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Neoplastic transformation of rat mammary cells exposed to 7,12-dimethylbenz[*a*]anthracene or *N*-nitrosomethylurea in cell culture

(epithelial cell chemical carcinogenesis *in vitro*/cell transplants)

JAMES RICHARDS AND S. NANDI

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

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ABSTRACT **Primary cultures of mammary cells from virgin Lewis rats were seeded at 5×10^5 cells per cm^2 in medium 199 supplemented with 10% fetal calf serum, insulin (5 μg/ml), prolactin (5 μg/ml), estradiol (5 ng/ml), progesterone (0.5 μg/ml), and hydrocortisone (0.5 μg/ml). On the second or third day of culture, cells were exposed to either 7,12-dimethylbenz[*a*]anthracene (0.1 μg/ml for 24 hr) or *N*-nitrosomethylurea (80 μg/ml for 2 hr). The cells were later assayed for transformation by transplanting 10^6 or 10^5 cells into gland-free mammary fat pads of 3-week-old female hosts. Untreated cells produced only normal mammary outgrowths when transplanted. Cells treated with 7,12-dimethylbenz[*a*]anthracene or *N*-nitrosomethylurea produced abnormal outgrowths in 11% of the transplants. These abnormal outgrowths ranged from rapidly growing adenocarcinoma to alveolar and ductal hyperplastic lesions. The results indicate that rat mammary epithelial cells can be transformed by exposure to chemical carcinogens in culture and thus represent a potential *in vitro* model for epithelial cell transformation.**

Cell culture systems have been used extensively to study the process of transformation (1–3). The majority of these studies have used cells classified as fibroblasts which, when transformed, usually give rise to sarcomas if transplanted into appropriate hosts. However, the commonly occurring cancers in man and animals are epithelial in origin (4, 5). Thus, *in vitro* systems are needed to analyze the transformation processes in epithelial cells. Toward this end, the current study was undertaken in an attempt to develop an *in vitro* transformation system with rat mammary epithelial cells. The rat mammary gland is very sensitive to chemical carcinogen induction of adenocarcinomas *in vivo* (6). 7,12-Dimethylbenz[*a*]anthracene (DMBA) and *N*-nitrosomethylurea (NMU) were chosen for use because they are known carcinogens for the rat mammary gland (7, 8). A primary mammary cell culture system was used in which the percentage of epithelial cells synthesizing DNA was maximized (9). Because there exists no current means of distinguishing between normal and neoplastic mammary cells in culture, transplantation of the cells into gland-free mammary fat pads was used to assay for transformed cells. Cultured mammary adenocarcinoma cells from tumors induced in rats with DMBA were also transplanted into gland-free fat pads to determine the growth behavior of known transformed cells in this transplant assay.

MATERIALS AND METHODS

Animals. Virgin Lewis female 50- to 60-day old rats (Simonsen, Gilroy, CA) were used as tissue donors. Eighteen-day-old female Lewis rats were used as hosts for transplants.

Table 1. Growth of cultured tumor cells in cleared fat pad*

Ovarian† dependency of original tumor	Transplants, no.	Tumors, no. (time, wk)‡	Outgrowths, no.	Character of outgrowths§
Independent	26	26 (3)	0	—
Independent	20	20 (4)	0	—
Independent	18	10 (11)	10	Ab-D
Dependent	10	7 (12)	6	Ab-L
Not tested	8	8 (13)	5	C & N-D
Dependent	8	8 (13)	2	N-L
Independent	8	1 (20)	5	Ab-L
Independent	8	0 (20)	7	Ab-L & N-D

* After injection of 10^5 cells in 10 μl of medium. Transplants were kept for 20 weeks.
† Ovarian dependency was determined at the time the biopsy piece was taken for culture. Animals were ovariectomized and the remaining portion of the tumor was monitored for growth.
‡ Time after transplanting to produce a tumor 1 cm^2.
§ Ab, abnormal; N, normal; D, ductal; L, lobular; C, cystic.

Host animals were anesthetized with Nembutal (6 mg/ml at 5 ml/kg of body weight), and the fourth inguinal mammary glands were cleared of parenchyma by the method of DeOme *et al.* (10). At the same time, 10^5 or 10^6 mammary cells were injected into the center of the cleared mammary fat pad in 10 μl of culture medium.

Culture. Primary rat mammary cultures were prepared as described (9). The fourth inguinal mammary glands were collagenase-dissociated (10 mg/10 ml of medium 199 per 1 g of tissue) until all visible tissue pieces were gone. The cell suspension was passed through a Nitex filter with a 150-μm pore size. By means of differential centrifugation, small gland fragments were separated from the single cells. These fragments were used to establish the cultures. The cells were plated at 5×10^5 cells per cm^2 in medium 199 with 10% fetal calf serum, insulin (5 μg/ml), prolactin (5 μg/ml), estradiol-17β (5 ng/ml), progesterone (0.5 μg/ml), and hydrocortisone (0.5 μg/ml). Cultures were also prepared from mammary adenocarcinomas induced in 50-day-old Lewis female rats by a single oral dose of DMBA (20 mg/100 g of body weight) in 1 ml of sesame oil.

Carcinogen Exposure. DMBA was prepared fresh in dimethyl sulfoxide; the dimethyl sulfoxide concentration never exceeded 0.1% of the medium. NMU was prepared immediately before use in Hanks' balanced salt solution (pH 7.0). Cultures were exposed to either DMBA at 0.1 μg/ml (for 24 hr in medium) or NMU at 80 μg/ml (in Hanks' balanced salt so-

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Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; NMU, *N*-nitrosomethylurea; HAN, hyperplastic aveolar nodule.

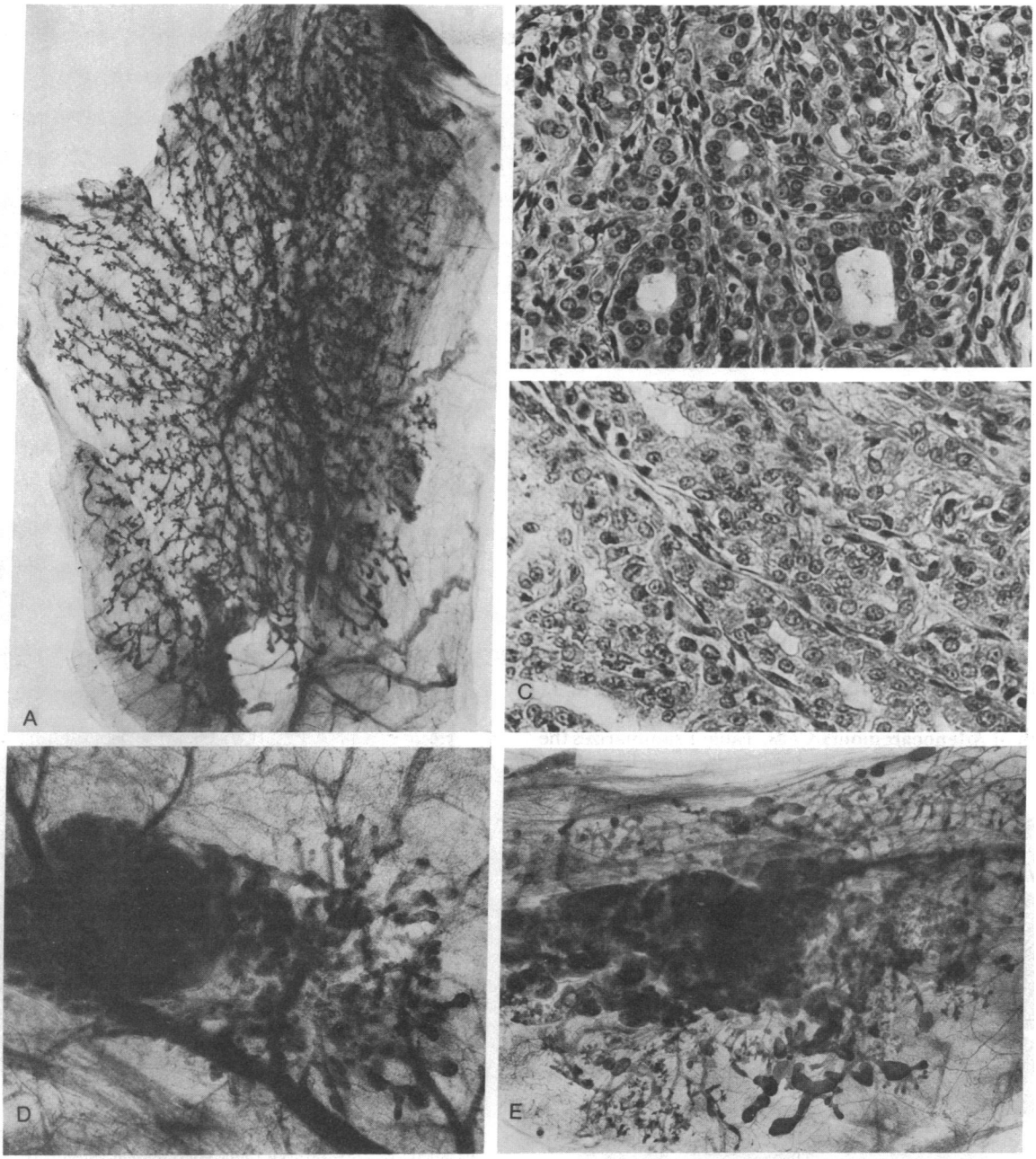

FIG. 1. (*A*) Whole mount of a typical normal outgrowth 10 weeks after injection of 10^5 cultured normal mammary cells into gland-free fat pad. (×5.5.) (*B*) Histological section of a mammary adenocarcinoma induced *in vivo* with DMBA. (×300.) (*C*) Histological section of the adenocarcinoma produced when cells from the tumor in Fig. *B* were cultured and transplanted into gland-free fat pads; note that the same basic histologic appearance was retained. (×300.) (*D*) Whole mount showing both a tumor (left) and a normal outgrowth (right) produced by transplanting cultured tumor cells into a gland-free fat pad. (×8.) (*E*) Whole mount showing both a tumor (center) and abnormal outgrowths produced by transplanting cultured tumor cells into a gland-free fat pad. (×4.5.)

lution for 2 hr) on the second or third day of culture, the period when the greatest percentage of epithelial cells are synthesizing DNA (9). Control cultures were treated with either dimethyl sulfoxide or Hanks' solution. Cultures were fed every 48 hr. On the eighth day of culture, carcinogen-treated and untreated control cells were removed from the culture dishes by gentle scraping. The cells were washed, tested for viability by trypan blue exclusion, and counted. The cells were stored at the appropriate concentration in medium at 4° and injected into the mammary fat pads within 5 hr.

Transplant Assay for Transformed Cells. Cultured normal mammary cells, carcinogen-treated cells, or cultured mammary adenocarcinoma cells were injected into the left and right gland-free fat pads of the host. Host animals were examined weekly for tumor growth. Animals were killed at various times after transplantation or, if tumor-bearing, when the tumor reached 1.0 cm^2. The fourth fat pad containing the transplant and a portion of autochthonous thoracic gland were excised, spread on Tissue-Tech baskets, fixed in 10% formalin for 24 hr, defatted in acetone, and stained with hematoxylin. The transplantation behavior of the adenocarcinoma cells was used to determine the expected outgrowth behavior of transformed cells produced by carcinogen treatment *in vitro*.

Table 2. Tumors and abnormal outgrowths from transplanted carcinogen treated cells

Treatment	Cells transplanted, no.	Transplants, no.	Abnormal outgrowths, no.	Tumors, no.
NMU				
Exp. 1*	10^6	11	0	1
	10^5	5	1	0
Exp. 2	10^6	24	2	1
Exp. 3	10^5	28	1	0
Total		68	4	2
DMBA				
Exp. 4*	10^6	7	1	0
	10^5	8	2	1
Exp. 5	10^6	40	3	1
Total		55	6	2

* Exp. 1 was terminated at 5 mo and Exp. 4 was terminated at 7 mo. All other experiments were terminated 11 mo after transplantation.

RESULTS

Growth of Untreated Cells. All transplants of untreated cells produced only normal mammary outgrowths similar to mammary trees of nonpregnant rats (Fig. 1*A*). The transplants filled the fat pads with normal mammary structure within 10 weeks. Neither abnormal mammary structures nor tumors were observed in more than 100 such transplants kept up to 11 months.

Growth of Adenocarcinoma Cells. Table 1 summarizes the data for transplants of cultured adenocarcinoma cells. The tumors showed a great deal of variability with respect to how rapidly they produced tumors after transplantation, and there was no relationship between the ovarian dependency of a tumor and its growth rate or types of outgrowths it produced. The transplanted tumors did, however, maintain the same basic histological appearance as the original tumors (Fig. 1 *B* and *C*). With the exception of the two very rapidly growing tumors, all other tumors gave rise to some form of outgrowth. These outgrowths were usually abnormal but varied from normal-appearing ductal structures (Fig. 1*D*) to very abnormally organized extensions of tumor cells (Fig. 1*E*).

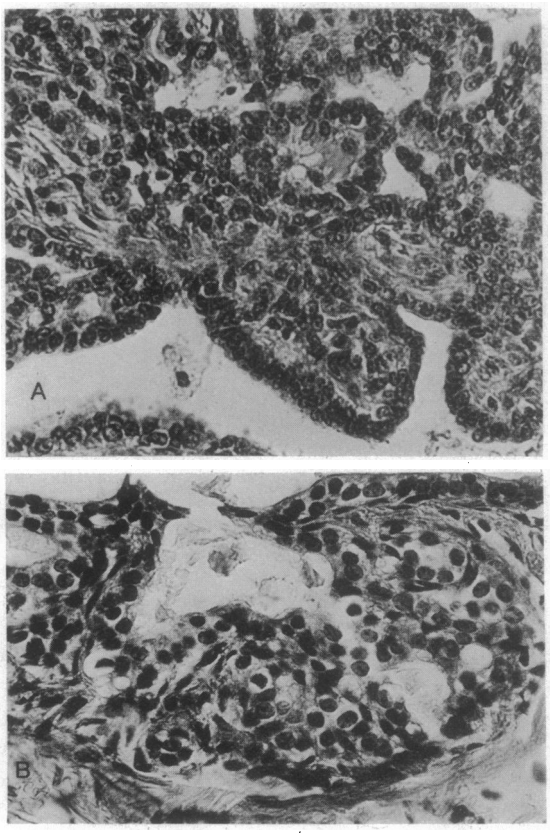

FIG. 2. (*A*) Histological section of an adenocarcinoma arising from cultured mammary cells treated with NMU and transplanted into a gland-free fat pad. (×300.) (*B*) Adenocarcinoma similar to that in *A* but resulting from treatment with DMBA. (×300.)

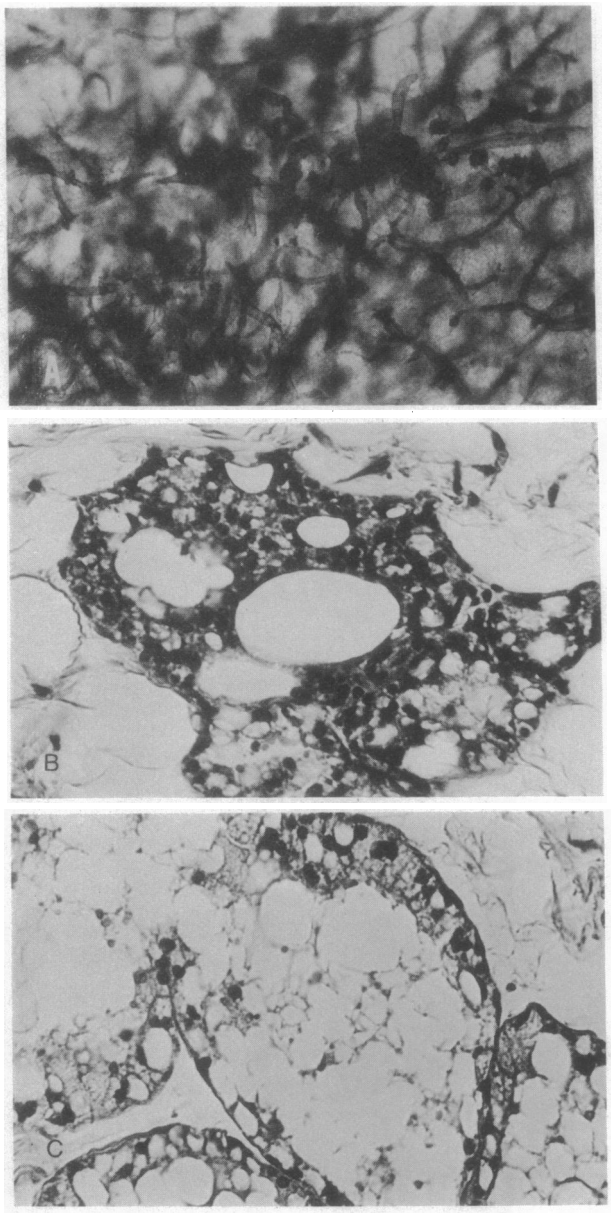

FIG. 3. (*A*) Whole mount of an area of abnormal ductal and lobular structures produced by transplanting mammary cells exposed to NMU in culture. (×9.) (*B*) Histological section from outgrowth shown in *A*. This cross section of abnormal duct contains the possible beginnings of a cribiform tumor. (×300.) (*C*) Histological section from the outgrowth in *A* showing abnormal cystic structures. (×300.)

Growth of Carcinogen-Treated Cells. The results of transplanting carcinogen-treated cells are summarized in Table 2. In 11% of the transplants, treatment with either DMBA or NMU resulted in the production of tumors (Fig. 2) or abnormal outgrowths (Fig. 3) similar to those produced by transplanting

cultured adenocarcinoma cells. The majority of lesions produced involved hyperplasia in the form of lobulo-alveolar units (Fig. 4), but a few ductal abnormalities were also detected in the whole mounts (Fig. 5).

DISCUSSION

The ability of normal cultured rat mammary cells to produce a normal mammary gland when transplanted into gland-free fat pads provides a useful means of assaying for transformed mammary cells. Unlike the mouse mammary cells used by Daniel and DeOme (11), the normal rat cells produced only normal mammary gland structures.

DMBA-induced mammary adenocarcinoma cells were used as a model for the transformed cells that might be produced in culture by carcinogen treatment. Besides producing tumors when transplanted, the cultured tumor cells were also observed to give rise to other outgrowth forms. The various outgrowths may represent populations of cells within the tumor that have been selected for by the culture and transplantation techniques or cells freed by the culture dissociation method from cell-to-cell growth restrictions within the tumor. The appearance of these outgrowths adds another dimension to the use of the fat pad to assay for transformed cells, in that lesions other than frank tumors might be produced by carcinogen treatments.

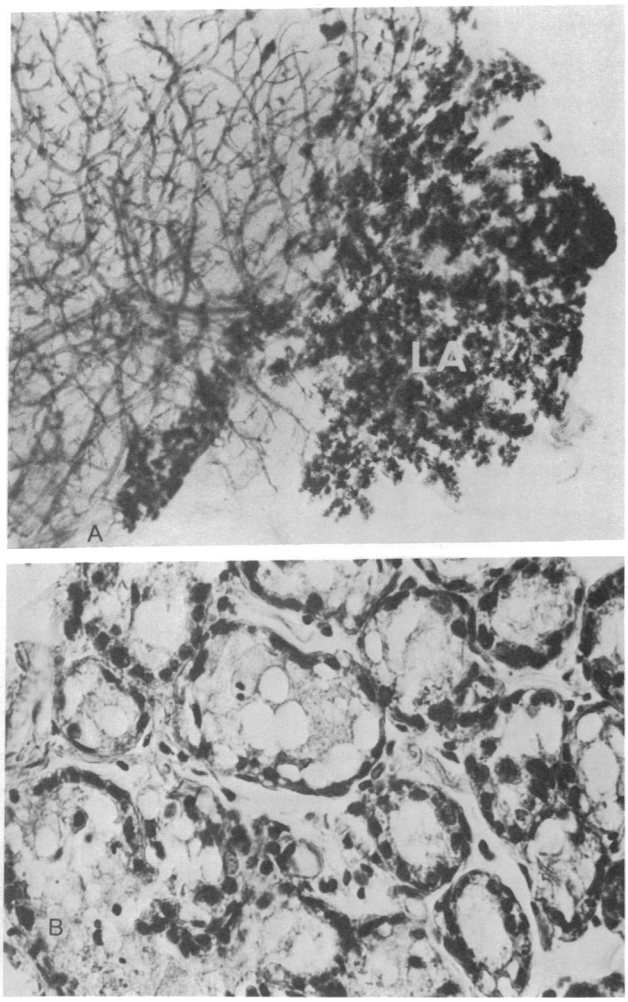

FIG. 4. (*A*) Whole mount of an outgrowth produced by mammary cells treated with DMBA in culture and transplanted into a gland-free fat pad. Note the hyperplastic lobulo-alveolar (LA) portion of the outgrowth. (×4.) (*B*) Histological section of the LA outgrowth in *A*. The LA units appear normal and secretory, similar to a late pregnant state. (×300.)

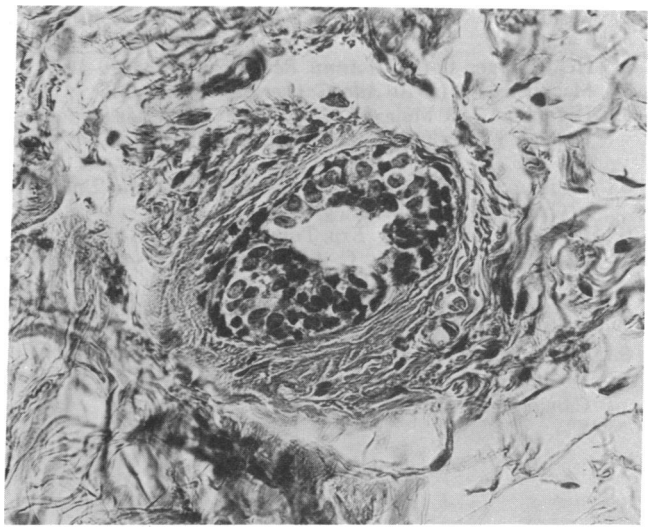

FIG. 5. Histological section of an abnormal hyperplastic duct produced by cells exposed to DMBA in culture and transplanted. (×300.)

Our results show that rat mammary cells can be transformed by treatment with chemical carcinogens in cell culture. The tumors and other lesions produced by carcinogen treatment of mammary cells in culture were of epithelial origin. This also indicates that the cells in culture are metabolizing DMBA to the ultimate carcinogen. Previous studies (12) had indicated that the cultured rat mammary cells were able to metabolize DMBA to water-soluble products and to form a cytotoxic metabolite. NMU, which does not require metabolic activation (13), was also able to transform these cells *in vitro*.

The majority of lesions produced by the carcinogens were lobulo-alveolar units and frequently were secretory. In the mouse, such lesions have been termed "hyperplastic alveolar nodules (HAN)" and are considered to be preneoplastic (10). In the rat, DMBA produces structures analogous to the HAN. However, depending on the method of exposing the mammary gland to the carcinogen, the production of HAN and the production of adenocarcinomas can be separated (14), indicating that the HAN may not be a precursor of the development of the tumor. The HAN cells are a transformed population in that they have undergone a heritable change in response to carcinogenic treatment (15). It may be that different cell populations are responsible for the production of tumors and HANs in the rat. There has been a great deal of discussion over the origin of mammary tumors in the rat (14, 16–19). Whether the tumors arise from ductal or alveolar cells, or from a preneoplastic state such as the HAN, is currently unresolved.

The incidence of transformation in our system was low. However, because of the need to assay transformation by transplantation into isologous hosts, it is difficult to state whether the small number of transformed cells detected was a result of only a few cells being transformed in culture or whether many cells were transformed but never expressed themselves in the animal. Work involving the transplantation of tumor cells mixed with normal cells in various ratios indicates that normal cells can exert an inhibitory effect on the tumor cells, preventing or delaying their emergence (20).

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