Establishment and characterization of new mammary adenocarcinoma cell lines derived from double transgenic mice expressing GFP and neu oncogene

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Abstract. A new murine cell line, named GFPneu, was established from a mammary adenocarcinoma arising in double transgenic MMTVneu × CMV-GFP mice. Breast tumours develop in 100% of females after 2 months latency, as a result of the over-expression of the activated rat *neu* oncogene in the mammary glands. All tissues, and in particular the breast tumours, express the GFP protein. This cell line was tumorigenic when inoculated into nude mice and the derived tumours showed the same histological features as the primaries from which they were isolated. Their histopathology reproduces many characteristics of human breast adenocarcinomas, in particular their ability to metastasize. The GFP marker allows us to visualize the presence of lung metastases in fresh tissues immediately, to confirm the histopathology. From a lung metastatic fluorescent nodule, we derived a further cell line, named MTP-GFP, which we also characterized. These two cell lines could be useful to study the role played by the *neu* oncogene in the maintenance of the transformed phenotype, in the metastatic process, to test novel therapeutic strategies to inhibit primary tumour growth and to observe the generation of distant metastases.

INTRODUCTION

Breast cancer in women is a major health problem, causing a great deal of suffering and a high number of deaths. Currently, early diagnosis followed by surgery is the only way to cure the disease. Although breast cancer cells are responsive to many endocrine and chemotherapeutic agents, treatment of advanced disease is unsatisfactory and metastatic breast cancer is still associated with poor prognosis (Kiljn *et al.* 1992). In the last few years, a number of technical

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advances have paved the way to the possibility of using gene transfer approaches for the treatment of neoplastic diseases (Lo & Johnston 2003; Miller 2004; Snyder 2004). However, the implementation of clinical protocols is costly and time consuming, as a large number of patients must be treated and followed up for years. For this reason, the availability of an experimental model to test single and combination therapies could be invaluable, if some correlation between the results found in animals and in humans could be established.

Over-expression of the *Erb-B2* gene (the human homologue of the rat *neu* oncogene), as a result of genomic amplification, often occurs in human breast tumours and is recognized as a factor contributing to a poor prognosis in a subset of these neoplasms (Maguire & Greene 1990; Dougall *et al.* 1994; Porter-Jordan & Lippman 1994; Hynes & Stern 1998). In order to investigate new approaches to the therapy of mammary carcinomas, our laboratory pioneered the use of a transgenic mouse model (MMTV*neu*) of the breast carcinoma, which has many similarities with its human counterpart, including their ability to generate distant metastases (Sacco *et al.* 1995, 2000). Tumours arising in MMTV*neu* mice express oestrogen receptors, which are present in a subset of human tumours also, and show chromosomal changes similar to ones found in human breast tumours. This transgenic lineage has been used as a model to test different therapeutic strategies (Sacco *et al.* 1995, 1996, 1998a, 1999, 2000, 2001, 2002, 2003). As *in vivo* studies are difficult to perform, the availability of a cell line representative of this kind of neoplasia would allow the preliminary evaluation of new therapeutic protocols (Sacco *et al.* 1998b).

Here we describe the isolation and characterization of a novel breast cancer cell line named GFP*neu* derived from double transgenic mice [MMTV*neu* × CMV-GFP (Okabe *et al.* 1997)] and a further cell line (MTP-GFP), derived from a lung metastatic nodule grown after inoculation of GFP*neu* cells into nude mice. These maintain the same features as the primary tumours (epithelial-like morphology, high level of *neu* expression and high tumorigenicity when inoculated into nude mice), providing the material to investigate the pathways leading to *neu* dependent transformation *in vitro*. Moreover, the presence of the GFP marker allows a very rapid detection of the presence of very small metastatic nodules in fresh samples (such as lung metastases at very early stages), which can then be confirmed by histopathological analysis, making possible their tracing *in vivo*, using imaging systems.

This characteristic, in addition to all the features previously mentioned, renders these cell lines very useful for following the *in vivo* evolution of metastases. This will allow the investigation of different therapeutic approaches, suggesting new strategies for tumour therapy, using an *in vitro* system that can be directly correlated with the corresponding *in vivo* model.

MATERIALS AND METHODS

MMTV*neu* transgenic and GFP*neu* double transgenic mouse lineage production

The production of MMTV*neu* transgenic mice as well as the histological features of breast tumours have been previously described (Lucchini *et al.* 1992; Sacco *et al.* 2000). CMV-GFP transgenic mice were a kind gift from Dr Okabe (Okabe *et al.* 1997). From the breeding of heterozygous MMTV*neu* and CMV-GFP mice we obtained double transgenic CD-1 GFP-*neu* mice.

Isolation of GFP*neu* cell line

Breast tumours from 4-month old GFP*neu* double transgenic female mice were removed and rinsed separately three times in phosphate-buffered saline (PBS) with 100 U/ml penicillin and

100 µg/ml streptomycin. Each tumour was then minced into small pieces (0.5 mm each) with a sterile scalpel and was transferred into trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma Pharmaceuticals) solution at 37 °C for 30 min. The cell suspension was then centrifuged at 300 g for 5 min and the pellet of cells was plated and cultured until the cells formed a subconfluent monolayer. Preliminary experiments had been performed to define the optimal *in vitro* growth conditions by growing the cells in different culture media (Dulbecco's Modified Eagle's Medium, RPMI1640 or William's E Medium; Gibco-BRL) supplemented with 10% or 15% foetal calf serum (Sigma). Several cell lines were derived from independent tumours. In particular, one clone was maintained in culture for 20 passages and was fully characterized.

Analysis of tumorigenic potential in nude mice

The tumorigenicity of the GFPneu cell line was assayed *in vivo* in nude mice. For this purpose, cells were trypsinized, re-suspended in PBS and were inoculated subcutaneously in nude mice (Harlan Nossan) at 1×10^6 cells in 0.1 ml/mouse (five mice per group) and left to grow for 1 month. Tumour size was monitored weekly by calliper measurements. For histopathological analysis, a complete necropsy was performed, as previously described (Sacco *et al.* 2000). Tumours, lungs and other tissues were immediately observed under a fluorescence-inverted microscope (Nikon eclipse TE 200). All the tissues were then fixed by immersion in 10% formalin for at least 24 h at room temperature and were then processed into paraffin wax. Serial sections (3–5 µm) were stained with haematoxylin and eosin for conventional evaluation.

Isolation of MTP-GFP cell line from a lung metastatic nodule

One metastatic nodule, 4 mm in diameter and having originated in the inoculation of GFPneu cells, was excised from a lung lobe of a nude female mouse, rinsed three times in PBS with antibiotics (Pen/Strep) and then minced into small pieces and processed as described in previous discussions for the GFPneu cell line. The cells were then plated and cultured in complete William's E medium (Gibco-BRL) as previously described. The cell population was named MTP-GFP. The *in vivo* lesion in nude mice was evaluated as described for the GFPneu cell line.

Analysis of neu expression

Expression of *neu* oncogene was evaluated by Northern blot analysis of total RNA, extracted from both cell lines, from a primary double transgenic tumour as positive control and from a non-transgenic mammary gland as negative control; this was as previously described (Sacco *et al.* 1998b).

Cell morphology and *in vitro* growth characteristics

The morphology of GFPneu and MTP-GFP cells was observed under an inverted microscope (Nikon eclipse TE 200) after cytological staining with 20% Giemsa for 20 min.

Plating efficiency

Cells were trypsinized, counted and seeded in 35-mm plates (2500, 1200, 600, 300, 150, 75 cells, each in six plates). Two independent experiments were performed with six replicates. After 7 days, adherent colonies were stained with 20% Giemsa for 20 min and were counted. The plating efficiency was calculated as the number of colonies/number of cells seeded $\times 100$.

Population doubling time

A growth curve was plotted by plating 10^5 cells per well on six-well plates (Falcon) and then counting them after 24, 48, 72 and 96 h of culture. Each experiment was performed four times.

Soft-agar assay

Anchorage-independent growth was studied by a clonogenic assay determined in a bilayer soft agar system modified by Hamburger *et al.* (1978). Cells at the 20th passage were plated in 0.3% agar at three different concentrations: 10^3 , 10^4 and 5×10^4 cells for 60-mm plates. Formation of clones was evaluated by light microscopy after 7, 14 and 21 days. Colonies consisting of more than 50 cells were counted as agar-growing colonies.

Karyotype analysis

Cells in logarithmic growth (70% confluent) were exposed to colcemid (10 µg/ml, Gibco, Scotland) for 2 h at 37 °C, were harvested using trypsin/EDTA and were centrifuged. The pellet was treated with hypotonic KCl (75 mM) for 15 min at 37 °C. Cells were fixed in three passages of methanol : acetic acid solution (3 : 1) and were spread on slides. The chromosome complement of cells was established after scoring at least 100 metaphases stained according to the conventional Giemsa technique.

Spectral karyotyping and comparative genome hybridization analysis

Spectral karyotyping (SKY) was performed as previously described (Weaver *et al.* 2002). Briefly, chromosome preparations were hybridized overnight with a SKY kit containing chromosome painting probes that recognize all mouse chromosomes. Images were acquired using an epifluorescence microscope (DMRXA, Leica, Wetzlar, Germany) connected to an imaging interferometer (SD200, Applied Spectral Imaging, Migdal HaEmek, Israel). Chromosomes were unambiguously identified using a spectral classification algorithm. Six to 10 metaphases were analysed for each tumour. For comparative genome hybridization (CGH), DNA was prepared using high salt extraction and phenol purification and was labelled by nick translation using biotin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). Genomic DNA from strain-matched mice was prepared and labelled with digoxigenin-12-dUTP (Boehringer Mannheim). Hybridization was performed on karyotypically normal metaphase chromosomes (FVB strain) using an excess of mouse Cot1-DNA (Gibco-BRL, Gaithersburg, MD). The biotin-labelled sequences were visualized with avidin-fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA, USA) and the digoxigenin-labelled sequences were detected with a mouse derived antibody against digoxigenin, followed by a secondary rhodamine conjugated antimouse antibody (Sigma-Aldrich, Milwaukee, WI). Quantitative fluorescence imaging and CGH analysis were performed using Leica Q-CGH software (Leica Imaging Systems, Cambridge).

Fluorescein-activated cell sorter analysis

Cells were detached with trypsin/EDTA solution (Sigma), washed in PBS and then fixed overnight at -20 °C in 70% ethanol. After washing twice in PBS, cells were stained with 0.5 ml of DNA-prep stain solution (50 µg/ml propidium iodide; 4 KU/ml of RNase Type III-A bovine pancreas) (DNA-prep Coulter Kit, Beckman Coulter). After 10 min of incubation in the dark, the cells were analysed using flow cytometry (EPICS ELITE, Coulter). Chicken erythrocytes were used as haploid DNA content control.

Telomere length measurement

The length of telomeres was estimated using the comparative Telomere peptide nucleic acid (PNA) Kit/FITC (Dako, Glostrup, Denmark) for flow cytometry. Briefly, cell suspensions consisting of a 1 : 1 mixture of sample and control cells were prepared and then hybridized in the presence or absence of fluorescein-conjugated PNA telomere probe at RT, overnight in the dark. The 1301 tetraploid cell line, which has very long telomeres (> 30 kilobases) was employed as control. The

stained samples were then analysed by flow cytometry. The average telomeric fluorescence per chromosome/genome in the sample cells with respect to the telomeric fluorescence per chromosome/genome in the control cells (100% of fluorescence) was calculated using the following equation:

$$(\text{mean FL1 sample cells with probe} - \text{mean FL1 sample cells without probe}) \times \text{DNA index of control cells} \times 100$$

$$\text{RTL} = (\text{mean FL1 control cells with probe} - \text{mean FL1 control cells without probe}) \times \text{DNA index of sample cells}$$
 where RTL is the relative telomere length; FL1 is the channel of the FITC fluorescence; the index is the ploidy (index $2n = 1$).

Apoptosis detection

The cell monolayer was treated with etoposide at the concentration of 10 µg/ml and 100 µg/ml. After 24 h of treatment; cells were incubated for 30 min in the dark with 50 µg/ml of propidium iodide (PI) (Sigma, St. Louis, MO). They were then rinsed twice with PBS to remove PI and were harvested by standard trypsinization. The collected cells were washed twice with PBS, pelleted by centrifugation and were resuspended in PBS for flow cytometry analysis. Apoptotic and necrotic cells were detected as a PI^{dim} and PI^{bright} clusters, respectively.

RESULTS

A novel breast adenocarcinoma cell line (named GFPneu) has been derived from a breast tumour growing in double transgenic mice (MMTVneu × CMV-GFP); from a lung metastatic nodule grown after inoculation of GFPneu cells into nude mice, a further cell line (MTP-GFP) was isolated. Cell of both lines showed an epithelial-like morphology typical of adenocarcinoma cells; they were polygonal and their nuclei had many visible nucleoli (Fig. 1a and b). Cell population growth was influenced by the culture medium used. Optimal growing conditions were obtained by using William's E medium supplemented with 15% foetal calf serum (Sigma), Non-essential amino acids (SIGMA), 100 U/ml penicillin and 100 µg/ml streptomycin (GYBCO-BRL). The *in vitro* growth characteristics of both cell lines are reported in Table 1. Cell population doubling time was very similar in both cell lines at 30 h and 28 h, respectively, in GFPneu and MTPGFP, whereas plating efficiency was higher in GFPneu than MTP-GFP at each cell density. The clonogenicity assay showed an equivalent growth levels at 10^4 and 5×10^4 cells, whereas it was higher in MTP-GFP than GFPneu at 10^3 cells.

In order to investigate whether the malignant potential was maintained by the two cell lines, 1×10^6 cells/mouse were inoculated subcutaneously into five nude female mice and were allowed to grow for 30 days. Tumours arose as solid masses and became palpable between 7 and 15 days postinoculation, reaching an average size of 800 mm³ and 870 mm³ for GFPneu and MTP-GFP, respectively, at the moment of sacrifice. We observed that the two cell lines were growing *in vivo* at approximately the same rate (Fig. 2a). The level of *neu* expression *in vitro* in both the cell lines was comparable to its expression in a primary transgenic tumour *in vivo*, as shown in Fig. 2(b).

Tumours, lungs and other organs were removed and were immediately observed under an inverted fluorescence microscope. The entire tumour masses were of a very intense green. Whereas in all the other tissues, fluorescence was not detected, all the lungs showed several green fluorescent masses visible at $\times 4$ and $\times 10$ magnifications (Fig. 1c and d). This is very sensitive observation, as it is possible to recognize as little as a single cell in a fresh tissue.

Figure 1. Morphology of GFPneu cells and derived tumors. Morphology of GFPneu cells observed by an inverted microscope ($\times 200$ magnification) with fluorescence for GFP (a) and after staining with 20% Giemsa solution for 20 min (b) a fluorescent nodule growing from the GFPneu cell line, inoculated subcutaneously in nude female mice observed under an inverted fluorescent microscope at $\times 4$ (c) and $\times 10$ (d) magnifications. Histopathological analysis of paraffin embedded sections of tumours derived from the *in vivo* inoculation of GFPneu cells (e) compared to spontaneously arising tumours of MMTVneu transgenic mice (g) a lung metastatic nodule growing in a nude mouse transplanted with GFPneu cells (f) and in a MMTVneu mouse (h).

The histopathological analysis (Fig. 1e) of these GFPneu and MTP-GFP derived adenocarcinomas showed the same histological characteristics as the spontaneously arising breast neoplasias observed in MMTVneu transgenic mice (Fig. 1g). Lung metastases were observed in four out of five mice inoculated with GFPneu cells, and in five out of five mice inoculated with MTP-GFP cells, with an average of three metastatic nodules per lung. The histological evaluation of lung metastases (Fig. 1f) confirmed what was observed in fresh samples, with the same morphology

Table 1. *In vitro* growth characteristics of GFPneu and MTP-GFP cells

	GFPneu	MTPGFP
Population doubling time (h)	30	28
Percentage plating efficiency (number of cells plated)		
75	9.3	4.0
150	10.6	3.7
300	10.6	7.3
600	13.0	8.3
1200	13.3	8.3
2500	14.0	8.0
Percentage agar clonogenicity (number of cells plated)		
10 ³	1.30	2.28
10 ⁴	1.95	1.58
5 × 10 ⁴	1.56	1.20

observed in lung metastatic nodules in transgenic mice (Fig. 1h). Metastatic spread was not observed in other tissues.

Metaphase spread analysis showed that the chromosome number was heterogeneous in both cell lines. It ranged from 37 to 50, with diploid cells being around 55%. It is interesting to note that about 25% of cells showed 39 chromosomes, probably reflecting the loss of a specific chromosome. To further substantiate this observation, we performed CGH and SKY analysis on the GFPneu and MTP-GFP cultures (Fig. 3a). Both analyses consistently revealed the presence of cytogenetic abnormalities in the lung metastasis cell line whereas the primary breast tumour cell line shows no rearrangements. In the MTP-GFP populations we detected the loss of chromosome 4 and partial gains on chromosomes 14 and 15 [(Del4 B-E), +14(E), +15(A-C)]. As a result of the previous rearrangements, the MTP-GFP cell line lost one copy each of the *cdkn2a* and *cdkn2b* loci mapping to MMU 4C, and gain of *c-myc* mapping to 15D.

Flourescein-activated cell sorter analysis results were compatible with karyotype analysis. The DNA index was calculated as the ratio between the mean channels of G₀/G₁ peak of GFPneu sample to that of the haploid control sample (avian erythrocyte nuclei). Both GFPneu and MTP-GFP cell lines had a high percentage of diploid cells (about 70%, as also determined by the karyotypic analysis (55% diploid +25% with 39 chromosomes) there was also a number of cells with a higher chromosome number that overlapped the G₂ + M peak (Data not shown).

Analysis of telomere length, carried out using the Dako kit (Fig. 3b–e), revealed that both cell lines, GFPneu and MTP-GFP, have long telomeres (79.6% ± 1.9% and 96.7% ± 7.9% compared to the control 1301 cell line, which was considered as having 100% of length). As expected, the metastasis-derived cell line has longer telomeres than GFPneu, also explaining the greater stability of cancer and metastatic cells with respect to normal cells.

Both cell lines were then treated with an anticancer drug, etoposide, and the percentages of apoptotic and necrotic cells were analysed. After exposure to 100 µg/ml etoposide, the percentage of necrotic and apoptotic cells in both lines increased. In particular, the percentage of apoptotic metastatic cells increased from 13.23% in the control to 21.27% after treatment and the percentage of necrotic cells from 2% (control cells) to 23.16%.

Thus, the apoptotic GFPneu cell percentage increased from 7.94% to 23.35%, showing a higher sensitivity of this cell line with respect to the metastatic one, entering the apoptotic pathway after chemical treatment. Also, the percentage of necrotic cells increased from 0.67% to 9.2% after exposure to 10 µg/ml etoposide (Fig. 4).

Figure 2. *In vivo* growth of GFPneu and MTP-GFP cell lines and evaluation of neu oncogene expression. The *in vivo* growth of the two cell lines was evaluated after subcutaneous inoculation of 1×10^6 cells into nude mice (panel a). Tumours originated from the two cell lines were monitored weekly by measuring their size with calipers. Nodule dimensions were used to compute tumour volumes, calculated with the following formula: $(d^2 \times D)/2$ where d is the lower and D the higher tumour diameter. *neu* Oncogene expression (panel b) was evaluated by Northern blot analysis of total RNA extracted from the two cell lines (lane 3, GFPneu, lane 4 MTP-GFP) and compared with a negative control (lane 1, non-transgenic mammary gland) and a positive control (lane 2, breast tumour from a double transgenic female mouse).

DISCUSSION

Mice transgenic for oncogenes are a valuable model for studying the multi-step pathway involved in the pathogenesis of neoplastic diseases (Muller *et al.* 1988; Cardiff *et al.* 1989; Hanahan 1989; Pattengale *et al.* 1989). They provide spontaneously growing tumours in which at least one of the primary determinants is clearly defined. In this regard, we have extensively characterized breast neoplasias arising in transgenic mice (MMTVneu), and this has several features in common with its human neoplastic counterpart; we exploited it as an appropriate model for gene therapy studies (Sacco *et al.* 1995, 1996, 1998a, 1999, 2000, 2001, 2002, 2003). The present investigation describes two new bi-transgenic murine cell lines derived from a breast adenocarcinoma arising in these mice crossed with CMV-GFP mice and from their corresponding lung metastases. Amplification and over-expression of the *Erb-B2* mutated gene (*neu* oncogene)

Figure 3. CGH analysis and telomere length analysis. Genomic imbalances detected by CGH in the MTP-GFP cell line (panel a). Genomic imbalances that resulted in gains are plotted on the right side of the ideogram, and losses are displayed on the left. We could identify loss of chromosome 4 and partial gains of chromosomes 14 and 15. GFP *neu* and 1301 (panels b and c) and MTP-GFP and 1301 (panels d and e) cells were processed using the protocol of the comparative telomere PNA kit/FITC (Dako) and analysed by flow cytometry in three independent experiments. Panels b and d: cells without FITC staining (or only PI stained); panels c and e: cells double stained (FITC/PI) as requested by kit protocol for telomere length calculation. G1 FITC emission values are indicated. The 1301 human leukaemia tetraploid cell line was used as reference.

Figure 4. Apoptosis sensitivity. GFPneu and MTP-GFP cells were exposed for 24 h to etoposide, 10 µg/ml or 100 µg/ml, then were stained with propidium iodide and analysed by flow cytometry. Apoptotic and necrotic cells were detected as a PI^{dim} and PI^{bright} clusters, respectively. R1: living cells; R2: apoptotic cells; R3: necrotic cells.

are a characteristic of one-third of human breast adenocarcinomas (Maguire *et al.* 1990; Dougall *et al.* 1994; Hynes *et al.* 1998). Because these cells maintain a high level of *neu* expression *in vitro*, comparable with the level of expression in primary tumours of MMTV*neu* transgenic mice, both GFPneu and MTP-GFP cell lines could be suitable tools to test *in vitro* experimental protocols of tumour growth inhibition targeted to the *neu* function. This notion is further supported by the observation that both cell lines have very similar *in vitro* growth features.

We provide here new reagents for stepwise analysis of possible therapeutic strategies. The efficacy of therapeutic agents can be first assayed in this cell line *in vitro*, and their different susceptibilities to undergo apoptosis, mediated by anticancer drugs, could also be useful for screening purposes. Then the inhibition of primary tumour growth and metastasis can be rapidly evaluated by treating *in vivo*-transplanted nude mice. The possibility of visualizing the presence of green cells in fresh tissues in a very sensitive way will allow a rapid screening of the effects of novel treatments on lung metastasis. This intermediate step can be followed by *in vivo* studies in the correspondent transgenic model in which tumours arise spontaneously and in which longer follow-ups (several months) can be performed.

These cell lines will allow us to perform basic studies on the molecular and cellular components of this tumour type in order to develop new therapeutic strategies against breast cancer and to evaluate more mechanistic approaches in therapy. The transgenic model will then be used only for the more promising therapeutic approaches, saving time, costs and animal suffering.

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REFERENCES

- Cardiff RD, Sinn E, Muller W, Leder P (1989) Transgenic oncogene mice. *Am. J. Pathol.* **39**, 495–501.
- Dougall WC, Quian X, Peterson NC, Miller MJ, Samanta A, Greene MI (1994) The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene* **9**, 2109–2123.
- Hamburger A, Salmon SE, Kim MB, Trent JM, Soehnlen BJ, Albert DS, Schmidt HD (1978) Direct cloning of ovarian carcinoma cells in agar. *Cancer Res.* **38**, 3438–3444.
- Hanahan D (1989) Transgenic mice as probes into complex systems. *Science* **246**, 1265–1275.
- Hynes NE, Stern DF (1998) The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim. Biophys. Acta* **1198**, 165–184.
- Kiljn JGM, Berns PMJJ, Bontelbal M, Alexieva-Figusch J, Foekens JA (1992) Clinical breast cancer: new developments in selection and endocrine treatment of patients. *J. Steroid Biochem. Molec. Biol.* **43**, 211–221.
- Lo S, Johnston SR (2003) Novel systemic therapies for breast cancer. *Surg. Oncol.* **12**, 277–287.
- Lucchini F, Sacco MG, Hu N, Villa A, Brown J, Cesano L, Mangiarini L, Rindi G, Kind S, Sessa F, Vezzoni P, Clerici L (1992) Early and multifocal tumors in breast, salivary, harderian and epididymal tissues developed in MMTV-Neu transgenic mice. *Cancer Lett.* **64**, 203–209.
- Maguire HC, Greene MI (1990) Neu (c-erbB-2), a marker in carcinoma of the female breast. *Pathobiology* **58**, 297–303.
- Miller KD (2004) Recent translational research: antiangiogenic therapy for breast cancer – where do we stand? *Breast Cancer Res.* **6**, 128–132.
- Muller WJ, Pattengale PK, Wallace R, Leder P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-Neu oncogene. *Cell* **54**, 105–115.
- Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimura Y (1997) ‘Green mice’ as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313–319.
- Pattengale PK, Stewart TA, Leder A, Sinn E, Muller W, Tepler I, Schmidt E, Leder P (1989) Animal models of human disease. Pathology and molecular biology of spontaneous neoplasms occurring in transgenic mice carrying and expressing activated cellular oncogenes. *Am. J. Pathol.* **135**, 39–61.

- Porter-Jordan K, Lippman ME (1994) Overview of the biologic markers of breast cancer. *Hematol. Oncol. Clin. North Am.* **8**, 73–100.
- Sacco MG, Mangiarini L, Villa A, Macchi P, Barbieri O, Sacchi MC, Monteggia E, Fasolo V, Vezzoni P, Clerici L (1995) Local regression of breast tumors following intramammary ganciclovir administration in double transgenic mice expressing neu oncogene and herpes simplex virus thymidine kinase. *Gene Ther.* **2**, 493–497.
- Sacco MG, Benedetti S, Duflot-Dancer A, Mesnil M, Bagnasco L, Strina D, Fasolo V, Villa A, Macchi P, Faranda S, Vezzoni P, Finocchiaro G (1996) Partial regression, yet uncomplete eradication of mammary tumors in transgenic mice by retroviral mediated HSV-TK transfer 'in vivo'. *Gene Ther.* **3**, 1151–1156.
- Sacco MG, Barbieri O, Piccini D, Zoppé M, Zucchi I, Frattini A, Villa A, Vezzoni P (1998b) *In vitro* and *in vivo* antisense-mediated growth inhibition of a mammary adenocarcinoma from MMTV-neu transgenic mice. *Gene Ther.* **5**, 388–393.
- Sacco MG, Gribaldo L, Barbieri O, Turchi G, Zucchi I, Collotta A, Bagnasco L, Barone D, Montagna C, Villa A, Marafante E, Vezzoni P (1998a) Establishment and characterisation of a new mammary adenocarcinoma cell line derived from MMTV neu transgenic mice. *Breast Cancer Res. Treat.* **47**, 171–180.
- Sacco MG, Benedetti S, Mira Catò E, Caniatti M, Ceruti R, Scanziani E, Pirola B, Villa A, Finocchiaro G, Vezzoni P (1999) Retroviral-mediated IL-4 gene therapy in spontaneous adenocarcinomas from MMTV-neu transgenic mice. *Gene Ther.* **6**, 1893–1897.
- Sacco MG, Caniatti M, Mira Catò E, Frattini A, Chiesa G, Ceruti R, Adorni F, Zecca L, Scanziani E, Vezzoni P (2000) Liposome-delivered angiostatin strongly inhibits tumor growth and metastatization in a transgenic model of breast cancer. *Cancer Res.* **60**, 2660–2665.
- Sacco MG, Mira Catò E, Ceruti R, Soldati S, Indraccolo S, Caniatti M, Scanziani E, Vezzoni P (2001) Systemic gene therapy with anti-angiogenic factors inhibits spontaneous breast tumor growth and metastatization in MMTVneu transgenic mice. *Gene Ther.* **8**, 67–70.
- Sacco MG, Soldati S, Mira Catò E, Cattaneo L, Pratesi G, Scanziani E, Vezzoni P (2002) Combined effects on tumor growth and metastasis by anti-estrogenic and antiangiogenic therapies in MMTV-neu mice. *Gene Ther.* **19**, 1338–1341.
- Sacco MG, Soldati S, Indraccolo S, Cato EM, Cattaneo L, Scanziani E, Vezzoni P (2003) Combined antiestrogen, anti-angiogenic and anti-invasion therapy inhibits primary and metastatic tumor growth in the MMTVneu model of breast cancer. *Gene Ther.* **10**, 1903–1909.
- Snyder RD (2004) New concepts in breast cancer therapy. *Intern. Med. J.* **34**, 266–269.
- Weaver Z, Montagna C, Xu X, Howard T, Gadina M, Brondie SG, Deng CX, Ried T (2002) Mammary tumors in mice conditionally mutant for *Brca1* exhibit gross genomic instability and centrosome amplification yet display a recurring distribution of genomic imbalances that is similar to human breast cancer. *Oncogene* **21**, 5097–5107.