subsequently were at 1-week intervals for up to 4 weeks. In one experiment, phorbol 12-tetradecanoate 13-acetate [TPA (Sigma)] was added to the serum-free medium (final concentration, 0.01 μg/ml) and fed to the cultures for 10 days after four treatments with MNU at 100 μg/ml. TPA was prepared in Me_2SO at a stock concentration of 1 mg/ml. The control cells were exposed to equivalent amounts of Me_2SO and were cultured for the same periods of time as MNU-treated cultures. All procedures were performed in a dark tissue culture hood.

Transplantation Assay. The timing of the transplantation was standardized as follows. When the MNU-treated cells seemed to have recovered from the cytotoxic effect of the final MNU treatment (8–11 days), the cells were fed with the serum-free medium supplemented with 10% porcine serum (Sterile Systems, Ogden, UT). The following day the cells were removed from collagen gels by 0.1% collagenase treatment and plated in 100-mm tissue culture plates (Corning) in the presence of 10% porcine serum. After 2 days cells were removed with trypsin, and 5×10^5 cells in a volume of 0.01 ml were injected into each mammary epithelial cell-free (cleared) inguinal fat pad of 3-week-old BALB/cCrgl female mice (16). In one experiment (Table 1, experiment 1), a palpable tumor was observed at 6 weeks after transplantation. In all other cases the host mice were injected i.p. 8–10 weeks after transplantation with 0.75 ml of 0.5% trypan blue, and the following day the fat pads were examined for the presence of abnormal mammary outgrowths (11). To test for their transformed potentials, small pieces (≈1 mm^3) from selected tumors and hyperplasias were secondarily transplanted into intact and cleared mammary fat pads of syngeneic female mice, respectively (11, 18). Mice with tumors were terminated either at the time of the fat pad examination or when the tumor was ≈2 cm in diameter. Mice without tumors were terminated at 2–6 months after the primary or secondary transplantation. At termination, both of the injected inguinal mammary fat pads, bearing outgrowths, and one of the thoracic mammary glands were removed from each host, spread in a Tissue Tek capsule (Miles), fixed in Tellyesniczky's fixative, defatted in acetone, stained in iron hematoxylin, and examined in methyl salicylate under a dissecting microscope. Fat pads prepared in this way were called "whole mounts." Samples of selected tumors and hyperplasias were cut out of the whole mounts, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin for histological examination.

Table 1. Incidence of primary mammary carcinomas and preneoplasias

Exp.	Carcinogen* treatment	No. transplants	Carcinoma† %	Carcinoma† No. palpable	Carcinoma† No. subgross	Preneoplasia % HANs	Preneoplasia % DHs	Normal outgrowths, %
1	4 × 100‡	30	70	5 (6–8)	16 (8)	30	7	7
	Control	25	0			0	0	100
2	3 × 50	20	30	2 (8)	4 (8)	30	15	25
	3 × 100	18	0			22	28	50
	3 × 200	10	0			10	0	90
	Control	19	0			0	0	100
3	1 × 100	18	6	0	1 (10)	17	0	83
	2 × 100	29	79	22 (8–10)	1 (10)	14	0	7
	3 × 100	15	13	0	2 (8)	53	13	27
	4 × 100	16	31	0	5 (8)	44	19	13
	Control	19	0			0	0	100
4	1 × 500	10	0			0	10	90
	Control	18	0			0	0	100

Incidence is expressed as the percentage of total transplants that contained mammary lesions. (Some transplants contained more than one type of lesion; thus, the total incidence may be ≥100%.)

*The first number refers to the number of MNU treatments. The second number is the concentration of MNU in μg/ml used in each treatment. Control groups were exposed to Me_2SO alone, which was the solvent for MNU.

†Subgross tumors produced palpable tumors within the following 2–12 weeks. The numbers in parentheses represent the time in weeks at tumor detection.

‡This group was exposed to TPA (0.01 μg/ml) for 10 days prior to transplantation.

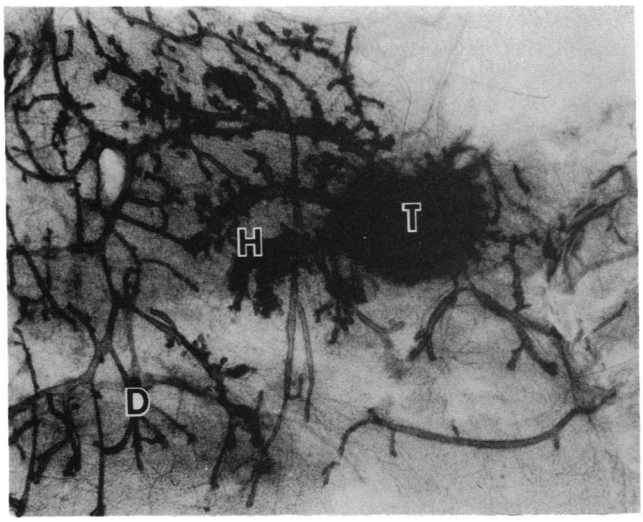


FIG. 1. Whole mount of mammary outgrowths showing a primary subgross tumor at the injection site (T). Some hyperplasias (H) are also observable along with normal ductal outgrowths (D). These outgrowths arose from mammary epithelial cells that were treated with MNU in culture and transplanted into a cleared fat pad. (×5.)

RESULTS

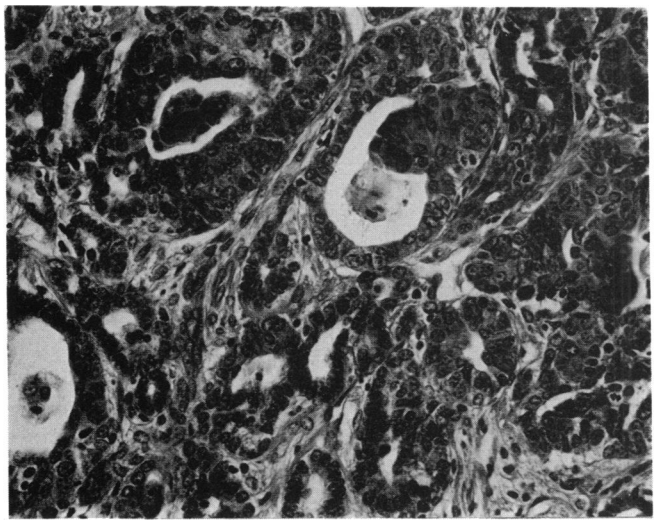
Control. The control mouse mammary epithelial cells, exposed only to Me_2SO (solvent for MNU) and maintained in culture for the same period as carcinogen-treated cells, produced only ductal outgrowths when transplanted into cleared fat pads (Table 1). These outgrowths were morphologically similar to the normal mammary ducts found in the adult virgin hosts, as shown in ref. 11.

MNU Treatment: Incidence and Phenotype of Tumors. Since the half-life of MNU *in vitro* is known to be extremely short (8 min in a culture medium; ref. 19), the cultures were treated within 5 min of preparation. Eleven to 14 days after the final MNU treatment, they were removed from culture and injected into the cleared mammary fat pads of syngeneic mice. They produced tumors at the injection site at high incidences (Table 1). The tumor incidence is expressed as the percentage of the total transplants that developed a tumor at the injection site in the fat pad. We observed the highest incidence of tumors (79%) from the cells exposed twice to MNU at 100 μg/ml. This concentration of MNU was slightly cytotoxic for the cells under our culture conditions (11). There was no correlation between tumor incidence and the frequency of treatment at this dose. Significantly, one exposure to MNU at 100 μg/ml was sufficient to induce tumorigenic transformation in 6% of transplants. Three exposures of cells to 50 μg/ml, a lower cytotoxic dose, were also sufficient to induce tumors in 30% of the transplants. On the other hand, when the cells were treated three times with 200 μg/ml or one time with 500 μg/ml, extensive cell colony degeneration was observed in culture; upon transplantation, these cells failed to develop any tumors and gave rise only to a relatively low incidence (10%) of preneoplastic lesions.

In the first experiment, TPA (0.01 μg/ml) was added to the serum-free medium for 10 days after four treatments with MNU at 100 μg/ml. The cell colonies underwent a change in morphology from stellate colonies composed of cuboidal cells to colonies composed of spindle-shaped cells after the addition of TPA (20). Upon transplantation into cleared mammary fat pads, these cells produced a relatively high incidence (70%) of tumors when compared to cells that were treated four times with the same concentration of MNU but not exposed to TPA (31%). The tumors from TPA-treated cultures had similar morphologies to those of tumors from cultures not treated with TPA.

In some cases, palpable tumors appeared within 6–10 weeks after transplantation of MNU-treated cells. In other cases, subgross tumors were visible at the injection sites (Fig. 1) 8–10 weeks after transplantation and then produced palpable tumors within the next 2–12 weeks (Table 1). In both cases, the tumors had extensive central necroses surrounded by a thin layer of living cells. They were histologically carcinomas (Fig. 2) and were classified as a mixture of type A and B carcinomas according to Dunn's (21) classification. Squamous metaplasia and lymphocytic infiltration were often observed in these carcinomas, similar to that seen in mouse mammary tumors induced by chemical carcinogens *in vivo* (22).

Secondary transplants of 12 primary tumors from MNU-treatment groups (Table 1, experiments 1–3) were made into intact fat pads of syngeneic 3-month-old virgin hosts. All produced tumors between 1 and 8 weeks. These tumors displayed similar histological morphologies to that observed in the original tumors. The secondary transplants usually



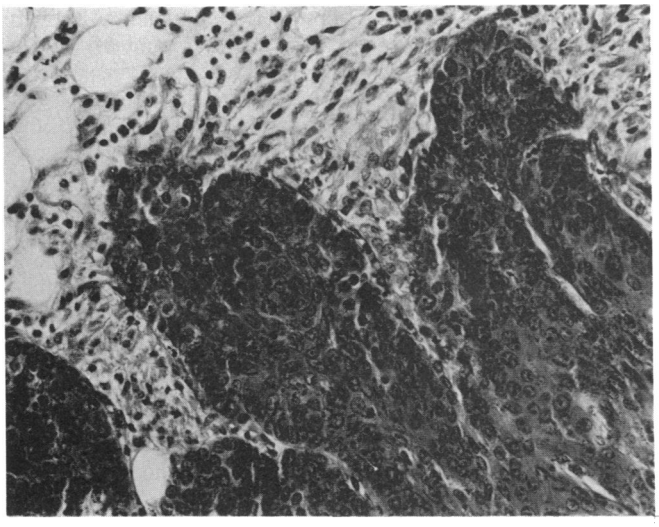


FIG. 2. (*Left*) Histological section of the primary tumor shown in Fig. 1. Note the carcinoma (mostly type A) contains abundant mitotic figures. (×50). (*Right*) Histological section of another primary carcinoma (type B) showing finger-like invasive cellular extensions. Note abundant mitotic figures and lymphocytic infiltration. This carcinoma was palpable within 6 weeks after transplantation of mammary cells treated with MNU. (×50.)

produced solid carcinomas unlike the primaries, which had extensive central necroses.

Some of the primary and secondary carcinomas invaded the adjoining muscle tissues of the body wall by the time they were ≈2 cm in diameter. All mice bearing carcinomas were examined for the presence of metastatic foci in other organs, but none were detected.

MNU Treatment: Incidence and Phenotype of Preneoplasias. Most of the preneoplastic lesions induced by MNU were hyperplastic alveolar nodules (HANs), but ductal hyperplasias (DHs) were also seen infrequently (Table 1). Three treatments at 100 μg/ml induced relatively high incidences of preneoplastic lesions.

When selected hyperplasias, five HANs and seven DHs, were secondarily transplanted into cleared mammary fat pads, all of them grew and maintained their abnormal morphologies. Two HANs and one of the DHs produced palpable mammary carcinomas within 6 months of secondary transplantation. The tumors were classified as type B carcinomas when examined histologically.

DISCUSSION

One of the long-term goals of our laboratory has been to develop a reproducible neoplastic transformation system that will allow *in vitro* analyses of the mechanism(s) of normal-to-malignant transformation of mammary epithelial cells. Toward this end, in a recent report (11), we demonstrated that MNU treatment of mammary cells, grown in the serum-free medium containing prolactin, progesterone, and linoleate, resulted in a high incidence of preneoplastic transformation. In the present study, we have made several significant changes in this transformation protocol and induced a reproducible high efficiency of neoplastic transformation *in vitro*.

First, in the present study, Pronase digestion after collagenase treatment during tissue dissociation, used normally for breaking up large epithelial cell clumps, was replaced by mixing. This mechanical disruption procedure may have been less harmful to cells than enzymatic digestion, generating more uniform and hormone-responsive cell clumps as judged by their consistent response. This resulted in a higher cellular proliferation rate at the time of MNU treatments, which in turn may have increased the cells at risk for transformation. Second, MNU was dissolved in increased amounts of Me_2SO in order to rapidly solubilize different amounts of MNU, which made the final Me_2SO concentration higher. Although the control cells that received the same amount of Me_2SO produced only normal mammary ducts when transplanted, Me_2SO, by increasing cell permeability, might have played some role in neoplastic transformation by MNU. Third, the cultures were treated with MNU within 5 min of preparation, a more rapid procedure than the one used previously (11). Since the half-life of MNU *in vitro* is extremely short (19, 23), this change in procedure resulted in a considerably reduced handling time of MNU. This may have helped to expose more cells to active MNU molecules, thus increasing the possibility of neoplastic transformation. Fourth, in the current studies, the MNU dose was varied from 50 to 500 μg/ml and the cells were exposed one to four times. These changes in MNU doses and number of exposures, compared to our earlier fixed treatment (three times at 100 μg/ml), may have contributed also in inducing a high incidence of mammary carcinomas. Finally, the timing between the last MNU exposure and transplantation was standardized so that the cells were kept for a reduced period in culture compared to our previous protocol (11). Since the serum-free medium used was developed for growth only of normal and not of neoplastic mammary cells (24), the reduction in time in culture after the final carcinogen exposure might have helped to enhance recovery of proportionately more tumorigenic cell populations vis-á-vis normal cells. This possibility is further supported by the observation that, in two cases (data not shown), we have transplanted half of the MNU-treated cells into cleared fat pads and passaged the other half in collagen gels and grown them in the serum-free medium for 4 more weeks. When the passaged cells were transplanted, they produced a high incidence (≈100%) of preneoplastic lesions, but no primary carcinomas were observed. In both cases, the cells transplanted without passaging produced a high incidence (70% and 13%; Table 1, experiments 1 and 3) of primary carcinomas.

The above discussion suggests the possibility that the modifications in the transformation protocol, compared to our earlier report (11), might have been the reason for high efficiency *in vitro* neoplastic transformation observed in the current study. However, at this time, it is not clear as to which one or combination of these changes in the transformation protocol has been essential for these results. Elucidation of the essential change(s) will require further in depth analysis of all the parameters involved.

Single or multiple exposures to MNU *in vitro* can induce neoplastic transformation of mouse mammary epithelial cells. A single treatment with MNU at the moderately cytotoxic dose of 100 μg/ml was sufficient to neoplastically transform these cells in our culture system. Since the half-life of MNU *in vitro* is extremely short and the tumors developed shortly after transplantation, all of the necessary tumorigenic conversions must have occurred *in vitro*. Among the various doses examined in the present study, two treatments with MNU at 100 μg/ml produced the highest incidence of tumors. In our system, no apparent additive effect of MNU was observed at this concentration when cells were exposed more than twice. A similar result was reported by Nettesheim and coworkers (25) in their *in vitro* transformation system for rat tracheal epithelial cells.

Tumor-promoting agents such as TPA were not required for neoplastic transformation with MNU *in vitro*, although TPA used in one experiment increased the tumor incidence when given after carcinogen treatments. Although we have shown earlier that TPA stimulated growth of mouse mammary tumor cells in our culture system (19), it is not certain at this time whether TPA-enhanced proliferation of neoplastic cells was the reason for the higher tumor incidence in this group. Several laboratories also have reported that tumor promoters could increase the efficiency of transformation of cultured cells (26–28). Further experimentation is thus necessary to define the role for TPA in this system.

Some of the carcinomas were palpable as early as 6 weeks and others were observed subgrossly 8–10 weeks after transplantation. These subgross tumors produced palpable tumors when left in the fat pads for longer periods (up to 12 more weeks). Apparently, the mouse mammary epithelial cells did not have to traverse through an intermediate preneoplastic stage (3) to express the neoplastic phenotype, since all tumors appeared at the site of injection within a short time after transplantation.

The majority of lesions induced in the serum-free medium containing prolactin and progesterone were preneoplastic HANs or mammary carcinomas of similar morphology along with some squamous metaplasia. A similar observation was earlier reported by Medina (22, 29) from his *in vivo* studies in which mice bearing pituitary isografts (which secreted a high level of prolactin) produced predominantly HANs or tumors with squamous metaplasia when treated with a chemical carcinogen. Thus, *in vitro*-induced transformants in the present study parallel the *in vivo* results. This also supports our earlier suggestion (11) that the growth conditions of mammary cells at the time of carcinogen exposure may

determine the phenotype of the preneoplastic and neoplastic lesions induced.

In conclusion, the *in vitro* transformation system described herein has several unusual and significant aspects: (*i*) neoplastic transformation is induced in serum-free culture without extensive passaging or extended time in culture unlike other *in vitro* mammary transformation systems, (*ii*) carcinomas are induced at a high efficiency (up to 79%), (*iii*) they appear with extremely short latencies (as short as 6 weeks), (*iv*) mammary carcinomas induced have the similar phenotype as those induced *in vivo*, and (*v*) neoplastic cells are induced without undergoing an apparent preneoplastic stage. The study of the mechanisms underlying the neoplastic transformation of mammary epithelial cells at cellular and molecular levels is now feasible using this well-controlled transformation system. Such studies may help to elucidate the cellular lineage for various types of mammary tumors observed *in vivo*, the effects of hormones or growth factors on neoplastic transformation, and the role of genes, including oncogene(s), in mammary carcinogenesis.

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1. Welsch, C. W. (1985) *Cancer Res.* **45,** 3415–3443.
2. Medina, D., Socher, S. H., Smith, G. H., Dusing-Swartz, S. K., Arthur, L. O. & Butel, J. S. (1982) in *Biological Carcinogenesis*, eds. Rich, M. A. & Furmanski, P. (Dekker, New York), pp. 169–182.
3. Medina, D. (1978) in *Breast Cancer 2: Advances in Research and Treatment*, ed. McGuire, W. L. (Plenum, New York), Vol. 2, pp. 47–102.
4. Chang, S. E., Keen, J., Love, E. B. & Taylor-Papadimitriou, J. (1982) *Cancer Res.* **42,** 2040–2053.
5. Stampfer, M. R. & Bartley, J. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2394–2398.
6. Ethier, S. P. (1985) *J. Natl. Cancer Inst.* **74,** 1307–1318.
7. Greiner, J. W., DiPaolo, J. A. & Evans, C. H. (1983) *Cancer Res.* **43,** 273–278.
8. Schaefer, F. V., Tonelli, Q. J., Dickens, M. S., Custer, R. P. & Sorof, S. (1983) *Cancer Res.* **43,** 3310–3315.
9. Telang, N. T., Banerjee, M. R., Iyer, A. P. & Kundu, A. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5886–5890.
10. Richards, J. & Nandi, S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3836–3840.
11. Guzman, R. C., Osborn, R. C., Bartley, J. C. Imagawa, W., Asch, B. B. & Nandi, S. (1987) *Cancer Res.* **47,** 275–280.
12. Yang, J. & Nandi, S. (1983) *Int. Rev. Cytol.* **81,** 249–286.
13. Richards, J., Larson, L., Yang, J., Guzman, R. C., Tomooka, Y., Osborn, R. C., Imagawa, W. & Nandi, S. (1983) *J. Tissue Cul. Methods* **8,** 31–36.
14. Imagawa, W., Tomooka, Y. & Nandi, S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4074–4077.
15. Imagawa, W., Tomooka, Y., Yang, J., Guzman, R. C., Richards, J. & Nandi, S. (1984) in *Cell Culture Methods for Molecular and Cell Biology*, eds. Barnes, D. W., Sirbasku, D. A. & Sato, G. H. (Liss, New York), Vol. 12, pp. 127–141.
16. Guzman, R. C., Osborn, R. C., Yang, J., DeOme, K. B. & Nandi, S. (1982) *Cancer Res.* **42,** 2376–2383.
17. DeOme, K. B., Osborn, R. C., Miyamoto, M. J. & Guzman, R. C. (1980) in *Cell Biology of Breast Cancer*, eds. McGrath, C., Brennan, M. J. & Rich, M. A. (Academic, New York), pp. 79–91.
18. DeOme, K. B., Faulkin, L. J., Jr., Bern, H. A. & Blair, P. B. (1959) *Cancer Res.* **19,** 515–525.
19. McCalla, D. R., Reuvers, A. & Kitai, R. (1968) *Can. J. Biochem.* **46,** 807–811.
20. Guzman, R. C., Osborn, R. C., Richards, J. E. & Nandi, S. (1983) *J. Natl. Cancer Inst.* **71,** 69–73.
21. Sass, B. & Dunn, T. B. (1979) *J. Natl. Cancer Inst.* **62,** 1287–1293.
22. Medina, D. (1974) *J. Natl. Cancer Inst.* **53,** 213–221.
23. Jensen, E. M., LaPolla, R. J., Kirby, P. E. & Haworth, S. R. (1977) *J. Natl. Cancer Inst.* **59,** 941–944.
24. Imagawa, W., Tomooka, Y., Hamamoto, S. & Nandi, S. (1985) *Endocrinology* **116,** 1514–1524.
25. Pai, S. B., Steele, V. E. & Nettesheim, P. (1983) *Carcinogenesis* **4,** 369–374.
26. Hsiao, W.-L. W., Gattoni-Celli, S. & Weinstein, I. B. (1984) *Science* **226,** 552–555.
27. Dotto, G. P., Parada, L. F. & Weinberg, R. A. (1985) *Nature (London)* **318,** 472–475.
28. Hsiao, W.-L. W., Wu, T. & Weinstein, I. B. (1986) *Mol. Cell. Biol.* **6,** 1943–1950.
29. Medina, D. (1976) *Cancer Res.* **36,** 2589–2595.