OVEREXPRESSION OF ERBB-2/NEU IS PARALLELED BY AN INHIBITION OF MOUSE MAMMARY EPITHELIAL CELL DIFFERENTIATION AND DEVELOPMENTAL APOPTOSIS

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Abstract

The erbB-2/neu oncogene is frequently overexpressed in many different tumors in humans including breast and ovary. The oncogene encodes a receptor tyrosine kinase closely related to the epidermal growth factor receptor. We studied effects on differentiation and cell death of ErbB-2/Neu during mammary gland development in transgenic mice expressing an activated, oncogenic rat erbB-2/neu gene controlled by the mammary gland specific promoter from mouse mammary tumor virus (MMTV-LTR). Transgenic animals develop mammary cancer after repeated pregnancies and lactation. We present evidence that overexpression of ErbB-2/Neu in these mice is restricted to tumor cells. Tumor cells fail to differentiate and express milk proteins such as β-casein and whey acidic protein (WAP) during lactation. Epithelial cell apoptosis during normal involution is characterized by non-random DNA degradation into oligonucleosomal fragments. Tumor cells were mostly refractory to this developmentally controlled programmed cell death. Distinct areas within tumors, however, showed spontaneous cell death as measured by *in situ* TUNEL staining that co-localized with caspase-3 like activity. Our results indicate that the control of developmental cell death during involution is disturbed in erbB-2/neu induced tumors although cell death and caspase activation can take place.

Introduction

Transgenic mice represent useful models for assessing tissue-specific effects of oncogenes *in vivo*. The neu oncogene was initially isolated from a chemically induced rat neuroblastoma (Shih *et al.*, 1981). It encodes a 185 kDa transmembrane protein that bears close homology to the epidermal growth factor receptor (Shih *et al.*, 1981). Transgenic mice were generated that express the activated erbB-2/neu oncogene under the transcriptional control of a mammary gland specific promoter. This promoter is contained in the long terminal repeat of the mouse mammary tumor virus (MMTV-LTR) (Muller *et al.*, 1988). Several lines of transgenic animals were generated, one of them develops tumors after repeated pregnancies and lactation periods.

Transforming oncogenes may affect the phenotype of a cell in many different ways. For several oncogenes it was shown that their expression can inhibit differentiation of various cell types, e.g. erythroleukemia cells (Coppola and Cole, 1986) muscle cells (Olson *et al.*, 1987) and mammary epithelial cells (Strange *et al.*, 1989; Jehn *et al.*, 1992).

After weaning the mouse mammary gland undergoes a reductive remodeling termed involution. Milk protein synthesis gradually decreases and a significant collapse of lobulo-alveolar structures occurs. The process of involution is dominated by two events: a loss of epithelial cells by programmed cell death (PCD) (Walker *et al.*, 1989; Strange *et al.*, 1992) and a proteolytic degradation of the extracellular matrix (Talhouk *et al.*, 1991) to which the epithelial cells and neighboring fibroblasts contribute in a seemingly coordinated manner (Talhouk

et al., 1991; Lefebvre *et al.*, 1992; Strange *et al.*, 1992). PCD or apoptosis in the involuting mammary gland results in the removal of about 80% of the secretory epithelial cells.

Recently, a family of intracellular proteases termed caspases was identified as key regulators of apoptosis. Caspases form a tightly regulated proteolytic network within the cell which orchestrates the regulation and execution of apoptosis (reviewed in Thornberry and Lazebnik, 1998). Depending on the cell type, apoptosis can be induced by a wide variety of stimuli. The signaling pathways induced by these stimuli often converge at the level of mitochondria or subsequently when Asp-Glu-Val-Asp (DEVD)-specific caspase-3-like caspases, also termed executioner caspases, are activated (Green and Kroemer, 1998). Active caspases cleave a limited set of cellular proteins at aspartate thereby activating or inactivating cellular proteins that are critically required for cell death or survival. Presently, the role of caspases during mammary gland involution has not been studied in detail and only very limited information is available.

The aim of the study presented here was to analyze the consequences of ErbB-2/Neu expression on mammary epithelial cell differentiation and involution in transgenic animals. We present evidence that tumor cells overexpressing erbB-2/neu fail to terminally differentiate at the end of pregnancy and during lactation and are mostly refractory to the developmentally controlled apoptosis during involution. Remarkably, distinct areas were identified within tumors that were characterized by extensive cell death and caspase activity, both, at lactation and during involution. These data suggest that tumor cells maintain their capacity to undergo caspase-dependent cell death but mostly escape a developmentally regulated apoptosis during post-lactational involution.

Material and Methods

Animals

ErbB-2/Neu transgenic animals (Muller *et al.*, 1988) were obtained from Charles River (Sulzfeld, Germany). This strain of mice develops tumors after repeated pregnancies and lactation periods. Mice were continually mated until tumors appeared. Involution was induced after 5 to 7 days of lactation by removing the litter. Mammary gland tissue was prepared from tumor-bearing mice and from wildtype FvB control mice, fixed in freshly prepared 4% paraformaldehyde phosphate–buffered saline and embedded in paraffin. 4 μm thick sections were stained with hematoxylin and eosin or used for *in situ* hybridization or immunohistochemistry. Part of the tissue was frozen in liquid nitrogen and stored at –70°C.

Immunohistochemistry, TUNEL staining and *in situ* hybridization

For immunohistochemistry, 4 μm thick sections were treated with 0.6% H_2O_2 in methanol for 30 min at room temperature to inactivate endogenous peroxidase. Non- specific binding was blocked with normal goat serum (Kirkegaard & Perry,

Gaithersburg, MD USA). Sections were incubated overnight at 4°C with Neu (Santa Cruz, # *sc-284-G*) or β-casein (a gift from E. Reichmann) specific antibodies or with CM1 (a gift from A. Srinivasan) (Srinivasan *et al.*, 1998) that recognizes active caspase-3. In the case of CM1, antigen-antibody complexes were detected with the dextran-based, biotin free horseraddish peroxidase conjugate (EnVision™+ System; DAKO, Glostrup, Denmark) using 3-amino-9-ethylcarbazole as substrate. For ErbB-2/Neu and β-casein we used a peroxidase-coupled avidin biotin system to detect antigen-antibody complexes using diaminobenzidin (DAB) as substrate.

For terminal transferase reactions (TUNEL), a previously described procedure was employed with several modifications (Feng *et al.*, 1995). In brief, 10 μm thick sections were treated with proteinase K (10 μg/ml) for 15 min at 25°C. Digoxigenin-labeled dUTP (Roche Diagnostics) was incorporated in the terminal transferase reaction and subsequently detected using fluorescein-coupled anti-digoxigenin Fab fragments (Roche Diagnostics).

In situ hybridizations were performed with the RNA color kit for non radioactive *in situ* hybridization (Amersham). A WAP containing pKS plasmid was linearized with Sal I for sense or Hind III for antisense probe. Labeled probe was prepared by incubating 1 μg linearized plasmid with 25 units of RNA polymerase in the presence of fluorescein-11-UTP for two hours at 37°C. Histological sections were pretreated by incubating with proteinase K solution (10 μg/ml) and washed with PBS. To reduce the background slides were incubated for 10 min in acetylation-acetic anhydride/triethanolamine buffer and washed in PBS just after pretreatment. Prehybridization was performed in hybridization buffer (1 x SSC, 1 x Denhardt's solution, 300 μg/ml herring testes DNA and a rate enhancement compound) (Amersham) containing 50% formamide for 1 to 6 hours at 37°C. Sections were hybridized in the same buffer containing probe overnight at 37°C. Washing steps were performed with 1 x SSC and 0.1% SDS at 40°C for 2 x 5 min followed by 0.2 x SSC, 0.1% SDS at 55°C for 2 x 10 min. Sections were incubated for 1 hour with anti-fluorescein alkaline phosphatase conjugate at a dilution of 1:1000 in TBS in the presence of 0.5% BSA. After washing, slides were incubated with NBT (Nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolylphosphate) and reactions were developed in the dark for about 10 min. Slides were rinsed in distilled water and mounted with Aquatex (Merck).

Extract preparation and Western blot analysis

Frozen tissue (approximately 300 mg) was homogenized with a polytrone on ice in 600 μl solution A (10 mM Hepes-potassium chloride pH 7.9, 1.5 mM magnesium chloride, 10 mM potassium chloride, 1 mM β-mercaptoethanol, 1 mM DTT) in the presence of protease inhibitors (5 μg/ml leupeptin, 50 μg/ml aprotinin, 1 mM amino-hexanoic acid, 5 μg/ml antipain, 5 μg/ml pepstatin, 5 μg/ml chymostatin, 5 mM benzamidine, 0.125 mM PMSF). ZR-75-1 cells were washed twice in phosphate-buffered saline and resuspended in one volume solution A in the presence of protease inhibitors. After incubation on ice for 10 min extracts were vortexed for 10 sec and centrifuged (Sigma 202-MK) at 3‘500 rpm for 5 min at 4°C. The supernatant was centrifuged at 13‘000 rpm at 4°C for

10 min and the cleared supernatant was taken as cytoplasmic extract. Protein concentration was determined using the Pierce BCA protein assay kit. Extracts were stored at –70°C. For Western blot analysis 15 µg cytoplasmic protein were separated on a 12% SDS polyacrylamide gel. After transfer to nitrocellulose caspase-3 like protease was detected with CM1 antibody using the western blot chemiluminescence reagent plus following the manufacturer's instructions (NEN, Life Science Products, Boston, USA). Non-specific binding was blocked with PBS, 5% milk and 0.2% Tween 20 prior to incubation with the first antibody.

DNA fragmentation analysis

DNA fragmentation analysis was performed as described by Strange *et al.* (1992). Tissue samples (approximately 50 mg) were homogenized with a polytrone and incubated with 0.5% sodium dodecyl sulfate and proteinase K (0.2 µg/µl) in TE-buffer (10 mM Tris, 1 mM EDTA) for 1 hour at 50°C. DNA was isolated by phenol/chloroform and chloroform extraction followed by ethanol precipitation. After RNase A treatment 20 µg DNA were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed.

Results

Histological analysis of erbB-2/neu transgenic mammary glands

Mammary glands from wildtype and transgenic animals were prepared at lactation and at 3 days of involution and analyzed histologically (Figure 1). The wildtype mammary gland at lactation is characterized by a well defined lobulo-alveolar structure consisting of single layers of secretory epithelial cells with only few adipocytes (panel A). By day 3 of involution alveolar structures collapse and epithelial cells are in the process of being removed (panel B). In transgenic animals epithelium-derived tumors develop after repeated cycles of pregnancy and lactation. At early stages of tumor development neoplastic areas are surrounded by morphologically normal epithelial structures and the tumors are encapsulated by a basement membrane typical for a carcinoma *in situ* (panels C and D). At lactation alveoli contain secretory material (panel C) and after weaning an apparently normal regression and restructuring takes place in the epithelium around the tumors (panel D).

ErbB-2/Neu is predominantly expressed in tumor cells and is negatively correlated with milk protein gene expression

The transforming erbB-2/neu oncogene in transgenic animals is under the transcriptional control of the mouse mammary tumor virus promoter suggesting an elevated level of expression during late pregnancy and throughout lactation. Mammary tissue was prepared from wildtype and transgenic animals at lactation and at day 3 of involution and expression of the transgene was determined immunohistochemically with an antibody that recognizes the endogenous mouse and the transgenic rat ErbB-2/Neu protein (Figure 2). No protein was detected in wildtype glands, both, at lactation and at day 3 of involution (panels A and B, respectively). In transgenic animals a strong

cytoplasmic and membrane staining was observed at lactation (panel C) and at day 3 of involution (panel D) that strictly correlated with morphologically transformed cells. This restriction of ErbB-2/Neu expression to tumor cells suggests that additional events contribute to the control of transgene expression and to the selection and proliferation of tumor cells.

Mammary gland development is characterized by terminal differentiation of secretory epithelial cells and milk production during late pregnancy and lactation. Whey acidic protein (WAP) and β-casein are highly expressed during lactation and the expression decreases during involution. WAP and β-casein were used as markers for terminal differentiation of secretory epithelial cells. Immunohistochemical stainings for β-casein were performed with mammary gland sections derived of wildtype animals and tumor bearing transgenic animals at lactation and at 3 days of involution. Strong β-casein-specific signals were detected in normal areas of the gland at lactation (panel E) that were maintained until day 3 of involution (panel F). Similar results were obtained with sections derived of wildtype animals (data not shown). Tumor cells completely failed to express this milk specific protein at lactation and during involution (panels E and F). *In situ* hybridizations were performed with a WAP specific probe. Using an antisense probe strong signals were detected at lactation in morphologically normal areas of transgenic glands while no WAP expression was found in tumor cells (panel G). At day 4 of involution a similar distribution of signals was observed as at lactation (panel H, tumor area shown in inset). Hybridizations with a corresponding sense probe confirmed the specificity of the signals (panels I and K). These results indicate that ErbB-2/Neu expression negatively correlates with β-casein and WAP gene expression, two markers for terminal differentiation of secretory epithelial cells.

Tumor cells are refractory to developmental apoptosis

In wildtype animals after weaning, secretory mammary epithelial cells die by programmed cell death that can be visualized on histological sections with a terminal transferase (TUNEL) assay. PCD was monitored in the mammary gland of wildtype and transgenic animals at lactation and at day 3 of involution. In wildtype animals DNA fragmentation increased from a very low level at lactation (Figure 3, panel A) to a substantial number at day 3 of involution (panel B), when a maximum abundance of apoptotic cells is apparent (C. Vallan and R.J., unpublished data). Similarly, no DNA fragmentation was observed in morphologically normal areas in transgenic animals at lactation (panel C) but was evident at day 3 of involution in morphologically normal regions of the gland (panel D). With the exception of a limited number of small and distinct areas the tumors of transgenic animals were free of TUNEL positive nuclei at lactation and during involution indicating that these cells are mostly refractory to the developmentally controlled elimination of supernumerary cells (panel D, see also Figure 5).

Non-random DNA-degradation into oligonucleosomal fragments, a cardinal marker of many apoptotic processes, was previously shown to occur in dying mammary epithelial cells (Strange *et al.*, 1992; Feng *et al.*, 1995). Genomic DNA was isolated from wildtype and transgenic animals at lactation and at day 3 of involution. Electrophoretic analysis revealed a DNA ladder corresponding

to oligonucleosomal fragments in the DNA derived of involuting mammary glands of FvB (Figure 3E, lane 2) and MORO mice (lane 8). No apparent DNA fragmentation was found in DNA derived of corresponding lactating mammary glands (lanes 1 and 7, respectively). DNA fragmentation analysis of mammary glands of two independent tumor bearing mice revealed extensive degradations but no or only a limited fraction appearing as oligonucleosomal fragments (lanes 3-6). This degradation was obvious at lactation (lanes 3 and 5) and during involution (lanes 4 and 6) suggesting that a typical apoptotic fragmentation of DNA is restricted to the DNA of wildtype glands during involution while a significant, most likely random degradation of DNA predominates in tumors at lactation and involution. The fact that an expected TUNEL staining was found in normal areas of transgenic glands suggests a simultaneous occurrence of apoptotic and non-apoptotic cell death (see also Fig. 5 and text below).

Apoptosis during involution is paralleled by caspase-3-like activity

It was previously shown that programmed cell death often correlates with an elevation of a caspase-3 like activity in several systems. Here we explored caspase-3 like activity in normal and transgenic animals with the CM1 antibody that specifically recognizes active caspases (Srinivasan *et al.*, 1998). At lactation no signal was detected with the CM1 antibody, both, in wildtype and in morphologically normal mammary tissue of transgenic animals (Figure 4, panels A and C). At day 3 of involution CM1 positive cells were detected in the regressing epithelium of wildtype glands (panel B) and in morphologically normal epithelium of transgenic glands (panel D). In the same glands of transgenic animals tumor areas were mostly free of CM1 specific staining (panels C and D).

Western blot analyses were performed with proteins derived of mammary gland tissue from wildtype, tumor free transgenic mammary glands and tumor tissue at lactation and at 3 days of involution. In extracts of cultured mammary epithelial cells that were induced for programmed cell death the CM1 antibody detects a specific band of about 28 to 30 kDa (Figure 4E, lanes 7 and 8), as was previously described by Srinivasan *et al.* (1998). An involution-specific signal of 28-30 kDa was also found in extracts from tumor free transgenic glands (lane 4) and wildtype glands (lane 6) that was absent in mammary tumor extracts (lane 2).

By analyzing tumor sections derived from several animals we consistently observed distinct areas containing CM1 positive cells. In order to investigate whether tumor cells in these areas undergo cell death we performed CM1 stainings and TUNEL assays from serial sections (Figure 5). A very striking overlap of CM1 and TUNEL positive areas was detected and the tumor in these areas was characterized by many cells that had mostly lost contact to neighboring cells. As a consequence these cells appeared more loosely packed suggesting an extended removal of tumor cells in these areas. As no characteristic oligonucleosomal fragmentation was seen in tumors (Figure 3E, lanes 3-6) it is not clear whether this cell death is apoptotic or not. The signals leading to the elimination of tumor cells are presently unclear but they seem to be independent of the developmental stage of the gland as such areas were

found both at lactation (Fig. 5) and during involution (data not shown). In fact, tumors with a diameter of more than about 5 mm were characterized very often by large cell free regions that were filled with blood (data not shown). It is likely that the observed TUNEL and CM1 positive areas contribute to the formation of these cell free areas within the tumor.

Discussion

The development of tumors is based on an imbalance between cell division and cell death. The consequence is net growth of the tumor cell mass. Numerous defined genetic perturbations that accumulate during tumor development facilitate and promote tumor growth. One such genetic alteration that is often observed in human cancer is overexpression of the erbB-2/neu gene. Although ErbB-2/Neu is molecularly well characterized, the exact mechanism how it promotes tumor growth is not completely understood. Here we show in a transgenic animal model of breast cancer that ErbB-2/Neu overexpression is restricted to tumor cells, that these cells fail to differentiate during pregnancy and lactation and are uncoupled from developmentally regulated programmed cell death that occurs in form of apoptosis in the involuting mammary gland.

The tumor cell specific expression of the erbB-2/neu transgene is a remarkable observation since the transgene is under the control of the glucocorticoid hormone-dependent MMTV-LTR that was previously shown to be active during lactation in normal epithelial cells (Kennedy *et al.*, 1982; Ross and Solter, 1985). Analysis at the mRNA level of numerous distinct erbB-2/neu transgenic mouse strains revealed a strain dependence for transgene expression in tumors and normal tissue (Muller *et al.*, 1988; Bouchard *et al.*, 1989; Guy *et al.*, 1996). For the mouse strain used in this study it is likely that additional genetic events are required in mammary epithelial cells that directly or indirectly enable or potentiate the MMTV-LTR promoter activity. Furthermore, the stochastic appearance of tumors with a relatively long latency points to a selection and growth advantage of cells which express high levels of ErbB-2/Neu. A consequence may be a glucocorticoid independent expression of ErbB-2/Neu in tumor cells. Indeed, we found high constitutive levels of ErbB-2/Neu expression in tumors but not in morphologically normal transgenic mammary tissue of fully regressed glands which would support this hypothesis (data not shown).

Glucocorticoid receptor activity is required for mammary epithelial cell differentiation (Topper and Freeman, 1980, Doppler *et al.*, 1989). Expression of mammary epithelial cell differentiation markers such as WAP and β-casein was below the level of detection in tumor cells of ErbB-2/Neu transgenic animals at the stage of lactation whereas the normal transgenic epithelium surrounding the tumor expressed high levels of these milk specific proteins. We have evidence that members of the AP-1 family of proteins such as c-Fos and JunD are highly expressed in tumor cells throughout lactation and involution (H.L. and R.J., unpublished observation). As previously shown, overexpression of several oncogenes such as src, ras and mos is incompatible with the differentiation of mammary epithelial cells (Jehn *et al.*, 1992). Furthermore,

overexpression of oncogene products such as Src, Ras and Mos lead to elevated AP-1 (Fos/Jun) transcription factor activity (Jehn *et al.*, 1992). Inhibition of the glucocorticoid receptor by increased AP-1 levels either through direct physical interaction or by competition for limiting co-factors such as CREB binding protein (CBP/p300) has been well described (reviewed in Gottlicher et al., 1998). Therefore, it may be that elevated levels of Fos and Jun proteins contribute to the inability of ErbB-2/Neu transformed tumor cells to differentiate.

The fact that mammary epithelium derived tumor cells do not differentiate as assessed by WAP and β-casein expression indicates that ErbB-2/Neu overexpression directly or indirectly uncouples cells from normal developmental controls. No mammary gland involution associated induction of DNA-fragmentation was found neither with TUNEL analysis nor with analysis of total DNA on agarose gels. Furthermore, involution associated caspase activation was strongly decreased in tumor samples as compared to wildtype and transgenic control samples.

Despite the lack of involution associated induction of apoptosis in tumor cells, spontaneous cell death was observed in tumors at lactation as well as during involution. This form of cell death is characterized by a more random DNA fragmentation that is reminiscent of necrotic cell death. However, cell death is also associated with induction of caspase activity as measured *in situ* by the CM1 antibody that specifically recognizes activated caspase-3-like proteases. These findings demonstrate that cell death and caspase activation can take place in ErbB-2/Neu transformed tumor cells. This spontaneous form of cell death may be either due to ErbB-2/Neu signaling mediated homeostatic imbalances or to a local deficiency of nutrients and oxygen supply.

In conclusion, we present evidence that ErbB-2/Neu mediated tumorigenesis involves an uncoupling of the developing tumor cells from mammary intrinsic developmental controls that regulate mammary gland homeostasis and mammary epithelial cell differentiation and apoptosis. However, caspase-mediated cell death can take place within the tumor. Tumor therapies that aim at a direct induction of tumor cell death by specific stimulation of caspase activity are presently being investigated.

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References

BOUCHARD, L., LAMARRE, L., TREMBLAY, P.J. and JOLICOEUR, P., Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. *Cell*, **57,** 931-936 (1989).

COPPOLA, J.A. and COLE, M.D., Constitutive c-myc oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. *Nature*, **320,** 760-763 (1986).

DOPPLER, W., GRONER, B. and BALL, R.K., Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat β-casein gene promoter constructs in a mammary epithelial cell line. *Proc.Natl.Acad.Sci.USA*, **86,** 104-108 (1989).

FENG, Z., MARTI, A., JEHN, B., ALTERMATT, H.J., CHICAIZA, G. and JAGGI, R., Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. *J.Cell Biol.*, **131,** 1095-1103 (1995).

GREEN, D. and KROEMER, G., The central executioners of apoptosis: caspases or mitochondria? *Trends Cell Biol*, **8,** 267-71 (1998).

GOTTLICHER, M., HECK, S. AND HERRLICH, P., Transcriptional cross-talk, the second mode of steroid hormone receptor action. *J.Mol.Med.*, **76,** 480-9 (1998).

GUY, C.T., CARDIFF, R.D. and MULLER, W.J., Activated neu induces rapid tumor progression. *J.Biol.Chem.*, **271,** 7673-7678 (1996).

JEHN, B., COSTELLO, E., MARTI, A., KEON, N., DEANE, R., LI, F., FRIIS, R.R., BURRI, H.P., MARTIN, F. and JAGGI, R., Overexpression of Mos, Ras, Src and Fos inhibits mouse mammary epithelial cell differentiation. *Mol.Cell.Biol.*, **12,** 3890-3902 (1992).

KENNEDY, N., KNEDLITSCHEK, G., GRONER, B., HYNES, N.E., HERRLICH, P., MICHALIDES, R. and VAN OOYEN, A.J., Long terminal repeats of endogenous mouse mammary tumour virus contain a long open reading frame which extends into adjacent sequences. *Nature*, **295,** 622-4 (1982).

LEFEBVRE, O., WOLF, C., LIMACHER, J.M., HUTIN, P., WENDLING, C., LEMUR, M., BASSET, P. and RIO, M.C., The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. *J.Cell Biol.*, **119,** 997-1002 (1992).

MULLER, W.J., SINN, E., PATTENGALE, P.K., WALLACE, R. and LEDER, P., Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*, **54,** 105-115 (1988).

OLSON, E.N., SPIZZ, G. and TAINSKY, M.A., The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation. *Mol.Cell.Biol.*, **7,** 2104-2111 (1987).

ROSS, S.R. and SOLTER, D., Glucocorticoid regulation of mouse mammary tumor virus sequences in transgenic mice. *Proc Natl Acad Sci U S A*, **82,** 5880-4 (1985).

SHIH, C., PADHY, L.C., MURRAY, M. and WEINBERG, R.A., Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature*, **290,** 261-4 (1981).

SRINIVASAN, A., ROTH, K.A., SAYERS, R.O., SHINDLER, K.S., WONG, A.M., FRITZ, L.C. and TOMASELLI, K.J., In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ*, **5,** 1004-16 (1998).

STRANGE, R., AGUILAR-CORDOVA, E., YOUNG, L.J.T., BILLY, H.T., DANDEKAR, S. and CARDIFF, R.D., Harvey-ras mediated neoplastic development in the mouse mammary gland. *Oncogene*, **4,** 309-315 (1989)

STRANGE, R., LI, F., SAURER, S., BURKHARDT, A. and FRIIS, R.R., Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development*, **115,** 49-58 (1992).

TALHOUK, R.S., CHIN, J.R., UNEMORI, E.N., WERB, Z. and BISSELL, M.J., Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture. *Development*, **112,** 439-449 (1991).

THORNBERRY, N.A. AND LAZEBNIK, Y., Caspases: enemies within. *Science*, **281,** 1312-6 (1998).

TOPPER, Y.J. and FREEMAN, C.S., Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol.Rev.*, **60,** 1049-1106 (1980).

WALKER, N.I., BENNETT, R.E. and KERR, J.F., Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am.J.Anat.*, **185,** 19-32 (1989).

Figure Legend

Figure 1 Morphology of wildtype (panels A and B) and erbB-2/neu-transgenic mammary glands (panels C and D). Sections of mammary gland tissue were stained with hematoxylin and eosin. Panels A and C show sections of lactating mammary glands, panels B and D sections of mammary glands at day 3 of involution. The bar represents 50 μm.

Figure 2 ErbB-2/Neu, β-casein and WAP expression in mammary glands of wildtype (panels A and B) and erbB-2/neu transgenic mice (panels C to K) at lactation and during involution. Immunohistochemical analyses were performed with Neu specific (A to D) or β-casein specific antibodies (E and F). *In situ* hybridizations were carried out with a WAP specific antisense probe (panels G and H) or sense probe (panels I and K). The insets (panels H and K) depict tumor areas from the same sections. Sections were derived of mammary glands at lactation (panels A, C, E, G, I), 3 days of involution (panels B, D, F) and 4 days of involution (panels H and K). Panels C through K represent sections from tumor bearing animals. The bar represents 50 μm.

Figure 3 TUNEL assays performed on mammary gland sections of wildtype FvB (panels A and B) and transgenic animals (panels C and D) at lactation (A and C) and 3 days of involution (B and D). The bar represents 50 μm. Panel E represents a DNA fragmentation analysis. Mammary glands were derived of wildtype FvB mice (lanes 1 and 2), tumors from transgenic mice (lanes 3 to 6) and Moro mice (lanes 7 and 8). DNA was isolated from glands at lactation (lanes 1, 3, 5 and 7) and 3 days of involution (2, 4, 6 and 8). The DNA was separated electrophoretically on a 1.5% agarose gel and stained with ethidium bromide.

Figure 4 Caspase-3 like protease activity in mammary glands of wildtype (panel A and B) and transgenic animals (panels C and D). Panels A to D show the result of an immunohistochemical analysis with CM1 antibody that is specific for active caspase-3 like proteases. Sections were derived of glands at lactation (panels A and C) and day 3 of involution (panels B and D). The bar represents 50 μm. Panel E: Cytosolic extracts prepared from mammary glands of transgenic animals with (lanes 1 and 2) or without tumors (lanes 3 and 4) and wildtype animals (lanes 5 and 6) at lactation (lanes 1, 3 and 5) and day 3 of involution (lanes 2, 4 and 6) were analyzed by Western blot analysis using a CM1 specific antibody. As positive control ZR-75-1 cells are shown before (lane 7) and after treatment for 48 hours with cisplatin (CP) (lane 8). The positions of apoptosis-associated bands are indicated with open arrowheads.

Figure 5 Existence of TUNEL positive and CM1 positive cells in ErbB-2/Neu-derived tumor areas. Serial sections were prepared from tumors at lactation and analyzed for TUNEL positive cells by terminal transferase assay (panel A) and for active caspase-3 by immunohistochemistry (panel B). Shown are corresponding areas of serial sections. The bar represents 25 μm.

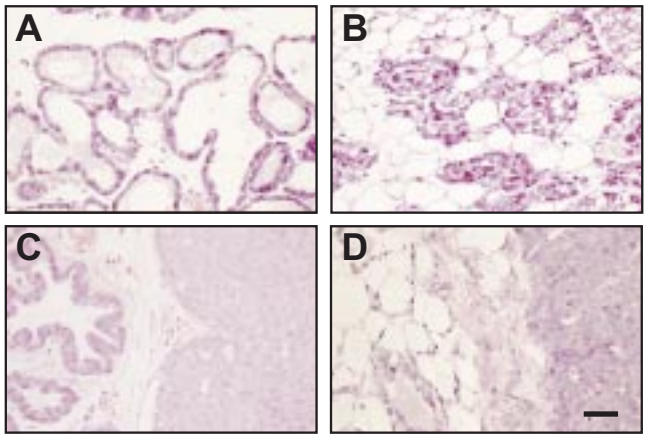


Figure 1

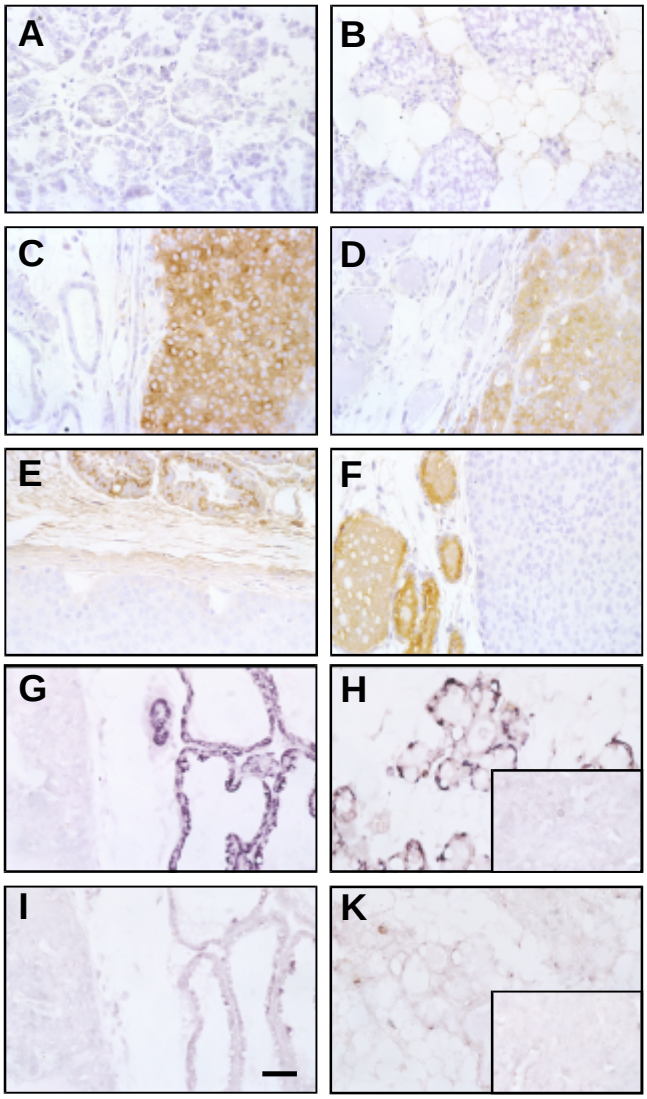
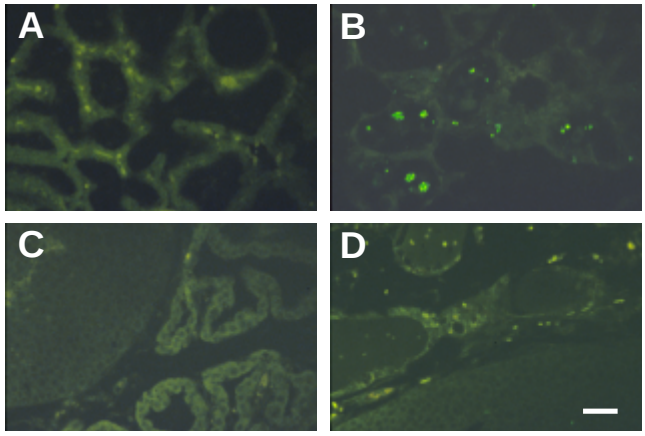


Figure 2



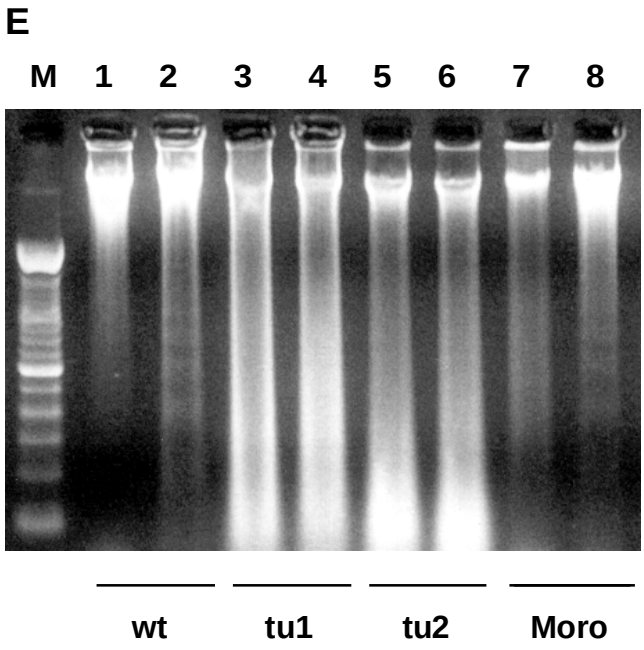


Figure 3

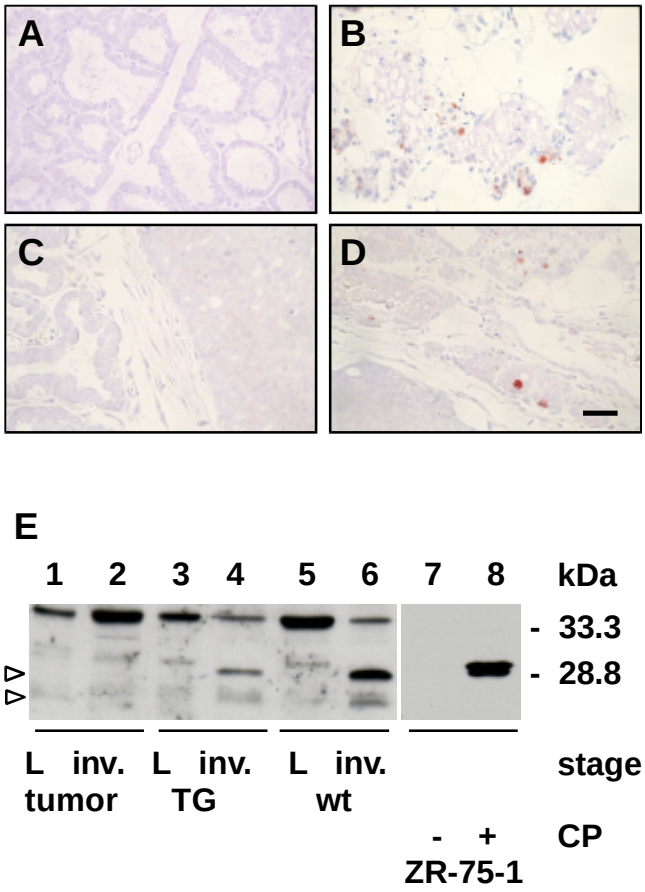
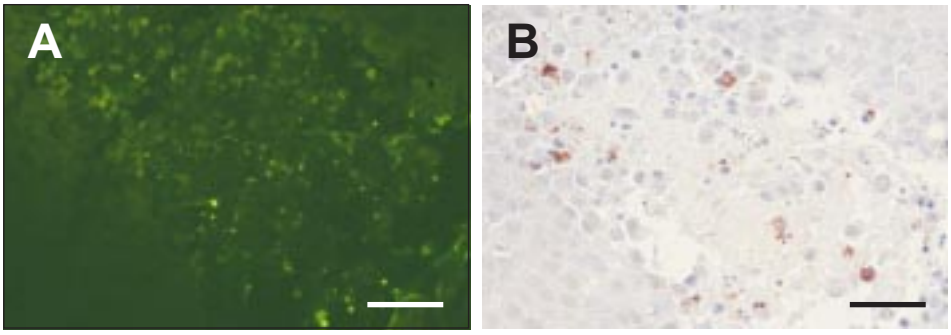

A
B
C
D
E
1 2 3 4 5 6 7 8
kDa
- 33.3
- 28.8
L inv. L inv. L inv.
tumor TG wt
stage
- +
ZR-75-1
CP

Figure 4



Figure