Murine c-erbB2: an oncogene that could be a target for vaccination therapy?

And the effect of HER2/ c-erbB2/ NeuT expression on cell signaling and milk protein formation in mouse mammary epithelial cells

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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aus Bern, BE

Bern, 2006

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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1 Summary

ErbB2 is an orphan receptor tyrosine kinase which can dimerize with other ligand-activated members of the EGF receptor family to signal in pathways inducing cell proliferation. Frequently overexpressed in breast cancer and other human cancers, homologs of ErbB2 are oncogenes in different animal species which have been studied for their contribution to the development of carcinomas.

The first part of this project was aimed at developing a method for vaccination of mouse, so that a transplanted tumor expressing the endogenous mouse c-erbB2 would be rejected. Initially, it was necessary to prepare a functional expression clone of mouse c-erbB2. Then the question of how to break the natural tolerance against an immune response against the self-antigen, mouse c-erbB2, had to be approached. Several protocols were attempted as described below, but I was unsuccessful in obtaining the intended protective effect against transplanted tumor.

In the second part of this work, we have examined phenotypes induced by several ErbB2 homologs in Line 31E mouse mammary epithelial cells which are capable of differentiation *in vitro* to undergo dome formation and to produce milk protein in response to lactogenic hormones. Included in this comparative study are the functional clone of the mouse proto-oncogene c-erbB2, a human homolog overexpressed in breast cancer, HER2, and the mutated rat homolog, NeuT, which is known to be oncogenic.

Line 31E mammary epithelial cells were infected with retroviral pBabe^{puro} constructs of the different ErbB2 homologs. Typical features of epithelial intercellular organization, such as density of tight junctions and dome formation, were disturbed by ErbB2 expression. While a dominant negative mutant of HER2 had no effect on the epithelial cells, both transepithelial monolayer resistance and dome formation were reduced by all three of the functional ErbB2 homologs, most dramatically by NeuT. While expression of both the mouse proto-oncogene c-erbB2 and HER2 resulted in significant inhibition of β-casein mRNA and protein levels after lactogenic hormone treatment, NeuT completely abrogated β-casein production and caused oncogenic transformation as evidenced by large colonies in soft agar and Matrigel suspension culture. While the cells expressing the homologs remain acutely responsive to EGF ligand in terms of Akt/PKB, ERK 1/2 and PKCα phosphorylation, an elevated basal phosphorylation in the absence of ligand was not apparent for PKCα.

2 Aims of the study

Part I:

Our goal was to test the hypothesis that the natural tolerance to the endogenous mouse c-erbB2 could be overcome by vaccination with the xenogeneic human homologue HER2. Vaccination experiments have already been shown to be successful in transgenic mice constitutively overexpressing the oncogenic rat homologue NeuT under the control of an exogenous promoter [2]. However, in this case, the immunity achieved was of course specific for the rat gene in mutated form, ie NeuT. Here we try to adapt this approach to achieve a theoretical vaccination against HER2 in human breast cancer. To test this concept, we immunized mice with HER2, a closely related, but foreign homolog, then challenged with a mouse tumor transplantation model expressing the endogenous c-erbB2 of the mouse. Ultimately, using this system, we also were unable to demonstrate an effective immunization or protective effect.

Part II:

The aim in part II is to characterize the phenotypes of endogenous mouse c-erbB2 (the normal endogenous gene occurring in healthy mice), of human HER2 (deriving from a tumor) and of NeuT (derived from a rat tumor) in mouse mammary cells in culture. Cell signaling and *in vitro* differentiation in response to lactogenic hormones were investigated.

3 Introduction

3.1 Human Breast Cancer

3.1.1 Background

In Switzerland, cancer is the most frequent cause of death below the age of 75 [3]. The dominating malignancy in women is the mammary carcinoma with 30% of all cancer, leading to more than 1300 cases of death per year [3,4]. Breast cancer is diagnosed in about 4000 women in Switzerland per year and in about 250'000 in Europe and its occurrence is steadily increasing *(websites 1 and 2)*. The aetiology is unknown but numerous risk factors have been described so far. Only 5% of mammary carcinomas follow a hereditary pattern, mutations in tumor-suppressor genes (BRCA-1 and BRCA-2) can be detected in most of these cases. Other risk factors are early menarche, late menopause, childlessness, hormone substitution in menopause, obesity, smoking and excessive alcohol consumption [4]. However, the majority of patients with breast cancer (up to 95%) develop the disease "spontaneously", with no risk factor being explicitly identified (*website 2*). The mortality is around 25% for breast cancer in Switzerland. Following the first treatment approximately half of the patients develop metastatic disease. The standard treatments for breast cancer so far have been chemotherapy, radiation, surgery and hormone therapy. These treatments are given with the goals of curing the cancer and/or limiting its spread, and providing relief from symptoms (*website 1*).

The adult mammary epithelium is organized into ducts and lobules. The ducts end in a highly branched structure, the terminal ductal lobular unit (TDLU). A TDLU consists of multiple individual units referred to as mammary acini. Each acinus has a central lumen, a single layer of polarized luminal epithelial cells surrounded by myoepithelial cells, and a basement membrane [5]. Breast cancer emerges through a multistep process progressing from hyperplasia to premalignant change, *in situ* carcinoma, and invasive breast cancer [6]. In ductal breast cancer the tumor arises from the lining of the milk ducts, as opposed to the lobules of the breast in lobular breast cancer [7]. Early stages of breast cancer (hyperplasia and ductal carcinoma *in situ* (DCIS)) are characterized by an increased proliferation of epithelial cells, a loss of acinar organization and filling of the luminal space. However, a lack of acinar organization and the acquisition of invasive behaviour are later events involved in progression towards malignancy [5].

3.1.2 The HER2 positive mammary carcinoma

Several types of cancers overexpress HER2. In breast cancer the protein is overexpressed owing to gene amplification in 30% of invasive ductal breast cancers [8]. HER2 amplification is detected in a high frequency (80–85%) of comedo-type DCIS tumours, which are non-invasive, premalignant mammary tumours [5]. Furthermore, HER2 is overexpressed in 20-25% of ovarian cancers, 35-45% of all pancreatic adenocarcinomas, and up to 90% of colorectal carcinomas [9]. In breast cancer, overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors, implying that HER2 confers a strong proliferative advantage to tumour cells. Paradoxically, a higher degree of HER2 overexpression is reported in early forms of breast cancer relative to more advanced invasive carcinomas, suggesting that alterations in HER2 alone are insufficient for breast tumour progression from a relatively benign to a more malignant phenotype [7].

Since in this study we are comparing ErbB2 receptors of different species which have received different designations, we decided to make clear distinctions: For easier distinction we will refer to them as follows: human ErbB2 and mouse c-erbB2 for the physiological non-mutated version of each of the species, as HER2 to designate the human homologue deriving from a tumor where it is overexpressed and for the sake of simplicity, oncogenic rat Neu will be referred to as NeuT.

3.1.3 Herceptin, a monoclonal antibody

An approved treatment in HER2 positive breast cancer is the recombinant humanized monoclonal antibody (mAb) trastuzumab (Herceptin®) targeting the HER2 receptor. Trastuzumab is used in therapy either alone (response rates of 26% first-line, resp. 15% post chemo) or in combination with cytotoxic chemotherapy (response rate up to 80%) [10]. The therapy is generally well tolerated and is not associated with chemotherapy-related side effects, such as alopecia, myelosuppression and severe nausea or vomiting [11]. If trastuzumab is given in combination with chemotherapy, and in particular with anthracyclines, an elevated incidence of cardiotoxicity is observed. There is evidence that this side effect reflects an exacerbation of anthracycline-induced cardiotoxicity [11]. Recent work

revealed that the inhibition of ErbB2 tyrosine kinase activity in rat cardiomyocytes causes MAPK-dependent structural damage in the myocyte contractile apparatus, leading to contractile dysfunction of the heart [12]. However, the cardiotoxicity is reversible and can usually be managed with standard medical treatment [11]. The clinical benefit from trastuzumab seems to be outstanding in the history of cancer treatment [13]. Trastuzumab was tested as an adjuvant to chemotherapy and in a one year treatment after surgery and chemotherapy. It was shown to dramatically reduce the risk of recurrence in women with HER2-positive breast cancer. Furthermore, the overall events (recurrence, second primary cancer or death before recurrence) were reduced by almost 50% in the trastuzumab treated group versus control, as was reported in recently published phase III trials [14,15].

It has been shown experimentally that the activity of trastuzumab is limited to ErbB2 homodimers. Binding of the mAb to ErbB2 homodimers rapidly downregulates PI3K/Akt, MAPK, Src and STAT signaling, and blocks the proliferation of tumor cell lines in nude mice [16]. However, the possible mechanisms of action of trastuzumab are numerous and can be attributed to extracellular and intracellular effects [17]. In contrast, trastuzumab can block neither the ligand-induced formation of ErbB2 heterodimers nor the heterodimer-induced activation of downstream signaling pathways. This characteristic could be a possible explanation for resistance to therapy [16]. In order to improve the inhibiting effect, pertuzumab, another humanized monoclonal antibody against ErbB2 has been developed and is in phase II trials at present. Pertuzumab is supposed to bind ErbB2 on the domain II dimerization arm *(see chapter 3.3.1 and Fig. 2 for details)* thereby preventing the formation of ligand-induced ErbB2 heterodimers [16].

In fact, 70% of patients are unresponsive from the start of trastuzumab treatment and nearly all eventually become unresponsive during treatment. These failures could depend on impairment of the machinery responsible for receptor downregulation, as it is known for autocrine TGFα production. By *in vitro* expression of exogenous TGFα in breast cancer cells, a dramatic reduction in trastuzumab-induced HER2 endocytosis, downregulation and cell growth inhibition could be observed. These findings were supported by selective cases *in vivo*, by comparing breast cancer neoplastic tissue before and after trastuzumab treatment. TGFα expression is not significantly induced upon treatment with trastuzumab [18].

3.1.4 Small-molecule tyrosine-kinase inhibitors

The small-molecule tyrosine-kinase inhibitors (TKIs) represent a distinct type of ErbB2 inhibitors that compete with ATP in the tyrosine-kinase domain of the receptor. So far two dual kinase inhibitors, blocking simultaneously both receptors, ErbB2 and EGFR, are being tested on ErbB2 positive breast cancer: the reversible inhibitor Lapatinib is being tested in phase III trials and the irreversible inhibitor CI-1033 has reached phase II trials. Both TKIs were shown to suppress tumor growth via the same pathways as with trastuzumab interaction. Although clinical efficacy of these inhibitors have been shown, the effects in patients can often not compete with the optimistic predictions from preclinical studies [16].

3.2 Part I – A xenogeneic HER2 DNA vaccination approach

3.2.1 Why a tumor vaccination should be investigated

Conventional cancer treatments including surgery, chemotherapy, and radiation are highly invasive and sometimes restricted to a palliative effect [19]. A targeted strategy such as a cancer vaccine has a much greater selectivity for tumor tissue and a decreased risk of collateral toxicity [20]. Many antigen-based vaccines have been successful in animal models in which they have been tested almost exclusively in tumor prevention. A reason for the failure of some of these strategies in humans is because they are being tested initially as therapeutic agents against advanced disease and usually after the failure of standard therapy [21].

The oncogenic potential of HER2, together with its elevated expression in tumors, cell surface localization, and immunogenicity in some patients, make this oncoprotein an ideal target for immunotherapeutic approaches [22]. Downregulation of HER2 and later EGFR with monoclonal antibodies was the first demonstration of targeted therapy [20]. Natural immunity at the level of T and B cells has been observed in patients with ErbB2 overexpressing tumors confirming the immunogenicity of ErbB2. ErbB2-specific CTLs can be detected in breast cancer patients, but in most cases do not prevent disease progression, which could be explained by the induction of tolerance. Antibody immunity to ErbB2 has been shown in some patients with early stage breast cancer suggesting that such autoantibodies are induced

by the native molecule and they do not simply reflect an increased tumor load or advanced stage. Progression of the disease even seems to suppress antibody production. The existence of ErbB2-specific T cell immunity in patients suggests that tolerance to ErbB2 has been circumvented. Many clinical studies have shown that it is possible to induce T-cell responses against ErbB2 peptides in cancer patients, but without engendering a clinical response. Peptide-based vaccines generally do not elicit antibody responses [9], which are known to be crucial in supporting T cell responses to inhibit ErbB2 positive breast cancer growth [23]. Disis et al. [24] stimulated the preexisting immunity of some ErbB2-positive breast cancer patients by vaccination with HER2-peptides and the adjuvant GM-CSF without causing autoimmunity. The non-occurrence of autoimmunity suggests that ErbB2-specific antibodies and T cells generated due to ErbB2 overexpression do not recognize basal ErbB2 expression on normal epithelial cells [24]. Immunizations with naked plasmid DNA present a number of advantages over peptide-based approaches. Peptide-based vaccines offer only a limited number of epitopes, whereas DNA vaccination allows the involvement of multiple different antigenic epitopes and a broad range of MHC restriction. Prior knowledge of host haplotype is therefore not necessary. Plasmid DNA encoding tumor-associated antigens, once reaching the cell nuclei, can persist as a circular episome and is not integrated into the host's genome. This results in a stable long-term expression of the encoded proteins by the host's cells and a permanent stimulation of the immune system, generating a long-lasting immunity. Another advantage is the generation of antigen-specific cellular (including CD4+ and CD8+ T cells) as well as humoral immune responses because the encoded antigen is processed through both endogenous and exogenous pathways. Peptide epitopes are presented by major histocompatibility complexes (MHC) class I as well as class II. In addition, DNA vaccines are cheap and easy in production and large scale-purification [19].

3.2.2 The hypothesis of breaking tolerance to endogenous ErbB2

The prerequisite for a successful cancer vaccination is breaking tolerance to the tumor-associated self-antigen [19]. In FVB mice transgenic for the oncogenic rat NeuT expressed under the MMTV-LTR promoter, vaccination with NeuT transfected allogeneic fibroblasts induced both T-cell and humoral immune responses and prevented tumor formation [2]. Interestingly the results of Pilon et al. [25] were not in accordance with the theory of a requirement of both cellular and humoral immune responses to block ErbB2 overexpressing tumors [23]. Pilon et al. focussed on inducing only a cellular response to ErbB2 in order to

circumvent the postulated harm resulting from an antibody reaction causing cardiac toxicity. In prospect of an *in vivo* application of the DNA vaccine, the tyrosine kinase activity was impaired through mutation, a modification which has been shown not to reduce immunogenic activity of ErbB2 [26]. The vaccination of non-transgenic mice with human cytoplasmic ErbB2 DNA with a deficient tyrosine kinase was revealed to engender an equivalent anti-tumor activity against human ErbB2 overexpressing tumor cells as to wild-type ErbB2 DNA, as long as supported by either GM-CSF or IL-2 DNA, but in contrast did not induce antibody response [25].

A promising vaccination approach is the injection of xenogeneic DNA. A xenogeneic homologue which is highly crossreactive, but not identical to the self antigen presents xeno-specific determinants that are non-self in the context of the recipients' immune surveillance; the immunological response to these determinants will therefore not suffer from the disadvantage of tolerance. A strong CD4 T cell response should be engendered to these determinants. According to basic immunology, this strong CD4 T cell response ought to stimulate a subset of primary CD8 or cytotoxic T cells (as well as B cells) showing specificity for the respective classes of self determinants shared with the xenogeneic product used as the vaccine immunogen. If the tumor antigen is a protein with an extracellular domain, this strategy profits from a B-cell response additionally. The efficacy of immunization against a self antigen using its xenogeneic version has been illustrated for prostatic acid phosphatase (PAP) [27], EGFR [28] as well as for ErbB2 [29]. All three examples are shared tumor antigens, expressed by both normal and malignant tissue. Prostate cancer patients vaccinated with dendritic cells loaded with mouse PAP protein all developed T cell immunity to mouse PAP and 50% of the patients showed cross-reactive T cells to the endogenous human PAP, which shares 81% homology with the mouse protein on an amino acid level. A subset of the patients with cross-reactivity could even profit from a clinical stabilization of their previously progressing prostate cancer. In contrast, vaccination with the identical human self antigen engendered little or no immunogenicity [27]. In the xenogeneic vaccination approach against EGFR, Lu et al. [28] relied on 88% of sequence homology at the amino acid level between human and mouse EGFR. In non-transgenic mice, human EGFR DNA induced both protective and therapeutic antitumor activity against an EGFR-positive tumor challenge, via humoral and cellular immune responses [28]. A xenogeneic DNA vaccination approach, consisting of human ErbB2 DNA without any adjuvants, resulted in autoimmunity and inhibition of mammary carcinoma development in a NeuT transgenic mouse model. The sera of these vaccinated mice revealed high titers of antihuman ErbB2 antibodies which didn't

crossreact with NeuT. No induction of specific CTL activity was observed in splenocytes. The vaccine was also given to healthy non-transgenic mice where it elicited autoreactive antibodies specific for the endogenous mouse c-erbB2. This data shows that xenogeneic DNA immunization can break tolerance against the endogenous mouse c-erbB2. Thus, tumor inhibition was explained by autoantibodies reducing the number of normal mammary epithelial cells at risk for transformation [29].

3.2.3 Proposed requirements for eliciting strong CD4/CD8 responses

For achieving the most efficient stimulation of CD4 and CD8 T cells, two leading opinions are currently discussed. On the one hand, it is believed that dendritic cells are the most powerful antigen presenting cells and that a fibroblast-based vaccine requires cross-presentation (i.e. transfer of the immunogen from the fibroblast to the dendritic cell in vivo) [30]. Ochsenbein and Zinkernagel et al. [31] however, argue that cross-presentation is unimportant as long as the fibroblasts are injected directly into a secondary lymphoid organ including the spleen and lymph nodes. The importance of targeting ErbB2 tumor antigen to antigen-presenting cells (APCs) in order to elicit a strong cell-mediated antitumor immunity has recently been demonstrated in non-transgenic mice [32]. Generally, adjuvants are believed to be crucial components of all cancer vaccines whether they are composed of whole cells, defined proteins or peptides. Adjuvants can activate APCs to stimulate T cells more efficiently [21]. Our vaccine design does not include any adjuvant since direct presentation of the antigen to the spleen is thought to be sufficient a stimulus for APCs. Without an appropriate priming of CTLs by CD4 T cells, the CTLs can remain anergic and nonresponsive [31,33]. Anergy is described as a state of antigen-specific nonresponsiveness in which a T or B cell is present, but functionally unable to respond to antigen. According to Ochsenbein [34], the crucial parameter that drives anti-tumoral immune responses is the antigen dose that reaches secondary lymphoid organs over time. If the antigen dose is low, the threshold for the activation of CTL within the lymphoid organ may not be reached, resulting in ignorance of the antigen. On the other hand a persistent high antigen dose, far above the threshold for CTL activation may induce tolerance. Tolerance describes situations where CTL have encountered antigen, but are not properly activated or they are actively suppressed by regulatory mechanisms. Antigen that is not cleared and persists at high levels in the lymphoid organ first activates T cells to proliferate and expand to a peak. T cells do not continue to expand further,

but undergo a contraction phase, resulting in the deletion of all T cells specific for that antigen [34].

To date no one has ever shown that one can effectively induce immune response to ErbB2 using the mouse endogenous peptides. The analogy to the work of the Gimmi group [2], who achieved a specific immune response against the rat *NeuT* antigen in transgenic mice, fails because it was the rat *NeuT* which served as the homologue to endogenous human HER-2. The mouse model for all such experiments to date have expressed a foreign exogenous transgene incorporated into the genome under the control of an exogenous promoter [2].

3.2.4 Our HER2 vaccination strategies

We have the clear aim of overcoming the natural tolerance to mouse endogenous ErbB2 in a non-transgenic FVB mouse model. The transplantable tumor model is the Met-1 murine mammary tumor line transfected with and expressing at high levels the mouse c-erbB2. To facilitate this breaking of tolerance we decided to vaccinate with a xenogeneic homologue, the human HER2. The degree of homology between human and mouse c-erbB2 protein has been shown to be above 85%. We tried essentially three different protocols to stimulate the immune system of the mice. In a first trial, Protocol A, we injected intramuscularly naked plasmid DNA carrying human ErbB2. Muscle cells are transfected by the gene and produce a protein product. The role of somatic tissues that express plasmid protein may be to serve as a reservoir for that antigen which is then transferred to the APCs. It has been shown that the mechanism of priming in this method for vaccination uses the MHC from bone marrow-derived APCs, which are efficient at providing all of the necessary signals for priming T cells [35]. In a subsequent series of experiments (Protocol B), we tested FVB fibroblasts as syngeneic carrier cells, transfected *ex vivo* with the xenogeneic homolog and injected directly into the spleen. Finally, Protocol C consisted of intrasplenic injections of human ErbB2 DNA. The enhanced immune stimulatory effects of the modifications in Protocols B and C have been described by Ochsenbein et al. and others [31,32,34], as mentioned above. Protocol C was refined by the application of electroporation. Gazdhar et al. [36] showed that the physical method of *in vivo* electroporation is a safe and effective tool for non-viral gene delivery to the lungs. Furthermore, it has been demonstrated that electroporation can significantly enhance the immunogenicity of a DNA vaccine, resulting in greatly improved antibody responses in macaques [37]. The benefit of electroporation has also been demonstrated in intramuscular

injections of a NeuT DNA vaccine in NeuT-transgenic FVB mice [38]. Boosters with the same dose and the same electroporation treatment in the gastrocnemius muscle of the hind leg was described to be effective in rat [37]. As endpoint for all these protocols, we used tumor protection against the ErbB2 overexpressing transplantable mouse mammary tumour Met-1. While our work was underway we found ErbB2 signaling had been described to act in downregulating components of the MHC class I machinery, resulting in an immune escape phenotype of the ErbB2 overexpressing cells [39,40]. Since this property would negatively affect our vaccination strategy, we engineered a mutation in the HER2 wild type gene, resulting in a deletion of the tyrosine kinase activation loop causing its inactivation. In the following, we refer to the deleted version of the gene as mutHER2. Early passage FVB fibroblast cultures serving as vaccine carriers were thus transfected with and expressing mutHER2 at high levels.

3.3 Part II – The cell differentiation and signaling pathway project

3.3.1 Molecular properties of EGF receptors

Receptor tyrosine kinases represent a subgroup of the protein kinase family, which is the largest enzyme family in humans. Protein kinases catalyze the following reaction:

$$MgATP^{1-} + \text{protein-OH} \rightarrow \text{protein-}OPO_3^{2+} + MgADP + H^+$$

Based upon the nature of the phosphorylated –OH group, these enzymes are classified as protein-serine/threonine kinases and protein-tyrosine kinases [41]. Protein-tyrosine kinases can be further subdivided into receptor tyrosine kinases, described in more detail below, and cellular, or non-receptor tyrosine kinases. The cellular tyrosine kinases are located in the cytoplasm or nucleus or are anchored to the inner leaflet of the plasma membrane. Src and Jak are exponents of this family [42]. Receptor tyrosine kinases (RTKs) are a superfamily of membrane receptors which catalyze the phosphorylation of tyrosine residues and elicit cellular effects in a minute timescale. RTKs share a common architecture of an extracellular region hosting the ligand-binding domain, a single pass transmembrane domain (approx. 20 amino acids) and the intracellular catalytic/effector domain incorporating both ATP- and substrate-binding sites. The intra- and extracellular domains are usually very large portions of

400-700 residues each [43]. The epidermal growth factor (EGF) or ErbB receptors are members of the RTK family type I. The family consists of four members: EGF receptor (EGFR), also termed ErbB1 (after the v-erbB oncogene of avian erythroblastosis virus)/HER-1, ErbB2/Neu/HER2 (human EGF receptor 2), ErbB3/HER-3 and ErbB4/HER-4 [41]. The four ErbBs share an overall structure of two cysteine-rich domains in their extracellular region, and a kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites [7]. ErbB3 has a tyrosine kinase domain that is highly homologous to those of the other family members but its kinase activity is impaired [41]. An overview of receptor structures is presented in Figure 1.

X-ray crystallographic analysis revealed the structures of the ErbB receptors' ectodomains in their active and inactive conformation and shed light on the mechanism of receptor activation. The extracellular component of all four ErbB proteins consists of domains I–IV. The activating growth factor, which binds to domains I and III, selects and stabilizes a conformation that allows a dimerization arm to extend from domain II to interact with domain II of an ErbB dimer partner. Crystallographic structures show that EGF and TGFα bind to domains I and III of a single receptor and do not span between two receptors. Ligand binding alters the disposition of domains II and III but not the relative orientation of domains I and II. In the non-activated receptor, domain II binds to domain IV, and the dimerization arm is buried and unable to interact with an adjacent receptor [44] (*Fig. 2*).

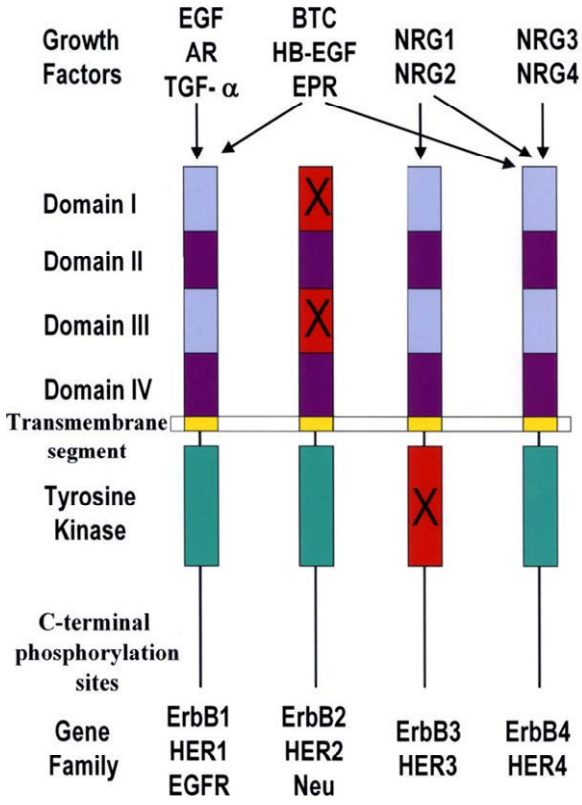


Fig. 1: Epidermal growth factor family of ligands and the ErbB gene family. The topology of the receptor proteins is indicated. The inactive ligand-binding domains of ErbB2 and the inactive kinase domain of ErbB3 are denoted with an X. Binding specificities of the EGF-related peptide growth factors are shown with arrows. There are four categories of ligands that bind ErbB family receptors. AR = amphiregulin, TGFa = transforming growth factor-a, BTC = betacellulin, HB-EGF = heparin-binding EGF, EPR = epiregulin, NRG = neuregulins. Figure from Roskoski [41].

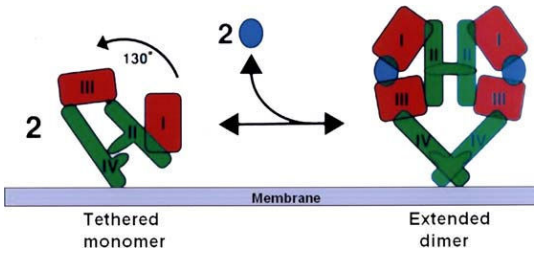


Fig. 2. Ligand induced conformational changes in the EGF receptor ectodomain. The inactive (tethered) EGF receptor ectodomain is shown on the left, and the active form is shown on the right. Homologous large (L) domains in the receptor (domains I and III) are colored red, and cysteine-rich (CR) domains (domains II and IV) are green. Ligand is colored blue. Individual domains are labeled. The mutual linking of the two domain II dimerization arms across the dimer interface can be observed in the center of the structure. The speculated position of the plasma membrane is depicted as a gray bar. EGF binding requires a 130° rotation of a rigid body containing domains I and II about the axis of the domain II/III junction. Figure from Burgess et al. [44].

The conversion of the inactive to active receptor involves a major rotation of the ectodomain, since the two ligand-binding sites on domains I and III are too far apart for a single ligand to bind to both simultaneously. The stoichiometry for epidermal growth factor binding to activated receptor is 2 mol EGF to 2 mol EGFR. The ectodomain of ErbB2, in the absence of ligand, resembles the extended conformation of activated ErbB1 suggesting that ErbB2 is autoactivated and thus constantly ready to dimerize. The ErbB2 ligand binding site is both obstructed and mutated which could serve as an explanation why no specific ligand for this receptor has ever been identified [44].

3.3.2 The physiological role of the ErbB signaling network

The importance of ErbB receptors in development is proven from the analysis of genetically modified mice. As an example, ErbB2 null mice die at midgestation due to trabeculae malformation in the heart, a phenotype that is shared by ErbB4 knockout mice. ErbB receptors also play essential roles in the adult organism, as well characterized in the proliferation and differentiation of the mammary gland, processes which are mostly undergone postnatally. In the mammary gland all four ErbB receptors are expressed in cell type- and developmental stage-specific patterns. ErbB2 is expressed in all developmental stages and together with ErbB4 it appears to have key roles in lobuloalveolar differentiation and lactation. ErbB receptors are activated by a number of ligands, referred to as EGF-related peptide growth factors, summarized in *Fig. 1*. These include EGF, amphiregulin (AR) and transforming growth factor α (TGFα), which bind specifically to EGFR, and betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which show a dual specificity for EGFR and ErbB4. The neuregulins (NRG) make a third ligand family. NRG1, also known

as neu-differentiation factor (NDF) or heregulin, and NRG2 both bind ErbB3 and ErbB4, whereas NRG3 and NRG4 bind solely ErbB4 [45]. All the ErbB family ligands have in common an EGF domain with six conserved cysteine residues characteristically spaced to form three intramolecular disulphide bridges. In general, EGF-related peptide growth factors are synthesized as glycosylated transmembrane precursors which are proteolytically cleaved from the cell surface to become the mature growth factor. EGF and betacellulin, which directly bind to the EGFR or ErbB4, mediate signaling through heterodimers of ErbB2 and ErbB3 in the absence of the EGFR [46]. Despite intensive efforts no direct ligand for ErbB2 has yet been discovered. The primary function of ErbB2 seems to be as a coreceptor. In fact, ErbB2 is the preferred heterodimerization partner for all other ErbB family members and plays a role in the potentiation of ErbB receptor signaling [45]. In earlier times, the description of an apparently ErbB2-specific ligand has been reported. It has been isolated and characterized as neu-activating factor (NAF), a 15-17 kDa protein, from a transformed human T-cell line. NAF is clearly distinguishable from the two other proposed homologous growth factors for ErbB2, the neu-differentiation factor (NDF 44 kDa) and heregulin (45 kDa) [47], which were later found to be identical, subsequently renamed neuregulin-1 (NRG1) [45]. The NAF ligand was reported to specifically interact with the ErbB2 extracellular domain, resulting in ErbB2-specific tyrosine kinase activation, homodimerization, internalization, and concomitant potentiation of the growth of ErbB2 expressing cell lines. NAF was reported to induce ErbB2 tyrosine phosphorylation in a dose-dependent manner *in vitro* and *in vivo*, as well as its down modulation from the cell surface [47]. Intriguingly, information about this proposed specific ErbB2 ligand breaks off in subsequent publications.

With the exception of EGF, which is found in many body fluids, ErbB ligands generally act over short distances as autocrine or paracrine growth factors. In this respect ErbB ligands display distinct expression patterns that are organ- and developmental stage-specific. In the case of the mammary gland, EGFR ligands and NRG-1 are co-expressed at various developmental stages. Ligand binding results in receptor dimerization, leading to activation of the intrinsic tyrosine kinase and autophosphorylation/transphosphorylation of specific, C-terminal tyrosine residues that provide docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. These include adaptor proteins such as Shc and Grb2, but also kinases such as Src and PI3K. The ability of ErB ligands to induce not only receptor homodimers but also heterodimers expands ErbB signaling potential and signal diversification. ErbB2-containing heterodimers display increased ligand affinity due to a decelerated off-rate that can be correlated with prolonged

activation of downstream signaling pathways. Furthermore biological responses such as proliferation, morphological differentiation and migration/invasion are enhanced in cells expressing ErbB2. Olayioye et al. [45] could prove that receptor phosphorylation is modulated by the dimerization partner. In addition, despite similar levels of total phosphotyrosine, oncogenic ErbB2 homodimers were significantly more potent in binding Shc than ErbB2 transphosphorylated by EGF. Thus, the signal elicited by a receptor heterodimer is not simply the sum of the signaling properties of the individual dimerization partners, but is rather due to unique properties acquired by the heterodimer [45]. Examination of the intracellular and extracellular domains of the ErbBs provides a satisfying explanation as to why a horizontal network of interactions is crucial to the ErbB signaling pathway: ErbB3 is devoid of intrinsic kinase activity, whereas ErbB2 seems to have no direct ligand. Therefore, in isolation neither ErbB2 nor ErbB3 can support linear signaling.

All ErbB family members couple via Shc and/or Grb2 to the mitogen-activated protein kinase (MAPK) pathway. Most active ErbB dimers bind and activate the phosphatidylinositol-3'-OH kinase (PI3K) [7], a lipid kinase that phosphorylates 3' hydroxyl residues in the inositol rings of certain lipids. These phospholipids act as second messengers to recruit and activate downstream targets, including the serine/threonine kinase Akt. This phospholipid-binding targets Akt to the cell membrane, where it is phosphorylated and activated [48]. The potency and kinetics of PI3K activation can vary, however, probably because PI3K couples directly with ErbB3 and ErbB4, but indirectly with ErbB1 and ErbB2 [7]. Interestingly ErbB2 appears unable to recruit the p85 adaptor subunit of PI3K. Thus, to activate this pathway, ErbB2 has either to heterodimerize with another ErbB receptor containing a p85 binding site, or activate the pathway indirectly, for example through Ras signaling. ErbB3 contains six docking sites for the p85 adaptor subunit of PI3K and efficiently couples to this pathway. Furthermore, inactivation of ErbB2 leads to loss of ErbB3 phosphorylation. These observations suggest that ErbB3's role in cancer cells might be to act as a partner of overexpressed ErbB2 promoting activation of the PI3K/Akt pathway [49]. Simultaneous activation of linear cascades, such as the MAPK pathway, the stress-activated protein kinase cascade, protein kinase C (PKC) and the Akt pathway translates in the nucleus into distinct transcriptional programmes. These involve not only the proto-oncogenes *fos*, *jun* and *myc*, but also a family of zinc-finger containing transcription factors that includes Sp1 and Egr1, as well as Ets family members such as GA-binding protein (GABP) [7]. The MAPK signaling pathway is a four-level cascade, in which each kinase activates the following kinase substrate through a complex network, enabling the cell to maintain diversity

and specificity while responding to various extracellular cues. The MAP kinases JNK and p38 are activated by a large spectrum of stress-related stimuli, whereas ERK is largely activated by growth factor signals [50]. The output of the ErbB network ranges from cell division and migration (both associated with tumorigenesis) to adhesion, differentiation and apoptosis [7]. A simplified overview of the processes involved in the ErbB signaling network, is depicted in Figure 3.

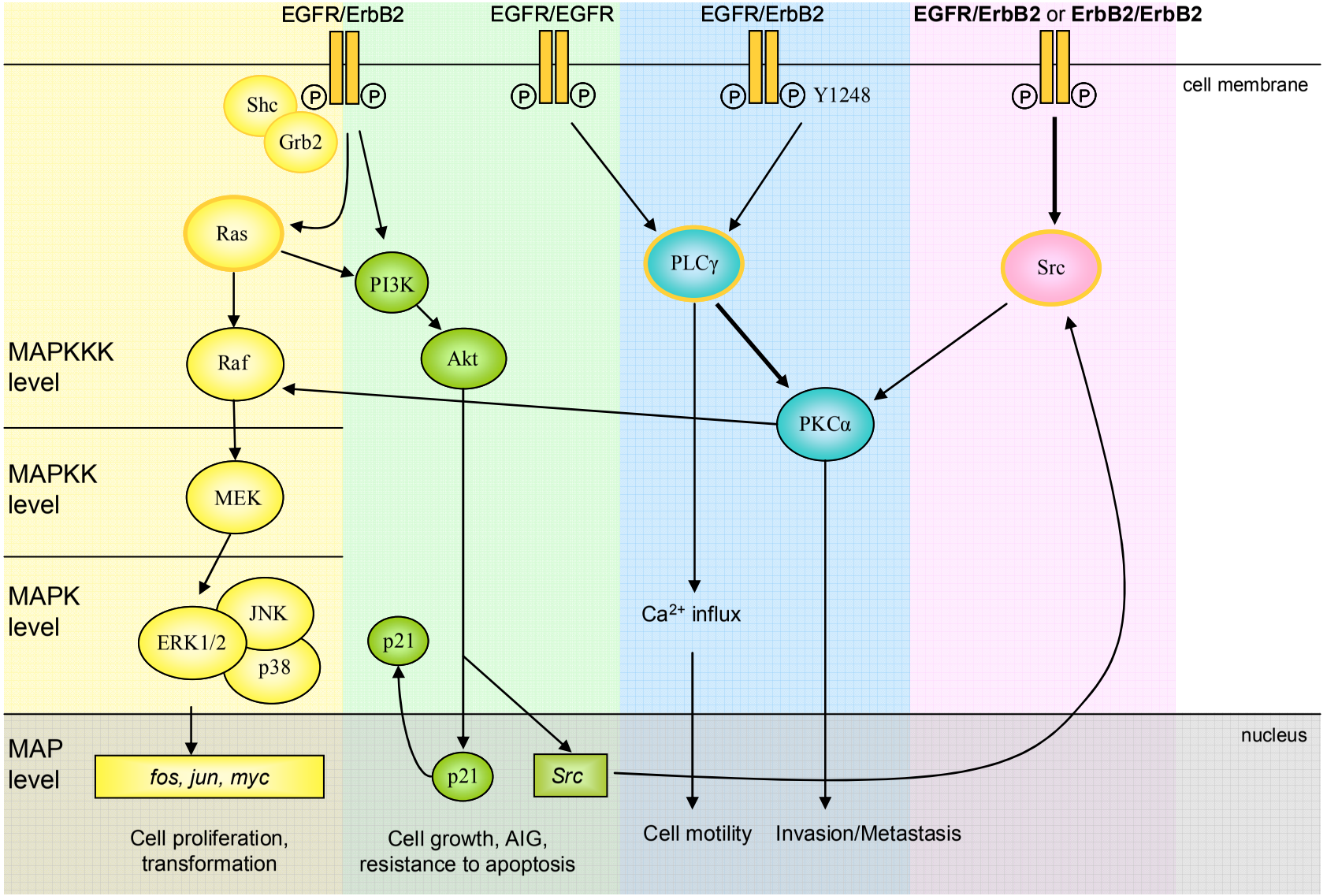


Fig. 3: ErbB2 signaling as desribed under ErbB2 overexpression. Broad arrows indicate predominantly activated pathways. Src is activated through ErbB2 overexpression, either by ErbB2 homodimers or strong signaling of EGFR/ErbB2 heterodimers, as indicated by bold letters. Golden circles highlight proteins binding directly to ErbB receptors. This figure shows data described in references [7,45,50,60,65,66,72].

ErbB RTKs also function as signal integrators, cross-regulating different classes of membrane receptors [51]. Cross-regulation mechanisms of ErbB receptors can be split in two major groups. Either ErbB receptors are phosphorylated by other kinases, which is the case for cytokine receptor transactivation, or EGF receptors autophosphorylate as a consequence of increased kinase activity due to ligand binding. Ligands availability in these situations is provided by metalloproteinase-mediated cleavage of an EGF-like pro-ligand. This second mechanism is observed with G protein-coupled receptor (GPCR) agonists as well as by Wnt proteins binding to frizzled (Fz) receptors, stimulating EGFR activity and activating the MAPK cascade [49]. Cross-regulation of cytokine receptors represents another mechanism for controlling and enhancing tumor cell proliferation. Cytokine receptors lack kinase activity and associate with Janus tyrosine kinases (Jak) to transduce signals. As an example, activation of the growth hormone (GH) receptor activates Jak2 which phosphorylates a tyrosine residue in the cytoplasmic domain of ErbB1 leading to Grb2 association and stimulation of the MAPK pathway. But RTKs and cytokine receptors can also negatively regulate each other as demonstrated on the inhibiting effect of EGF on prolactin (Prl) receptor signaling in the mammary gland. Hynes et al. have found that continuous EGFR activation in mammary cells inhibits Prl-induced Jak2 activation and therefore suppresses the cells in responding to prolactin. ErbB2 and Prl receptor cross-regulation has also been observed. It was recently shown in an ErbB2 overexpressing breast tumor cell line that Prl-mediated activation of Jak2 led to an enhancement of ErbB2 tyrosine phosphorylation [51].

3.3.3 Mutations in ErbB2

Originally two different unrelated mutations were found in Neu, the rat ErbB2 homologue, each leading to the onset of transformation. The oncogenic variant of Neu was called NeuT [46]. It was Bargmann et al. [52] who first found a single point mutation in the transmembrane domain of Neu in chemically induced rat neuroectodermal tumors. These findings demonstrated that alterations in the transmembrane segment of rat neu may also have profound effects on its biologic activity and transforming ability. The single-point mutation (GTG to GAG) results in the valine to glutamic acid amino acid substitution at position 664 in the transmembrane domain of the receptor. This point mutation apparently mimics ligand induction of receptor activation and results in the activation of the receptor tyrosine kinase with constitutive receptor dimerization. The described mutation is the only difference found between the oncogenic and proto-oncogenic forms of neu. Later, in tumors from transgenic

MMTV/Neu mice, a deletion was found in the extracellular region of Neu, resulting in a constitutively activated receptor capable of transforming Rat-1 fibroblasts.

V-ErbB, originally identified as one of the oncogenes carried by avian erythroblastosis virus was shown to encode a homologue of the human EGF receptor [49]. In human cancers, activation of wild-type ErbB2 occurs through overexpression, generally due to genomic amplification of the ErbB-2 proto-oncogene [8]. It is speculated that high levels of the wild-type receptor might promote spontaneous homodimerization, thus causing constitutive ErbB2 activation and signaling [49]. At that time, no activating point mutations of ErbB2, similar to what has been described for NeuT, had been found in human tumors [53-55].

Recently somatic mutations in the kinase domain of human ErbB2 have been identified in a small subset of primary lung tumors (NSCLCs) [56]. IHC staining for ErbB2 revealed no differences between tumors with or without ErbB2 mutations, leading to the presumption that overexpression probably does not accompany the mutation [56]. Further mutations in the human ErbB2 kinase domain have been detected in gastric, colorectal and breast carcinomas by Lee et al. [57]. Again, the mutations were detected with a frequency of below 5% of the analyzed cases. Lee et al. identified the two amino acids L755 and V777 to be affected in the frequently mutated sites. No ErbB2 amplification was detected in cancers with ErbB2 mutations, matching the previous findings in the lung tumors [56,57]. To date it is still unknown how these mutations influence ErbB2 activity [16,57]. Apparently, with Neu both mutation and overexpression result in stabilization of receptor dimers, which are essential for maintaining the tyrosine kinase in its active state.

Based on the observation that deletion of the whole non-catalytic C-terminal tail of ErbB2 abrogated its oncogenic function, the five autophosphorylation sites contained in the C-terminal part have been examined. It has been shown that oncogenicity is conferred due to a single autophosphorylation site, the most C-terminal tyrosine Y1248. The Y1248 phosphoresidue of ErbB2 alone regulates the association of SHC with ErbB2. These results indicate that the multiplicity of autophosphorylation sites on a receptor tyrosine kinase is not essential for transformability. Oncogenic activation of Neu appears to utilize the same biochemical pathway, but the ligand-induced initiation event is replaced by the point mutation mentioned above [58]. Overexpression of ErbB2 has been shown to occur in a significant portion of breast, ovarian, bladder, gastric, and other human malignancies [49].

Although a point mutation corresponding to that found in the neu oncogene has not been found in the HER2 gene in human tumors, a polymorphism at codon 655 **(**encodes either isoleucine (Ile; ATC) or valine (Val;GTC)) in the transmembrane domain of human ErbB2

has been reported in healthy individuals as well as in neuroectodermal tumors. Xie et al. could correlate this polymorphism with a decreased risk to develop breast cancer in a population-based study in Chinese women. Whether the polymorphic ErbB2 proteins differ in their ability to transform cells and/or have different tyrosine kinase activities has not been determined [59].

3.3.4 The alterations in ErbB receptor dimerization in cancer cells

Although ErbB2 homodimers alone may contribute to malignancy, a number of observations suggest that ErbB2 does indeed cooperate with other ErbB receptors during tumor development. Many human tumors that contain ErbB2 also exhibit autocrine stimulation of EGFR via expression of one of its numerous ligands [45]. Seton-Rogers et al. describe that homodimerization of either EGFR or ErbB2 alone cannot induce cell migration. Induction of migration in response to ErbB2 and TGFβ requires secretion of at least one EGFR ligand. It is possible that endogenous EGFR ligands stimulate migration through induction of heterodimers of EGFR with other ErbB family members, whereas homodimers do not induce a migratory response [60]. Heterodimerization and/or transactivation between ErbB2 and EGFR results in a high-affinity receptor for EGFR ligands like EGF or TGFα. Elevated expression of both receptors can be detected in primary human breast cancers. These observations suggest that these two closely related RTKs may collaborate in mammary tumorigenesis [61]. The association of frequent co-expression of both ErbB2 and ErbB3 transcripts, as detected in human breast tumors has been also observed in Cardiff's ErbB2 transgenic MMTV mice [48]. If ErbB2 phosphorylation is suppressed without suppressing dimerization with ErbB3, generally considered the most active heterodimer with regard to *in vitro* growth and transformation, signaling may continue [62,63]. In a murine hematopoietic progenitor cell line, Pinkas-Kramarski et al. [63] found that ErbB3 is devoid of any biological activity but both ErbB1 and ErbB2 can reconstitute its extremely potent mitogenic activity. They could show that heterodimers amongst ErbB1-3 are more potent than the respective homodimers, and ErbB3-containing complexes, especially the ErbB2/ErbB3 heterodimer, are more active than ErbB1 complexes. Pinkas-Kramarski et al. propose the following hierarchy of receptor crosstalk: ErbB2/ErbB3 > ErbBl/ErbB2 > ErbBl/ErbB1 > ErbB1/ErbB3 [63] *(see also Fig. 4).*

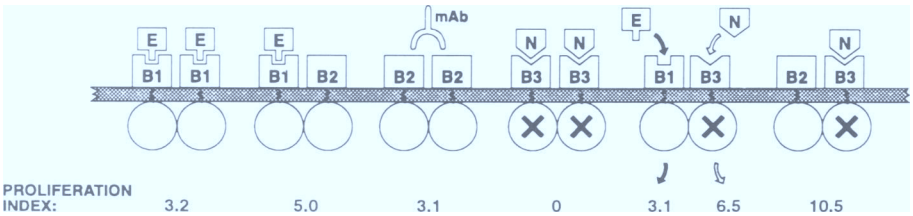


Fig. 4. Schematic illustration of various combinations of ErbB proteins and their respective proliferative potentials. The ligands depicted are: E = EGF; N = NRG1, mAb = a monoclonal antibody that binds to ErbB2. The crosses in the cytoplasmic domain of ErbB3 indicate an impaired tyrosine kinase activity. At the time of publication the fourth ErbB family member (ErbB4) was still unknown. The proliferation index represents the signal obtained in MIT assays after a 48 h-long incubation with saturating concentrations of the corresponding ligand, and it is calculated relative to the effect of IL-3. Note that heterodimers of ErbBl and ErbB3 can be activated by either ligand (arrows). Figure taken from Pinkas-Kramarski et al. [63].

Homodimeric receptor combinations are less mitogenic and transforming than the corresponding heterodimeric combinations, and ErbB2-containing heterodimers are the most potent complexes. The NRG-induced ErbB2–ErbB3 heterodimer is the most transforming and mitogenic receptor complex [7]. Indeed, the fact that ErbB2 does not constitutively display maximal activity when expressed in cells, and that it must be overexpressed to quite high levels to cause cell transformation, suggests that ErbB2 homodimerization is rather weak.

Antibodies with different epitopes could potentially change the orientation of ErbB2 homodimers or heterodimers to one another, substantially changing phosphorylation and downstream signaling. Antibodies to ErbB2 are predicted to change the rotational conformation of dimers relative to one another and in this way either inhibit or activate ErbB2 signaling. If antibodies suppress intramolecular phosphorylation without down-regulating membrane localization, effective suppression of ERK signaling may not occur [62].

Approximately 30% of human breast tumors overexpress EGFR and this overexpression is correlated with a loss of estrogen responsiveness and a poorer prognosis. Much evidence suggests that EGFR is involved in later stages of human breast cancer and may play a role in the metastatic process [64].

However, the two main signaling cascades are the PI3K/Akt and the ras-Raf-MAPK pathway. How they can be deregulated in ErbB2 overexpressing tumor cells is described in more detail in the following two chapters.

3.3.5 The PI3K/Akt pathway under ErbB2 overexpression

PI3K is strongly activated in ErbB2 initiated tumor cell lines derived from transgenic mice and a specific PI3K inhibitor is able to block anchorage independent growth in the same model. These findings of Amundadottir et al. [65] demonstrate that ErbB2 mediated tumor cells require PI3K for the transformed phenotype. ErbB2 activates the PI3K/Akt pathway and confers resistance to apoptosis induced by tumor necrosis factor. Akt detaches from the inner surface of the plasma membrane, where it is initially activated, and relocalizes to the nucleus within 30 min of activation by growth factors. ErbB2-mediated cell growth requires the activation of Akt which localizes $p21^{Cip1/WAF1}$ to the cytoplasm. $p21^{Cip1/WAF1}$ is a member of the cyclin-dependent kinase inhibitors and is expressed during cellular senescence. However, the cell-growth-inhibiting activity of $p21^{Cip1/WAF1}$ is strongly correlated with its nuclear localization. $p21^{Cip1/WAF1}$ can also be localized in the cytoplasm where it has an important role in protecting cells against apoptosis. It has been shown that cytoplasmic $p21^{Cip1/WAF1}$ inhibits the MAPK cascade via ASK1 and therefore results in resistance to apoptosis. In this sense, overexpression of ErbB2 may enhance cell proliferation by inducing cytoplasmic localization of $p21^{Cip1/WAF1}$ which in turn inhibits MAPK signaling [66]. Akt kinase activity is frequently increased in breast cancers, where it is associated with poor prognosis and resistance to tamoxifen and radiotherapy. Akt may play important roles in ErbB2-mediated mammary tumorigenesis, because ErbB2 possesses seven consensus docking sites for PI3K and can therefore transduce multiple signals through the PI3K/Akt pathway. A simplified version of the PI3K/Akt pathway is depicted in Figure 5. Hutchinson et al. [48] have investigated the importance of Akt activation in ErbB2 mammary tumor progression using a bitransgenic mouse model designed to co-express activated Akt and activated ErbB2 each under an MMTV promoter. Concomitant

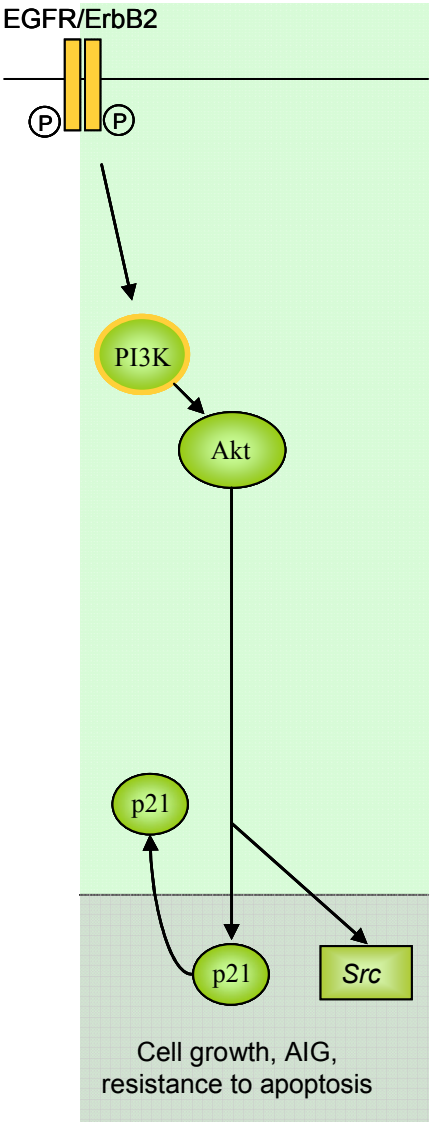


Fig. 5. The PI3K/Akt pathway activated through ErbB2. A simplified scheme.

activation of Akt accelerated ErbB2 mediated mammary tumor progression which correlated with enhanced cellular proliferation and elevated Cyclin D1 levels. On the contrary, these bitransgenic tumors showed lower levels of invasion and a significantly lower metastatic potential [48].

It has been argued that activation of the PI3K/Akt/mTOR pathway by ErbB2 overexpression predicts tumor progression in breast cancer [67]. Phosphorylation of Akt and mTOR (mammalian target of rapamycin) increases progressively from normal breast epithelium to hyperplasia and abnormal hyperplasia to tumor invasion [67]. The upstream molecules PI3K/Akt activate mTOR resulting in the initiation of protein translation. Phosphorylated Akt and mTOR were positively associated with ErbB2 overexpression *in vivo* as well as *in vitro* [67]. Signaling from the proto-oncogene ErbB2 has been shown to inhibit the adhesive function of the collagen receptor integrin α2β1 in human mammary epithelial cells. This anti-adhesive effect is mediated by the MAP ERK kinase 1/2 (MEK 1/2) and PI3K/Akt (protein kinase B, PKB) pathways. Hedjazifar et al. [68] could demonstrate that both pathways mediate suppression of matrix adhesion in a parallel fashion by causing the extracellular domain of the β1 integrin subunit to adopt an inactive conformation. In addition the PI3K/mTOR pathway was recognized as mediator of integrin inhibition but without causing conformational inactivation [68].

3.3.6 The MAPK pathway under ErbB2 overexpression

Oncogenicity of ErbB2 is almost fully dependent on an active Y1248, signaling via the Ras-MAPK (ERK) pathway. Mutation or deletion of tyrosine Y1248 in the non-catalytic C-terminal of ErbB2 abrogates downstream ERK activation, and implicate the MAP kinase pathway in transduction of the oncogenic signal of ErbB2 [58].

ErbB2 specifically activates the ERK/MAPK pathway, and relies on this pathway to mediate transformation and to maintain the transformed phenotype *(see Fig. 6)*. An inhibitor of MAPK kinase, the upstream activator of ERK, blocks phenotypic transformation by ErbB2 in the same model, confirming the importance in ERK signaling for transformation [65]. MAPKs are differentially activated by growth factors, hormones and cytokines. In addition, MAPK cascades may be activated by cellular stresses including irradiation, heat shock, osmotic imbalance, DNA damage, and bacterial products such as lipopolysaccharide. Activation of MAPKs in response to these stimuli controls gene expression, metabolism, cytoskeletal functions and other more complex cellular regulatory events, such as

mitogenesis, differentiation, survival and migration [69]. Although other signal transduction pathways are clearly important in ErbB2-mediated transformation, activation of ERK is a consistent downstream consequence of ErbB2 expression and is generally considered the principal driver of cell proliferation. Seton-Rogers et al. [60] were using a model of chimeras of ErbB1 and ErbB2 which were inducibly activated by means of homodimerization. In the absence of EGF, dimerization of either receptor per se was sufficient to promote proliferation. Both receptors induced activation of ERK and were competent to bind SHC and GRB2, a pattern consistent with their wild type counterparts. Activated ErbB2 alone was not able to induce transformation of human mammary epithelial cells (MCF10A) in terms of anchorage independency, invasion and migration. MCF10A cells were grown in a three-dimensional culture imitating acini (Matrigel, also described in chapter 3.3.9). Coactivation of ErbB2 and TGFβ signaling pathways induced invasive activity of MCF10A cells. ERK activation was required for the observed synergy between ErbB2 and TGFβ, but was not sufficient to induce migration or invasion, except when co-expressed with TGFβ [60].

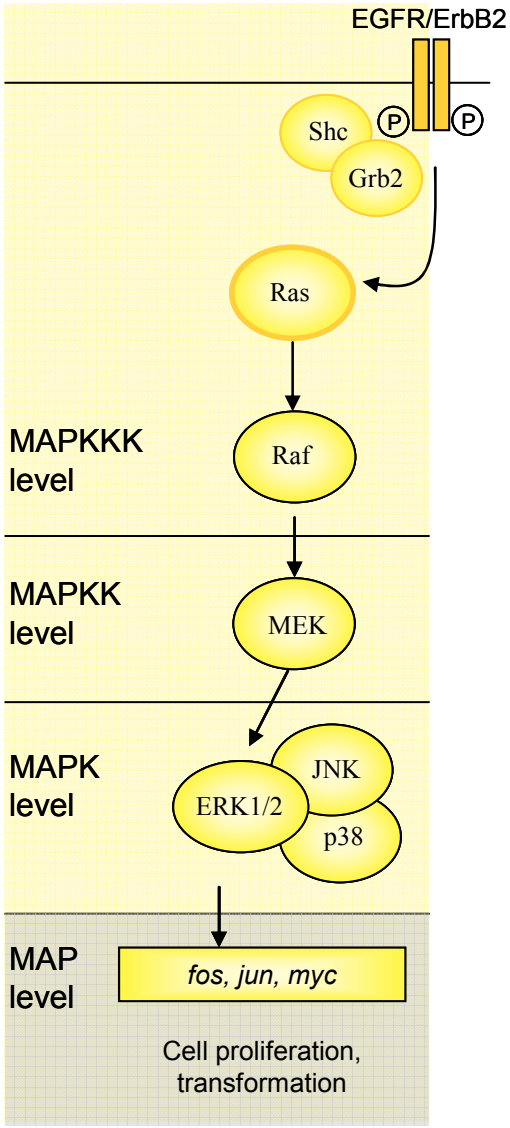


Fig. 6. The MAPK pathway activated through ErbB2. A simplified scheme.

Endogenous anti-HER2 antibodies induced in human by HER2 peptide vaccines are capable of blocking HER2 phosphorylation on tyrosine Y1248 and consequently blocking signaling through the MAP kinase ERK1/2 [62]. The suppression of ERK activity through HER2 antibodies [62] has another interesting implication since suppression of ERK in HER2-expressing cells restores sensitivity to tamoxifen. MAPK has been shown to phosphorylate Ser-118 in the estrogen receptor (ER), leading to ligand-independent ER activation with loss of the inhibitory effect of tamoxifen. Kurokawa et al. [70] could demonstrate that interruption of the MAPK cascade enhanced the inhibitory effect of tamoxifen on both ER-mediated

transcription *in vitro* and on tumor cell proliferation *in vitro* and *in vivo*. These findings were consistant in three different attempts of blocking the MAPK pathway, with either small molecule inhibitors of the HER2 kinase, inhibitors of the MAP kinase kinases (MEK-1/2) or via a dominant-negative mutant of MAPK [70]. Extensive research has documented the role of MAPK *in vitro* and in animal models in breast cancer, though data are inconclusive and sometimes contradictory with respect to proliferation, anoikis (apoptosis induced by loss of cell adhesion) and the association between MAPK, estrogens, and HER2. In therapy-related models, ErbB receptor inhibition combined with Gemcitabine results in elevated p38 and reduced ERK and AKT with ensuing apoptosis, but both ERK and p38 are elevated prior to paclitaxel-induced apoptosis. The expression, activation status, and the clinical role of p38 and JNK in breast cancer are unknown at present. p38 activation in breast carcinoma effusions is inversely linked to c-erbB2 expression, with no apparent relationship between this tyrosine kinase receptor and p-ERK or p-JNK. Davidson et al. [50] have previously shown that expression and phosphorylation of all three MAPK members is associated with clinical parameters of better outcome and better survival in ovarian carcinoma patients with effusions. These data have been reproduced in primary ovarian carcinomas. Davidson et al. have found a correlation between higher p38 activation ratio and poor disease-free survival and overall survival (OS). Notably, expression and activation of ERK did not correlate with any of the clinical parameters of aggressiveness or with survival in this cohort. Data regarding the role of phospho-ERK expression in primary breast cancer are contradictory [50].

3.3.7 The PKC pathway under ErbB2 overexpression

Protein kinase C (PKC) is a family of serine/threonine kinases that are involved in the signaling pathways that regulate cell proliferation, differentiation, apoptosis, motility and adhesion. The α-isozyme of PKC, PKCα, is widely expressed in various tissues. PKCα expression and activity have been found to be elevated in human breast cancer cells and are associated with an increased invasive or metastatic potential. In the classic model of PKC activation by receptor tyrosine kinases, ErbB2 (among other RTKs) phosphorylates and associates with phospholipase-γ (PLCγ), which may lead to the activation of PKCα *(see Fig. 7).* Tan et al. [71] showed that Src is co-immunoprecipitated with PKCα and that Src kinase activity contributes to PKCα activation by ErbB2 in human breast cancer cells. Overexpression or activation of ErbB2 upregulates and activates PKCα through Src, promoting breast cancer cell invasion. Downregulation of ErbB2 decreased expression and

activity of PKCα. These findings have been made *in vitro* as well as *in vivo* and suggest that Src kinase is another important regulator of PKCα activation in breast cancer cells [71]. The human c-Src proto-oncogene encodes an intracellular tyrosine kinase, pp60c-Src. c-Src was originally identified as a homologue of the transforming gene of the Rous sarcoma virus and is the prototype of a family of highly conserved genes that includes yes, fyn, fgr, lyn, lck, hck, blk, and yrk. Src family proteins have been implicated in many signal transduction pathways and in a wide variety of cellular functions. Activation of Src kinase has been linked to the development of many human neoplasias, especially those of the colon, breast, lung, and pancreas [72]. Src binds to tyrosine phosphorylated ErbB2 and is activated in ErbB2-overexpressing cancer cells [73]. A striking feature of c-Src association with the EGFR family is that it is restricted to the ErbB-2 receptor and cannot directly interact with the closely related EGFR [74]. Overexpression of c-Src alone is insufficient to transform murine fibroblasts in culture or to sustain tumor growth in intact animals. Studies in transgenic mice have demonstrated that c-Src is necessary for induction of mammary tumors by the polyomavirus middle T oncogene [64]. There appear to be two prominent roles of c-Src in this regard. One is to modulate receptor function by augmenting signals immediately downstream of the receptors and by regulating endocytosis, and the other is to affect

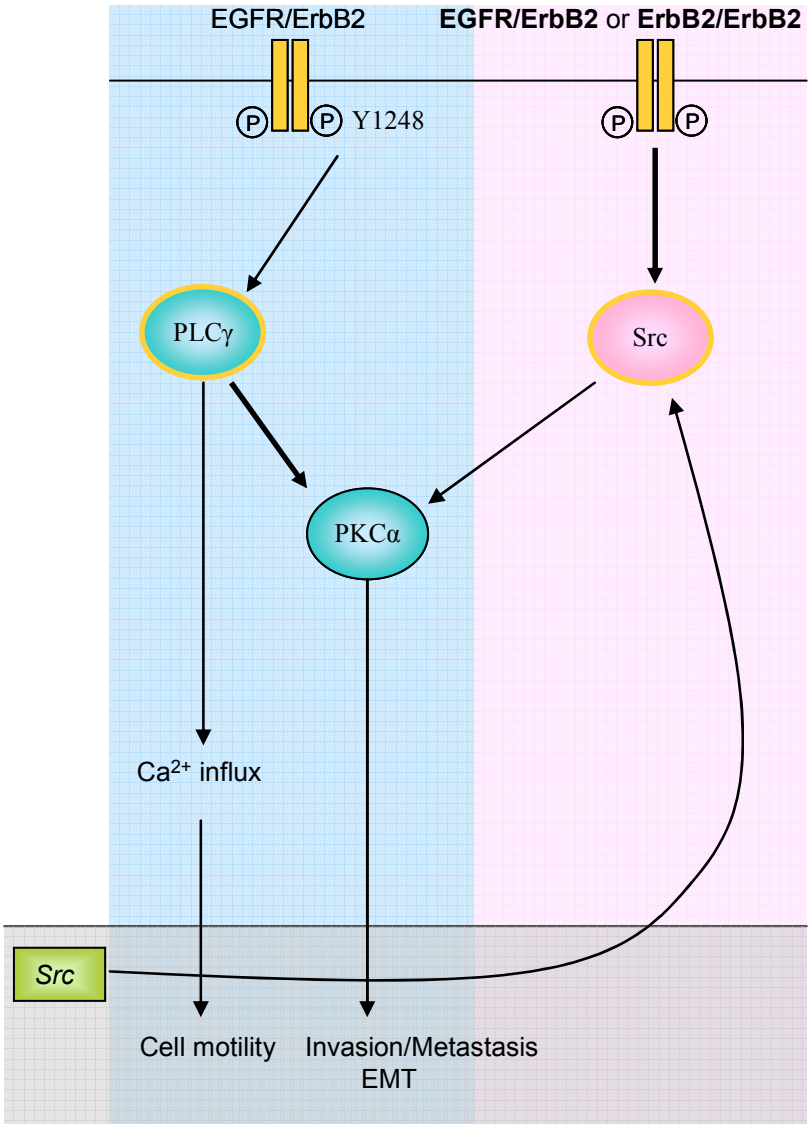


Fig. 7. The PKCα and Src signaling activated through ErbB2. A simplified scheme.

morphogenetic remodeling of the cell by phosphorylating proteins that associate with the actin cytoskeleton. Previous results suggest that an ErbB2–c-Src complex may play an important role in heregulin-stimulated anchorage-independent growth and antiapoptotic or survival mechanisms, but have less of an effect on anchorage-dependent growth [64]. Kim et al. [75] could show that c-Src interacts with a tyrosine residue on ErbB2 that is distinct from the five major autophosphorylation sites. They demonstrated that c-Src associates specifically with the kinase domain of ErbB2. Further investigation revealed that the ErbB2-dependent activation of c-Src results in a disruption of epithelial cell–cell contacts leading to cell dispersal and loss of cell polarity. The experiments were conducted on chimeric EGFR competent in c-Src recruitment. In contrast to the EGFR expressing normal epithelial kidney cells (MDCK), the chimera expressing cells exhibited a dramatic scattering and invasion phenotype in response to EGF. These observations correlated with the relocalization of phospho-MAPK to the cytoplasmic membrane and were dependent on a functional MEK/MAP kinase cascade [75]. Tan et al. [72] showed that activation of ErbB2 can lead to increased Src protein synthesis, through activation of the Akt/mTOR pathway, and decreased Src protein degradation through the inhibition of the calpain protease. These two novel mechanisms result in Src protein upregulation and activation, which play critical roles in ErbB2-mediated breast cancer invasion and metastasis. Furthermore, inhibition of Src activity by a Src-specific inhibitor, PP2, or a Src dominant-negative mutant dramatically reduced the oncogenic potential of ErbB2-mediated cancer as determined on cell invasion and metastasis. Tan et al. [72] could report the first experimental evidence that the higher metastatic potential of ErbB2-overexpressing breast cancer cells is mediated through and even requires increased Src activities.

3.3.8 The internalization of ErbB2

The principal process that turns off signaling by the ErbB network is ligand-mediated receptor endocytosis, and the kinetics of this process also depend heavily on receptor composition. On ligand binding, ErbB1 molecules cluster over clathrin-coated regions of the plasma membrane, which invaginate to form endocytic vesicles. These mature to early and late endosomes, while gradually decreasing their internal pH and accumulating hydrolytic enzymes that lead to receptor degradation. Importantly, the other three ErbB proteins are endocytosis impaired and are more often recycled back to the cell surface. Sorting to degradation is determined by the composition of the dimer: ErbB1 homodimers are targeted

primarily to the lysosome; ErbB3 molecules are constitutively recycled; and heterodimerization with ErbB2 decreases the rate of endocytosis and increases recycling of its partners [7]. Sorting in the early endosome seems to depend on the pH stability of the ligand-receptor interaction. EGF interaction is relatively pH resistant and targets EGFR to lysosomes whereas transforming growth factor α (TGFα) readily dissociates from the receptor at endosomal pH, resulting in receptor recycling. Subsequent rerouting of the receptor to the plasma membrane may, therefore, play a major role in signal potentiation [45].

Cbl protein, an E3 ubiquitin ligase that promotes ubiquitinylation and degradation of activated receptors plays a critical role in antibody-induced HER2 downregulation, as in the case of Trastuzumab. Antibody stimulation induces HER2 receptor activation and phosphorylation in tyrosine 1112 known to be responsible for the recruitment of Cbl. Upon binding, Cbl catalyses the transfer of ubiquitin molecules to HER2. Ubiquitinylated receptor–Cbl complexes are endocytosed by clathrin-coated pits, fuse into early endosomes and move to lysosomes where the receptor is destroyed. In the absence of such antibodies, phosphorylated HER2 only weakly associates with Cbl and it is thus resistant to Cbl-induced downregulation. HER2 heterodimerization with other members of the EGFR family prevents HER2/Cbl association and avoids the lysosomal fate, favouring recycling, and this accounts for HER2 heterodimers enhanced signaling potency. The initiation steps of HER2 endocytosis could be attributed to the autocrine production of TGFα, a mechanism known for governing EGFR downregulation. Valabrega et al. [18] show that metastatic cells examined after Trastuzumab treatment acquire the ability to express TGFα. Tumor cells often produce TGFα that acts either in an autocrine or in a paracrine manner. TGFα has been found to have synergistic interaction with Neu regarding the formation of multifocal mammary tumors in the mammary epithelium of bitransgenic mice co-expressing TGFα and Neu. The development of the tumors was correlated with the tyrosine phosphorylation of Neu and the recruitment of c-Src to the Neu complex. A probable biochemical explanation for this observation is that TGFα activates Neu-associated tyrosine kinase activity by transphosphorylation through the activated EGFR [61].

3.3.9 The HER2-transformed phenotype of the mammary gland

Human mammary epithelial cells overexpressing HER2 have been shown to undergo two major changes in cellular behaviour with high relevance to tumorigenesis; firstly, the epithelial-to-mesenchymal transition (EMT), and secondly, anchorage independent growth (AIG). In EMT, the epithelial characteristics of the cells, such as cell polarity, the presence of adherens junctions, tight junctions and desmosomes, and expression of many epithelium-specific proteins, are lost and replaced with a more or less fibroblastic phenotype [76] characterized by a change to spindle-like cell morphology and a gain of mesenchymal markers [60]. AIG is usually detected by the classical parameter of growth capacity in soft agar. In a model of mammary epithelial cells overexpressing a chimaeric NeuT, Jenndahl et al. [76] support the finding that EMT is a critical phenotypic change required for AIG in ErbB2 signaling. Activated ErbB2-induced EMT has been shown to be mediated and dependent on c-Src in normal epithelial kidney cells (MDCK) [75]. When HER2 is overexpressed in an infected human breast cancer cell line (MDA-MB-231), it appears to synergize with the TGFβ pathway to potentiate pro-invasion, angiogenesis, and EMT [77]. ErbB receptor heterodimer signaling is known to stimulate cell motility through induction of PLCγ, activating either IP_3, DAG and calcium or PKCα [78]. In HER2-overexpressing cells, PKCα induction is potentiated through Src [71], as described in chapter 3.3.7. Dittmar et al. [78] have shown that ErbB2 initiates the cell migration program by modulating the time course of PLCγ activation and that this effect is dominantly regulated by the EGFR transphosphorylated tyrosine residue 1248 of ErbB2, upon EGF interaction. Furthermore, EGFR signaling either in homo- or heterodimer configuration has revealed to be crucial for PLCγ activation [78].

An *in vitro* model of the *in vivo* architecture of mammary acinar structures is represented by human mammary epithelial cells forming growth-arrested acinar structures when cultured within a three-dimensional matrix rich in laminin and collagen IV suspension (Matrigel). Muthuswamy et al. [5] investigated the effect of overexpressed chimaeric ErbB2 in such a model system and found evidence that simple overexpression of ErbB2 is not enough to induce invasive phenotypes. The chimaeric ErbB receptors consist of the extracellular and transmembrane domains of the nerve growth factor receptor and ErbB1 or ErbB2 cytoplasmic domains linked to the synthetic-ligand-binding domain from FK506-binding protein (FKBP). The chimaeric ErbB receptors can be dimerized with the bivalent FKBP ligand AP1510.

Treatment with AP1510 induced tyrosine phosphorylation of the chimaeras but not of endogenous ErbBs. EGF stimulation, required for the proliferation of the cell model, did exclusively activate the endogenous ErbBs and not the chimaeras. Activation of ErbB2, but not ErbB1, homodimers has led to altered mammary acini structures that share properties with ductal carcinoma in situ, such as loss of proliferative suppression and repopulation of lumen. ErbB2 activation also disrupted tight junctions and the cell polarity of polarized epithelial cells. Interestingly, the epithelial cells did not invade the basement membrane or display anchorage independence. Thus, ErbB2 activation did not induce a fully transformed phenotype. The findings suggest that activation of ErbB2 can induce early stages of mammary carcinogenesis but is not sufficient to induce invasive phenotypes. It has been suggested that additional genetic or epigenetic events are needed for mammary epithelial cells to acquire invasive properties [5]. These findings are supported by Seton-Rogers et al. [60] observing ErbB2 activation in similar three-dimensional cell cultures imitating human acini and using Muthuswamys' chimaeric ErbB receptors.

Complex organisms have evolved cellular mechanisms to suppress the proliferation of cells at risk for oncogenic transformation. The two main mechanisms are apoptosis or programmed cell death, and cellular senescence. Cellular senescence is an irreversible growth arrest and a major failsafe that cells must overcome to reach the malignant phenotype. This mechanism was first discovered to occur in normal cells after a limited number of cell divisions, driven by telomere shortening and was termed 'replicative senescence'. Meanwhile stimuli having little or no impact on telomeres were shown to induce senescence in normal cells, and the term for the mechanism was broadened to 'cellular senescence'. These stimuli include DNA damage through oxidation, chromatin remodelling and strong mitogenic signals such as the activated forms of Ras, Raf or MEK. These stimuli induce senescence after only a few cell divisions. Cellular senescence is controlled by several tumor-suppressor genes such as p53 and pRB, *in vitro* as well as *in vivo*. Cells derived from p53 knockout mice failed to senesce in response to such stimuli and the animals developed cancer at an early age. Furthermore, a genetic manipulation that causes premature senescence of mammary epithelial cells suppresses the development of breast cancer in mice subsequently exposed to the mouse mammary tumor virus [79]. Cellular senescence causes many changes in gene expression, only some of which are involved in the growth arrest. Upon senescence, human fibroblasts also become resistant to apoptosis and overexpress genes encoding secreted proteins such as growth factors which in turn stimulate neighbouring cells to grow. Growth stimulation was observed only with preneoplastic, but not normal, human epithelial cells. One hypothesis for

these opposing effects is that cellular senescence is antagonistically pleiotropic, contributing to age-related tumorigenesis [79]. Expression of tumor-promoting factors by senescent cells is mediated in part by senescence-associated cyclin-dependent kinase inhibitors such as $p21^{Cip1/WAF1}$. Senescent cells show characteristic changes in morphology, such as enlarged and flattened cell shape and increased granularity. Tumor cells have not lost the ability to senesce, but can be even induced to undergo senescence by genetic modifications, chemotherapy, radiation, retinoids or TGFβ. Treatment-induced senescence was shown to be one of the key determinants of tumor response to therapy *in vitro* and *in vivo* [80]. Senescence has also been described as 'living cell death' because cells maintain the integrity of their plasma membranes [81], remain metabolically and synthetically active, but cannot divide even if stimulated with mitogens [80]. Senescence is accompanied by the induction of senescence-associated β-galactosidase (SA-β-gal) activity, which is commonly used as a marker for senescence [81]. Trost et al. [82] have found that tightly regulated overexpression of the oncogenic rat homologue NeuT in human breast carcinoma cells does not stimulate proliferation but provokes premature senescence, accompanied by induction of $p21^{Cip1/WAF1}$, SA-β-gal and the typical morphologic alterations. The same behaviour was observed with retrovirus-mediated overexpression of NeuT in low-passage murine embryonic fibroblasts. A tetracycline-regulated NeuT expression system was used by Trost et al. [82], which results in $p21^{Cip1/WAF1}$ localization to the nucleus in the growth arrested cells. Previous studies using constitutively overexpressing cell lines observed localization of $p21^{Cip1/WAF1}$ to the cytoplasm where it enhanced cell proliferation [66] ($p21^{Cip1/WAF1}$ is described in more detail in chapter 3.3.7). Furthermore, specific inhibition of the MAP kinase p38, but not ERK1/2 or PI3K, reversed the phenotypic alterations and functional inactivation of $p21^{Cip1/WAF1}$ prevented growth arrest and the senescent phenotype. It has been documented that the biological outcomes of oncogenic signaling via the Ras-Raf-MEK-ERK (MAPK) cascade critically depend on the strength and duration of the signal. Thus, the induction of senescence in the regulated expression system, but not in the constitutively overexpression system, could be due to a similar effect. However, Trost et al. [82] observed the features of senescence after 2-7 days of NeuT induction. The authors speculate that subcellular relocalization of $p21^{Cip1/WAF1}$ to the cytoplasm due to Akt may be an essential secondary step to escape cellular senescence [82].

Mammary gland functional differentiation occurs with distinct morphological and molecular changes of the epithelial cells and allows for the production and secretion of milk. Thus, the secretory alveolar cells represent the final cellular state of the life-spanning multistep differentiation process within the mammary gland. These differentiation steps

taking place during pregnancy and lactation are defined and characterized by the sequential activation of genes encoding the milk proteins such as β-casein, whey acidic protein (WAP) and α-lactalbumin. Mammary gland development in response to lactogenic hormones *in vitro* was known to be accompanied by a distinct morphological change of the cells forming structures called domes. Zucchi et al. [83] have correlated the dome formation *in vitro* with differentiation processes occurring in the mammary gland at pregnancy. Dome formation was shown to be inducible by the lactogenic hormones hydrocortisone (HC) and prolactin (PRL) and could be paralleled with the expression of the milk protein β-casein, which is a specific marker of the initial stage of mammary gland functional differentiation [83].

Hynes et al. [84] have reported that induction of β-casein in HC11 mouse mammary epithelial cells by the lactogenic hormones insulin, prolactin and the glucocorticoid dexamethasone can be suppressed by EGFR activation but not oncogenic human ErbB2 or NeuT signaling. In order to investigate the exact function of ErbB2 during normal breast development, Jones and Stern [85] inactivated endogenous ErbB2 signaling through expression of a dominant negative truncated ErbB2 transgene during mouse mammary gland development. They found that normal ErbB2 signaling is required for the terminal stages of lobuloalveolar development and lactation as measured by the observation of inhibited alveolar growth and non-secreting lobuloalveoli under transgene expression [85]. The influence of oncogenic rat NeuT on mouse mammary gland development has also been studied in transgenic mice overexpressing NeuT. Expression is controlled by the MMTV-LTR promoter, leading to an elevated level of expression during late pregnancy and throughout lactation. Transgene expression was assessed at lactation and early involution and revealed to be restricted to morphologically transformed cells whereas no endogenous mouse c-erbB2 was detected in wild-type glands. NeuT expressing tumor cells completely failed to express the milk proteins β-casein and WAP at lactation or during involution [1].

Senescence can be induced in primary or early passage cells by MEK activation and reversed by blocking p38, but not ERK1/2 or PI3K. Constitutive overexpression of NeuT is critical for overcoming senescence, strength and duration of signaling play a pivotal role. With NeuT overexpression, EMT via Src is required for AIG. Cell motility is mediated through Y1248-activated PLCγ (EGFR involvement crucial) and PKCα (potentiated through Src). ErbB2 and not ErbB1 homodimer signaling is responsible for loss of proliferative suppression and repopulation of lumen, but *per se* is unable to induce invasive behaviour. Normal ErbB2 signaling is required for the development of the mammary gland, which is accompanied *in vitro* by dome formation, inducible by HC and PRL and paralleled by β-

casein expression. EGFR, but not overexpressed mutated HER2 or NeuT, suppresses β-casein production. NeuT expression in tumor cells blocks the differentiation of the gland. Again, an expression-level dependent function can be attributed to ErbB2 receptors in mammary gland development.

In order to learn about the influence of ErbB2 on normal, polarized mammary epithelial cells, we decided to study the differentiation *in vitro* of the Clone 31 mouse mammary epithelial cells in culture, using retroviral constructs to infect with either human or murine ErbB2 or the oncogenic rat NeuT.

4 Materials and Methods

4.1 The animal work

4.1.1 The animal model

Vaccination studies were performed in female FVB inbred mice originally obtained from Charles River, Germany. The mice have the MHC status H-2q. All experiments have been conducted in accordance with the Swiss Tierschutzverordnung. The transplanted tumors grew until the size reached the termination criterion of 200 mm^2, when the mice were sacrificed.

4.1.2 Surgery of the mice

For injections into the mammary gland and the spleen mice were anesthetized with a liquid triple narcotics (*see Table 1*) injected i.p. Animals were kept under anaesthesia for maximal 15 minutes. Narcosis was reversed with a subcutaneous injection of an antidote mixture *(Table 1).* Access to the mammary gland was gained by fixing the anesthetized animal on the back with tape bands and incising the skin in the region of the inguinal right mammary gland. Injections of Met-1 cells were done directly into the fat pad of the mammary gland where the subsequent formation of a bulge indicated a well-targeted application.

For transplantation of tumor tissue fragments, the fat pad of the inguinal mammary gland was incised with sharp forceps in order to form a pocket in which the tissue fragment was then enveloped.

Table 1. Composition of anaesthetics and antidote

Narcotics			**Corresponding antidotes**		
	Conc. in mixture [mg/ml]	Dosage per FVB mouse (~20g) in 50ul mixture, in µg		Conc. in mixture [mg/ml]	Dosage per FVB mouse (~20g) in 80ul mixture, in µg
Medetomidin HCl 1mg/ml (Domitor®)	0.25	12.5	**Atipamezol HCl** 5mg/ml (Antisedan®)	1	80
Climazolam 10mg/ml (Climasol®)	2.5	125	**Sarmazenil** 1mg/ml (Sarmasol®)	0.2	16
Fentanyl 0.05mg/ml (Fentanyl-Janssen®)	0.025	1.25	**Naloxon HCl** 0.4mg/ml (Narcan®)	0.24	19

For dissecting the spleen, the mouse was laid on its right side, a 10mm long skin flap was cut out and the spleen could then be localized by eye through the peritoneum. Through a fine cut in the peritoneum (approx. 4mm) the spleen was gently extracted for about 1cm with a flat forceps. The application was performed by inserting the needle along the tongue shaped organ and progressively retracting it while injecting, in order to prevent congestion in the dense tissue. With this technique the spleen can hold an application volume of 30-50ul.

The injections into the gastrocnemius muscle were performed in the non-anaesthetized animal without dissection of the muscle, except for the injections followed by electroporation. In the case of the applications supported by electroporation, the treatment was done under narcosis and the muscle was dissected for precise localization of the injection site and to ensure conduction of the current.

Incisions were sewed with non-resorbing Ethilon II 4-0 FS-2 Ethicon Polyamid 6 monofil suture, Johnson & Johnson.

Blood samples were taken from the tail by cutting a 1mm piece off the tip [86]. Repeated blood retrieval was limited to 4 samples per animal spread over 2 months. For each sample 100ul of whole blood was collected in a glass microcapillary and stored overnight at 4°C in an uncoated centrifuge microtube for coagulation. The sample was then centrifuged twice 10 minutes at 13'000 rpm in an Eppendorf microfuge and the serum aliquoted and frozen at -20°C.

4.1.3 *In vivo* electroporation

In order to improve gene transfer in our naked DNA vaccination protocols, we applied *in vivo* electroporation to the spleen and muscle of anaesthetized mice with the kind help of Dr. Amiq Gazdhar. Electroporation conditions had been previously optimized by Gazdhar et al. [36] to

four pulses (200 V/cm, 20 ms at 1 Hz) using flat plate electrodes. The organ has been electroporated immediately after injection of the plasmid DNA at the site of injection *(Fig. 8)*.

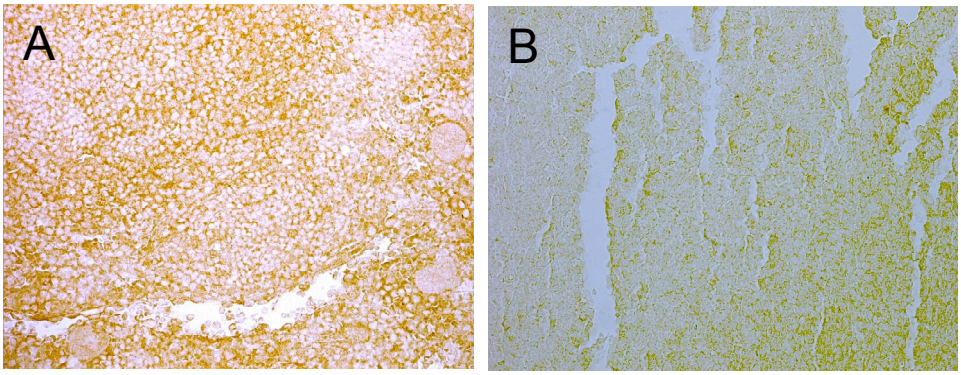


Fig. 8. Electroporated spleen after injection of 80ug mutHER2 plasmid **(A)** or 80ug pBabe^{puro} vector **(B)**; stained with anti-ErbB2/HER2 antibody (Upstate). 40x magnification.

4.2 The tumor model

Mouse mammary primary tumor cells from the FVB Met 1-line, transgenic for wild type PyV-mT [87], were the generous gift of Dr. Robert D. Cardiff (Centre for Comparative Medicine, University of California, Davis, USA) and were maintained *in vitro* and *in vivo* as well. For *in vivo* studies tumor cells were injected directly into the lower right mammary gland of female FVB mice. Cells were administered as suspension in Eagles Dulbecco-Modified cell culture medium lacking foetal calf serum. The following characteristics have been observed *in vivo*:

Met-1:

a) Tumor development was reproducible, growth has been observed in 24 out of 25 animals.

b) Low doses, 10'000 - 25'000 cells develop tumors faster than larger (50'000 cells).

c) When primary tumors are surgically removed, some regrow at the site, others do not.

Met-1 infected with either mouse c-erbB2 or human ErbB2:

a) Infection with ErbB2 did not alter the growth rate or morphology of the Met-1 tumor.

b) With Met-1 mouse pBabe^{puro} ErbB2, 10'000 cells initiated tumors more rapidly than 30'000 cells but 3'000 cells did not produce 100% tumors.

c) With Met-1 human pBabe^{puro} ErbB2, 30'000 cells were found to be above the threshold for producing 100% tumors.

d) The tumors remain transplantable.

Expression of the antigen has been checked by IHC, as shown for c-erbB2 in Figure 9.

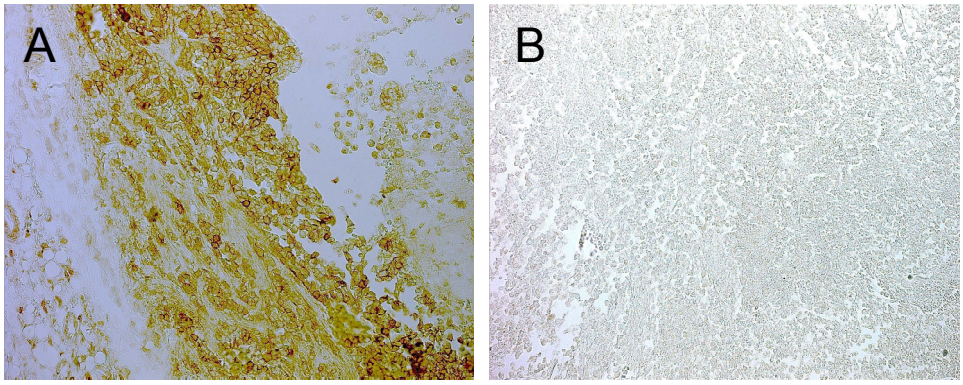


Fig. 9. Met-1 primary tumor infected with c-erbB2 **(A)** or pBabe^{puro} vector **(B)**; staining was done with anti-ErbB2/HER2 antibody (Upstate); 20X magnification.

4.3 Cell cultures and cell lysates

3T3 FVB fibroblasts were prepared in our laboratory and used at approximately the 50th passage. These had the MHC status H-2q, so they are syngeneic to the FVB mice used in the experiments. The mouse mammary epithelial (31E) and stem cell (30F) lines were established from BALB/c mice and characterized by Dr. Ernst Reichmann in our laboratory as described previously [88]. Cells were cultured in Eagle's Dulbecco-Modified medium enriched by 10% foetal calf serum (EDM10). Cells prepared for injection in the mice were resuspended in serum-free EDM in order to minimize unspecific immunological reactions. The cells used in the EGF-stimulation experiments had been deprived of EGF one week before and deprived of serum 20-24 hrs prior to stimulation with EGF. Stimulation has been done with 40 ng/ml EGF for 0, 2, 5, 15 and 30 min.

For generation of cell lysates, confluent cells from a 5 cm plate were washed with phosphate buffered saline and incubated at 4°C for 10 min in 1 ml of modified RIPA lysis buffer (see Appendix B). Following Dounce homogenisation, samples were centrifuged at 20'000 *g* for 10 min and the collected supernatant represented the cytosol containing also membrane proteins. After determination of protein concentration using the Bradford-based Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany), the lysates were

diluted to 1X SDS-PAGE sample buffer (80mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol and 0.02% bromphenol blue) and boiled for 2 min. Total cell lysates were collected by lysing cells directly with 1X SDS-PAGE sample buffer (1 ml for a 10 cm cell culture dish) and boiling the lysates for 2 min.

4.4 In Vitro Differentiation Assays, soft agar and Matrigel growth assays

In order to induce functional *in vitro* differentiation of Line 31E mouse mammary epithelial cells expressing the various ErbB2 constructs, co-cultures were prepared with Line 30F cells as described in Reichmann et al. [88]. After culture for 48 hrs in medium supplemented with EGF (10 ng/ml), cells were incubated for 24 hrs in EDM with 3% foetal calf serum, 5 ug/ml insulin, 1 ug/ml hydrocortisone and 5 ug/ml ovine prolactin. Thereafter, supernatants were discarded and cells lyzed using RIPA lysis buffer (Appendix B) for determination of β-casein in Western blots using a specific antibody against mouse β-casein.

For determination of the electrical resistance across the monolayers of Line 31E cells infected with the different ErbB2 constructs, cells were grown to confluence on cellulose nitrate filter inserts (diameter 2.2 cm). Resistance measurements were obtained with the Millipore (Bedford, MA., USA) Millicell-ERS instrument, essentially a millivolt/ohmmeter that provides an alternating voltage source to minimize cell damage.

Culture in soft agar was performed by mixing gently 50'000 cells in EDM10 at room temperature with EDM10/Noble agar at 42°C to give a final concentration of 0.37%. 3 cm culture dishes which had been covered with 0.8 ml EDM10 containing 1% Noble agar as a hard agar under-layer, were overlayed with 1.5 ml of the cell suspension. Incubation was for 6 days at 37°C in a conventional CO_2 incubator. Culture in Matrigel (Growth factor depleted; Collaborative Research) was performed according to the manufacturers instructions by diluting an ice cold cell suspension (50'000 cells) in EDM10 (2.5 parts) with Matrigel (1 part), plating in 3 cm culture dishes which were subsequently incubated for 6 days at 37°C. Images were obtained using a 5X objective in phase contrast.

4.5 SDS-PAGE and immunoblot analysis

Electrophoretic separation of proteins was performed by discontinuous Laemmli SDS-PAGE using the Mini-PROTEAN® II electrophoresis cell (Bio-Rad) with an electrophoretic separation length of 7 cm. For immunoblot analysis 10% polyacrylamide gels were poured using research-grade 29:1-Acrylamide/ Bisacrylamide (Serva, Heidelberg, Germany). To ensure that equal amounts of protein were loaded, protein concentration was determined in advance by using a Bradford-based assay (Bio-Rad Protein Assay, Bio-Rad Laboratories GmbH, Munich, Germany) and/or estimated by Coomassie staining of gels run in parallel and confirmed in some cases by Ponceau S staining of Western blot membranes.

For Western Blot analysis, proteins were separated by SDS-PAGE as described above and transferred onto nitrocellulose membranes (Schleicher & Schuell Inc., Dassel, Germany) using a semidry electroblotter (Bio-Rad). After transfer, the membranes were incubated for 1h in blocking solution (20 mM Tris pH 7.6, 140 mM NaCl, 0.05% Tween-20, 5% w/v non-fat dry milk) and then washed twice for 5 min with TBS-T (20 mM Tris pH 7.6, 140 mM NaCl, 0.05% Tween-20). Primary antibodies were diluted with TBS-T containing 2% w/v BSA and the membranes were incubated overnight at 4°C with gentle agitation. After three 5 min-washes with TBS-T, the membranes were incubated for 1h at room temperature with the secondary antibody diluted 1:20'000 in TBS-T containing 2% w/v BSA. After further washing the membranes three times for 5 min with TBS-T, the immunoreactive bands were visualized by chemiluminescence (using SuperSignal® West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA). A CCD camera system (Lumi-Imager™, Boehringer Mannheim, Germany) was used to detect the signal.

In the case of reprobing membranes for a protein of different size from the protein detected first line, membranes were washed twice for 10 min with TBS-T and incubated again for 1h in blocking solution, then proceeded with the next primary antibody as described above. Alternatively, stripping was accomplished by incubation for 30 min at 50°C with gentle agitation in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS), washing twice for 10 min with TBS-T followed by incubation for 1h in blocking solution prior to reprobing for a different protein.

4.6 Isolation of RNA and Northern blotting

Total RNA was prepared from tissue samples and cells using the guanidinium-thiocynate extraction method described by Chomczynski and Sacchi [89]. In total, 5 μg of total RNA was denatured with glyoxal, electrophoresed on a 1% agarose gel and blotted to nitrocellulose. Equal loading was controlled by examination of ribosomal RNA bands following staining with acridine orange as previously described [90]. Probes for hybridization were prepared using the Random Primed Labeling Kit (Roche Diagnostics, Rotkreuz, Switzerland) including ^{32}P-dCTP (800 Ci/mM; Amersham International, Little Chalfont, England) in the reaction and hybridization was carried out as previously described [90].

4.7 Antibodies

A specific antibody against mouse β-casein was generously provided by Dr. Ernst Reichmann (Pediatric Surgery Research, University Hospital, Zurich, Switzerland). Affinity-purified antibodies specific for c-erbB2 (mouse, human and rat specific; Cat. No. 06-562), phospho Tyr-1248 HER2 (only human-specific), Akt/PKB (Cat. No. 07-416), phospho Ser-473 Akt/PKBα (Cat. No. 05-736), phosphorylated ERK 1/2 (Cat. No. 05-481), and phospho Ser-657 PKCα (Cat. No. 06-822) were obtained from Upstate (Lake Placid, NY, USA). Horse radish peroxidase-conjugated secondary antibodies against rabbit IgG (Cat. No. 12-348) and mouse IgG (Cat. No. 12-349) were obtained from Upstate.

4.8 Immunohistochemistry

Animals were sacrificed and the spleen, gastrocnemius muscle or primary tumor removed and fixed in 4% formaldehyde overnight. The next day the tissues were dehydrated through graded ethanols and toluene and embedded in paraffin for sectioning. From every block of tissue, fixed in formalin and embedded in paraffin, serial sections of 4 μm were cut. Serial sections were dewaxed in xylene and rehydrated through graded ethanols. Sections were heated in 10 mM citrate buffer pH 6.0 in a microwave oven for 10 min at defrost power (till the temperature reached 90 – 95°C) followed by 2 min at low power (to maintain temperature at 90 – 95°C), to allow the antigens to be unmasked. For quenching endogenous peroxidase the sections were then incubated in 3% hydrogen peroxide diluted in 50% methanol for 10

min at room temperature. Incubation for 1 hour with 6% horse serum in a humidified chamber was then performed to block non-specific binding.

Sections were stained with anti-ErbB2 antibody (1:1000, Upstate, Lake Placid, NY, USA) overnight at 4°C. Negative controls were performed with 6 % horse serum (blocking solution) as primary antibody. Incubation with the secondary antibody, biotinylated polyclonal goat-anti-rabbit-immunoglobulin (DAKO A/S, Denmark) in a dilution of 1:300, was done for 45min. This was then followed by a conventional Avidin-Biotin-horseradish peroxidase complex staining procedure, finally detecting with diaminobenzidine as substrate. Slides were counterstained in haematoxylin. The sections were evaluated under a light microscope (Leitz DMRD) and pictures taken with a Leica DMRB/DC200 (CCD camera) and imported in Adobe Photoshop 7.0.

4.9 The Constructs

4.9.1 The rat NeuT plasmid

The rat NeuT in pBabe^{puro} vector we received as a kind gift from Prof. Nancy Hynes (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). The accession number for rat NeuT sequence at the National Center for Biotechnology Information (NCBI) was NM_017003.1.

4.9.2 The mouse c-erbB2 RIKEN plasmid

We obtained the mouse c-erbB2 gene in a pFLCI vector in a collaboration with the Genome Exploration Research Group RIKEN, Japan. The NCBI database Accession Number for mouse c-erbB2 at that time was BC046811.

By comparing to the human and rat homologs, we found it to contain a substitution (addition of 81 bp) causing a frameshift and a non-functional product. That this error was in fact present in the RIKEN clone could only be confirmed by comparison with a later submitted mouse c-erbB2 sequence that was entered into the NCBI as this project was midway along. With time, growing knowledge about the sequence of mouse c-erbB2 revealed a more and more realistic picture of the true sequence found in nature. Ultimately, the NCBI predicted a virtual sequence combining the various versions submitted so far. The alignment

of the initial mouse version (accession number BC046811) against this predicted version of mouse c-erbB2 (acc. no NM_001003817*) (Fig. 10)* as well as against human HER2 (acc no NM_004448.2) *(Fig. 11)* finally revealed a cloning artefact in the mouse sequence. The insert is not a repeated sequence of mouse c-erbB2, but is spurious.

With Western blot using a commercially available anti-ErbB2 antibody (Upstate) it was impossible for us to detect the RIKEN construct when it was cloned in an expression vector: Although this antibody was prepared with a peptide from a region (amino acid 1243-1255) that comes after the frameshift (which is at approximately AA 734), and therefore no signal would be expected, it does detect a weak band, which can also be detected in normal Line 31E mouse epithelial cells.

```
Mouse c-erbB2 (old) = BC046811 containing the addition
Mouse c-erbB2 (new) = NM_001003817


c-erbB2(old)    2192 CCTAATCAAACGAAGGCGACAGAAGATCCGGAAGTATACCATGCGTAGGC   2241
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
c-erbB2(new)    2201 CCTAATCAAACGAAGGCGACAGAAGATCCGGAAGTATACCATGCGTAGGC   2250

c-erbB2(old)    2242 TGCTGCAGGAGACCGAGGTGAGGCGTGCCGAGGGCCTCCTAGCACCCCCT   2291
                     |||||||||||||||
c-erbB2(new)    2251 TGCTGCAGGAGACCG---------------------------------   2265

c-erbB2(old)    2292 TGGCTTTGCAGCTAGCTGGGCATTCACCCTACCACCCTCTCGCTCCAGCT   2341
                                                                 ||||
c-erbB2(new)    2266 ---------------------------------------------AGCT   2269

c-erbB2(old)    2342 GGTGGAGCCGCTGACGCCCAGTGGAGCTGTGCCCAACCAGGCTCAGATGC   2391
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
c-erbB2(new)    2270 GGTGGAGCCGCTGACGCCCAGTGGAGCTGTGCCCAACCAGGCTCAGATGC   2319
```

Fig. 10. Nucleotide sequence alignment of initial versus predicted mouse c-erbB2.

```
Mouse c-erbB2 (old) = BC046811 containing the addition
Human HER2          = NM_004448.2

mousec-erbB2    2176 TGGTGGTCATTGGAATCCTAATCAAACGAAGGCGACAGAAGATCCGGAAG   2225
                     .|||||||.||||.|||||.|||||.|||.|||..|||||||||||||||
humanHER-2      2241 GGGTGGTCTTTGGGATCCTCATCAAGCGACGGCAGCAGAAGATCCGGAAG   2290

mousec-erbB2    2226 TATACCATGCGTAGGCTGCTGCAGGAGACCGAGGTGAGGCGTGCCGAGGG   2275
                     ||.||.|||||.||.||||||||||.||.|
humanHER-2      2291 TACACGATGCGGAGACTGCTGCAGGAAACGG-------------------   2321

mousec-erbB2    2276 CCTCCTAGCACCCCCTTGGCTTTGCAGCTAGCTGGGCATTCACCCTACCA   2325

humanHER-2      2322 ---------------------------------------------------   2321

mousec-erbB2    2326 CCCTCTCGCTCCAGCTGGTGGAGCCGCTGACGCCCAGTGGAGCTGTGCCC   2375
                                 |||||||||||||||||||.||.||.|||||..|||||
humanHER-2      2322 ------------AGCTGGTGGAGCCGCTGACACCTAGCGGAGCGATGCCC   2359

mousec-erbB2    2376 AACCAGGCTCAGATGCGGATCCTAAAGGAGACAGAGCTAAGGAAGCTGAA   2425
                     ||||||||.|||||||||||||||.||.|||||.|||||.|||||||.||||
humanHER-2      2360 AACCAGGCGCAGATGCGGATCCTGAAAGAGACGGAGCTGAGGAAGGTGAA   2409
```

Fig. 11. Nucleotide sequence alignment of initial mouse c-erbB2 versus HER2.

Since the RIKEN clone is the only isolate available of the full length mouse c-erbB2 (4.7 kb), and our own initial efforts to clone the full-length mouse c-erbB2 were unsuccessful, we decided to correct the cloning error by doing a site-directed replacement of missing sequences. C-erbB2 in the pcDNA vector was subcloned into pBluescript SK(±) (Stratagene, La Jolla, CA, USA). A pBluescript subclone was restricted with Bst1107I (which cuts c-erbB2 at position 2105) and BsiWI (cuts at position 2444) to remove a fragment of 339 bp spanning the erroneous 81 bp addition region. RNA isolated from a pool of 15 day mouse embryo liver, lung, intestine, kidney and heart was employed to prepare cDNA with Transcriptor Reverse Transcriptase (Roche, Cat. No. 03 531 317 001) using the primer sequence AGGCAGCCATAGGGCATAAG to obtain a fragment extending beyond the entire excised 339 bp sequence. A PCR reaction (Expand High Fidelity Plus PCR System; Roche, Cat. No. 3 300 242) was then performed with the cDNA using primers designed to contain restriction sites for Bst1107I (sense) and BsiWI (antisense). Finally, the PCR fragment obtained was restricted with Bst1107I and BsiWI *(Fig. 12)*, purified and ligated into the previously restricted pBluescript c-erbB2. The resulting full-length "corrected" c-erbB2 was sequenced and the alignment to HER2 showed that the 81 bp addition had been removed successfully *(Fig. 13)*. Finally we subcloned the corrected mouse c-erbB2 into the pBabe^{puro} retroviral vector. Biological activity was observed in cell culture indicating a functional protein and a protein of the correct size could be detected with a Western blot using commercially available anti-ErbB2 antibody (Upstate).

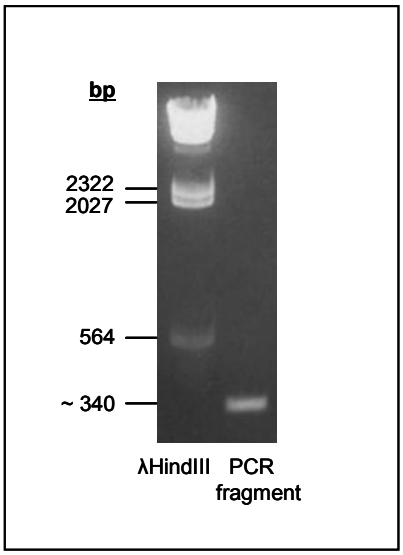


Fig. 12. Bst1107I and BsiWI restricted PCR fragment ~339 bp.

```
c-erbB2seq  = from sequenced full-length corrected mouse c-erbB2
Human HER-2 = NM_004448.2

c-erbB2seq      2006 TGGTGGTCATTGGAATCCTAATCAAACGAAGGCGACAGAAGATCCGGAAG   2055
                     .|||||||.||||.|||||.|||||.|||.|||..|||||||||||||||
humanHER-2      2241 GGGTGGTCTTTGGGATCCTCATCAAGCGACGGCAGCAGAAGATCCGGAAG   2290

c-erbB2seq      2056 TATACCATGCGTAGGCTGCTGCAGGAGACCGAGCTGGTGGAGCCGCTGAC   2105
                     ||.||.||||||.||.||||||||||||.||.||||||||||||||||||||
humanHER-2      2291 TACACGATGCGGAGACTGCTGCAGGAAACGGAGCTGGTGGAGCCGCTGAC   2340

c-erbB2seq      2106 GCCCAGTGGAGCTGTGCCCAACCAGGCTCAGATGCGGATCCTAAAGGAGA   2155
                     .||.||.||||||..||||||||||||||.||||||||||||||.||.||||
humanHER-2      2341 ACCTAGCGGAGCGATGCCCAACCAGGCGCAGATGCGGATCCTGAAAGAGA   2390
```

Fig. 13. Nucleotide sequence alignment of sequenced corrected mouse c-erbB2 versus HER2.

4.9.3 The pGEX2 construct

In an attempt to obtain an antibody with a higher specificity against mouse c-erbB2 than the commercial antibody from Upstate, we decided to engineer a GEX construct containing a highly antigenic sequence of mouse c-erbB2 RIKEN. In order to determine roughly the best antigenic region in HER2/c-erbB2 protein [91], which is expected to be the extracellular domain, we examined the hydropathy plot of this protein (*Fig. 14*) [91]. The representation of hydrophobic and hydrophilic tendencies within the protein depicts the extracellular domain to be approximately from amino acid 1 to 600.

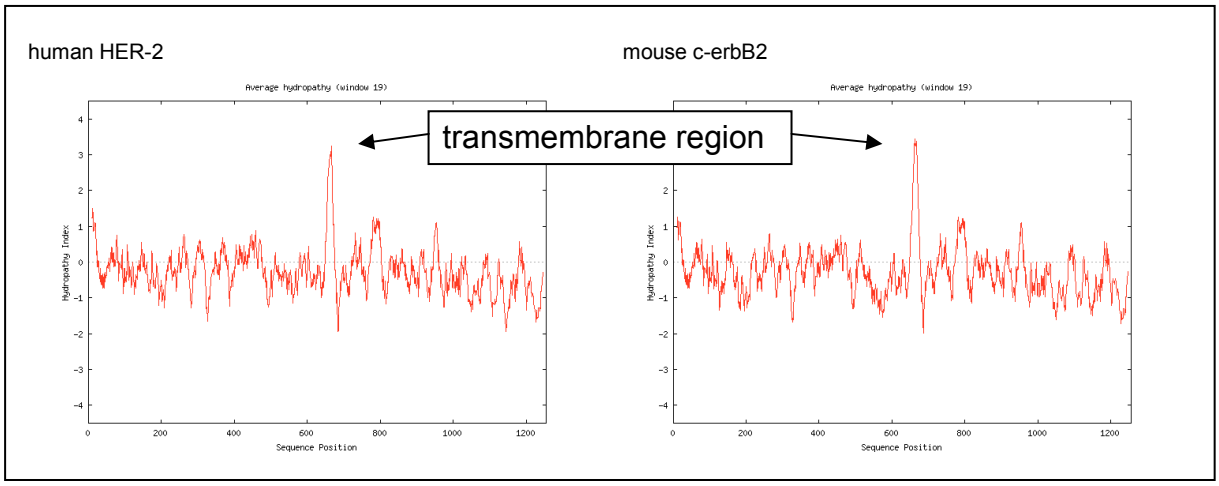


Fig. 14. Kyte-Doolittle hydropathy plots of HER2 and c-erbB2.

We then designed two primers spanning 380bp within this extracellularly exposed region. With a sense primer containing a BamHI restriction site and an antisense primer containing an EcoRI restriction site a fragment of 380bp was obtained through PCR (RIKENPCRsmall). This fragment was then ligated into the BamHI/ EcoRI cut polylinker of the pGEX2 vector and sequenced. The sequenced 380bp insert was compared to the published database version of mouse c-erbB2 and showed an identity of a 100% (mouse sequence nucleotides 1107-1486). In addition RIKENPCRsmall has of course the usual degree of identity towards human, i.e. 86%, see *Fig. 15*.

DNA

```
human: 1150 ttctacggacgtgggatcctgcaccctcgtctgccccctgcacaaccaagaggtgacagc 1209
             || ||||| ||||||||||||||| || ||||| |||| | |||||||||||||| |||||
mouse: 1094 ctccacggaagtgggatcctgcactctggtctgtcccccgaacaaccaagaggtcacagc 1153


human: 1210 agaggatggaacacagcggtgtgagaagtgcagcaagccctgtgcccgagtgtgctatgg 1269
             ||||| |||||||||||||||||||| ||||||||||||||||||  |||| ||||||||
mouse: 1154 tgaggacggaacacagcggtgtgagaaatgcagcaagccctgtgctggagtatgctatgg 1213


human: 1270 tctgggcatggagcacttgcgagaggtgagggcagttaccagtgccaatatccaggagtt 1329
             |||||||||||||||| | |||| || ||||||  | ||||||| ||||||||||||||
mouse: 1214 tctgggcatggagcacctccgaggggcgagggccatcaccagtgacaatatccaggagtt 1273


human: 1330 tgctggctgcaagaagatctttgggagcctggcatttctgccggagagctttgatgggga 1389
             |||||||||||||||||||||||||||||||||||| |||||||||||||||||||| |
mouse: 1274 tgctggctgcaagaagatctttgggagcctggcatttttgccggagagctttgatgggaa 1333


human: 1390 cccagcctccaacactgccccgctccagccagagcagctccaagtgtttgagactctgga 1449
             |||  ||||| | |||||| ||  |||||||||| |||||||||| || || |||||
mouse: 1334 cccctcctccggcgttgccccactgaagccagagcatctccaagtgttcgaaaccctgga 1393


human: 1450 agagatcacaggttacctatacatctcagcatggccggacagcctgcctgacctcagcgt 1509
             |||||||||||||||||||||| |||||||||| || ||| | |  |||||||| ||
mouse: 1394 ggagatcacaggttacctatacatttcagcatggccagagagcttccaagacctcagtgt 1453


human: 1510 cttccagaacctgcaagtaatccggggacgaattctgcacaatggcgcctactcgctgac 1569
             |||||||||||| |  || || |||||||| ||||| ||  |||| || |||||  ||||
mouse: 1454 cttccagaaccttcgggtcattcggggacggattctccatgatggtgcttactcattgac 1513


identities in DNA fragment seq: 84.7%
overall identity in full-length seq: 85%
```

Protein

```
humanHER-2       300 PYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGMEH    349
                     |||||||:|||||||||.:|||||||||||||||||||.|||||||||
mousec-erbB2     301 PYNYLSTEVGSCTLVCPPNNQEVTAEDGTQRCEKCSKPCAGVCYGLGMEH    350

humanHER-2       350 LREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQV    399
                     ||..||:||.|||||||||||||||||||:|:|..|||:||.|||
mousec-erbB2     351 LRGARAITSDNIQEFAGCKKIFGSLAFLPESFDGNPSSGVAPLKPEHLQV    400

humanHER-2       400 FETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLG    449
                     |||||||||||||||:|..||||||||:||||||||:||||||||||||
mousec-erbB2     401 FETLEEITGYLYISAWPESFQDLSVFQNLRVIRGRILHDGAYSLTLQGLG    450

humanHER-2       450 ISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANR    499
                     |..|||||||||||||||.|||||||:||||||||||||||||:.||
mousec-erbB2     451 IHSLGLRSLRELGSGLALIHRNTHLCFVNTVPWDQLFRNPHQALLHSGNR    500

identities in protein fragment seq: 123/143 = 86%
overall identity in full-length seq: 87.7%
```

Fig. 15. pGEX2-construct of RIKEN PCR small – comparison to human. The highlighted part in yellow represents the 380 bp insert.

Furthermore, the new bacterial pGEX2 construct has been checked for translation into protein and concentration by induction with IPTG in E. coli DH5alpha bacteria and finally via Western blot. The insert 400bp ~ 14kDa protein sequences fused to the glycogene-S-transferase gene 710bp ~ 26kDa is detectable as an approximately 40kDa protein, see *Fig. 16*.

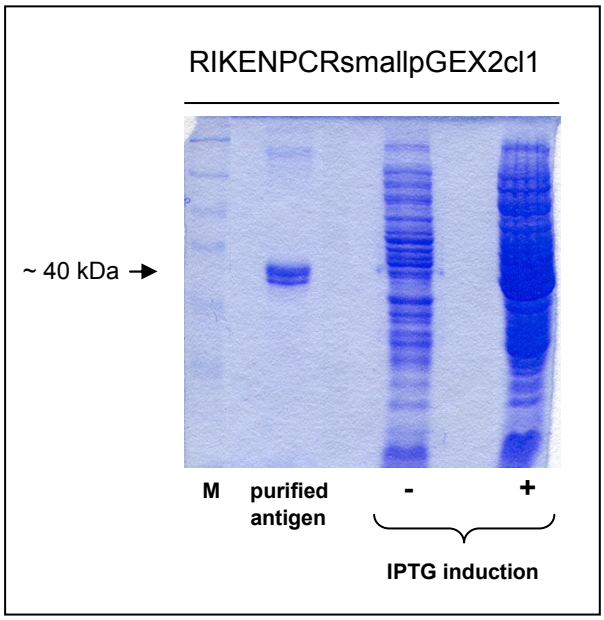


Fig. 16. IPTG induced and purified RIKENPCRsmallpGEX2cl12. Lane M = molecular size standard.

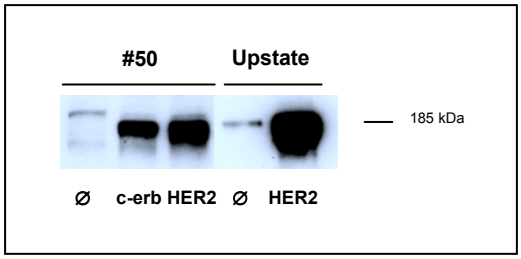


Fig. 17. Lysates all Met-1 cells; mouse #50 antiserum diluted 1:500; c-erb = mouse c-erbB2.

The new RIKENPCRsmallpGex2 clone 12 construct was used to immunize two male FVB mice (#50 + #51) by injecting the purified antigen in an oil emulsion intradermally in aliquots at various injection sites scattered over the back of the animals. A booster dose has been given in week 3 performing the same procedure as described. Blood was collected from these mice in week 6 and the serum was analyzed for specific antibodies via Western Blot *(Fig. 17)*.

In order to gain larger amounts of antibody, we immunized two rabbits with a dose of 100ug purified antigen followed by 5 repetitive boosters according to the procedure described above. Antibody from rabbit serum was affinity purified on small columns of Affigel 10 which had been covalently coupled with PAGE-purified antigen. Antibody elution was with glycine buffer, pH 2.5, rapidly neutralized to pH 7 after collection of fractions. Antibody was checked via Western blot see *Fig. 18A*.

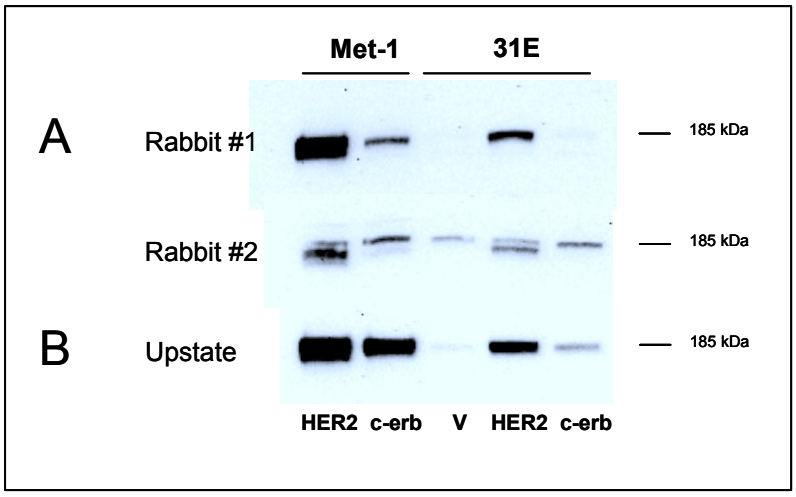


Fig. 18. Expression of HER2 wt and c-erbB2 in Met-1/ 31E cells. **A** detected with rabbit antisera RIKENPCRsmallpGEX2 1:1000. **B** detected with commercial a-ErbB2/HER2 rabbit polyclonal antibody, Upstate, cat.no 06-562. Concentrations of lysates were measured photometrically. c-erb = infected with mouse c-erbB2; V = pBabe^{puro} vector.

4.9.4 The human HER2 plasmid

The human HER2 plasmid pcDNA3 we received as a kind gift of Dr. Ming-Fong Lin, University of Nebraska Medical Center, Omaha, NE, USA [92]. The sequence of the HER2 gene is published and filed in the NCBI database currently under the accession number NM_004448.2 (total length 4624 bp CDS 239-4006). This version was originally submitted by Yamamoto et al. [93] in 1986, and derived from tumor. Interestingly, when compared to the sequence of the physiological human ErbB2 gene (acc. no DQ047380) the Yamamoto sequence does not show any mutations, supporting the presumption that the oncogenicity of HER2 from this human tumor sample did not rely on a mutation in the gene. We then subcloned HER2 into the retrovirally promoted pBabe^{puro} vector.

4.9.5 Expression of c-erbB2 and HER2 in cultured cells

We initially used the c-erbB2 and HER2 constructs in the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) under the regulation of a CMV promoter. Since after transfection, both genes were gradually lost upon prolonged culture or *in vivo* passage and since the efficiency of gene transfer was rather low, we changed the vector system to the retrovirally promoted pBabe^{puro} vector. Together with the rat NeuT plasmid we now dispose of a comparable set of

plasmids which carry the homologs of ErbB2 as inserts. Expression of pBabe^{puro} constructs of c-erbB2 and HER2 in the cell lines Met-1 and Line 31E is stable as verified in Western blot analysis of cell lysates *(Fig. 18B)*. The weak detection of the mouse c-erbB2 probably reflects a lower degree of sensitivity by the Upstate antibody, though the mouse construct was consistently less strongly expressed. Transcription of ErbB2 has been detected by Northern blot analysis *(Fig. 19)*. The labelled probe for Fig. 19 was prepared from HER2 template.

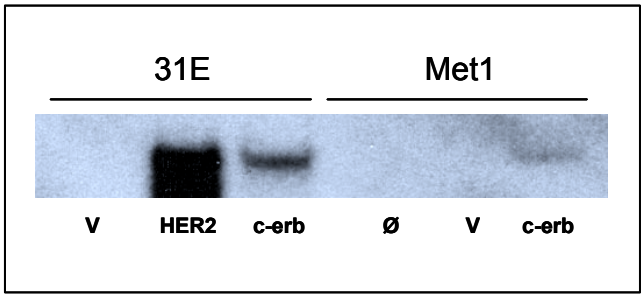


Fig. 19. RNA of HER/ErbB2 by Northern blot analysis. Ø = non-infected; V = infected with pBabe^{puro} vector; c-erb = infected with mouse c-erbB2.

4.9.6 Cloning a dominant-negative mutated human HER2

According to the literature, HER2 expression can downregulate components of the MHC class I antigen-processing machinery [39,40], resulting in an immune escape phenotype of oncogene-transformed cells. This effect presumably occurs due to signaling of the HER2 receptor. The downregulation of MHC class I products becomes manifest after times as short as 24 hrs, making this effect a possible hindrance for HER2 DNA vaccination strategies. Therefore, we decided to construct for use as antigen, and also as a negative control for the transformation phenotype of HER2, a mutant HER2 gene, lacking the sequence coding for the ATP-binding site of this tyrosine kinase receptor protein. This mutation results in an inactivation of the tyrosine kinase activity and consequently blocks the transforming activity otherwise inherent in the immunization.

With four specially designed primers we used PCR to produce two fragments, i.e. the coding regions on either side of the ATP-phosphate binding loop GSGAFG, at amino acid positions 727-732 (nucleotides 2181-2196) [41]. The primers were designed to contain either a restriction site for SalI or HindIII, see *Fig. 20*, and produced fragments of 2.3kb or 1.9kb respectively *(Fig. 21)*.

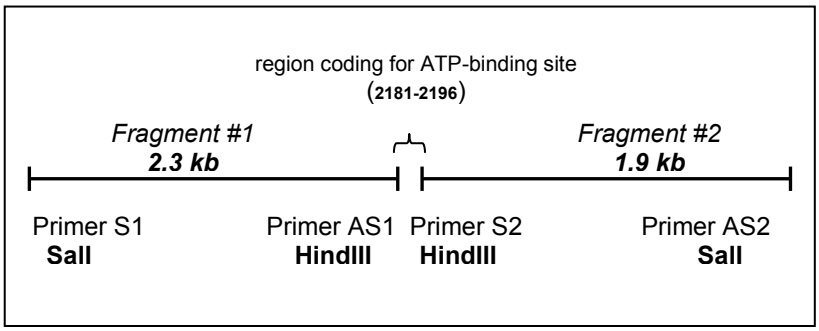


Fig. 20. Human HER-2 insert size approx. 4250 bp (full-length 1-4624).

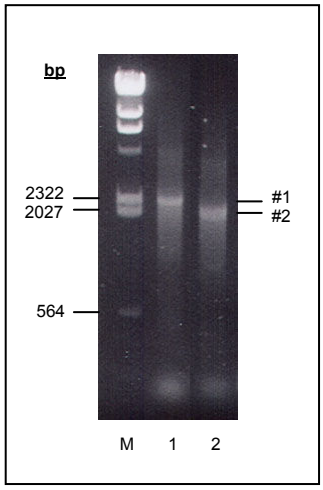


Fig. 21. PCR of human HER-2 **M** marker λHindIII; **1** primer pair S1/AS1; **2** primer pair S2/AS2 #1 fragment of ~2.3kb #2 fragment of ~1.9kb.

Upon ligation of the two SalI and HindIII restricted fragments, the mutant HER2 gene (mutHER2) was then subcloned into the retrovirally promoted pBabe^{puro} vector, restricted with SalI. The sequence lacked the amino acids 725-733 and had AA 734 converted from Valine to Leucine *(Fig. 22),* adding to our antigen one more possible foreign determinant in the context of the mouse immune system.

```
mutHER-2          6 TPSGAMPNQAQMRILKETELRKVK---------LYKGIWIPDGENVKIPV    46
                    |||||||||||||||||||||||||         :|||||||||||||||||
HER-2 db        701 TPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPDGENVKIPV   750
```

Fig. 22. Protein alignment of mutHER2 (sequenced mutated HER2) and HER2 db (database version of HER2 acc no NM_004448.2) showing the deleted region and the non-matching Leucine. The ATP-phosphate binding loop is depicted in red letters.

Virtual translation of the sequenced fragment into protein predicts a 1245 AA protein. The pBabe^{puro} mutHER2 was used to infect cultured FVB fibroblasts and expression was checked via Western blot analysis *(Fig. 23)*. The mutation does not interfere with recognition by the commercial antibody (Upstate) against c-erbB2 (prepared against peptides AA 1243-1255) and against phospho-ErbB2 (pTyr 1248).

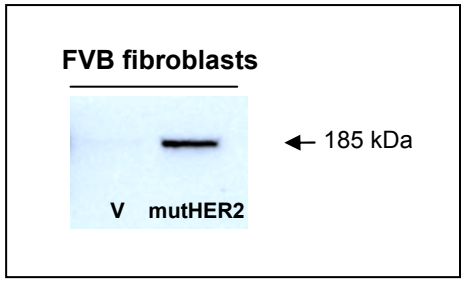


Fig. 23. Expression of mutHER2 in early passage FVB fibroblasts. Detection with commercial anti-ErbB2/HER2 Ab (Upstate) 1:1000.

4.9.7 Retrovirus Constructs

Retroviral constructs of HER2, dominant negative mutant HER2 and mouse c-erbB2 were all prepared in pBabe^{puro} [94]. For HER2, the insert was restricted with HindIII and blunted with Klenow enzyme. For the mouse c-erbB2 RIKEN "corrected" clone, restriction was with SacI, followed by blunting with T4 polymerase, and isolation of the insert by a second restriction with EcoRV. Finally, the blunted inserts were ligated into a blunted SnaBI pBabe^{puro} site. Helper-free infectious virus was produced using amphitrophic Phoenix packaging cells [95] kindly supplied by Dr. G. Nolan (Stanford University Medical Center, Stanford, CA). Line 31E cells were infected with approximately $4*10^5$ infectious units on $2*10^6$ cells. For infection Polybrene was used at 8 ug/ml. Successfully infected cells were selected with 4 days treatment with puromycin (Sigma) at 2.5 ug/ml. Expression was tested with Northern and Western blots, and biological activity confirmed by cell biological assays.

4.10 Immunological methods

4.10.1 Vaccination protocols

Protocol A: DNA into muscle

4 weekly injections have been performed of each 100ug naked DNA HER2 wt plasmid (pcDNA3 vector, in 50ul water) into the gastrocnemius muscle of mice. The injections have been done alternately in the muscle of the left and the right leg. The control animals have been injected with 100ug of the empty vector. The mice were challenged two weeks after the last vaccination by injecting Met-1 tumor cells transfected with mouse c-erbB2 (25'000 cells) in the right mammary gland. In a variant of this protocol, some of the mice have been boosted with 100ug naked DNA HER2 wt plasmid into the gastrocnemius muscle two weeks after the challenge. Tumor growth has been monitored by measurements during the following month *(Appendix C, graph 1).*

The experiment has been repeated with 5 weekly injections but no challenge. Two weeks after the last vaccination dose two mice of each group were sacrificed and the spleen has been removed for isolating lymphocytes, analyzed in the following for the presence of specific cytotoxic T lymphocytes by intracellular INFy staining as well as in a Cr-Release

Assay. The positive control run together with the samples were lymphocytes from LCMV-infected mice being used in another study, the generous gift of Prof. A. Ochsenbein and Dr. M. Matter (Department of Clinical Research, University of Berne, Switzerland). Blood was taken before the first vaccination and one week after the last injection and analyzed for specific antibodies against HER2.

Protocol B1: fibroblasts into spleen (with fibroblast booster i.p. day 18)

In this protocol FVB 3T3 fibroblasts infected with HER2 wt were injected directly into the spleen ($1*10^6$ cells in 50ul) followed by a booster dose of the same cells i.p. on day 18 ($3*10^6$ cells). 4 weeks after the intrasplenic vaccination tumors became visible by eye in the region of the spleen. Histology of the primary tumor emerging from the spleen showed clearly sarcoma cells and indicated that the aetiology of the tumours was mouse c-erbB2 transformed 3T3 fibroblasts. Long-term culture following transfection with an oncogene can favour cell transformation, so we vaccinated the next set with fibroblasts irradiated with 5000rad until growth arrest. Radiation time was titrated. Mice have been boostered i.p. with the same cells ($2*10^6$) on day 10, but no challenge has been performed. Irradiated fibroblasts didn't transform and the spleens could be used for analysis of activated and/or specific CTLs. Lymphocytes isolated from the spleen 21 days after i.s. vaccination were analyzed for CTL against HER2 wt by intracellular INFy staining and Cr-Release Assay. The positive control run together with the samples were lymphocytes from an LCMV-infected memory mouse as been used in another study. Tumor growth has not been determined in these groups.

Protocol B2: low passage fibroblasts inf mutHER2 into spleen

The achievement of a mutated HER2 together with FVB fibroblasts of an early passage made the irradiation of the vaccine unnecessary, it prevented transformation successfully. The first group of animals was challenged with endogenous mouse c-erbB2 *(Appendix C, graph 2)*, and the second with xenogeneic HER2 wt *(graph 3)*. Whole serum as well as immunoprecipitation-purified serum has been analyzed for specific antibodies by Western blot.

The injections of FVB fibroblasts into the spleen were often followed by rapid death, in most cases instantly after injection of the cells and always accompanied by signs of respiratory stress. Intrasplenic injections of fibroblasts seemed to cause more frequent deaths than injections of naked DNA. These observations together led to the presumption of an embolus reaching the lungs. In an investigation of the abdomen of two mice a focal

hemorrhagic necrosis of the left liver lobe with signs of autolysis could be detected. This high death rate during surgery was the reason why we abandoned this protocol and concentrated on intrasplenic naked DNA injections.

Protocol C1: DNA into spleen + electroporation

Each animal received 80ug of naked mutHER2 DNA (30ul) into the spleen followed by electroporation of the injection site. There was no booster dose in this protocol. Mice were challenged in week 3 with either endogenous mouse c-erbB2 *(Appendix C, graph 4)* or xenogeneic HER2 wt *(graph 5)*. Serum was analyzed by Western blot.

Protocol C2: DNA into spleen with boost into muscle + electroporation

The same protocol as C1 described above, except that mice were boostered in the gastrocnemius muscle with electroporation of the muscle and that all the animals have been challenged with HER2 wt. The schedule of first dose, booster and challenge was either week 0 – 2 – 4 *(Fig. 24)* or 0 – 3 – 6 *(Appendix C, graph 6)*. Serum was analyzed by Western blot.

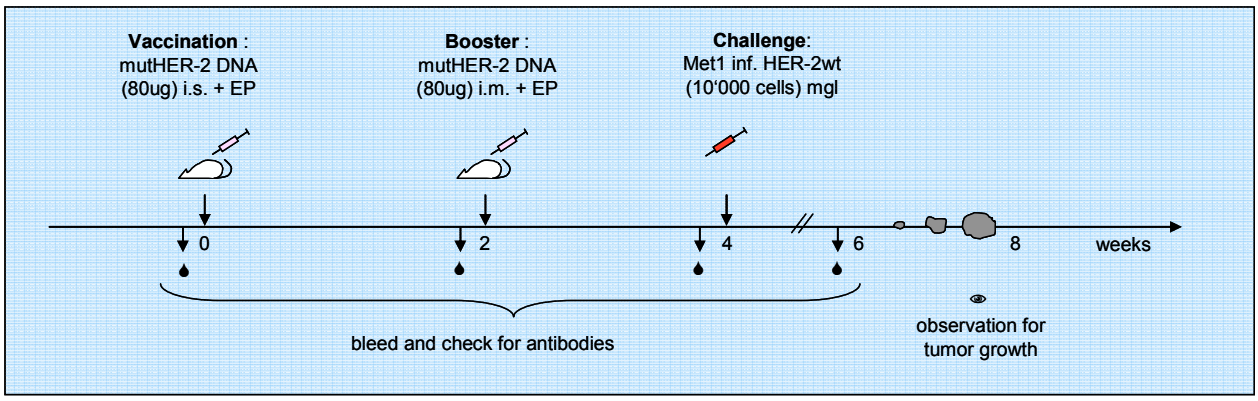


Fig. 24. Protocol C2: DNA into spleen with boost into muscle + electroporation. EP = electroporation; mgl = injected into the mammary gland.

Protocol D: 31E cells into peritoneum with boost i.p.

This last immunization attempt has been designed to represent just a proof of principle and does not claim to stand as a model for pre-clinical treatment in human. $4*10^6$ 31E cells infected with either mutHER2 or $pBabe^{puro}$ were injected into the peritoneal cavity. An identical dose has been injected as booster two weeks later. One group of animals was challenged with Met-1 tumor expressing c-erbB2, the other group with Met-1 expressing HER2 in week 5 *(see chapter 5.1, Fig. 25)*.

4.10.2 Isolation of lymphocytes

Mice were sacrificed and a fold of skin was cut from its right side slightly towards the back. The spleen was carefully separated from the pancreas in order to prevent any enzymatic damage of spleen cells. The spleen was then removed and put in Eagle's Dulbecco-Modified culture medium supplemented with 2% foetal calf serum (EDM2%) on ice. The spleen gets poured on top of a sterile wire grid placed in a Petri dish and smashed with the plunger of a sterile syringe until most of the tissue has passed through the grid. The cell suspension gets centrifuged in a quick spin of 500rpm at room temperature to remove the remaining tissue debris. The cell suspension is centrifuged with 1200rpm at 4°C for 5min and the pellet resuspended in Iscove's Modified Dulbecco's Medium supplemented with 10% foetal calf serum and 0.05mM β-mercaptoethanol (IMDM 10% βME). In the mean a number of $20*10^6$ lymphocytes could have been isolated per spleen by this procedure.

4.10.3 The Intracellular Interferon γ Staining

In this protocol, lymphocytes are first activated *in vitro*, stained for the surface antigens CD8 and CD4, then fixed with paraformaldehyde to stabilize the cell membrane and reversibly permeabilized with the detergent saponin to allow anti-interferon γ (INFγ) antibodies to stain intracellularly. The *in vitro* stimulation of the lymphocytes is required since cytokine levels are typically too low in resting cells. For detection of intracellular INFγ that has been produced in response to *in vitro* stimulation, it is necessary to block secretion of INFγ with protein transport inhibitors such as BrefeldinA, which keeps the INFγ inside the endoplasmic reticulum. INFγ, CD8 and CD4 are finally detected by flow cytometry.

Briefly, $1*10^6$ lymphocytes isolated as described in 4.10.2, resuspended in IMDM 10% βME and supplemented with 100 U/ml IL-2 (received as a gift from Prof. Ochsenbein, DKF Bern) and 20 ug/ml BrefeldinA (Sigma, Germany) were restimulated for 5 hours on adherent stimulator cells in a 48-well plate ($1*10^5$ cells/ well). Two different stimulator cells were tested, FVB fibroblasts transfected with HER2 wt and Met-1 tumor cells transfected with mouse c-erbB2. A set of non-restimulated samples (on normal FVB fibroblasts) has been run in parallel in order to spot the effect of the specific stimulus. All samples have been run in duplicate.

Following restimulation and transfer to a round bottom 96-well plate, lymphocytes were stained with the antibodies CD8-PE and CD4-PE (eBioscience) diluted 1:600 in FACS buffer. Membranes and bound antibodies were fixed with 1% paraformaldehyde (Merck-Schuchardt, Munich, Germany) before permeabilizing the cells with 0.1% saponin (Sigma, Germany). Intracellular INFγ was stained with anti INFγ-FITC antibody diluted 1:300 (eBioscience). Detection was done by flow cytometry in 500 ul FACS Buffer in 5 ml Polystyrene Round-Bottom Tubes (Falcon 35 2058, Becton Dickinson).

As internal controls served unstained lymphocytes, each dye used for the FACS scan separate as well as FACS buffer only. The positive control consisted of splenocytes from an LCMV infected mouse as used in another study. Restimulation was done with peptide p33 (main epitope of LCMV activating CD8) or p13 (activating CD4).

4.10.4 The Chromium Release Assay

Stimulator cells were FVB fibroblasts transfected with HER2 wt or untransfected as control. For better antigen presentation stimulator cells have been stopped in proliferation by irradiating with 5000 rad. Radiation time has been titrated. $0.5*10^6$ stimulator cells were cultured for 24 hours in 35mm mini culture dishes. Lymphocytes are prepared and resuspended to $2.5*10^6$ cells/ml in IMDM 10% βME (see 4.10.2) and supplemented with IL-2 at 50 U/ml. Stimulator cell cultures were supplied with $2.5*10^6$ lymphocytes each and incubated at 37°C for five days. An IL-2 control (without stimulator cells) has been run in parallel in order to exclude unspecific lymphocyte stimulation through IL-2. Irradiated target cell suspensions, FVB fibroblasts transfected with HER2 wt or untransfected as control were plated out to 7000 cells per 96-well on day 4. On day 5 target cells were loaded with chromium-51 (Amersham Biosciences, Germany) approximately 12 uCi/ $2*10^5$ targets at 37°C for 3h. The 5 days stimulated lymphocytes are given onto the target cells in a row of dilutions and were incubated at 37°C for 6 hours. Specific killing was observed in Kontrogel Scintillator Cocktail (Kontron Analytic, Zurich, Switzerland) using a liquid scintillation counter (Packard, Downers Grove, IL, USA). The positive control consisted of splenocytes from an LCMV infected mouse, see 4.10.3. Cell wall dissolving NP40 0.1% was used as maximum release control. Minimum release was assessed with samples containing no lymphocytes. A polystyrene control without cells was run in parallel.

4.10.5 Antibody detection

Western Blots with 31E cells infected with wild-type HER2 or non-infected were probed with mouse sera in a dilution of 1:200. The positive control consisted of a strip with the same lysates probed with commercial anti-ErbB2 antibody (Upstate).

5 Results and Discussion

5.1 Part I – A xenogeneic HER2 DNA vaccination approach

An overview of the tested immunization protocols is displayed in Table 2. The outcomes of these experiments will be very briefly summarized below.

Protocol A: No specific CD8(+) and CD4(+) T lymphocytes could be detected by intracellular INFy staining and no specific killing was observed in a Cr-Release Assay in vaccinated mice (data not shown). The analyzed sera didn't show any specific antibodies against HER2. In order to enhance the immune stimulatory effect of the vaccine, we changed the application route to a direct presentation of the antigen to secondary lymphoid organs, such as the spleen or the mesenteric lymphnodes, as described in Protocols B1-D.

Protocol B1: The FVB fibroblasts as vaccine carrier had to be irradiated prior to injection into the spleen in order to prevent formation of sarcoma tumors. Lymphocytes isolated from the spleen 21 days after i.s. vaccination and analyzed for CTL against HER2 wt did not show any significant difference between vaccinated and control group. Neither intracellular INFy staining nor Cr-Release Assay could detect specific T lymphocytes in vaccinated mice. Tumor growth has not been determined in these groups.

Protocol B2: Since we employed the dominant negative mutated HER2 in FVB fibroblasts of an early passage, irradiation of the immunizing cells was unnecessary. In fact this modification of the procedure prevented the transformation of fibroblasts successfully. Control animals #26-32 displayed an interesting, significantly reduced tumor growth compared to the vaccinated group (animals #33-38), see Appendix C, graph 3. The probability for the null-hypothesis 20 days after the challenge is low ($P = 0.0089$), as calculated with the Mann-Whitney U-Test. This was our first observation suggesting the vaccination procedure

might in fact favor the growth of tumor. The high death rate during surgery was the reason why we abandoned this protocol and concentrated on intrasplenic naked DNA injections.

Protocols C1 and C2: Sera have been repeatedly analyzed for specific antibodies using Western blotting. Specific antibodies were detected in a small subset of the mice, but were neither statistically significant nor reproducible. Tumor growth was comparable between vaccinated and control groups *(see Appendix C, graph 6).*

Protocol D: In this vaccination attempt, designed as a proof of principle, we see more frequent tumor development in the vaccinated mice than in the control groups, see Figure 25. The calculated probability for the null-hypothesis is low, P = 0.0139 for the Met-1 c-erbB2 challenged group and P = 0.119 for the Met-1 HER2 group (both at day 40 after challenge). We were unsuccessful in measuring specific antibodies in the serum.

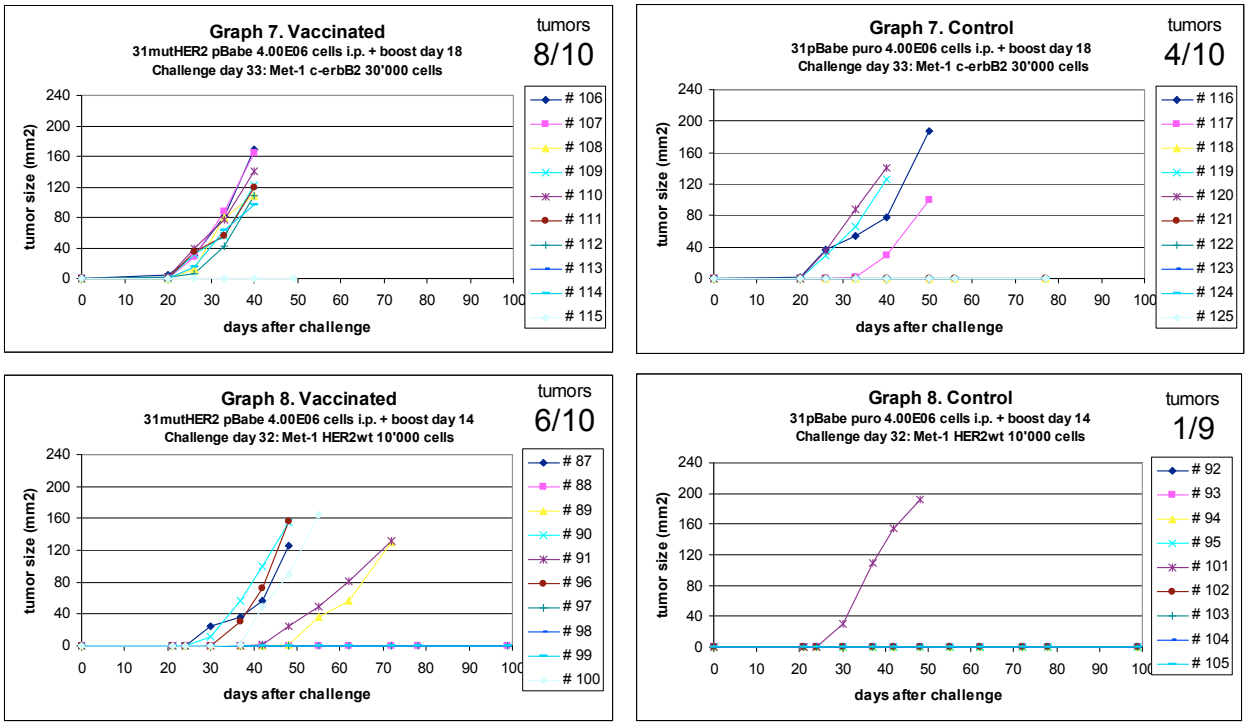


Fig. 25. Growth curves for protocol D: The tumor challenge in graph 7 is c-erbB2 infected Met-1 tumor, whereas in graph 8 Met-1 is infected with HER2 wt.

The observation in Protocols B2 and D, where vaccinated mice developed tumors more rapidly than the control animals, is nonetheless interesting. The explanation may be due to a mechanism in priming specific T lymphocytes in the secondary lymphoid organ. In fact, such a phenomenon has been previously described by Ochsenbein [34]. The ability of CTLs to extend tolerance to the antigen, especially when the encounter between antigen and CTLs is persistently high was discussed [34]. In such a situation, T cells stimulated to maximal

proliferation can consequently undergo a contraction phase, resulting in the deletion of all T cells specific for that antigen [34] (discussed in more detail in chapter 3.2.3). It is theoretically possible that the fibroblasts in Protocol B2, probably due to their size, reside longer in a dense tissue such as the spleen whereas naked DNA, taken up by APCs is processed rapidly and persists for a shorter time in the organ, thus limiting the contact to T cells. This could explain also why no such phenomenon was observed with the protocols of naked DNA injection into the spleen (Protocols C1 and C2). In Protocol D, epithelial cells infected with human HER2 were injected into the peritoneal cavity. The antigen, once cross-presented to APCs, is priming T cells in the various secondary lymphoid organs located in the gastrointestinal tract. The gut-associated lymphoid tissue or GALT is equipped with a roughly equivalent number of lymphocytes to those in the spleen. The ErbB2 peptides are predominantly presented in mesenteric lymph nodes and Peyer's patches, lymphoid follicles located in the mucosa of the small intestine. Thus, induction of tolerance in protocol D might be due to the high dose of vaccine (twice $4*10^6$ cells) and the potent priming occurring in the GALT. Tumor cells escaping the immune system by either downregulation or loss of ErbB2 receptors are less likely to play a role in our system since expression of ErbB2 in Met-1 cells has been monitored in cultures over a long period and was found to be stable.

While we have performed many experiments and have suffered many disappointments, the vaccination approach would probably justify much further work. One possible line of further investigation would be the injection of more than one foreign ErbB2 homolog antigen. This possibility might generate a stronger immune response and could be seen as an improvement of our tested vaccination schedules. The idea would be to engineer the same kinase deficient mutant in rat NeuT. Mice would be vaccinated first with mutHER2, receive a first boost with mutated NeuT and a second boost with both mutated genes combined.

Another possible improvement of the vaccine design would include the use of syngeneic dendritic cells (DCs) as hosts for the antigenic DNA. DCs are believed to be the most potent antigen processing cells [96,97] and numerous successful vaccination studies relied on this system [98,99]. A recent publication describes stronger immune responses to NeuT using adenovirus NeuT-transfected dendritic cells compared to plasmid DNA [100].

Table 2. Overview of the tested immunization protocols

Protocol	**Vaccine**	**Challenge** (Met-1 tumor cells infected with antigen)	**Growth observed**	**Sera analyzed for Ab**	**Immunological assays conducted**	**Specific immune response detected**	**Result positive control**
A	DNA i.m.	c-erbB2	Graph 1	WB	INFy Cr	no no	pos pos
B1	FVB fibos HER2 wt i.s. + irradiation	-	-	Aborted due to sarcomas			
		-	-	WB	INFy Cr	no no	pos pos
B2	FVB fibos mutHER2 i.s.	c-erbB2	Graph 2	WB	-		
		HER2 wt	Graph 3	IP			
C1	DNA i.s.+ EP	c-erbB2	Graph 4	IP			
		HER2 wt	Graph 5	-			
C2	DNA i.s. + boost i.m. + EP	HER2 wt	-	WB + IP			
		HER2 wt	Graph 6	IP			
D	31E i.p.	c-erbB2	Graph 7	WB			
		HER2 wt	Graph 8	WB			

Abbreviations: i.m. intramuscular injection, i.s. intrasplenic injection, EP *in vivo* electroporation, graphs of tumor growth see Appendix C, WB Western blot, IP Immunoprecipitated prior to analysis by WB, INFy Intracellular interferon y staining, Cr Chromium release assay

5.2 Part II – The cell differentiation and pathway project

The results obtained in this part are subject to a manuscript prepared for publication. See below.

Acknowledgements

I thank Dr. Daniel Kalbermatten for teaching me the surgery on the mice. I also thank Dr. Claudio Vallan for the assistance in FACS analysis and Dr. Matthias Matter for helping me with the intracellular INFγ staining and the Chromium release assay. A lot of useful advice came from Dr. M. Halpern, Prof. A. Ochsenbein as well as from Dr. Amiq Gazdhar, who helped me also with the electroporation technique. I especially thank Susann Saurer for all her efforts in the cloning procedures and for always having an open ear during my time in the Tiefenau lab. Furthermore I would like to thank Barbara Nesti, Christel Gremion, and Thecla Constantinou. In the end I thank my parents and my sister who supported me in many ways during the whole time of my thesis work.

Appendix A: Abbreviations

Akt	Protein kinase B (PKB), a serine/threonine kinase activated by PI3K
APC	Antigen-presenting cell
ASK1	Apoptosis-signal-regulating kinase 1
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
Erk	Extracellular signal-regulated kinase, a MAP kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Grb2	Growth-factor-receptor-bound protein 2
HC	hydrocortisone
IHC	Immunohistochemistry
IL-2	Interleukin 2
IP_3	Inositol-1,4,5-triphosphate
IPTG	Isopropyl-β-D-thiogalactosid (substrate of β-galactosidase)
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin, a 289kDa serine/threonine kinase
NSCLC	Non-small-cell lung carcinoma
$p21^{Cip1/WAF1}$	A senescence-associated cyclin-dependent kinase inhibitor
p38	High osmolarity glycerol response kinase (p38α, β, γ, δ), a MAP kinase as well as a stress-activated protein kinase (SAPK), responds to stress
PRL	prolactin
RTK	Receptor tyrosine kinase
SH2	Family of intracellular proteins which have in common a sequence of about 100 aa, known as the SH2 domain, standing for src-homology (examples: Grb/ GAP/ PLCy/ PI3-Kinase/ Src…)
SHC	Src homology 2 domain containing transforming protein
Src	An intracellular tyrosine kinase, $pp60^{c\text{-}src}$, human c-Src is a proto-oncogene
STATs	Signal transducer and activator of transcription proteins; signaling effectors often associated with EGFR activation
WAP	Whey acidic protein

Appendix B: Cell lysis buffer

Modified Radioimmunoprecipitation (RIPA) buffer

Base ingredients:

- 1X modified PBS (see below)
- 1% Triton X-100
- 0.25% Na-deoxycholate

Protease inhibitors:

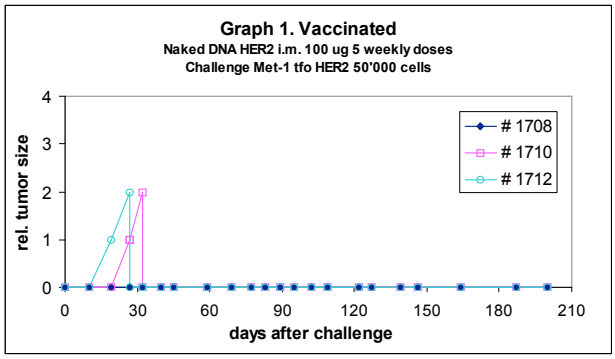
- 1 μg/ml leupeptin
- 1 μg/ml aprotinin
- 1 mM EDTA
- 1 mM EGTA
- 1 mM β-glycerophosphate
- 1 mM 6-aminohexanoic acid
- 1 mM PMSF (added just prior to use)

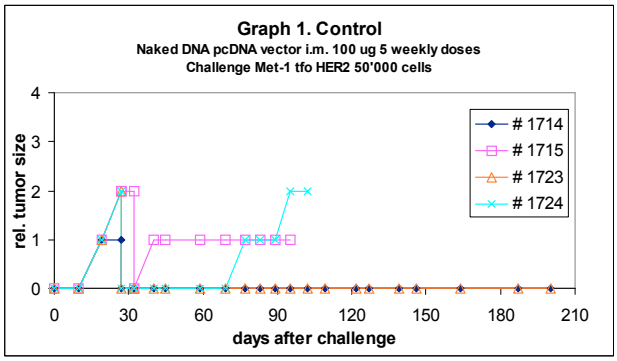
Phosphatase inhibitors:

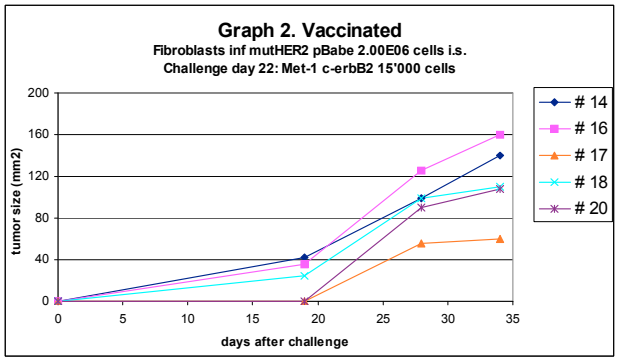
- 1 mM activated Na-orthovanadate
- 1 mM NaF

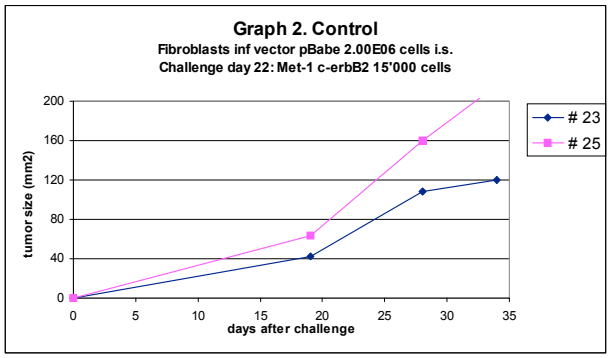
PBS (10X): 0.1 M Na_2HPO_4, 17.6 mM KH_2PO_4, 1.37 M NaCl, 26.9 mM KCl
(the pH was adjusted to 7.4 with HCl)

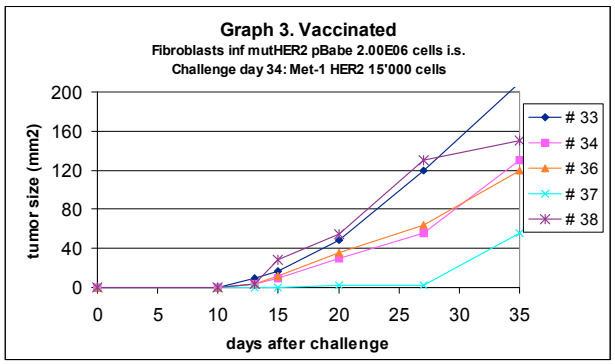
Appendix C: Tumor growth curves

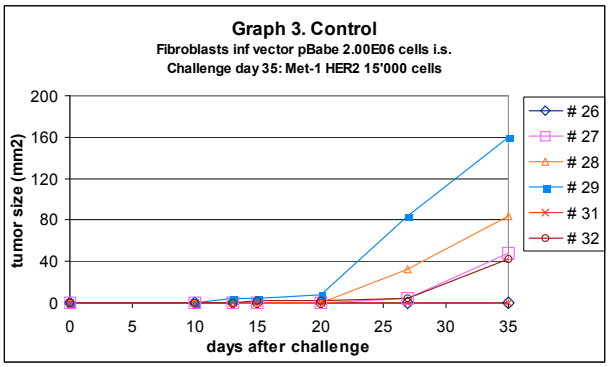


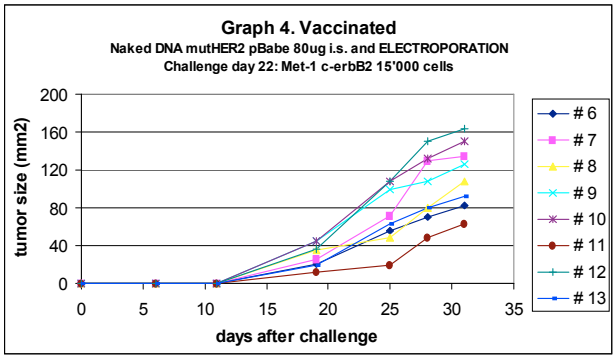


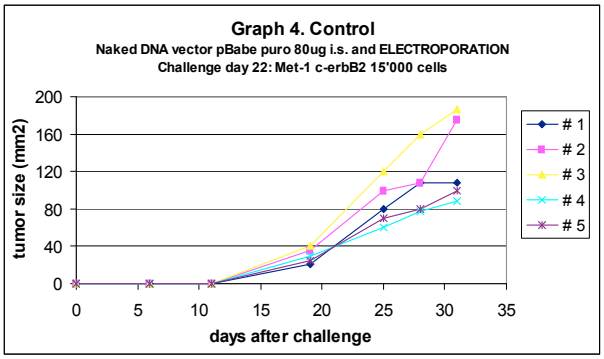


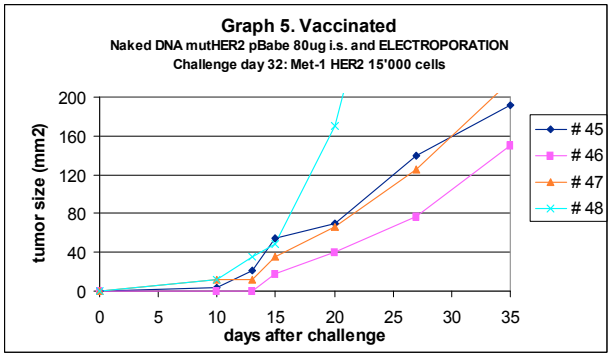


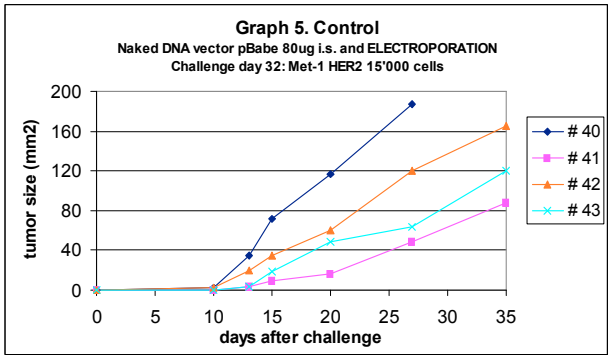


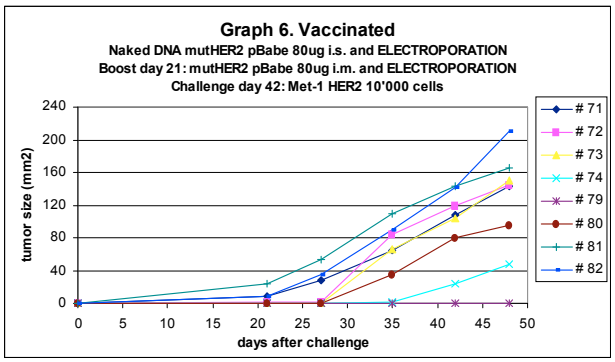


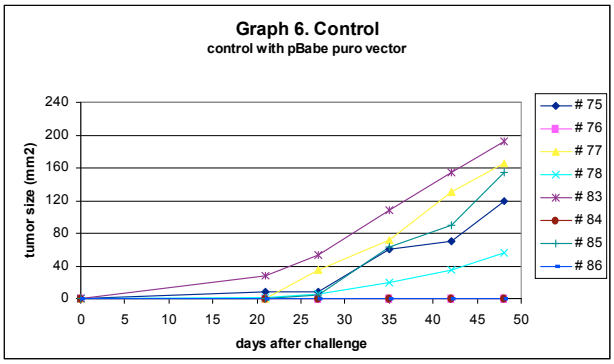

Graph 4. Vaccinated
Naked DNA mutHER2 pBabe 80ug i.s. and ELECTROPORATION
Challenge day 22: Met-1 c-erbB2 15'000 cells
tumor size (mm2)
days after challenge
6
7
8
9
10
11
12
13


Graph 4. Control
Naked DNA vector pBabe puro 80ug i.s. and ELECTROPORATION
Challenge day 22: Met-1 c-erbB2 15'000 cells
tumor size (mm2)
days after challenge
1
2
3
4
5


Graph 5. Vaccinated
Naked DNA mutHER2 pBabe 80ug i.s. and ELECTROPORATION
Challenge day 32: Met-1 HER2 15'000 cells
tumor size (mm2)
days after challenge
45
46
47
48


Graph 5. Control
Naked DNA vector pBabe 80ug i.s. and ELECTROPORATION
Challenge day 32: Met-1 HER2 15'000 cells
tumor size (mm2)
days after challenge
40
41
42
43


Graph 6. Vaccinated
Naked DNA mutHER2 pBabe 80ug i.s. and ELECTROPORATION
Boost day 21: mutHER2 pBabe 80ug i.m. and ELECTROPORATION
Challenge day 42: Met-1 HER2 10'000 cells
tumor size (mm2)
days after challenge
71
72
73
74
79
80
81
82


Graph 6. Control
control with pBabe puro vector
tumor size (mm2)
days after challenge
75
76
77
78
83
84
85
86

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To be submitted to *Int. J. Cancer.*

Differentiation Phenotypes of Mouse Mammary Epithelial Cells expressing different Homologs of ErbB2

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Keywords: mammary epithelial cells, mouse c-erbB2, HER2, NeuT, E-cadherin and Connexin 43

Abstract

ErbB2 is an orphan receptor tyrosine kinase which can dimerize with other ligand-activated members of the EGF receptor family to signal in pathways inducing cell proliferation. Frequently overexpressed in breast cancer and other human cancers, homologs of ErbB2 are oncogenes in different animal species which have been studied for their contribution to the development of carcinomas. We have examined the phenotypes induced by several such ErbB2 homologs in the Line 31E mouse mammary epithelial cell model which is capable of differentiation *in vitro* in response to lactogenic hormones to undergo dome formation and to produce β-casein. Included in this comparative study are the functional proto-oncogene mouse c-erbB2, HER2, a human homolog overexpressed in breast cancer and the mutated rat homolog, NeuT, which is known to be oncogenic.

Line 31 E mammary epithelial cells were infected with retroviral pBabe^{puro} constructs of the different *ErbB2* homologs. While the cells expressing the homologs remain responsive to EGF ligand in terms of Akt/PKB, ERK 1/2 and PKCα phosphorylation, a somewhat elevated basal phosphorylation level in the absence of ligand was apparent for Akt/PKB and ERK 1/2 with mouse *c-erbB2*-, *HER2*- and *NeuT*-infected cells, but not with the *dominant negative mutant HER2*. Typical features of epithelial intercellular organization, such as transmonolayer electrical resistance (TER, a measure of the density of tight junctions) and dome formation, were disturbed by ErbB2 expression. While the dominant negative mutant of HER2 had no phenotypic effect, both TER and dome formation were reduced to some degree by all three of the functional ErbB2 homologs, most dramatically by NeuT. While expression of both the mouse proto-oncogene c-erbB2 and HER2 resulted in significant inhibition of β-casein mRNA and protein levels when the mouse mammary epithelial cells received lactogenic hormone stimulation, NeuT completely abrogated β-casein production and caused oncogenic transformation as evidenced by large colonies in soft agar and Matrigel suspension culture.

Introduction

Several types of cancers overexpress ErbB2.[1] For example, invasive ductal breast cancer shows gene amplification in approximately 30% of all cases[2] while the comedo-type ductal carcinoma in situ, a non-invasive, premalignant mammary tumor, shows a much higher proportion (80–85%).[3] In general one can say that overexpression correlates with tumour size, probability of spread of tumour to lymph nodes, higher tumor grade, higher percentage of S-phase cells, aneuploidy and loss of steroid hormone receptor expression. Together these characteristics imply that ErbB2 overexpression probably confers a strong proliferative advantage to tumor cells since the output of the ErbB signalling network impacts functions ranging from cell division and migration (both associated with tumorigenesis) to adhesion, differentiation and apoptosis.[4]

Three sorts of *ErbB2* gene isolates have been made over the years: *Neu*, a mutated form of the rat *ErbB2* homolog was cloned from neuroblastomas,[5] *HER2*, not mutated, but repeatedly isolated from breast cancer biopsies,[6] and mouse *c-erbB2*, the normal mouse homolog.[7] Two different unrelated mutations have been found in Neu, the rat ErbB2 homolog, each leading to the onset of transformation. Firstly, Bargmann et al.[5] found a single point mutation in the transmembrane domain of Neu in chemically induced rat neuroectodermal tumors. Their findings demonstrated that alterations in the transmembrane segment of rat Neu can have profound effects on its biological activity and transforming ability. The single-point mutation (GTG to GAG) results in the valine to glutamic acid amino acid substitution at amino acid 664 in the transmembrane domain of the receptor. This point mutation apparently mimics ligand induction of receptor activation and results in the activation of the receptor tyrosine kinase with constitutive receptor dimerization. This mutation was the only difference found between the oncogene Neu and the rat proto-oncogene. Later, however, in tumors from transgenic MMTV/Neu mice, a second mutation was discovered in the extracellular region of Neu, resulting in a constitutively-activated receptor capable of transforming Rat-1 fibroblasts.[8]

In human cancers, activation of wild-type ErbB2 occurs through overexpression, generally due to genomic amplification of the *ErbB2* proto-oncogene.[2] Holbro and Hynes[9] postulate that high levels of the wild-type receptor are responsible for the oncogenic phenotype by promoting spontaneous homodimerization and constitutive ErbB2 activation. Recently, somatic mutations in the kinase domain of human ErbB2

have, however, been identified in a small subset (approximately 5%) of primary lung tumors (NSCLCs).[10]

Human mammary epithelial cells overexpressing HER2 have been shown to exhibit *in vitro* two major changes in cellular behaviour with high relevance to tumorigenesis; firstly, the epithelial-to-mesenchymal transition (EMT), and secondly, anchorage independent growth (AIG).[11, 12] In EMT, the epithelial characteristics of the cells, such as cell polarity, the presence of adherens junctions, tight junctions and desmosomes, and expression of many epithelium-specific proteins, are lost and replaced with those of a more or less mesenchymal phenotype.[11] An important characteristic of EMT is the down-regulation of E-cadherin expression. Furthermore, functional a disturbance in gap junctional communication between epithelial cells owing to phosphorylation and down-regulation of connexin 43 protein has been reported.[13-15] AIG is usually detected by the classical parameter of growth capacity in soft agar. Jenndahl et al.[12] argue that EMT is a critical phenotypic change required for AIG to develop in the course of ErbB2 signalling. When HER2 is overexpressed in an infected human breast cancer cell line (MDA-MB-231), it appears to synergize with the TGFβ pathway to potentiate pro-invasion, angiogenesis, and EMT.[16]

ErbB2-induced EMT has been shown to be mediated by and dependent on c-Src with normal epithelial kidney cells (MDCK).[17] The oncogenic behaviour of ErbB2-expressing mammary tumor cells seems to be a consequence of Src activation which, further down the pathway is also responsible for PKCα activation. Src and PKCα are present as a physical complex, and treatment with inhibitors of either Src or PKCα suppresses tumor cell invasion.[18]

Mammary gland functional differentiation occurs with distinct morphological and molecular changes of the epithelial cells and allows for the production and secretion of milk. Thus, the secretory alveolar cells represent the final cellular state of the life-spanning multistep differentiation process within the mammary gland. The differentiation steps taking place during pregnancy and lactation are characterized by the sequential activation of genes encoding the milk proteins such as β-casein, whey acidic protein (WAP) and α-lactalbumin. Mammary gland development in response to lactogenic hormones *in vitro* is known to be accompanied by distinct morphological changes in the monolayers forming structures called domes. Zucchi et al.[19] have correlated the dome formation *in vitro* with differentiation processes occurring in the mammary gland during pregnancy. Dome formation was shown to be inducible by the

lactogenic hormones insulin, hydrocortisone and prolactin preceding expression of the milk protein β-casein, which is a specific marker of the initial stage of mammary gland functional differentiation.[20]

In order to learn about the influence of ErbB2 on normal, polarized mammary epithelial cells in culture, we decided to study the differentiation *in vitro* of the Line 31 mouse mammary epithelial cells,[20, 21] using retroviral constructs of mouse *c-erbB2*, *HER2* and *NeuT* in order to study the behavioural phenotype exerted by the different isolates.

Materials and Methods

Cell Culture

The mouse mammary epithelial (31E) and fibroblast-like mammary stem cell (30F) lines were established from BALB/c mice and characterized by Dr. Ernst Reichmann in our laboratory as described previously.[20] Cells were cultured in Eagle's Dulbecco-Modified medium enriched by 10% foetal calf serum (EDM10) and supplemented with EGF (10 ng/ml). The cells used in the EGF-stimulation experiments had been deprived of EGF one week before and subjected to serum starvation (2% FKS) 20 - 24 hrs prior to stimulation with EGF. Ligand stimulation was performed with 40 ng/ml EGF for 0, 2, 5, 15 and 30 min.

For preparation of cell lysates, confluent cells from 5 cm plates were washed with phosphate buffered saline (PBS) and incubated at 4° C for 10 min in 1 ml of modified RIPA lysis buffer (PBS with 1% Triton X-100, 0.25% Na-deoxycholate, 1 ug/ml each leupeptin and aprotinin, 1 mM each EDTA, EGTA, β-glycerophosphate, 6-aminohexanoic acid, NaF and sodium-orthovanadate). PMSF was added to 1 mM immediately before use. Following Dounce homogenisation, samples were centrifuged at 20'000 x *g* for 10 min and the supernatants collected. After determination of protein concentration using the Bradford-based Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany), the lysates were diluted to 1X SDS-PAGE sample buffer (80mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol and 0.02% bromphenol blue) and boiled for 2 min before electrophoresis on 10% polyacrylamide gels.

In Vitro Differentiation Assays, Soft agar and Matrigel growth assays

In order to induce functional *in vitro* differentiation of Line 31E mouse mammary epithelial cells expressing the various ErbB2 constructs, co-cultures were prepared with Line 30F cells as described.[20] The co-cultures were established with densities of Line 31E to Line 30F in the ratio of 4:1. After culture for 48 hrs initially in medium supplemented with EGF (10 ng/ml), cells were changed for 24 hrs to EDM with 3% foetal calf serum, 5 ug/ml insulin, 1 ug/ml hydrocortisone and 5 ug/ml ovine prolactin. Thereafter, supernatants were taken and cells were lysed using RIPA lysis buffer for determination of β-casein in Western blots.

For determination of the electrical resistance across the monolayers of pure cultures of Line 31E cells infected with the different *ErbB2* constructs, cells were grown to confluence on cellulose nitrate filter inserts (diameter 2.2 cm) in 6-well plates as previously described.[21] Resistance measurements were obtained with the Millipore (Bedford, MA., USA) Millicell-ERS instrument, essentially a milli-volt/ohmmeter that provides an alternating voltage source to minimize cell damage.

Cell morphology of 31E cells on plastic substrate, expressing the different ErbB2 homologs was recorded using a 20x objective in phase contrast after 3 days and 6 days in culture with lactogenic hormones. Culture in soft agar was performed by mixing gently 50'000 cells in EDM10 at room temperature with EDM10/Noble agar at 42° C to give a final concentration of 0.37% Noble agar. 3 cm culture dishes which had been covered with 0.8 ml EDM10 containing 1% Noble agar as a hard underlayer, were overlayed with 1.5 ml of the cell suspension. Incubation was for 6 days at 37° C in a conventional CO_2 incubator. Culture in Matrigel (growth factor depleted; BD Biosciences, Bedford, MA, USA) was also performed according to the manufacturer's instructions by diluting an ice cold cell suspension (with 50'000 cells) in EDM10 (2.5 parts) with Matrigel (1 part), plating in 3 cm culture dishes which were subsequently incubated for 6 days at 37° C. Images of suspension culture colonies were obtained using a 5X objective in phase contrast.

SDS-PAGE and Immunoblot Analysis

Electrophoretic separation of proteins was performed by discontinuous Laemmli SDS-PAGE using the Mini-PROTEAN® II electrophoresis cell (Bio-Rad) with an electrophoretic separation length of 7 cm. For immunoblot analysis 10% polyacrylamide gels were poured using research-grade 29:1-Acrylamide/ Bisacrylamide (Serva, Heidelberg, Germany).

For Western Blot analysis, proteins were separated by SDS-PAGE as described above and transferred onto nitrocellulose membranes (Schleicher & Schuell Inc., Dassel, Germany) using a semidry electroblotter (Bio-Rad). After transfer, to ensure that equal amounts of protein were loaded, protein concentration was confirmed by Ponceau S staining of Western blot membranes. The membranes were incubated for 1h in blocking solution (20 mM Tris pH 7.6, 140 mM NaCl, 0.05% Tween-20, 5% w/v non-fat dry milk) and then washed twice for 5 min with TBS-T (20 mM Tris pH 7.6, 140 mM NaCl, 0.05% Tween-20). Primary antibodies were diluted with TBS-T

containing 2% w/v bovine serum albumin (BSA) (Fraction V, Sigma) and the membranes were incubated overnight at 4°C with gentle agitation. After three 5 min-washes with TBS-T, the membranes were incubated for 1h at room temperature with the secondary antibody, horse radish peroxidase-conjugated anti-mouse or anti-rabbit antibody diluted 1:10'000 in TBS-T containing 2% w/v BSA. After further washing the membranes three times for 5 min with TBS-T, the immunoreactive bands were visualized by chemiluminescence using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). A CCD camera system (Lumi-Imager™, Roche Diagnostics, Rotkreuz, Switzerland) was used to detect the signal.

For reprobing membranes for proteins of a different size, membranes were washed twice for 10 min with TBS-T and incubated again for 1h in blocking solution, before proceeding with the next primary antibody as described above. Alternatively, stripping was accomplished by incubation for 30 min at 50°C with gentle agitation in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS), washing twice for 10 min with TBS-T followed by incubation for 1h in blocking solution prior to reprobing for a different protein.

Antibodies

A polyclonal rabbit antibody against mouse β-casein was generously provided by Dr. Ernst Reichmann (Pediatric Surgery Research, University Hospital, Zurich, Switzerland).

Affinity-purified antibodies specific for c-erbB2 (mouse, human and rat specific), Akt/PKB phospho-Ser-473 Akt/PKBα, MAP kinase phospho ERK 1/2 (monoclonal 12D4), and PKCα phospho-Ser-657 were all obtained from Upstate (Chemikon, Lake Placid, NY). Horse radish peroxidase-conjugated secondary antibodies against rabbit IgG or mouse IgG were also obtained from Upstate.

Isolation of RNA and Northern blotting

Total RNA was prepared from tissue samples and cells using the guanidinium-thiocyanate extraction method described by Chomczynski and Sacchi.[22] 5 µg of total RNA was denatured with glyoxal, electrophoresed on a 1% agarose gel and blotted to nitrocellulose. Equal loading was controlled by examination of ribosomal RNA bands following staining with acridine orange, but immediately prior to blotting as previously described.[23] Probes for hybridization were prepared using the Random

Primed Labelling Kit (Roche) including ^{32}P-dCTP (800 Ci/mM; Amersham International, Little Chalfont, England) in the reaction and hybridization was carried out as previously described.

Constructs

The mouse c-erbB2 RIKEN plasmid

We obtained the mouse *c-erbB2* gene in the pFLCI vector in a collaboration with Dr Yoshihide Hayashizaki, Genome Exploration Research Group, RIKEN, Japan (Clone ID D030063B12).[7] The NCBI database Accession Number for mouse *c-erbB2* at that time was BC046811.

By comparing to the human and rat homologs, we found Clone D030063B12 to contain a substitution (addition of 81 bp) causing a frameshift and a non-functional product. This error could be established by comparison with an NCBI-predicted virtual sequence for mouse *c-erbB2* (Accession No. NM_001003817) that was entered into the NCBI database after this project had begun. See Table 1 for the alignment. The 81 bp insert is not a repeated sequence of mouse *c-erbB2*, but is spurious.

Since the RIKEN clone was the only isolate available of the full length mouse *c-erbB2* (4.7 kb), we decided to correct the cloning error by site-directed mutagenesis of the clone D030063B12. The RIKEN clone was first subcloned into pBluescript SK(±) (Stratagene, La Jolla, CA, USA). A pBluescript subclone was restricted with Bst1107I (restricts *c-erbB2* at position 2105) and BsiWI (cuts at position 2444) to remove a fragment of 339 bp spanning the erroneous 81 bp addition region. RNA isolated from a pool of 15 day mouse embryo liver, lung, intestine, kidney and heart was employed to prepare cDNA with Transcriptor Reverse Transcriptase (Roche) using the primer sequence AGGCAGCCATAGGGCATAAG to obtain a fragment which extended beyond the entire excised 339 bp sequence. A PCR reaction (Expand High Fidelity Plus PCR System; Roche) was then performed with the cDNA using primers designed to contain restriction sites for Bst1107I (sense) and BsiWI (antisense). Finally, the PCR fragment obtained was restricted with Bst1107I and BsiWI, purified and ligated into the previously restricted pBluescript *c-erbB2*. The resulting full-length "corrected" *c-erbB2* was sequenced and the alignment to human *HER2* showed that the 81 bp addition had been removed successfully. Finally, we subcloned the

corrected mouse *c-erbB2* into the pBabe^{puro} retroviral vector.[24] Biological activity was observed in cell culture indicating a functional protein and a protein of 185 kD could be detected with a Western blot using commercially available (Upstate) anti-c-erbB2 antibody.

The human HER2 plasmid

The human *HER2* plasmid pcDNA3 we received as a kind gift of Dr. Ming-Fong Lin, University of Nebraska Medical Center, Omaha, NE, USA.[25] The sequence of the human *HER2* gene is published and filed in the NCBI database under the Accession Number NM_004448.2 (total length 4624 bp; CDS 239-4006). This version was originally submitted to the NCBI by Yamamoto et al.[6] in 1986, and derived from a human mammary tumor. Interestingly, when compared to the sequence of the physiological human *ErbB2* gene (Accession No. DQ047380), the Yamamoto sequence does not show any mutations, thus, supporting the presumption that the oncogenicity of HER2 in this human tumor sample did not rely on a mutation in the gene.

Construction of a Dominant-negative mutated human HER2

We decided to construct as a negative control for the transformation phenotype of HER2, a dominant negative mutant of the *HER2* gene lacking the sequence coding for the ATP-binding site of this tyrosine kinase receptor protein. This mutation results in an inactivation of the tyrosine kinase activity and consequently blocks the transforming activity. With four specially designed primers we used high fidelity PCR to produce two fragments, i.e. the coding regions on either side of the ATP-phosphate binding loop GSGAFG, at amino acid positions 727-732 (nucleotides 2181-2196). The primers were designed to contain restriction sites for SalI and HindIII, respectively, and produced fragments of 2.3kb and 1.9kb, respectively, which when re-ligated restored a full-length *HER2* construct, coding for a protein lacking only 6 amino acids, with a single mutation replacing the valine at position 733 with alanine.

The rat NeuT plasmid

The rat *NeuT* in the pBabe^{puro} vector was the generous gift of Prof. Nancy Hynes (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland).

Retrovirus Constructs

Retroviral constructs of *HER2*, *dominant negative mutant HER2* (designated *MUT*) and mouse *c-erbB2* were all prepared in pBabe^{puro}.[24] For *HER2*, the insert was restricted with HindIII blunted with Klenow enzyme. For the mouse *c-erbB2* RIKEN "corrected" clone, restriction was with SacI, followed by blunting with T4 polymerase, and isolation of the insert by a second restriction with EcoRV. Finally, the blunted inserts were ligated into a blunted SnaBI pBabe^{puro} site. Helper-free infectious virus was produced using amphitrophic Phoenix packaging cells[26] kindly supplied by Dr. G. Nolan (Stanford University Medical Center, Stanford, CA). Line 31E cells were infected with approximately $4x10^5$ infectious units on $2x10^6$ cells. For infection Polybrene was used at 8 ug/ml. Successfully infected cells were selected with 4 days treatment with puromycin (Sigma) at 2.5 ug/ml. Expression was tested with Northern and Western blots, and biological activity confirmed by cell biological assays.

Results

We first examined effects of expression of the ErbB2 homologs on several signalling pathways one could expect to be perturbed. In order to obtain a baseline, Line 31E cells infected with the different *ErbB2* homologs were compared without EGF ligand stimulation in Western blots of parallel, identical PAGE runs. The Western blot in Fig. 1 demonstrates that with an antibody cross-reactive with all ErbB2 homologs, each of the constructs was well expressed. That equal loading was attained is apparent from the detection with Akt-specific antibody, while the two lower gels demonstrate that basal phosphorylation levels for Akt in the PI3 kinase pathway and ERK 1/2 in the MAP kinase pathway are elevated in Line 31E cells expressing the different ErbB2 homologs, most strikingly with *NeuT* infected cells. PKCα phosphorylation was not stimulated in the absence of EGF ligand stimulation (data not shown).

Fig. 2 illustrates the phosphorylation/activation of Akt, Erk 1/2 and PKCα following EGF ligand treatment for different times. Total Akt is shown in the first panel as an equal-loading control. Akt levels do not vary after treatment with the ligand, whereas Akt phosphorylation shows an increase within 2 minutes. Not surprisingly, the dominant negative mutant HER2 shows responses to EGF similar to cells expressing functional ErbB2, reflecting the inevitable effect owing to the endogenous EGF receptor-family members in Line 31 cells. In these normal epithelial cells, expression of EGF Receptor 1 (EGFR1), which is essential for any response by ErbB2 homologs, at the same time complicates the interpretation of the results observed in the signalling pathways. Phospho-ERK 1/2 shows a rapid response to EGF, while PKCα phosphorylation is slower, but more dramatic, extending beyond 30 minutes after stimulation (Fig. 2). The significance of the results shown in Fig. 2 is that while basal receptor signalling (Fig. 1) can be detected for all the ErbB2 homologs even without ligand added, stronger responses can be induced in the pathway following EGF stimulation.

The modest effects elicited in the PI3 kinase and MAP kinase pathways by expression of ErbB2 homologs do, however, show major consequences in the cell biological phenotype. Co-cultures of Line 31E and Line 30F cells (in the proportions 4:1) show characteristic *in vitro* differentiation behaviour in response to lactogenic hormones. Fig. 3 illustrates production of β-casein after 24 hrs of lactogenic hormone treatment. Co-cultures were grown first 2 days in the presence of EGF as a “priming” step, then changed to either hydrocortisone, insulin, prolactin (HIP) medium or just

Eagle's Dulbecco-modified medium, both in the absence of EGF. A Northern blot (Fig. 3A) shows β-casein mRNA with HIP-stimulated cells expressing only a neutral vector, but much less in mouse c-erbB2 or HER2-expressing cultures, while NeuT totally blocks expression of the β-casein. Unstimulated cells produce only tiny amounts of β-casein mRNA, but in the same proportions as the HIP treated cultures (Fig. 3A).

A Western blot employing β-casein-specific antibody (Fig. 3B), demonstrated the same result. No β-casein protein was detectable in cultures not receiving HIP, and no synthesis of the protein was apparent with 31E/HER2 or 31E/NeuT cells. Production by mouse c-erbB2-expressing cells was sharply reduced when compared to normal co-cultures or dominant negative mutant HER2-expressing co-cultures (Fig. 3B). Interestingly, a sharp inhibition of β-casein production occurred whenever HIP stimulation was combined with EGF.

Another measure of differentiation recognizable in Line 31E cells in pure culture is the production of "domes". Fig. 4A & B show that while both normal Line 31E and mutant HER2-expressing cells form these domes or bubbles caused by vectorial water transport in a polarized cell monolayer, cells expressing ErbB2 homologs lack this characteristic (Fig. 4C, D & E). Cells infected with mouse *c-erbB2* in phase contrast resemble early monolayers of normal Line 31E (Fig. 4D), while *HER2* and *NeuT*-infected cells exhibit clusters or foci of rounded cells on a normal-appearing monolayer (Fig. 4C & E). In fact, both *HER2* and *NeuT* infected Line 31E cells demonstrate in longer-term culture (6 days) cord-like structures made up of fibroblast-like cells overlying normal-appearing epithelial cells (Fig. 4F). This latter phenomenon is probably the result of epithelial-mesenchymal transition among a fraction of the infected cells.

The oncogenic effect of NeuT expression in Line 31E cells is demonstrated in Fig. 5 with colony formation in soft Noble agar. Matrigel suspension culture provided essentially the same results (data not shown). About 20% of the c-erbB2 and HER2-expressing cells form colonies of up to about 16 cells. While approximately 50% of the *NeuT*-infected Line 31E cells produce only the same tiny colonies seen with wild type *HER2* and mouse *c-erbB2* cells, larger, looser colonies consisting of up to hundreds of cells made up another 50% of the total. Giant colonies of thousands of cells were very rarely seen, as illustrated in the lower right panel. We also examined growth rate for Line 31E cells on the plastic dishes and compared with the ErbB2

homolog-expressing cells. There was no significant difference in the growth rates over a three-day period, although in long term culture, *NeuT*-infected cells achieved much higher maximum densities (data not shown).

Various authors have described an EMT-like phenotype in ErbB2-expressing cells.[11, 12] Since EMT is characteristically associated with downregulation of E-cadherin, we prepared total RNA from normal and ErbB2-expressing Line 31E cells and performed Northern blots, probing for E-cadherin. The results are shown in Fig. 6. Taking advantage of the loose adherence of the fibroblast-like over-growing cells which appear in late culture with HER2 and NeuT-expressing cells (see Fig. 4, lower right-hand picture) we were able to achieve an isolation and enrichment of these EMT-like cells. These were designated wild type HER2 “floaters” (wtfl) or NeuT “floaters” (NeuTfl), and were analyzed in the Northern blot next to the “parental” Line 31E/HER2 and 31E/NeuT samples (Fig. 6). A striking downregulation could be observed in the “floaters” which was, however, not seen in the adherent underlying “parental” cells. Similarly, when blots were probed for connexin 43, clear downregulation was apparent in the EMT-like cell population (Fig. 6). Ethidium bromide staining of 18 S ribosomal RNA in the total RNA serves as an equal loading control.

Table 2 provides biophysical confirmation of the morphological evidence. The transepithelial monolayer resistance (TER) reflects the density of tight junctions between the cells which hinder ion movement between electrodes placed on either side of the monolayer. The values shown are the product of the resistance values obtained (Ohms) multiplied by the area of the monolayer, and indicate that *HER2* and *NeuT*-infected Line 31E monolayers are essentially non-polarized; while mouse *c-erbB2* infection leads to partial de-polarization. It is interesting that the more adherent cultures of Line 31E/HER2 and 31E/NeuT show high levels of E-cadherin and connexin 43 expression in Northern blot as compared to very low expression in “floaters”. Even so, transmonolayer resistance measurements show a significant loss of functional cell polarization in both *HER2* and *NeuT*-infected cultures.

Discussion

Our Line 31E *in vitro* differentiation model is a cloned mouse mammary epithelial cell line. Our investigation has demonstrated similar phenotypic effects produced by different ErbB2 homologs, forming a sort of hierarchy in which the mutated NeuT shows the strongest oncogenic effects. The mouse c-erbB2 proto-oncogene exhibits nonetheless characteristics similar to other members of the ErbB2 group. The results presented above clearly demonstrate that expression of functional ErbB2 homologs influences signalling and biological phenotype. Modest changes in basal phosphorylation of Akt/PKB and of ERK 1/2 were evident in cells expressing mouse c-erbB2, HER2 and NeuT. However, the response to added EGF ligand remained intact. Our results show a hierarchy of effects with the different homologs consistent with the known fact of mutation in NeuT.[5] For the first time, the mouse c-erbB2 product has been investigated and found to be quite similar to HER2, both in terms of signalling characteristics, and phenotypic effects on over-expressing cells.

The main phenotypic effects of ErbB2 homolog expression were apparent changes in behaviour of the epithelial cell monolayer visible in phase contrast microscopy, but also biophysically in the lowered transmonolayer electrical resistance (Table 2). Most striking was the biological effect, a partial or complete block in lactogenic hormone responsiveness, i.e. in the formation of domes, and in synthesis of β-casein (Figs. 3 and 4). More directly related to oncogenic properties, only the NeuT expressing Line 31E cells demonstrated the ability to induce efficient colony formation, both in soft agar and in Matrigel. Neither senescence nor apoptosis was observed.

We observed an EMT-like effect in consequence of HER2 and NeuT expression in Line 31E cells. This effect was apparent in the appearance of a morphologically different, fibroblast-like cell type which could be isolated based on its weak adherence. Clones of these cells, however, consistently gave rise to cell populations comprising both the adherent epithelial and weakly adherent fibroblast-like phenotype. The fibroblast-like cell type exhibited downregulation of both E-cadherin and connexin 43. In this respect, our results shown in Fig. 6 closely corresponded to those reported recently by Jenndahl et al.[11] for E-cadherin. Similarly, disturbances in gap junctional communication and connexin 43 function have been previously reported as a consequence of ErbB2 expression.[13-15]

Hynes et al.[27] reported that the induction of β-casein in HC11 mouse mammary epithelial cells by the lactogenic hormones insulin, prolactin and the glucocorticoid, dexamethasone is not suppressed by oncogenic human ErbB2 or NeuT signalling, but is blocked by EGFR activation. Our results with Line 31E disagree in that NeuT caused a sharp inhibition in β-casein production, as evidenced with both Northern and Western blot methods. In our model as in theirs, however, EGF effected a total block in β-casein synthesis.

In order to investigate the function of ErbB2 during normal breast development, Jones and Stern[28] inactivated endogenous ErbB2 signalling through expression of a *dominant negative truncated ErbB2* transgene during mouse mammary gland development. They found that normal ErbB2 signalling is required for the terminal stages of lobuloalveolar development and lactation as measured by the observation of inhibited alveolar growth and non-secreting lobuloalveoli under transgene expression.[28] In other *in vivo* experiments, the influence of oncogenic rat NeuT on mouse mammary gland development has also been studied in transgenic mice overexpressing NeuT.[29] Expression of the oncogene in this model is controlled by the MMTV-LTR promoter, leading to its elevated expression during late pregnancy and throughout lactation. NeuT transgene expression was assessed at lactation and early involution and revealed to be restricted to morphologically transformed cells, whereas no endogenous mouse c-erbB2 was detected in wild-type glands. NeuT-expressing tumor cells completely failed to express the milk proteins β-casein and WAP at lactation or during involution.[29]

Acknowledgements

We thank Dr. Nancy Hynes (Friedrich Miescher Institut, Basel) for providing us with the pBabe^{puro} construct of *NeuT*, Dr. G. Nolan (Stanford University Medical Center) for giving the Phoenix retrovirus packaging cells and Dr. Ernst Reichmann (Research Laboratory, Clinic for Pediatric Surgery, University of Zurich) who kindly supplied rabbit antibody specific for mouse β-casein. Human *HER2* was the gift of Dr. Ming-Fong Lin (University of Nebraska Medical Center, Omaha, NE). Clone D030063B12 of mouse *c-erbB2* was provided in the context of a collaborative arrangement with Dr Yoshihide Hayashizaki, Genome Exploration Research Group, RIKEN, Japan.
We thankfully acknowledge the support of the Department of Clinical Research, University of Berne and of Professors Anne-Catherine Andres and Andrew Ziemiecki. We are grateful for the support of this project by the Werner and Hedy Berger-Janser Stiftung and the Stiftung für klinisch-experimentelle Tumorforschung.

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Figure Legends:

Fig. 1: The basal expression of ErbB2 homologs, PKB/Akt protein, phospho PKB/Akt (serine 473) and phospho-ERK 1/2 MAP kinase was assayed using Western blots. The levels expressed were evaluated for control, uninfected Line 31E cells (**Ø**), for *dominant negative mutant HER2* cells (**mut**), for mouse *c-erbB2* cells (**c-erb**), for *wild type HER2* cells (**HER2**) and for *NeuT* cells (**NeuT**). Four identical blots were prepared and probed with antibodies detected using chemiluminescence.

Fig. 2: Cultures of Line 31E cells infected with *dominant negative mutant HER2*, *wild type HER2*, mouse *c-erbB2* and *NeuT* were treated with EGF for different times between 0 to 30 minutes. A set of lysates was prepared as described in Materials and Methods. Four identical PAGE blots were prepared and expression of different pathway markers was evaluated with Western blots using specific antibodies to PKB/Akt protein, phospho PKB/Akt, phospho ERK 1/2 and phospho PKCα (Upstate). The panel showing PKB/Akt protein indicates equal loading for the other panels.

Fig. 3: The differentiation response of mammary Line 31E and 30F (4:1) co-cultures to lactogenic hormone stimulation is shown in "**A**". Line 31E cells infected with *β-galactosidase*, *wild type HER2*, mouse *c-erbB2* and *NeuT* retroviral vectors were treated with or without hydrocortisone, insulin and prolactin (HIP) for 24 hours. Samples were of total RNA resolved in a Northern blot and hybridized with a mouse β-casein-specific probe. In "**B**", co-cultures were analzyed using a Western blot with an anti-mouse-β-casein anti-serum. Lysates of uninfected (normal) Line 31E are shown in comparison with the *dominant negative mutant HER2*, mouse *c-erbB2*, *wild type HER2* and *NeuT*-infected co-cultures at 24 hours with no treatment (**Ø**) hormone stimulation, **HIP** either alone or with **EGF**.

Fig. 4: Morphology of uninfected, normal Line 31E cells and cells infected with the *ErbB2* homologs in phase contrast after 3 days in culture (**A** – **E**) and after 6 days in culture (**F**). Dome formation is seen at 3 days (after 24 hours HIP treatment) in normal and *dominant negative mutant HER2* cells, but not apparent in parallel cultures of cells expressing functional c-erbB2, though small local foci of rounded cells can be recognized in these cultures (**C** – **E**). An EMT-like effect is observed in cultures of *NeuT*-infected cells maintained for longer times in culture (**F**), in which

fibroblastic cells overgrow the even monolayer. Such changes can also be seen in *HER2 wild type* cultures, but are absent in mouse *c-erbB2* infected cultures.

Fig. 5: Soft agar colony growth of Line 31E cells expressing the various c-erbB2 homologs is illustrated after 6 days in culture. Photographs were taken with the 5X objective.

Fig 6: Northern blots are presented to show the steady state expression of E-cadherin and connexin 43 in uninfected, normal Line 31E cells (**Ø)** and at 6 weeks after infection with *dominant negative mutant HER2*, *wild type HER2* (**wt**) and *NeuT*. Lines of loosely attached "floater" cells could be isolated by washing cultures of wild type HER2 and NeuT cells with a stream of medium from a pipette. These cells re-attached and proliferated readily when sub-cultured, giving rise to the (**wtfl**) and (**NeuTfl**) cultures.

Table 1:

Mouse c-erbB2 (old) = BC046811 containing the addition
Mouse c-erbB2 (new) = NM_001003817

```
c-erbB2(old)    2192 CCTAATCAAACGAAGGCGACAGAAGATCCGGAAGTATACCATGCGTAGGC   2241
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
c-erbB2(new)    2201 CCTAATCAAACGAAGGCGACAGAAGATCCGGAAGTATACCATGCGTAGGC   2250

c-erbB2(old)    2242 TGCTGCAGGAGACCGAGGTGAGGCGTGCCGAGGGCCTCCTAGCACCCCCT   2291
                     ||||||||||||||||
c-erbB2(new)    2251 TGCTGCAGGAGACCG---------------------------------     2265

c-erbB2(old)    2292 TGGCTTTGCAGCTAGCTGGGCATTCACCCTACCACCCTCTCGCTCCAGCT   2341
                                                                 ||||
c-erbB2(new)    2266 ---------------------------------------------AGCT   2269

c-erbB2(old)    2342 GGTGGAGCCGCTGACGCCCAGTGGAGCTGTGCCCAACCAGGCTCAGATGC   2391
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
c-erbB2(new)    2270 GGTGGAGCCGCTGACGCCCAGTGGAGCTGTGCCCAACCAGGCTCAGATGC   2319
```

Table 2: Electrical resistance across monolayers of cultured Line 31E mammary epithelial cells

	Cell Type	Transmonolayer resistance* (Ohms x cm^2)
Line 31 E	Normal	420
	Mutant HER2	440
	Mouse c-erbB2 (Riken)	180
	HER2	60
	NeuT	50
	Neu T monolayer from floaters	15

* Cultures were grown on nitrocellulose filter inserts (2.2 cm diameter) for 5 days. Values shown were calculated from the average of 3 measurements taken across the membrane from apical to basal sides with the Millicell ERS instrument.

Figure 1

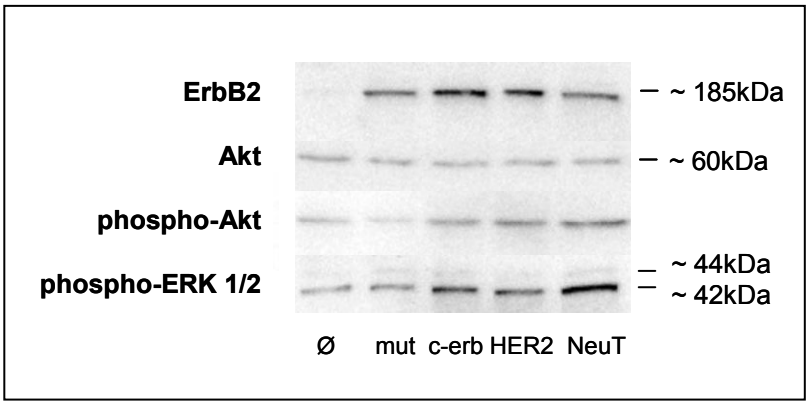
ErbB2
Akt
phospho-Akt
phospho-ERK 1/2
~ 185kDa
~ 60kDa
~ 44kDa
~ 42kDa
Ø
mut
c-erb HER2
NeuT

Figure 2

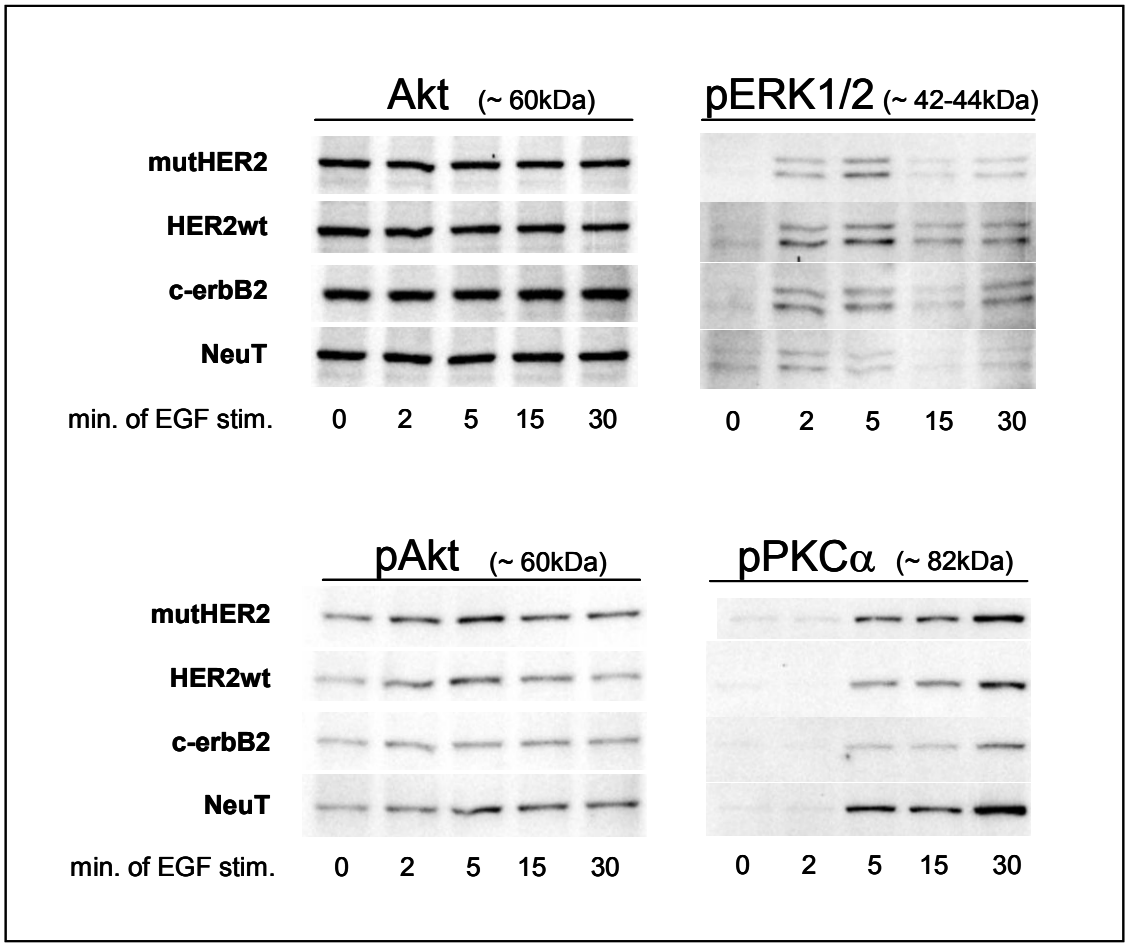
Akt (~ 60kDa)
pERK1/2 (~ 42-44kDa)
mutHER2
HER2wt
c-erbB2
NeuT
min. of EGF stim.
0 2 5 15 30
0 2 5 15 30
pAkt (~ 60kDa)
pPKCα (~ 82kDa)
mutHER2
HER2wt
c-erbB2
NeuT
min. of EGF stim.
0 2 5 15 30
0 2 5 15 30

Figure 3

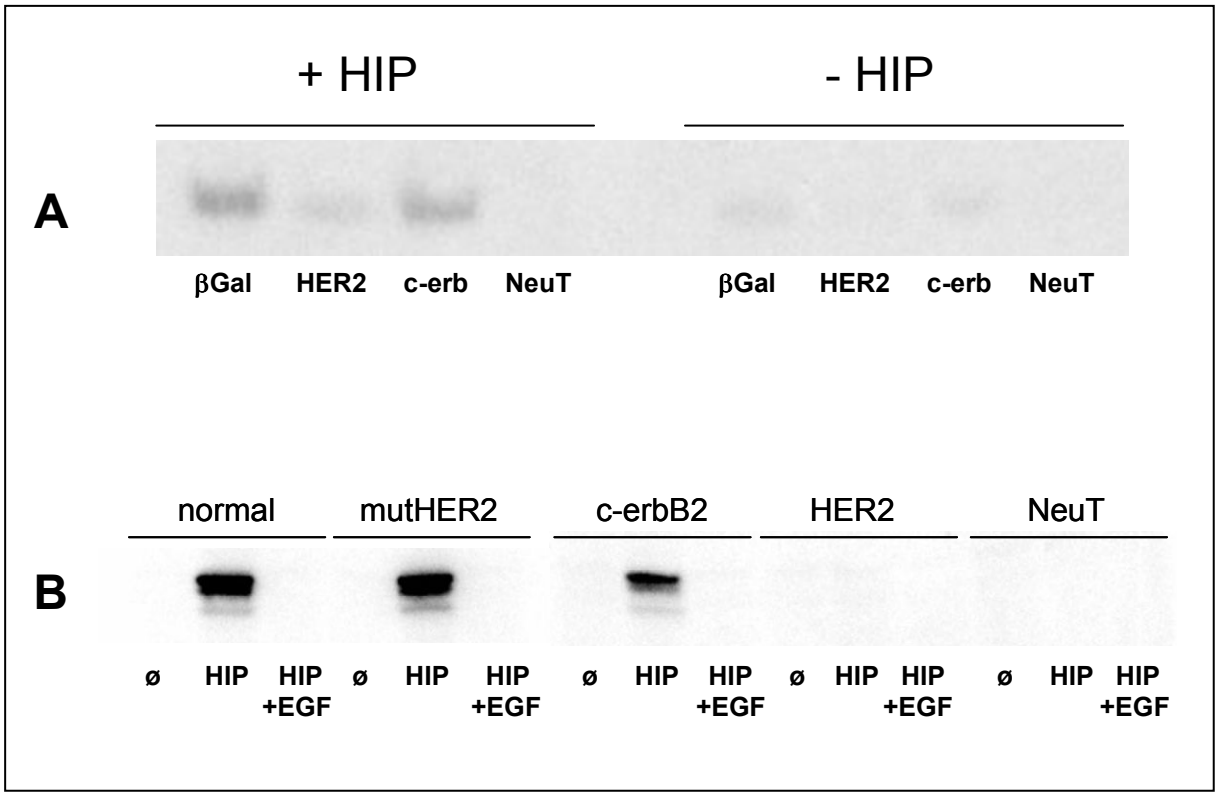
+ HIP
- HIP
A
βGal
HER2
c-erb
NeuT
βGal
HER2
c-erb
NeuT
normal
mutHER2
c-erbB2
HER2
NeuT
B
ø
HIP
HIP +EGF
ø
HIP
HIP +EGF
ø
HIP
HIP +EGF
ø
HIP
HIP +EGF
ø
HIP
HIP +EGF

Figure 4

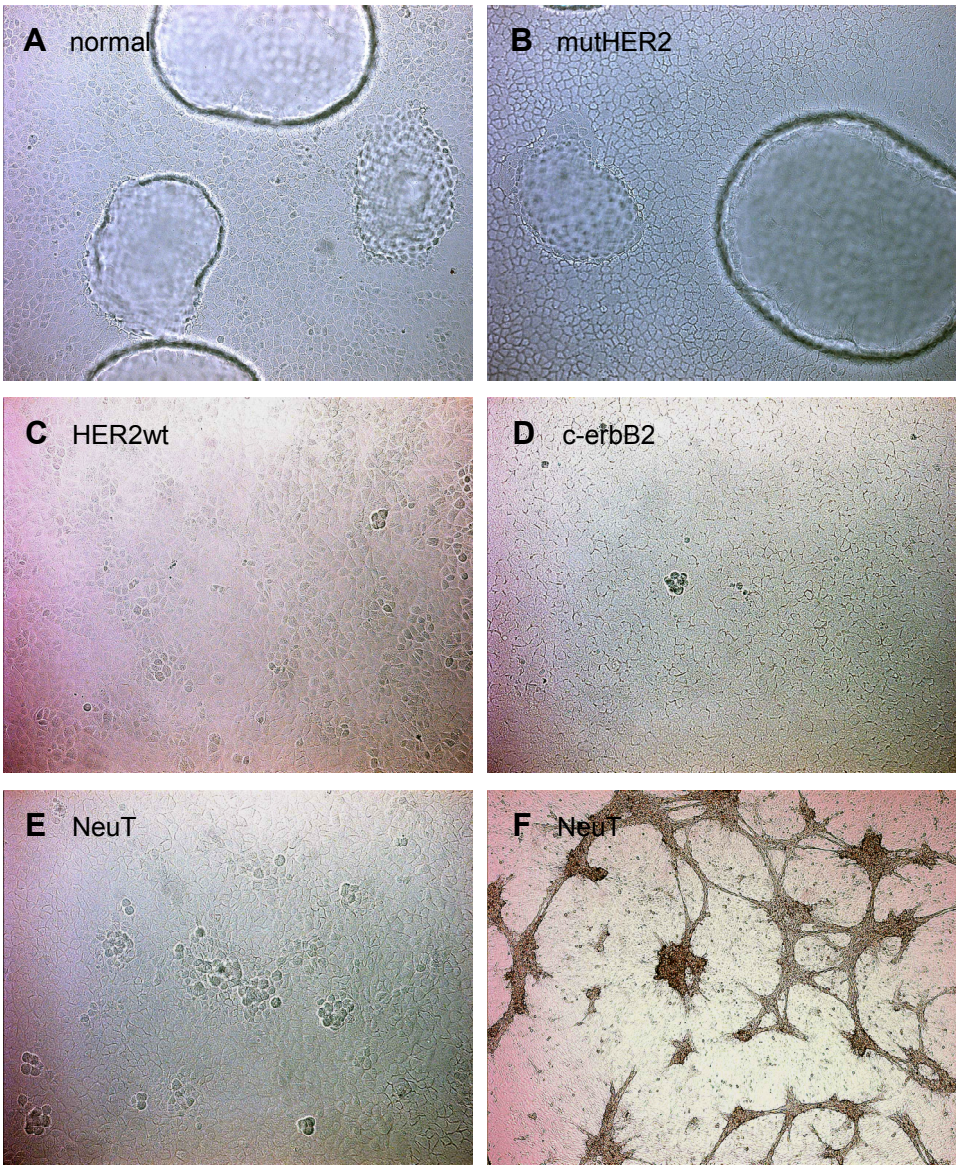

Figure 5

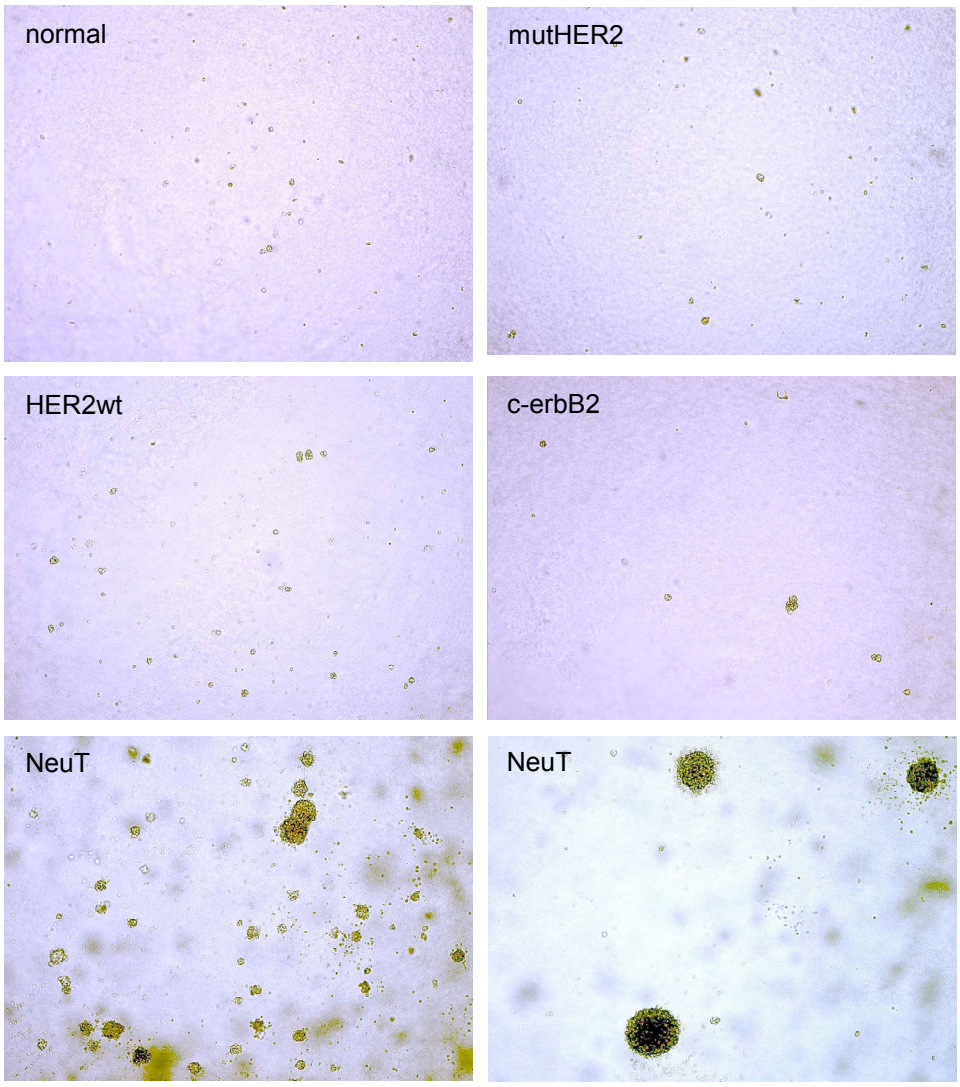

Figure 6