Gene Therapy (1998) 5, 388–393

In vitro and in vivo antisense-mediated growth inhibition of a mammary adenocarcinoma from MMTV-neu transgenic mice

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Oncogene-bearing transgenic mice develop various kinds of tumors depending on both the regulatory sequences and the specific oncogene used. These mice not only help to clarify the pathogenetic pathways leading to tumor formation, but can also be useful as models to test novel therapeutic strategies, including gene therapy. We have previously reported the establishment of an MMTV-neu (ErbB-2) transgenic mouse lineage, in which 100% of females develop breast tumors with many features similar to their human counterparts; these tumors are due to the overexpression of the activated rat neu oncogene in the mammary gland. From one such mouse we established a cell line of mammary adenocarcinoma named MG1361. We report here that the growth of this cell line can be inhibited in vitro and in vivo by transfection of a plasmid vector carrying an antisense anti-neu construct. This inhibitory effect is specific, as it is related to the expression of the antisense transgene (determined by RT-PCR), and to a reduction in neu mRNA and protein, as determined by Northern and Western blot analyses. Moreover, inoculation of cells carrying the antisense or the control vector in nude mice demonstrated that the morphological and biochemical effects elicited by the antisense construct resulted in a significantly slower rate of in vivo growth of tumor xenografts. Finally, significant mammary tumor growth inhibition was obtained after liposome-mediated direct inoculation of the same antisense vector in tumors spontaneously arising in MMTV-neu mice. Taken together, these findings suggest that targeting neu expression by an integrated large anti-neu antisense segment affects the in vivo growth of these tumors.

Keywords: *antisense therapy; mammary adenocarcinoma; MMTV-neu; transgenic mice; liposomes*

Introduction

Oncogene-bearing transgenic and tumor suppressor 'knock out' animals have been an invaluable tool for the evaluation of the molecular steps leading to the establishment of a full malignant phenotype.[1–3] Some of these animals are also reliable models for feasibility studies of novel therapeutic strategies, including gene therapy.[4,5] Among the advantages of using transgenic mice to evaluate tumor growth inhibition is the fact that at least one step leading to cell transformation is exactly defined. Therefore, the effect of targeting the oncogene action or restoring the tumor suppressor function can be accurately evaluated as they are certainly involved in the pathogenesis of the tumors arising in these mice.

We have recently described a transgenic mouse lineage in which overexpression of the activated rat *neu* oncogene was obtained in the mammary gland by the mouse mammary tumor virus long terminal repeat (MMTV-LTR). These mice develop breast tumors which have several features in common with human mammary tumors, including the ability to induce metastases to distant organs.[4–6]

Overexpression of the Erb-B2 gene (*neu* oncogene) often occurs in human breast tumors and appears to be a factor contributing to a poor prognosis in a subset of these neoplasms.[7,8] We reasoned that this mouse lineage could be an interesting model to test the effect of inhibiting the *neu* abnormal function through the use of an antisense strategy.

For this purpose we inhibited the expression of *neu* oncogene by means of antisense in an adenocarcinoma cell line (MG1361) derived from a breast tumor of these mice.[9] We show here that antisense expression inhibited the production of both *neu* mRNA and *neu* protein (p185) *in vitro*. We also show that this strategy results in a slower growth rate of stably transfected cells *in vitro* as well as *in vivo*. Furthermore, we were partially able to inhibit tumor growth *in vivo* by liposome-mediated intratumoral inoculation of the antisense construct in primary tumors from MMTV-*neu* transgenic mice. These results suggest that antisense-mediated *neu* oncogene inhibition could represent a new weapon for the therapy of human breast neoplasias.

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Received 5 September 1997; accepted 27 October 1997

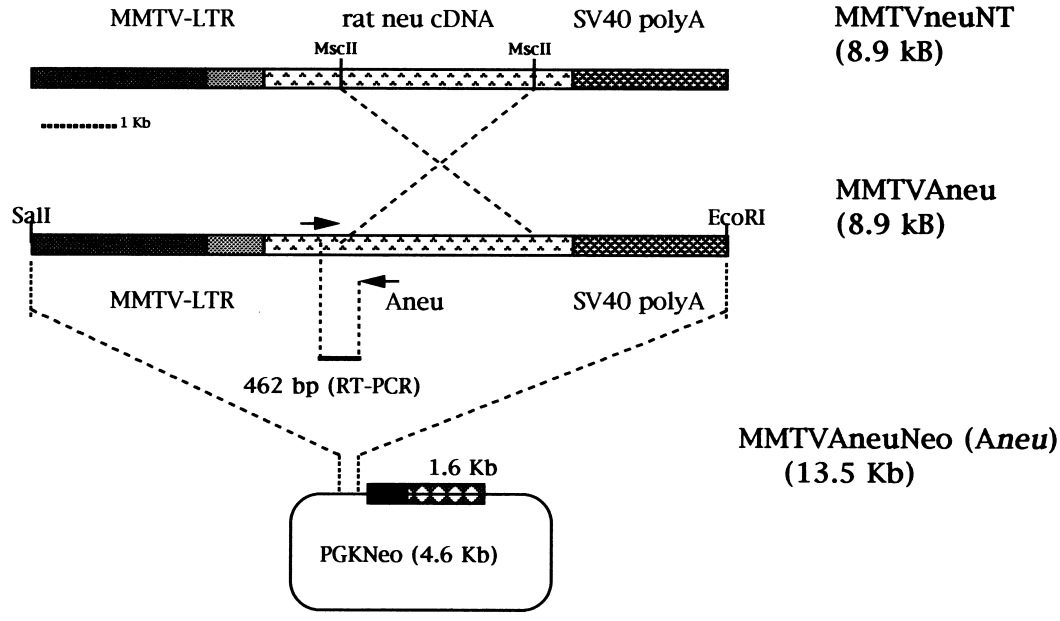

Figure 1 *Strategy to produce Aneu construct: a 2.6 kb MscII fragment from the MMTVneuNT construct was cut and re-cloned in the opposite direction giving rise to MMTVAneu construct. The whole insert from this construct was excised as a SalI–EcoRI fragment and cloned in the SalI–EcoRI sites of PGKNeo to give rise to the Aneu construct, which contains the MMTV-LTR, the Aneu and the PGKNeo cassette. Arrows indicate the position of the primers used for the RT-PCR. For the electroporation, both PGKNeo and Aneu plasmids were linearized at the SalI site.*

Results

In the studies presented here we developed an antisense strategy towards the inhibition of the *neu* oncogene expression. For this study we used a mammary adenocarcinoma cell line (MG1361) isolated in our laboratory from a spontaneously developed tumor in a MMTV-*neu* transgenic mouse.[9] As described, this cell line shows the typical characteristics of other mammary adenocarcinoma cell lines, and when inoculated *in vivo* in immunodeficient mice gives rise to mammary tumors with the same features as the primary tumor from which it was derived.

In order to test whether inhibition of tumor growth was possible by antisense technology, we electroporated MG1361 cells with the antisense construct A*neu* shown in Figure 1. As a negative control, a PGKNeo construct was used. Both plasmids were linearized and 5 μg of each construct were electroporated in 3×10^6 MG1361 cells.

After 10 days of G418 selection, surviving cells were tested for the integration of the electroporated DNA by PCR (data not shown) and then expanded. After several (five to 10) *in vitro* passages, cells transfected with the A*neu* construct showed a different morphology compared with PGKNeo cells and a different growth rate. In particular, PGKNeo electroporated cells showed the same morphology as the parental MG1361 line (Figure 2A), while the antisense-expressing cells showed a more flattened, fibroblast-like morphology (Figure 2B). The difference in the growth rate between the two electroporated lines (calculated as described in Materials and methods) was 2.2-fold slower for A*neu*, compared with PGKNeo cells.

Antisense expression was determined by RT-PCR. As shown in Figure 3, the 472-bp specific PCR product was amplified in the A*neu* cells (lane 1) but not in PGKNeo

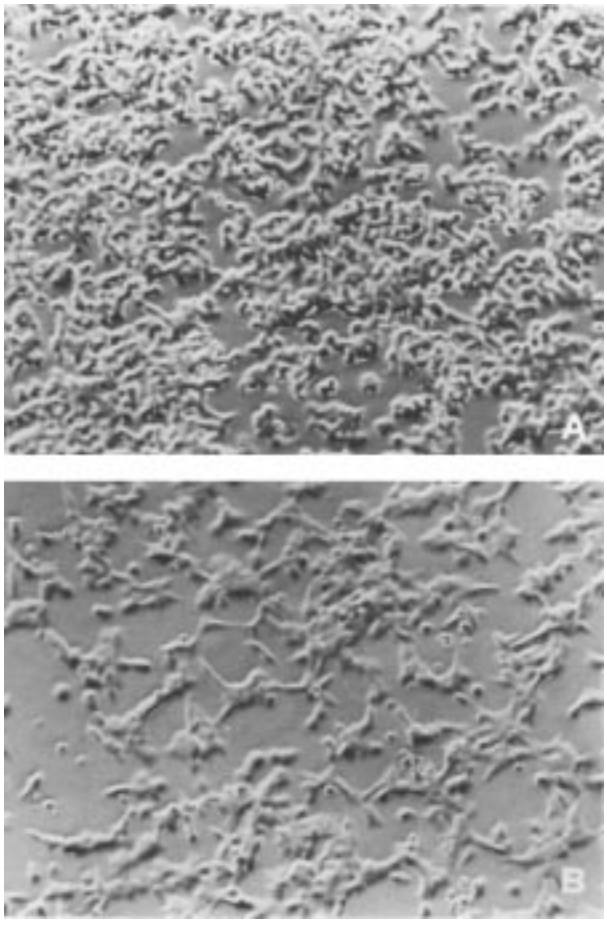

Figure 2 *Morphology of PGKNeo (A) and Aneu (B) electroporated cells observed through an inverted microscope with phase contrast optics (original magnifications × 200).*

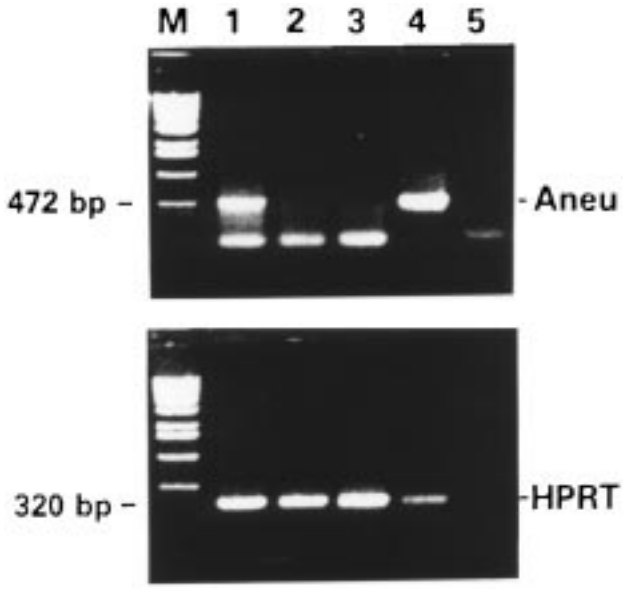

Figure 3 *Analysis of antisense Aneu (upper panel) and HPRT (lower panel) expression by RT-PCR. The RNAs were obtained from Aneu (lane 1), PGKNeo (lane 2) and wt MG1361 (lane 3) cells. Lanes 4 and 5 are positive and negative controls, respectively. M: 1 kb ladder marker (GIBCO-BRL, Bethesda, MD, USA).*

(lane 2) or in parental MG1361 cells (lane 3), while the constitutively expressed HPRT was present in all the cell lines. Lanes 4 and 5 represent the positive and negative controls of both PCR reactions.

We then evaluated the specific inhibition of the *neu* transgene expression by Northern blot. As shown in Figure 4, a specific *neu* signal was evident in the RNA from PGKNeo electroporated cells and *neu* tumors from transgenic mice (lanes 1 and 3) whereas the signal was undetectable in RNA from the A*neu* electroporated cells (lane 2) and control mammary gland (lane 4).

These findings were confirmed by Western blot analysis. As shown in Figure 5, high levels of neu protein (p185) were present in the PGKNeo cells (lane 3), while A*neu* electroporated cells showed clearly decreased protein levels (lane 4), comparable with those present in a normal epithelial cell line derived from a murine mammary gland (normal mouse mammary gland cell line (NMuMG); ATCC CRL 1636, Rockville, MD, USA) (lane 1). Embryonic stem cells were used as a negative (nonepithelial) control (lane 2).

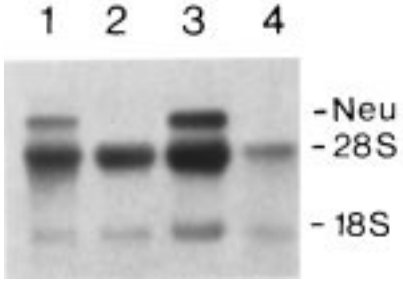

Figure 4 *Northern blot analysis of neu transgene expression. The following samples were analyzed: PGKNeo cells (lane 1), Aneu cells (lane 2), breast tumor from MMTVneu transgenic mouse (lane 3) and control mammary gland (lane 4).*

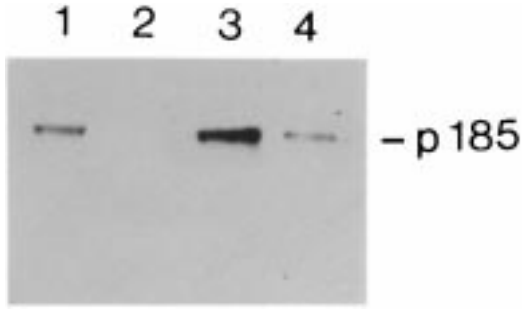

Figure 5 *Western blot analysis of p-185 neu protein in extracts from the following samples: NMuMG (lane 1), ES (lane 2), PGKNeo (lane 3) and Aneu (lane 4) cells.*

The relevance of these findings was evaluated in a physiological setting by *in vivo* experiments. For this purpose, 1 or 10×10^6 PGKNeo and A*neu* electroporated cells were inoculated subcutaneously in nude mice and allowed to grow to 0.5 cm^3. This is the maximal size that tumors are allowed to grow to under the rules on animal welfare adopted in our Institute. Whereas in repeated experiments with PGKNeo cells neoplasms arose as solid masses that became palpable approximately 10 days after inoculation and reached maximal size within 1 month. Tumor formation with 10^6 A*neu* cells was never observed during a follow-up of up to 2 months (Figure 6a). With 10^7 A*neu* cells, small tumor masses appeared only 50–60 days after inoculation, growing quite slowly and never reaching the volumes of those arising from the PGKNeo line, as shown in Figure 6b. The difference in the growth rate of these two populations is highly significant.

The *in vivo* tumor growth inhibition ability of the A*neu* construct was further investigated. Liposomes carrying the A*neu* plasmid were inoculated in 10 mammary glands of the right side of 1-month-old MMTV-*neu* mice, at which age tumor foci are already present.[4] The glands of the contralateral side were not inoculated. In addition, five mammary glands of the left side were injected with liposomes carrying PGKLacZ plasmid, while the contralateral ones were co-injected with liposomes carrying PGKLacZ and A*neu* plasmids. We have previously demonstrated that in our transgenic model the size of the tumor of one side does not differ significantly from the corresponding tumor of the opposite side.[4,5] Forty-five days after inoculation, tumors were resected and the weight of the left-side untreated tumors was compared with that of the treated neoplasias on the right side. Liposome-mediated gene transfer was highly effective, as all the PGKLacZ injected tumors (with or without the A*neu* plasmid) were stained an intense blue (Figure 7B and C), while the uninjected or the A*neu* only injected tumors were negative (Figure 7A). Although no complete remission was obtained with this protocol, statistical analysis of the weight ratio between untreated and treated tumors (see Materials and methods) demonstrated that untreated neoplasias were significantly larger than the treated ones (1.658 ± 0.1438 in treated mice *versus* 1.075 ± 0.1065 in control mice; $P < 0.01$).

Discussion

Oncogene bearing transgenic animals have been revealed as being a valuable tool in the study of both carcinogenesis and possible therapeutic approaches.[4,5,10,11]

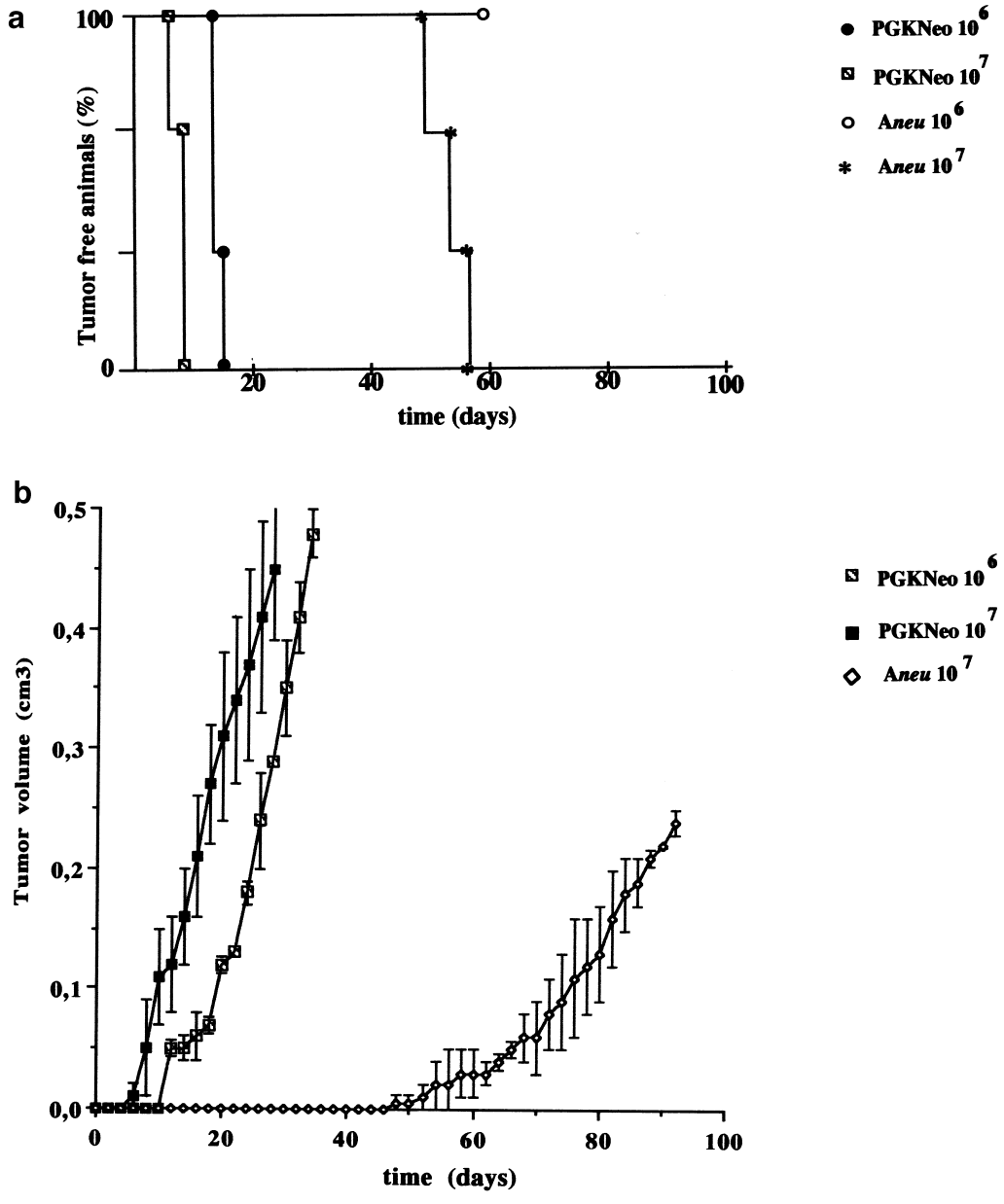

Figure 6 *In vivo tumorigenicity of PGKNeo and Aneu cells. The cells (10^6 or 10^7 resuspended in 0.3 ml) were inoculated subcutaneously in nude mice. At least three mice were injected for each experiment. (a) Percentage of animals with no detectable tumors. (b) Tumor growth in the three different groups. Tumors were measured every second day and left to grow until the masses reached the maximal size of 0.5 cm^3 or up to 3 months. Means ± s.e. are indicated.*

We have previously reported the generation of a transgenic mouse line which overexpresses an activated form of the rat *neu* oncogene in the mammary gland (MMTV-*neu* lineage).[4,6] In this lineage 100% of females develop multiple multifocal breast tumors by 12 weeks of age, suggesting that the tumorigenesis of the mammary epithelial cells is mostly due to the effect of the transgene, with little (if any) contribution of further genetic modifications, as required by the multistep theory of carcinogenesis.

A cell line was derived from one of these tumors, and its growth properties defined.[9] This cell line was used in the present study which involved antisense inhibition of the *neu* oncogene expression.

Antisense technology has been widely studied as a means of treating cancer, mostly through the use of oligonucleotides, with little success, despite high expectations.[12–14] The main problems encountered are related to the difficulty of drug delivery, the intrinsic toxicity of naked DNA, the transient nature of the treatments and also the cost of the reagent. We have thus decided to explore the possibility of using a stably integrated construct,[15,16] producing an antisense RNA corresponding to a large portion of the *neu* oncogene. To this end we have electroporated MG1361 cells with antisense or control plasmid and selected a population stably expressing A*neu*, as described in Results. This led to an effective inhibition of *neu* expression at both the RNA and protein level, which translated into changes in the morphology of the cells as well as in the growth rate *in vitro*. When

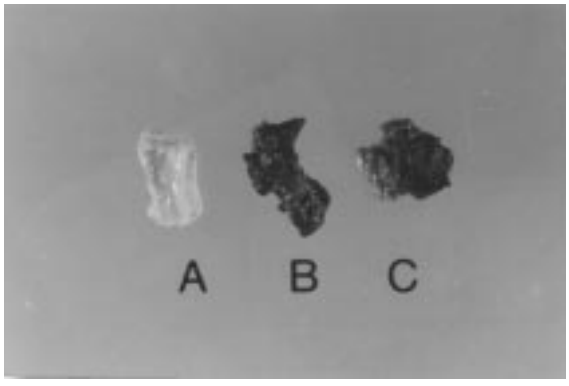

Figure 7 *β-Galactosidase staining of mammary tumors: uninjected control tumor (A), tumors inoculated with liposomes carrying PGKLacZ plasmid (B) and PGKLacZ + Aneu (C).*

these cells were inoculated in immune-compromised mice, a clear effect on tumor formation and growth was observed; while the PGKNeo-electroporated (control) cells behave in the same way as the parental line, no tumor was seen with the same amount of A*neu* cells (10^6 cells). In order to obtain observable tumor formation with A*neu* expressing cells, we had to inoculate 10 times the number of cells in nude mice. Even then, the growth rate of the tumors was greatly reduced, indicating that inhibition of the *neu* oncogene in these cells diminished their oncogenic potential by at least 20- to 100-fold.

The *in vivo* antisense-mediated tumor growth inhibition was further demonstrated by a liposome-mediated approach by transfecting the *Aneu* construct into tumors spontaneously arising in MMTV-*neu* transgenic mice. The size of inoculated tumors was significantly reduced after 45 days compared with the non-inoculated controls. This suggests that a single treatment with antisense vectors can partially control tumor growth. It is possible that further improvements could be obtained with more intense injection schedules.

We conclude that the use of a DNA vector with a large antisense segment can effectively inhibit the growth of cultured cells *in vitro* as well as *in vivo*. Cellular and biochemical parameters obtained with this antisense 'endocellular' approach are in agreement with results recently described in another breast cancer cell line treated with anti-*neu* oligonucleotides.[17] In our case, when the antisense vector was integrated, its effects were stable as the inhibition on tumor xenografts was maintained for several months even when an amount of cells one order of magnitude larger was injected into the immunocompromised host (see Figure 6). This suggests the possibility that gene therapy approaches with long antisense DNA fragment-carrying vectors can substantially affect tumor growth *in vivo*, avoiding problems associated with systemic toxicity of oligonucleotides. This possibility was confirmed in our experiments with primary tumors in MMTV-*neu* mice. As amplification and overexpression of the *neu* oncogene is a common occurrence in human mammary adenocarcinomas,[7,8] the results obtained in the present study raise the possibility that vector-mediated antisense expression could find a role in the combined treatment of human breast cancer.

Materials and methods

DNA constructs and electroporation

PGKNeo construct[18] containing the neomycin resistance gene under control of the PGK promoter, was used in electroporation studies as a control. MMTV-A*neu* construct was obtained by inverting a *Msc*II fragment of the rat *neu* cDNA of MMTV-*neu*NT construct (see Figure 1). A *Sal*I–*Eco*RI fragment from this MMTV-A*neu* construct was excised and cloned in the PGKNeo polylinker to produce the MMTV-A*neu*Neo construct (referred to as A*neu* construct) which was used in the current study. Five micrograms of each plasmid linearized at the *Sal*I site were electroporated (250 V, 500 μF in 0.8 ml PBS) in 3×10^6 MG1361 cells. G418 (Sigma, St Louis, MO, USA) selection (0.5 mg/ml) was started 24 h later and was continued for 10 days. Surviving colonies were pooled, tested by PCR, expanded and used for further analysis. PGKLacZ was produced by a blunt end cloning of a *Hin*dIII–*Bam*HI fragment from PCH110 eukaryotic assay vector (Pharmacia Biotech, Cologno Monzese, Italy) into the *Pst*I–*Xba*I sites of PGKNeo.

Cell morphology and proliferation

The production of MMTV-*neu* transgenic mice as well as the histological features of the tumors have been previously described.[4] The establishment, morphology and *in vitro* growth conditions of MG1361 cell line have been previously described.[9] The morphology of cells electroporated with PGKNeo or A*neu* was observed under an inverted microscope with phase contrast optics. In order to determine any difference in growth, cells were seeded in six-well tissue culture dishes at a density of 10^5 cells per well. The cells were left to grow and counted every second day for 2 weeks by direct counting in a hemocytometer chamber.

Analysis of neu RNA and protein expression

Total RNA was extracted from the control mammary gland, MG1361, PGKNeo and A*neu* electroporated cell lines with the RNA-easy kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Antisense and HPRT gene expression was determined by RT-PCR on DNase-treated RNA samples using the the following primers: A*neu*L: TCTGGTGTGTCCCCCGAATA and A*neu*R: AGAACTCATCGGAGCAGGGG for the antisense sequence, and HPRT1: TGCTCAAGGGGGGCTATAAG and HPRT2: TCCAACACTTCGAGAGGTCC for the HPRT gene.

Thirty-five amplification cycles were performed; the denaturation step was at 94°C for 30 s, the annealing step was at 55°C for 30 s and the polymerization step was at 72°C for 45 s.

The antisense construct inhibition effect on the expression of the *neu* oncogene was determined by Northern blot analysis performed according to standard methods.[19] Five micrograms of each RNA were resolved on a denaturating gel, blotted on to Hybond N^+ membrane and hybridized with a rat *neu* cDNA probe according to standard procedures.[19]

For Western blot analysis, cellular extracts from NMuMG (ATCC, CRL 1636), ES cells, PGKNeo and A*neu* cells were prepared using 300 μl of buffer (NaCl 150 mm, Tris-HCl 50 mm, pH 8.0; Triton X-100 1%, Antiprotease cocktail, Sigma-2714, 1:1000) per 10 cm dish. Typically,

50 μg of protein extract were separated by electrophoresis on a 6% SDS-polyacrylamide gel and transferred to HyBond ECL Membrane (Amersham, UK). The membrane was then incubated for 1–3 h with rabbit antibody against the neu protein p185 (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with anti-rabbit antibodies HRP conjugated for 30 min. After several washes, membranes were treated with Amersham ECL detection reagent and exposed to radiographic film.

Analysis of tumorigenicity in nude mice

The tumorigenicity of PGKNeo and A*neu* electroporated cells was assayed *in vivo* in nude mice. For this purpose, the cells were trypsinized, resuspended in PBS and inoculated subcutaneously in nude mice (Harlan Nossan, Correzzana, Italy) at 1 or 10×10^6 in 0.3 ml and left to grow until the tumor mass reached approximately 0.5 cm^3 (in the case of PGKNeo cells). At least three mice were injected for each experiment. A complete necropsy was performed for histopathological analysis, essentially as previously described.[4,6]

Staining for β-galactosidase activity

Tumors were fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 2 h at 4°C. After three washes in PBS, they were stained (5 mm $K_3Fe(CN)_6$, 5 mm $K_4Fe(CN)_6{\cdot}3H_2O$, 2 mm $MgCl_2$, 1 mg/ml X-gal (Promega) in PBS) overnight at 37°C.

Liposome-mediated DNA transfer

Plasmid DNA (A*neu* or PGKLacZ) was dissolved in HBS (20 mm Hepes, 150 mm NaCl, pH 7.4) to a final concentration of 30 μg/ml. This solution was mixed with an equal volume of DOSPER liposomal transfection agent prepared according to the manufacturer's instructions (Boehringer Mannheim, Monza, Italy). Fifty microliters of the DOSPER/A*neu* mixture were injected into the right-hand side mammary glands (n = 10) of 1-month-old MMTV*neu* transgenic females. The left side was not injected and used as internal control. In addition, five mammary glands of the left side and the five contralateral of the right side were injected respectively with 50 μl of DOSPER/PGKLacZ and DOSPER/PGKLacZ + DOSPER/A*neu*.

After 45 days, tumors were removed and weighed. The ratio between the left- and right-side tumors was calculated and compared with the ratio of a control group of untreated transgenic mice from the same litter. The statistical analysis of the data was carried out by Student's t test.

Acknowledgements

The technical assistance of Dario Strina and Enrica Mira Cato is acknowledged. We thank Prof R Dulbecco for encouragement, Dr Silvana Canevari for her useful suggestions and Ms Sophie Bevan for typing the manuscript. We thank Dr Cristina Mantegazza for help in statistical analysis. The financial support of AIRC to AV is gratefully acknowledged. MZ is supported by a grant from Telethon Italy. This is manuscript No. 14 of the Genome 2000/ITBA Project funded by CARIPLO.

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