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Stochastic Appearance of Mammary Tumors in Transgenic Mice Carrying the MMTV/c-*neu* Oncogene

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Summary

Transgenic mice carrying the activated c-*neu* oncogene under the control of the mouse mammary tumor virus (MMTV) long terminal repeat were produced. Epithelial hyperplasia of epididymis, seminal vesicles, and salivary glands, and dysplasia of harderian glands, were induced. Moreover, in females of our four lines, independent but multiple mammary tumors arose asynchronously, between 5 and 10 months of age, as stochastic events. Histologically, poorly differentiated adenocarcinomas, with intratumor necrosis and calcifications, arose adjacent to morphologically normal epithelium. High transgene expression was detected in all mammary tumors tested and in normal mammary glands before the appearance of the tumors. Together these results suggest that the expression of the activated c-*neu* oncogene was necessary but not sufficient to induce malignant transformation of the mammary epithelial cells. These tumors appear to be an adequate model for human breast cancers overexpressing c-*neu*.

Introduction

The rat c-*neu* oncogene encodes a 185 kd membrane glycoprotein (Padhy et al., 1982) related to, but distinct from, the EGF receptor (Schechter et al., 1984; Bargmann et al., 1986a). This oncogene can transform fibroblasts in vitro if activated (Hung et al., 1986; Bargmann et al., 1986b). The human c-*neu* has been found to be amplified in a significant proportion of human breast cancers, and this amplification seems to correlate with a more malignant phenotype (Slamon et al., 1987; Zhou et al., 1987; Varley et al., 1987; Berger et al., 1988) and with a specific tumor type, the comedo-type ductal carcinoma in situ (van de Vijver et al., 1988). However, the role of this oncogene in the initiation or the maintenance of these tumors still remains to be determined.

The transgenic mouse system is an attractive approach for studying the effect of an oncogene on tumor development within a given tissue. Several oncogenes have been introduced and expressed in a variety of tissues of transgenic mice (for a review, see Cory and Adams, 1988; Jaenisch, 1988). Transgenic mice expressing v-Ha-*ras* (Sinn et al., 1987; Tremblay et al., 1989), *myc* (Stewart et al., 1984) or *int*-1 (Tsukamoto et al., 1988) in the mammary gland were found to develop mammary tumors stochastically. However, those expressing the activated rat c-*neu* were reported to develop mammary tumors in a nonstochastic mode (Muller et al., 1988), in contrast to the apparent effect of this oncogene in human breast tumors.

To investigate a possible role for the c-*neu* oncogene in the development of mammary tumors, we constructed transgenic mice carrying the activated rat c-*neu* oncogene under the transcriptional control of the MMTV LTR.

Results

Construction of Transgenic Mice

Transgenic mice were generated by microinjecting a 8.2 kb Sac II–EcoRI chimeric DNA fragment containing the activated rat c-*neu* cDNA under the transcriptional control of the MMTV LTR (Figure 1). This MMTV/c-*neu* fusion gene readily transformed rat-1 cells in a focus-forming assay in vitro, and the resulting transformed cell lines grew in agar (data not shown).

Five transgenic founders were produced. In each of these founders, the MMTV*neu* sequences appeared intact and localized at an unique integration site (data not shown). Four founders transmitted the transgene to their progeny in a Mendelian fashion and one male founder (MN-16) was mosaic. Lines (MN-9, MN-10, MN-12, and MN-17) were established by mating transgenic founder mice to BALB/c mice.

Predominant Phenotype of Female Mice Carrying the MMTV*neu*T Transgene: Mammary Tumors

Development of mammary tumors was the most apparent phenotype observed in these transgenic mice. Mammary tumors arose after 5 to 10 months of age in a high proportion of females of four transgenic lines (Figure 2, Table 1). Affected mice generally had multiple tumors of different size arising independently and asynchronously. In almost every affected mouse, one or many of the ten mammary glands still remained tumor-free at the time the animals were sacrificed, while some tumors reached 2 cm in size (data not shown). Before the appearance of the tumors, transgenic females became pregnant frequently and could nurse their progeny normally. We do not know yet, however, whether these tumors will arise in virgin females. Established tumors continued to grow in the absence of novel pregnancy. To date, none of the transgenic males has developed mammary tumors.

Histological examination of these tumors revealed poorly

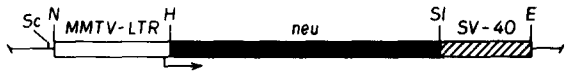

Figure 1. Schematic Representation of the Injected MMTV/*neu*T Fusion Gene

The arrow indicates the transcriptional start site located 110 bp upstream of the junction HindIII site. The restriction sites are: E, EcoRI; H, HindIII; N, NcoI; Sc, SacII; Sl, SalI.

differentiated typical adenocarcinomas arising adjacent to morphologically normal epithelium that sometimes showed microcalcifications without significant hyperplasia or dysplasia (Figure 3). In some areas of some tumors, a few papillary formations were occasionally seen. Intratumor necrosis and calcifications were seen frequently. These tumors, which were best classified as Dunn's type B adenocarcinomas (Dunn, 1959) are morphologically quite similar to human ductal breast carcinomas. They have a more uniform histological type than the mammary tumors found in transgenic mice carrying the MMTV-v-Ha-*ras* oncogene and classified as Dunn's type A and adenoacanthomas (Tremblay et al., 1989). In three out of five animals examined, we could document metastases in the lungs, one within a blood or lymphatic vessel (Figure 3C). Each of the 11 mammary tumors tested could be transplanted in nude mice, confirming their malignant nature (data not shown).

Additional Phenotypes Observed in MMTV*neu*T Transgenic Mice

Salivary and harderian gland tumors, enlarged or abnormal seminal vesicles, enlarged epididymis, and splenomegaly were also observed in these transgenic mice (Table 1). Mice from lines MN-10 and MN-12 developed a salivary gland tumor, which appeared hyperplastic histologically (data not shown). One of these tumors (MN250-12) tested in nude mice did not grow, suggesting it might not have been malignant.

Males of the four transgenic lines were fertile in the first 2 months of age, but most became sterile by 3–4 months.

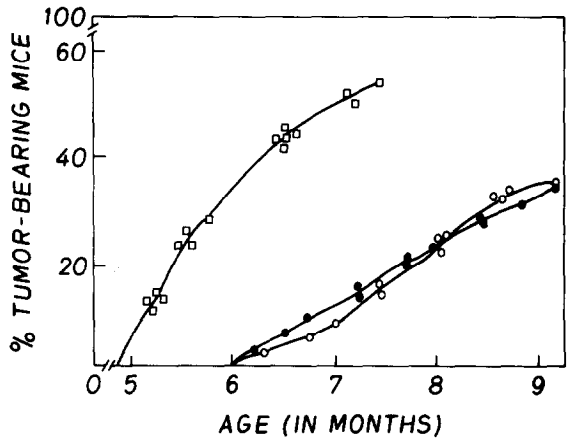

Figure 2. Cumulative Incidence of Mammary Tumors in Transgenic Females

The percentage of animals with tumors is plotted as a function of age. Data include 29, 35, and 34 females from line MN-9 (□), MN-10 (●), and MN-17 (○), respectively.

Bilateral enlarged epididymis (reaching three to four times their normal weight) were observed in all the few male mice sacrificed to date. Histologically, the lumen was often extremely dilated and focal areas of papillary epithelial hyperplasia were seen (data not shown). Seminal vesicles were either enlarged or of a reddish color and indurated. Histological examination revealed focal papillary hyperplasia and hypertrophy of the epithelium (data not shown). In one animal, a papillary tumor with cytological atypia suggestive of a malignant neoplasm was detected.

In five mice of line MN-10, an unilateral proptosis was observed (Table 1). This phenotype was identical to the one seen in transgenic mice carrying the MMTV/v-Ha-*ras* oncogene (Sinn et al., 1987; Tremblay et al., 1989), except that it was not bilateral. Dissection showed a harderian gland tumor with severe bilateral hyperplasia and dysplasia of the epithelium and possible malignant transformation (data not shown).

Table 1. Pathologies Associated with Transgenic Mice Carrying the MMTV/*neu*T Fusion Gene

Pathology	Sex	% of Mice Affected (no./total) in Line			
		MN-9	MN-10	MN-12	MN-17
Mammary tumor[a]	Female	50 (18/36)	44 (18/41)	28 (2/7)	35 (15/43)
	Male	0 (0/16)	0 (0/40)	0 (0/9)	0 (0/34)
Salivary gland tumor[a]	Female	0 (0/36)	5 (2/41)	0 (0/7)	0 (0/43)
	Male	0 (0/16)	0 (0/40)	33 (3/9)	0 (0/34)
Splenomegaly[b]	Female	100 (10/10)	100 (7/7)	100 (1/1)	100 (2/2)
	Male	0 (0/3)	0 (0/1)	0 (0/1)	–
Seminal vesicle and epididymis enlargement[b]	Male	100 (3/3)	100 (1/1)	100 (1/1)	–
Unilateral proptosis[a]	Female	0 (0/36)	5 (2/41)	0 (0/7)	0 (0/43)
	Male	0 (0/16)	8 (3/40)	0 (0/9)	0 (0/34)

[a] Only data from animals older than 5 months were included in this group.
[b] These lesions were observable at autopsy. Thus, only autopsied animals are included in this group.

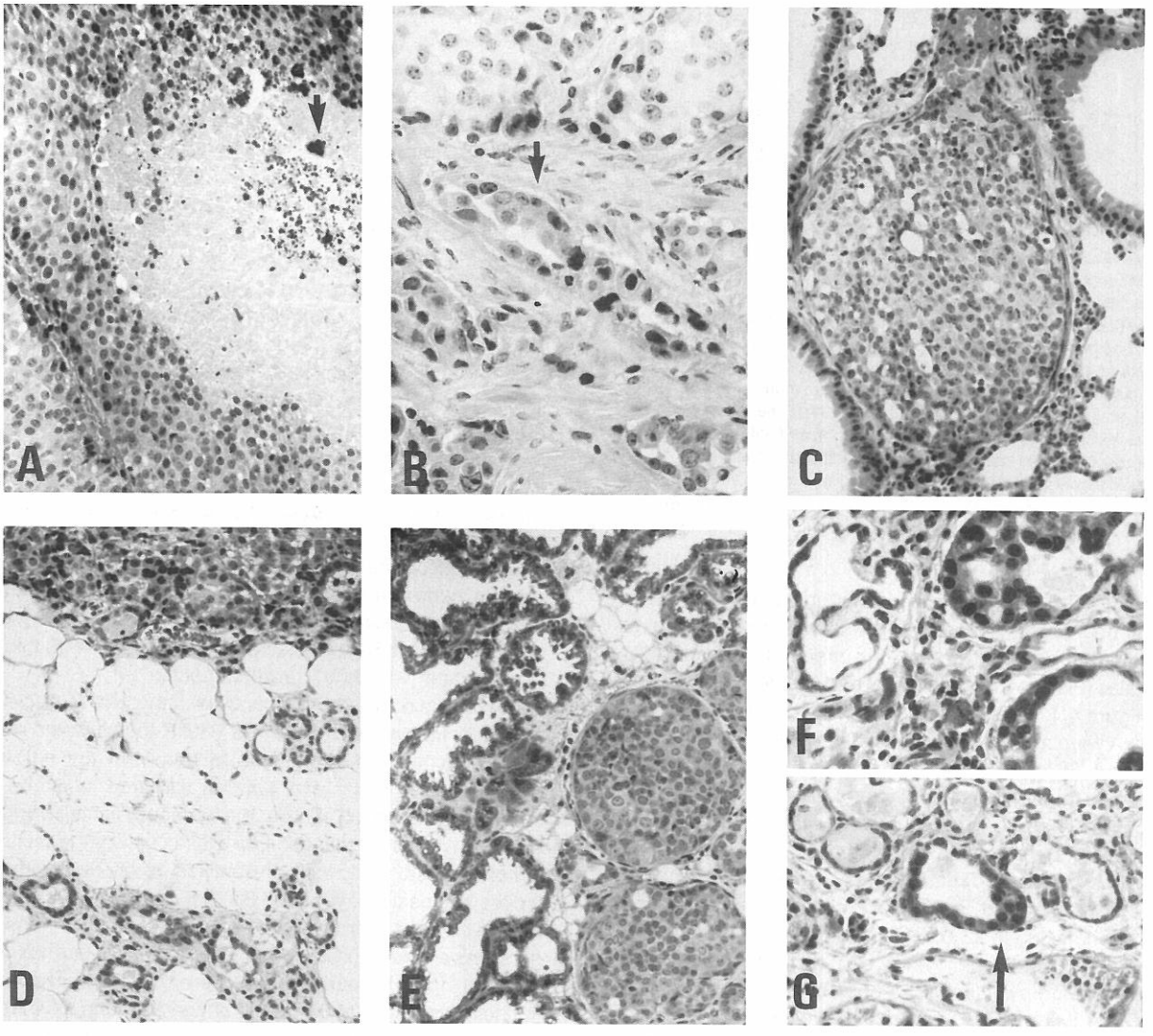

Figure 3. Histological Sections of Mammary Tumors Arising in Transgenic Mice (Hemotoxylin and Eosin Stain)

(A) Adenocarcinoma from transgenic mouse MN-158-9. Poorly differentiated adenocarcinoma without glandular structure. Note abundant necrosis and calcifications (arrow). This aspect is reminiscent of the comedo-type carcinoma in human breasts. 250×

(B) Adenocarcinoma from transgenic mouse MN-37-9. Infiltrating cords of cells with fibrosis. Poorly developed glandular formations are seen (arrow). 250×

(C) Intravascular lung metastasis in a mammary tumor-bearing mouse (MN-9) without grossly visible tumor in the lung. 250×

(D) Adenocarcinoma (up) adjacent to non-neoplastic parenchyma. (Mouse MN-158-9). 160×

(E) A focus of adenocarcinoma adjacent to non-neoplastic lactating acini. 160×

(F) Mammary adenocarcinoma of transgenic mouse MN-37-9. An area of in situ carcinoma or Pagetoid spread which is reminiscent of what is seen in human breasts. 250×

(G) Macroscopically normal gland in a transgenic mouse (MN-160-9) with seven distinct mammary tumors. Several dilated non-neoplastic ducts and acini with a focus of dysplasia or possible early in situ carcinoma (arrow). 250×

Although the incidence of these proliferations is low, it is significantly higher than that found in normal control mice, which strongly suggests that the c-*neu* oncogene is also responsible for the initiation or maintenance of these lesions, or both.

In a significant proportion of females sacrificed because of their massive load of mammary tumors, splenomegaly (spleen weight up to 1.7 g) was also observed. Histologically, a relatively well conserved white pulp was seen, but the red pulp was markedly hypercellular and composed of immature elements of several lineages (data not shown). This finding resembles the picture found in transgenic mice carrying the MMTV/v-Ha-*ras* fusion gene (Tremblay et al., 1989). This phenotype remains to be investigated fully to determine whether it is a paraneoplastic phenomenon or whether it is related to the expression of the transgene.

Expression of the Transgene

Transgenic RNA was first measured by the agarose gel

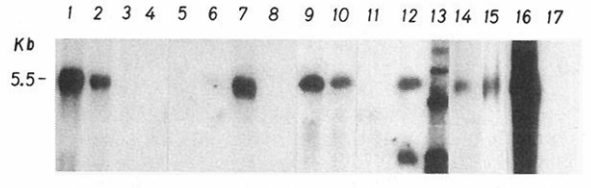

Figure 4. Northern Blot Analysis of the MMTV-*neu* Expression in Representative Normal and Tumor Tissues of Transgenic Mice

Hybridization was with a ^{32}P-labeled SV40 SalI–EcoRI fragment from MMTV*neuT* DNA. RNA (20 μg) from nontumoral tissues of mice from line MN-9: two independent lactating mammary glands at 4 months of age (lanes 1 and 2), brain (lane 3), harderian gland (lane 4), testis (lane 5), seminal vesicle (lane 6), epididymis (lane 7), and kidney (lane 8). RNA from tumors: two distinct mammary tumors from mouse MN-158-9 (lanes 9 and 10), salivary gland tumor from mouse MN-250-12 (lane 14), enlarged epididymis from mouse MN-291-9 (lane 15), enlarged seminal vesicle from mouse MN-250-12 (lane 16), and enlarged spleen from mouse MN-39-9 (lane 17). Negative control rat-1 cells (lane 11) and positive control MMTV*neuT*-transformed rat-1 cells (lane 12). HindIII-digested ^{32}P-labeled lambda DNA was used as a marker (lane 13).

transfer (Northern) procedure. In undiseased mice from our best-characterized line, MN-9, high transgene expression was detected in lactating mammary glands of females (Figure 4, lanes 1 and 2) and in epididymis of males (Figure 4, lane 7). Low but detectable levels of transgene RNA were also found in seminal vesicles of males (Figure 4, lane 6) and in muscles of females (not shown). Other tissues from males and females of this line were negative for transgene expression (Figure 4). In mice of line MN-12 and MN-10, high transgene expression was observed in salivary and harderian glands, respectively (data not shown). Elevated levels of transgene RNA transcripts were detected in 12 solid mammary tumors and in each salivary gland, epididymis, and seminal vesicle tumor tested (Figure 4, lanes 9, 10, and 14–16). In each case, the mRNA detected had the expected size, comigrated with the 5.5 kb RNA produced by the fibroblast rat-1 cells transformed with the same MMTV*neuT* plasmid (Figure 4, lane 12), and also hybridized with a c-*neu* probe (not shown). Moreover, its transcription initiation site, as measured by an RNAase protection assay, was at the correct LTR R region (data not shown). Using in situ hybridization with a ^{35}S-labeled SV40-specific RNA probe, we also confirmed that morphologically normal mammary epithelial cells from pregnant or lactating females expressed the transgene (data not shown).

Discussion

neu Expression Predisposes to the Development of Mammary Tumors

We report that mice carrying the MMTV/c-*neu* oncogene have a strong heritable predisposition to the development of mammary adenocarcinomas. In contrast to control nontransgenic mice, females of these lines spontaneously develop multiple tumors that arise independently and asynchronously, late after birth, and at multiple sites. In each mammary tumor analyzed, high levels of transgene *neu* mRNA were expressed, suggesting they are necessary for the initiation or the maintenance of these tumors, or both. However, the expression of this oncogene does not appear to be sufficient for the appearance of these tumors: indeed, high levels of transgene RNA could be detected in morphologically normal mammary glands of lactating or pregnant females before the appearance of visible tumors, and these tumors arose stochastically. This mode of development strongly suggests that another genetic event is required to transform these cells, in addition to the dominant expression of the *neu* oncogene. Mammary tumors arising in a similar stochastic fashion have previously been described in transgenic mice carrying *myc* (Stewart et al., 1984), v-Ha-*ras* (Sinn et al., 1987; Tremblay et al., 1989), or *int*-1 (Tsukamoto et al., 1988) expressed in mammary tissues.

The mammary tumors arising in our MMTV/c-*neu* transgenic mice were histologically uniform, resembling human ductal breast carcinoma. They showed necrosis and calcifications relatively frequently, a characteristic rarely seen in mammary tumors induced in mice carrying the v-Ha-*ras* oncogene (Tremblay et al., 1989), but frequently seen in human mammary tumors, more specifically in comedo-type ductal carcinoma, a histological tumor type recently found to be associated more frequently with *neu* amplification (van de Vijver et al., 1988).

Our results contrast significantly with those of Muller et al. (1988). In their transgenic line TG.NF, the activated rat c-*neu* oncogene was sufficient to transform the entire mammary epithelium and tumors appeared synchronously, at an early time, at the same frequency in males and females. An easy explanation for these conflicting results is not obvious. First, they are unlikely to be due to the mouse strains used. We used BALB/c mice, while CD-I or FVB mice were used by Muller et al. However, our transgenic mice bred with CD-I mice, for two generations, have not developed an early phenotype either and have remained tumor-free at 6 months of age (our unpublished data). Second, these different results are unlikely to be the consequence of a position effect of the transgene, since the same stochastic development of mammary tumors was observed in four of our transgenic lines, although at different frequency. Third, possibly these different results reflect differences in the structure of the transgene used by both groups, although the *neu* cDNA was obtained from the same plasmid (pSV2 *neuT* [Bargmann et al., 1986b]). Our MMTV*neuT* transgene contains the MMTV LTR immediately adjacent to the *neu* cDNA sequences. In contrast, in Muller et al.'s transgene, 600 bp sequences separate the MMTV LTR from the *neu* cDNA sequences. These 600 bp sequences that represent the rat 30 S sequences derived from Harvey murine sarcoma viral (MSV) genome are usually transcribed in Harvey MSV-infected cells (Gruss et al., 1981). They are also translated to generate a fusion p30 protein containing N-terminal residues encoded by the 30 S rat sequences in frame with the v-Ha-*ras* residues (Gruss et al., 1981; Dhar et al., 1982). The relevance of these 30 S sequences in the transformation potential of Harvey MSV has not yet been investigated in primary cells, although part of them were found to be dispensable for transformation of established NIH/3T3 cells

(Cichutek and Duesberg, 1986). Since these sequences have been captured and retained in the correct reading frame in the Harvey MSV, they might be implicated in oncogenesis, and might contribute to the different effects of the *neu* oncogene observed by both groups. Fourth, alternatively, other undetected modifications generated during the manipulations might have changed the biological properties of one of the transgenes. Direct comparison of both transgenes will be needed to understand the molecular basis of these different results.

Distinct Effects of *neu* in Various Tissues

When expressed in various tissues, the activated c-*neu* oncogene was found to have distinct effects. By itself, the *neu* oncogene had no transforming ability in any of the tissues where it was expressed. However, its expression was sufficient to induce epithelial cell proliferation (hyperplasia) in the epididymis and seminal vesicles, and hyperplasia and dysplasia in the harderian gland. In the mammary gland, its expression only acts as a predisposing factor to the development of malignant outgrowth. In contrast to *int-1*, whose overexpression in mammary epithelial cells leads to hyperplasia and a nonlactating state (Tsukamoto et al., 1988), *neu* overexpression in these cells did not induce hyperplasia and did not seem to interfere with their normal function. Together, these results illustrate the cell-specific effect of the *neu* oncogene.

An Animal Model for Human Breast Adenocarcinoma Overexpressing c-*neu*

The mammary tumors arising in these transgenic mice appear to be an adequate model for human breast adenocarcinoma overexpressing c-*neu*. The stochastic appearance of these murine mammary tumors, their histology, and their metastatic potential in the lung suggest that they might reflect the biology of some human breast cancers at the cellular level adequately. Therefore, these transgenic mice should be instrumental in studying the role of the c-*neu* oncogene in the development of mammary tumors and in designing new therapeutic approaches to this disease.

Experimental Procedures

Preparation of DNA for Microinjection

To construct the MMTV*neuT* fusion gene, we first introduced a HindIII linker into plasmid pA9 (Huang et al., 1981) at the BamHI site located at the junction between the MMTV LTR and the v-Ha-*ras* sequences. The 2.0 kb NcoI–HindIII MMTV LTR-containing fragment was then ligated with the 6.2 kb Hind III–EcoRI fragment of plasmid pSV2*neuT* (Bargmann et al., 1986b) (containing the activated rat c-*neu* cDNA and SV40 early region splicing site and polyadenylation signal) in NcoI–EcoRI-cleaved plasmid vector pJRD184 to generate plasmid MMTV*neuT*. The 8.2 kb fragment to be microinjected was obtained by cleavage of MMTV*neuT* DNA with SacII and EcoRI and was isolated and purified as described before (Hogan et al., 1986; Tremblay et al., 1989).

Construction of Transgenic Mice

One-cell (C57BL/6 × C3H) F2 embryos were collected, microinjected, and transferred into pseudopregnant CD-1 females essentially as described before (Hogan et al., 1986; Tremblay et al., 1989). From 350 injected and reimplanted eggs, 32 mice were born, 5 of which were later found to be transgenic by Southern hybridization analysis of tail DNA with a *neu*-specific probe (4.6 kb EcoRI–SalI fragment excised from MMTV*neuT* DNA). Positive mice were then bred to BALB/c mice (Charles River Laboratories, Canada).

Analysis of the Transgene RNA Transcripts

RNA was isolated by the method of Chomczynski and Sacchi (1987). It was separated on formaldehyde-agarose gels, transferred onto nylon membranes (Hybond), and hybridized as described (Singer and Jones, 1984).

Cell Transplantation to Nude Mice

Mammary tumors were dispersed in Dulbecco's modified Eagle's medium containing 5% calf serum (Gibco) by mechanical shearing onto a large mesh grid and injected subcutaneously into 40 day old nude mice. Approximately 10^7 cells were injected at each site.

Acknowledgments

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