Premature Senescence Is a Primary Fail-safe Mechanism of ERBB2-Driven Tumorigenesis in Breast Carcinoma Cells


Abstract
The receptor tyrosine kinase ERBB2 plays a central role in the development of breast cancer and other epithelial malignancies. Elevated ERBB2 activity is believed to transform cells by transmitting mitogenic and antiapoptotic signals. Here we show that tightly regulated overexpression of oncogenic ERBB2 in human breast carcinoma cells does not stimulate proliferation but provokes premature senescence, accompanied by up-regulation of the cyclin-dependent kinase inhibitor P21 (WAF1/CIP1). A similar effect was caused by retrovirus-mediated overexpression of oncogenic ERBB2 in low-passage murine embryonic fibroblasts. In contrast to previous observations based on constitutively overexpressing cell lines, P21 induced by tetracycline-regulated ERBB2 localizes to the nucleus in arrested cells. P21 up-regulation seems to be independent of the P53 tumor suppressor protein, and senescence-associated phenotypic alterations are reversed by specific inhibition of P38 mitogen-activated protein kinases. Functional inactivation of P21 by antisense oligonucleotides is sufficient to prevent cell cycle arrest as well as the senescent phenotype, thereby identifying the P21 protein as the key mediator of hypermitogenic cell cycle arrest and premature senescence in breast carcinoma cells. Our results may thus indicate that premature senescence represents an inherent anticarcinogenic program during ERBB2-driven mammary tumorigenesis. We propose a multistep model for the process of malignant transformation by ERBB2 wherein secondary lesions either target P21 or downstream effectors of senescence to bypass this primary fail-safe mechanism. (Cancer Res 2005; 65(3): 840-9)

Introduction
Breast cancer is the leading cause of death in female malignancies, currently affecting one of nine women (1). The proto-oncogene ERBB2 is overexpressed in approximately 30% of breast carcinomas (2). Elevated ERBB2 expression is a predictor of poor prognosis with early metastasis and short overall survival (3). ERBB2 is also implicated in the pathogenesis of a variety of other human cancers, in particular ovarian adenocarcinoma (2), non-small cell lung carcinoma (4), bladder cancer (5), and tumors of the gastrointestinal tract (6).

Elevated ERBB2 signaling is very effective in driving cells into malignant transformation, as shown by overexpression of wild-type cDNA, or of its activated form in the mammary gland and in the skin of transgenic mice (7–10). One of the major cellular end points of ERBB2 signaling is the cell cycle. Several studies showed that (a) up-regulation of ERBB2 results in enhanced proliferation and that (b) down-regulation of the ERBB2 signal leads to growth arrest, occasionally associated with increased apoptosis (11–14). However, it has recently become apparent that certain oncogenes can also induce growth arrest instead of a proliferative response. This puzzling feature is attributed to a primary fail-safe mechanism known as the premature senescence program (as opposed to replicative senescence due to the shortening of telomeric repeat sequences; reviewed in refs. 15–18). Whether premature senescence in response to overexpression of oncogenes represents a physiological cellular response is still a matter of debate, but it is intriguing to speculate about this condition as an inherent anticarcinogenic program limiting the transforming potential of proto-oncogenes in primary cells (19, 20). The induction of premature senescence by oncogenes is well documented for constituents of the RAS-RAF-mitogen-activated protein/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway. Oncogenic RAS, for example, is a potent inducer of premature senescence in low-passage fibroblasts (21–23), and activated forms of RAF can induce growth arrest in mammalian cells (24–26). Key mediators of growth arrest in the course of premature senescence seem to be the cyclin-dependent kinase inhibitors (CDKIs). The induction of different CDKI family members, especially P16 and P21, has been linked to the establishment of oncogene-driven growth arrest (23–27). Current data indicate that the differential biological outcomes of oncogenic signaling via the RAS-RAF-MEK-ERK cascade critically depend on the strength and duration of the signal, as well as on the integrity of important tumor suppressor genes, like TP53 or RB1 (reviewed in refs. 15–18). Oncogenic signaling may thus not necessarily lead to a proliferative response but may instead trigger the tumor-suppressive premature senescence program.

We intended to investigate the immediate effects of ERBB2 signaling using the tetracycline-based expression system (28). For this purpose, we conditionally expressed an oncogenic ERBB2 variant, NeuT, in MCF-7 breast carcinoma cells. MCF-7 cells have retained epithelial cell morphology and do not show endogenous ERBB2 amplification or overexpression (29). The NeuT cDNA has been widely used for the study of oncogenic ERBB2 signaling in a...
variety of in vitro and in vivo settings (7, 10, 29). It encodes the rat homologue of the ERBB2 receptor harboring an activating point mutation within the transmembrane domain (30), which promotes ligand-independent receptor dimerization, and constitutive signaling in murine and human cells. Here, we report that regulated expression of NeuT in our MCF-7 cell system clearly induced an antimitogenic response in several independent MCF-7/pTet-NeuT lines. Interestingly, this response was always accompanied by features of premature senescence that have not yet been described as a consequence of ERBB2 signaling. The same effects were observed in NeuT-overexpressing low‐passage murine embryonic fibroblasts (MEF). In addition, we found a marked up‐regulation of the CDK1 P21 in both cell types that was identified as the responsible mechanism for senescence in MCF-7 breast carcinoma cells because specific suppression of P21 induction abolished the antiproliferative effect of NeuT signaling. Taken together, our data for the first time implicates the receptor tyrosine kinase ERBB2 in premature senescence and highlights the importance of the CDK1 P21 in this process. Based on these observations we propose a model of ERBB2‐dependent mnemonic tumorigenesis wherein premature senescence may represent a primary fail-safe mechanism that has to be overcome by the acquisition of further mutations in the course of tumor progression in vivo.

Materials and Methods

Antibodies and Reagents. ERBB2- (sc-284), P21- (sc-397), P53- (sc-126), P-P38- (sc-7973), and actin- (sc-1616) specific antibodies were obtained from Santa Cruz (Heidelberg, Germany). The ERBB2-specific antibody used for communofluorescence staining was purchased from Calbiochem (Bad Soden, Germany; Ab-4) and the P38-specific antibody was obtained from Cell Signaling Technology (Frankfurt, Germany). Doxycycline (obtained as Soden, Germany; Ab-4) and the P38-specific antibody was obtained from Santa Cruz (Heidelberg, Germany). The ERBB2-specific antibody used for coimmunofluorescence staining was purchased from Calbiochem (Bad Soden, Germany) with 50°C/1 minute, 60°C/1 minute, 72°C/1.5 minutes; 21 cycles), P16 (94°C/1 minute, 60°C/1 minute, 72°C/1.5 minutes; 32 cycles), P27 (94°C/1 minute, 59°C/1 minute, 72°C/1.5 minutes; 26 cycles), pyruvate dehydrogenase (PDH; 94°C/1 minute, 58°C/1 minute, 72°C/1.5 minutes; 24 cycles). All programs additionally included an initial denaturation step (94°C/3 minutes) and a final elongation step (72°C/7 minutes). Forward (F) and reverse (R) primers used in this study were as follows: P21-F, 5'-GTCGTTACGAGGTGGTGAAG-3'; P21-R, 5'-GGGGTTTGATGTTGTTGAAGA-3'; P27-F, 5'-AAATGGTTTCAAGCGTTC-3'; P27-R, 5'-ACAGGATGTCCATCCTAGTA-3'; P21-F, 5'-GACTCTACCCCGACCGT-3'; P21-R, 5'-GCCATGTACTGCTCTGTTG-3'; PDH-F, 5'-GACCATTGAGACATGGAAACCA-3'; PDH-R, 5'-TGGACACGGTACAGACAAA-3'.

Real-time Quantitative PCR. Quantitative analysis of P21 expression was done on cDNA synthesized from doxycycline- and/or inhibitor-treated or untreated cells. Amplifications were carried out on a Light Cycler machine (Roche) using the Light Cycler Fast Start DNA Master SYBR Green I system (Roche) in a 10 μl reaction volume consisting of 1 to 2 μl of 1:50 diluted cDNA, 1× SYBR Green PCR Master Mix, 4 pmol forward and reverse primer and 3 mM MgCl2. Cycling conditions were 95°C/2 seconds, 62°C/5 seconds, 72°C/7 seconds, 45 cycles (for P21 amplification) and 95°C/2 seconds, 62°C/5 seconds, 72°C/15 seconds, 40 cycles (for PDH amplification). Both programs were preceded by an initial incubation for 10 minutes at 95°C. Relative quantification of cDNA concentrations was done using the data analysis function of the Roche Molecular Biochemicals Light Cycler software (V.3.5). P21 expression levels were calculated in relation to the expression level of PDH as the reference gene. Primers used for quantification of P21 transcripts were P21LC-F (5'-GGAAAGCACGATGTTGACCG-3') and P21LC-R (5'-GGCCGTTTGATGTTGTTGAA-3'). Northern Blot Analysis. Fifteen micrograms of total RNA were vacuum dried, denatured by incubation at 65°C for 10 minutes after resuspension in loading dye, and electrophoresed in 1% agarose gels containing 6.5% formaldehyde in 1 x 3-(N-morpholino) propane sulfonic acid buffer. Ribonucleic acids were blotted to positively charged nylon membranes (Hybond N+, Amersham, Freiburg, Germany) by neutral capillary transfer in 10 x SSC (1.5 mol/L NaCl, 0.15 mol/L sodium citrate, pH 7.0) overnight followed by UV cross-linking. After prehybridization in Express Hyb hybridization solution (Clontech) for at least 1 hour at 68°C the membrane was probed with α 32P-labeled NeuT cDNA fragment (NeuT-F, 5'-TAGAAGACCGCAA-GACTCTCTCTTT-3'; NeuT-R, 5'-TCATACAGTTACATCCAGGCCTAGTA-3') overnight at 68°C. Washing of the membrane was done according to the Express Hyb hybridization protocol.

Immunoblotting. Cells were washed twice with PBS, directly lysed in 400 μl lysis buffer [10 mmol/L Tris (pH 7.5), 140 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 mmol/L DTT] Protein concentrations were measured with Bio-Rad electroporation cuvettes) in a volume of 700 μL serum-free DMEM and 100 μL PBS containing 10 μg DNA (9 μg pDSpBl-NeuT/EGFP, 1 μg pcDNA3Neo/ rtTA2). Cells were plated in DMEM/10% FCS and selection with G418 (1 mg/ ml) was started after 2 days. Single-cell clones were isolated and expanded 2 weeks after transfection. Senescence-Associated β-Galactosidase Staining. Visualization of senescence-associated β-galactosidase (SA-β-gal) for detection of senescent cells was done as previously described (32). Relative numbers of stained cells were determined in independent countings and analyzed statistically.

RNA Extraction and Semiquantitative Reverse Transcription–PCR. Isolation of total RNA, removal of contaminating genomic DNA, and cDNA synthesis were done essentially as previously described (33). PCR amplification was done on 1 μl of the resulting cDNA as follows: P21 (94°C/1 minute, 60°C/1 minute, 72°C/1.5 minutes; 21 cycles), P16 (94°C/1 minute, 60°C/1 minute, 72°C/1.5 minutes; 32 cycles), P27 (94°C/1 minute, 59°C/1 minute, 72°C/1.5 minutes; 26 cycles), pyruvate dehydrogenase (PDH; 94°C/1 minute, 58°C/1 minute, 72°C/1.5 minutes; 24 cycles). All programs additionally included an initial denaturation step (94°C/3 minutes) and a final elongation step (72°C/7 minutes). Forward (F) and reverse (R) primers used in this study were as follows: P21-F, 5'-GTCGTTACGAGGTGGTGAAG-3'; P21-R, 5'-GGGGTTTGATGTTGTTGAA-3'; P27-F, 5'-AAATGGTTTCAAGCGTTC-3'; P27-R, 5'-ACAGGATGTCCATCCTAGTA-3'; P21-F, 5'-GACTCTACCCCGACCGT-3'; P21-R, 5'-GCCATGTACTGCTCTGTTG-3'; PDH-F, 5'-GACCATTGAGACATGGAAACCA-3'; PDH-R, 5'-TGGACACGGTACAGACAAA-3'.
Cytoplasmic proteins) were collected in separate tubes and quantified for 

PMSF] and subsequently incubated on ice for 10 minutes. After short cen-

titer retroviral stocks generated by transient calcium phosphate transfection of 

BOSC23 ecotropic packaging cells (35) with pBabe-puro vectors (36) containing 

Cruz, sc-3738).

Primary antibody (either the P21-specific antibody alone or in combination 

HRAS-V12 oncogene (a kind gift of Dr. S. Lowe, Cold Spring Harbor 

Laboratories, Cold Spring Harbor, NY; ref. 21) or NeuT. Viral supernatants were 

used to infect early-passage MEFs. To infect at different multiplicities of 

infection (MOI), viral supernatants were titered using NIH3T3 cells as described 

(36). Twenty-four hours after infection, infected cells were selected for 4 days in 

the presence of 2 μg/mL puromycin and plated on the 5th day postinfection for 

the corresponding assays; the 6th day from the beginning of the infection was 

called day 0, as described in ref. 21.

Growth Curves. For growth curves, 10,000 cells per well were plated into 

24-well plates. At the indicated times, cells were washed with PBS, fixed in 10% 

(pH 7.2) formalin, and rinsed with distilled water. Cells were stained with 0.1% 

crystal violet (Sigma) for 30 minutes, rinsed, and dried. Cell-associated dye was 

extracted with 1 ml 10% acetic acid. Aliquots were diluted and the absorbance 

at 590 nm was determined. Values were normalized to the absorbance at day 0 

for each cell type. Each time point was determined in triplicate; each growth 

curve was done at least twice.

Statistical Analysis. To study the influence of ERBB2 signaling on proliferation, the data obtained for doxycycline-exposed cells were divided by the respective data of solvent controls. Data shown in the results section are mean values and SDs of at least three independent experiments. For analysis of SA-β-gal staining, data shown in the results section are mean values and SDs of at least three independent experiments. Differences between doxycycline-

exposed cells and solvent controls were analyzed using the t test for paired data (two-sided test); SPSS10.0 software was applied.

SD of quantitative, real-time, reverse transcription–PCR (RT-PCR) data were calculated using a formula for propagation of error: SD(tot) = \sqrt{cv(tot)^2 \times means(tot)^2 + means(tot)^2} = means(P21)/means(PDH) and cv(tot) = \sqrt{cv(P21)^2 + cv(PDH)^2}, cv(P21) and cv(PDH) represent the variation of respective 
duplicate samples (cv = SD/means).

Results

Regulated Expression of Oncogenic ERBB2 in MCF-7 Cells.

To investigate the immediate effects of oncogenic ERBB2 activation in the course of tumorigenesis we established inducible MCF-7-

derived cell lines (tet-on, utilizing the rtTA2-M2 molecule; ref. 37). Three lines [MCF-7/pTetNeuT3 (NeuT3), MCF-7/pTetNeuT15 

(NeuT15), and MCF-7/pTetNeuT48 (NeuT48)] displayed stringent regulation of EGFP and NeuT by doxycycline administration, as 

assessed by fluorescence microscopy (data not shown) and Northern blot analysis using a NeuT-specific probe (Fig. 1A). To 

verify functional ERBB2 signal transduction in these cell lines, we 

confirmed NeuT-regulated expression of VEGF and MMP-2 as 

known transcriptional target genes of ERBB2 signaling (data not 

shown). Kinetic analysis of line NeuT48 revealed a strong induction of 

NeuT protein 8 to 10 hours after addition of doxycycline to the 

culture medium (Fig. 1B). A control cell line [MCF-7/pTetEGFP10 

(EGFP10)] harboring the empty pB-EGFP vector and the rtTA2 

expression plasmid displayed doxycycline-dependent EGFP expres-

sion but no induction of NeuT (data not shown and Fig. 1C).

Neut Expression Induces Growth Arrest in MCF-7 Cells. To 

study the effect of oncogenic ERBB2 signaling on proliferation of 

MCF-7 cells, NeuT-inducible cell lines were incubated with 

doxycycline for 48 hours. Cellular proliferation was quantified by 

FACS analysis and [3H]thymidine incorporation assay (data not shown). Fluorimetric determination of cellular DNA content 

revealed a dramatic decrease of cells in S phase following transgene induction: S-phase levels in NeuT-positive cell lines 2 

days after the addition of doxycycline to the culture medium were 

reduced to 51%, 52%, and 37%, respectively (Fig. 2A). In addition, 

we did bromodeoxyuridine-incorporation assays of noninduced
and induced NeuT48, as well as EGFP10 control cells. Induction for 8 days led to an accumulation of cells in the G2 phase of the cell cycle (Fig. 2B). The prolonged incubation resulted in a further decrease of actively proliferating cells with only ~5% of the cells still synthesizing DNA. This cell cycle arrest was a specific response to oncogenic ERBB2 signaling because doxycycline treatment of control cells did not affect proliferation (Fig. 2A and B).

**Nuclear P21 Accumulates upon Oncogenic ERBB2 Signaling.**

Cell cycle arrest is mediated by up-regulation of CDK1 expression. The CDKIs P16, P21, and P27 are key regulators of the eukaryotic cell cycle acting at defined restriction points to ensure proper growth, replication, and division. We therefore analyzed expression levels of P16, P21, and P27 in NeuT48 cells after doxycycline administration. RT-PCR revealed a strong increase of P21 RNA levels in doxycycline-treated NeuT48 cells, whereas no differential expression could be observed for P16 and P27 (data not shown). Western blot analysis of treated and untreated cells confirmed the increase of P21 protein in the MCF-7/pTetNeuT48 cells (Fig. 3A), whereas P53 as a potent transcriptional inducer of P21 was constantly expressed (data not shown). Because Zhou and coworkers recently showed that ERBB2 signaling (via AKT) redistributes P21 into the cytoplasm of NIH3T3 cells, thereby converting tumor-suppressive nuclear P21 into an antiapoptotic molecule (38), we looked for the subcellular localization of P21 in MCF-7/pTetNeuT48 cells. As shown by Western blotting of subcellular protein fractions (Fig. 3B) and by indirect immunofluorescence staining (Fig. 3C), up-regulation of P21 protein in response to oncogenic ERBB2 signaling is predominantly restricted to the nucleus.

**Prolonged Oncogenic ERBB2 Signaling Induces Premature Senescence in MCF-7 Cells.**

The CDKI P21 is a key mediator of the cellular premature senescence program characterized by cell cycle arrest, enlarged cell volume, and up-regulation of additional markers, like the SA-β-gal enzymatic activity (active at pH 6.0; ref. 32). We observed all of these features of premature senescence in independent NeuT cell lines following prolonged NeuT induction. In addition to P21 up-regulation and cell cycle arrest, all cell lines exhibited prominent phenotypic alterations in response to NeuT overexpression. Starting 2 days after induction of oncogenic ERBB2 signaling, cells developed cytoplasmic protrusions. Following this stage, cellular appearance changed to an enlarged, flat morphology with multiple vacuoles (Fig. 4A). SA-β-gal activity was assessed in NeuT48 cells after 7 days of doxycycline treatment. The percentage of SA-β-gal-positive cells was low (3.4%) in untreated NeuT48 cells, comparable to untreated (2.7%) and treated (4.0%) control EGFP10 cells. In contrast, more than half of the treated NeuT48 cells (51%) stained SA-β-gal positive (Fig. 4B), thereby giving further support to the assumption that the proliferative block induced by NeuT is part of the premature senescence program initiated in response to prolonged oncogenic ERBB2 signaling in MCF-7 cells.

**P38 Mitogen-Activated Protein Kinase Mediates the Induction of Premature Senescence.**

The ERBB2 protein connects to several different intracellular signal transduction pathways including phosphatidylinositol 3-kinase (PI3K)/AKT and the mitogen-activated protein kinase (MAPK) family influencing cellular physiology. Up-regulation of P21 as part of the premature senescence program in direct response to NeuT expression represents a novel and important finding. Premature senescence due to ectopic mitogenic signals is known to be mediated by members of the P38 MAPK family (22). To identify the signal transduction cascade(s) responsible for P21 induction and subsequent premature senescence in response to NeuT signaling we used small molecule protein kinase inhibitors designed to selectively block signaling constituents of protein kinase cascades. In quantitative real-time RT-PCR experiments, we were able to assign the NeuT-induced P21 up-regulation to the activation of the P38 MAPK cascade (Fig. 5A). Phosphospecific Western blots of induced cells consistently showed that the P38 MAPK pathway was engaged in active signaling upon NeuT induction (Fig. 5B). The importance of this cascade in regulation of P21 was reflected by corresponding phenotypic changes: Blocking P38 MAPK activation by inhibitor SB203580 selectively suppressed the senescent phenotype (Fig. 5C), whereas inhibition of ERK1/2, c-jun-NH2 kinase (JNK), and PI3K failed to do so and induced cells underwent the phenotypic conversion to premature senescence (as reflected by the occurrence of the cytoplasmic protrusions after 2 days of oncogenic ERBB2 signaling; data not shown). Our data show that active engagement of the P38 MAPK signaling module is necessary to initiate the cellular senescence program as a result of oncogenic ERBB2 activation in the MCF-7/pTet-NeuT cell lines and further supports the pivotal role of the P21 CDKI molecule in this process.

**Neut-Induced Cell Cycle Arrest Is Reversed by P21 Antisense Oligonucleotides.**

As shown above, overexpression of oncogenic...
ERBB2 strongly up-regulates P21 expression in a P38-dependent manner. To determine whether P21 is solely responsible for the NeuT-induced cell cycle arrest, we selectively inhibited P21 protein expression in our cells. NeuT48 cells were transiently transfected with P21 antisense or P21 sense oligonucleotides tagged by a fluorescent dye label to determine transfection efficiency. Fluorescence microscopy indicated high transfection efficiency (data not shown). NeuT was induced 7 hours post transfection by the addition of doxycycline-containing medium and maintained for 48 hours. As shown in Fig. 6A, Western blot analysis of P21 expression showed the efficiency and specificity of antisense treatment: A marked reduction of P21 in NeuT48 cells was observed only after P21 antisense treatment, whereas the strong increase in P21 protein levels remained unaffected in P21 sense and mock-transfected NeuT48 cells.

Oligonucleotide-transfected cells were analyzed by FACS to determine whether blocking P21 up-regulation could relieve the NeuT-induced cell cycle arrest. As shown in Fig. 6B, cell cycle arrest remained unaffected in mock-transfected NeuT48 cells (P21S, LIP). In contrast, the NeuT48 cells resumed active cycling when P21 up-regulation was blocked by antisense oligonucleotide treatment (Fig. 6B; P21AS). CDK1 P21 is thus identified as a key effector mediating cell cycle arrest and premature senescence in MCF-7 cells after oncogenic ERBB2 activation.

Oncogenic ERBB2 Signaling Also Induces Growth Arrest and Premature Senescence in Low-Passage Murine Embryonic Fibroblasts. To examine whether the antiproliferative response to oncogenic ERBB2 signaling can also be observed in nontransformed cells we expressed NeuT (as well as mutant HRAS as a positive control; data not shown and ref. 21) in low-passage MEFs. Retroviral transduction of NeuT resulted in an antiproliferative response of early-passage MEFs (Fig. 7A). Interestingly, the extent of this response depended on the expression level of the oncogene: MEFs infected with high titer retrovirus (MOI >10; therefore expressing high levels of NeuT) showed a stronger antiproliferative response compared with MEFs with lower NeuT expression (MOI = 1, Fig. 7A). In analogy to the results obtained for the MCF-7/pTet-NeuT cells, this proliferative block was accompanied by a marked increase of SA-β-gal positive cells in NeuT-expressing cultures (Fig. 7B). In addition, a NeuT-dependent up-regulation of P21 was observed in NeuT-transduced MEFs: Vector controls expressed much lower levels of P21 compared with the NeuT-transduced
fibroblasts (Fig. 7C). Taken together, these experiments suggest that activation of the premature senescence program accompanied by up-regulation of the P21 CDKI is not a cell type–specific phenomenon but represents rather a more general cellular response to oncogenic ERBB2 signaling.

Discussion

The receptor tyrosine kinase ERBB2 plays a central role in the development of several cancer types. Mitogenic signaling via the RAS-RAF-MEK-ERK cascade is believed to be the major pathway required for malignant transformation and tumor progression during ERBB2-driven tumorigenesis. The present study shows for the first time that regulated overexpression of oncogenic ERBB2 initially does not stimulate proliferation but induces cell cycle arrest and premature senescence in MCF-7 breast carcinoma cells. Similar effects were observed in low-passage murine embryonic fibroblasts. This antiproliferative effect is accompanied by up-regulation of the CDK inhibitor P21. As to be expected, the P21 protein localizes to the nucleus of arrested cells. In our system, induction of P21 by ERBB2 signaling is not dependent on P53 accumulation but is mediated by the P38 MAPKs. Using antisense oligonucleotides designed to specifically block P21 protein synthesis, we identify the P21 molecule as the key mediator of cell cycle arrest and premature senescence caused by oncogenic ERBB2 activation in breast carcinoma cells.

At first glance it may seem paradoxical that a strong mitogenic signal causes growth arrest rather than enhanced proliferation in tumor cells. However, based on several studies on ectopic oncogene

![Figure 3](https://example.com/figure3.png)

**Figure 3.** P21 is induced upon oncogenic ERBB2 signaling. A, protein extracts from untreated and doxycycline-treated NeuT-inducible cells (NeuT48) as well as control cells (EGFP10) were analyzed by Western blotting. Top, incubation of the blot with a P21-specific antibody. Actin-specific immunostaining is shown below as loading control. B, NeuT48 and EGFP10 control cells were grown for 48 hours in DMEM in the presence (+) or absence (−) of doxycycline. Fifty micrograms of cytoplasmic (C) and nuclear (N) protein extracts were subjected to SDS-PAGE and compartment-specific P21 levels were determined by immunoblotting. C, untreated (top) and doxycycline-treated (bottom) NeuT48 cells were probed with a P21-specific primary and a Cy5-coupled secondary antibody to determine cellular distribution of the CDKI (P21). The P21-specific antibody was omitted from control samples (neg) to assess unspecific binding of the secondary antibody. Cells were embedded in DAPI antifade after staining to visualize the nuclei. Photographs show Cy5 or DAPI excitation as indicated on the left.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** MCF-7 cells undergo premature senescence in response to prolonged oncogenic ERBB2 signaling. A, NeuT48 cells were cultured in the absence or presence of doxycycline for 7 days and stained for SA-β-gal (blue cytoplasmic stain). Note the enlarged, flat morphology of NeuT48 cells after 7 days of oncogenic ERBB2 signaling. B, relative amounts of senescent EGFP10 and NeuT48 cells in untreated (−dox) and 7 days doxycycline-treated cultures (+dox) was calculated after counting the number of SA-β-gal-positive cells (●). *, P < 0.05, t test for paired data comparing the respective cell lines with and without doxycycline treatment (two-sided test).
expression in primary cells, we classify the growth arrest in response to ERBB2 signaling as part of the premature senescence program. Premature senescence is suggested to be a basic cellular fail-safe mechanism designed to arrest cells at risk for tumorigenesis, (e.g., due to inappropriate oncogene activation or cytostatic stress; reviewed in refs. 15–18). Our data identifies premature senescence as a primary response to hypermitogenic ERBB2 signaling in breast carcinoma cells. In support of these findings, other members of the RAS-RAF-MEK-ERK cascade have been described to elicit growth arrest and premature senescence in several cellular settings (21, 23, 26, 27). Overexpression of activated RAS or RAF provokes changes analogous to those observed in our cell lines, including growth arrest, morphologic alterations, like flattening of the cells, increased SA-β-gal activity, as well as upregulation of negative cell cycle regulators. Consequently, all known inhibitors of CDKs provoke premature senescence when ectopically expressed in low-passage fibroblasts (39).

Our results provide evidence that premature senescence, defined by the criteria listed above, may be a general mechanism, as it is triggered by hypermitogenic ERBB2 signaling in nontransformed and tumor cells. Based on the assumption that MCF-7 cells are a legitimate model system to study the effects of ERBB2-mediated

Figure 5. Implication of P38 MAPK in premature senescence induced by oncogenic ERBB2 signaling. A, quantitative real-time RT-PCR analysis of P21 induction after treatment of NeuT48 cells with specific kinase inhibitors in presence (●) or absence (□) of doxycycline. Inhibitors of ERK1/2 (PD), P38 (SB), c-jun-NH2-kinase (SP) and PI3K (Wort) or DMSO (vehicle control) were added to the cells prior to doxycycline treatment. Relative P21 transcript levels in the absence of doxycycline were taken as reference and induction was calculated by comparing the corresponding P21 transcript levels after doxycycline stimulation. Results of a representative experiment. Bars, SD of mean calculated taking into account propagation of error as described in Materials and Methods. B, immunoblot analysis of expression levels and activation (i.e., phosphorylation; indicated by “p”) status of P38 MAPK in response to oncogenic ERBB2 signaling. Whole cellular protein extracts of NeuT48 cells grown in DMEM/10% FCS with or without doxycycline for the indicated period of time were analyzed (48-0, cells grown for 48 hours in the absence of doxycycline). Actin-specific staining is shown to demonstrate equal loading. C, phenotypic changes associated with oncogenic ERBB2 signaling preceding premature senescence are selectively suppressed by specific inhibition of P38. Cells were grown for 48 hours in the absence (− dox) or presence of doxycycline (+ dox). Where indicated, DMSO (solvent control) or an inhibitor of P38 (+ SB) was added before the induction of oncogenic ERBB2 signaling.

Figure 6. Suppression of P21 prevents cell cycle arrest induced by oncogenic ERBB2 signaling. A, Western blot analysis of P21 regulation in differentially treated NeuT48 cells. Cells were transfected with P21 antisense (P21AS), P21 sense (P21S) or LipofectAMINE (LIP) alone and oncogenic ERBB2 signaling was induced by subsequent doxycycline treatment for 48 hours in the indicated samples. Whole cellular protein extracts were subjected to SDS-PAGE and analyzed for P21 protein expression level. Actin staining of the immunoblot is shown as loading control. B, proliferative response of P21-antisense (P21AS), P21-sense (P21S) and mock (LIP)-transfected NeuT48 cells. The relative number of cells in S phase are represented by each column. The number of cells in S phase when cultured in the absence of doxycycline (□) were taken as reference and the values for corresponding doxycycline-treated cells (●) were normalized accordingly. *P < 0.05, t test for paired data comparing the respective cell lines with and without doxycycline treatment (two-sided test).
transformation (other mechanisms have apparently led to the malignant transformation of these cells that have retained some degree of epithelial differentiation) we believe that our findings may have important implications for the understanding of ERBB2-driven tumorigenesis. However, a detailed investigation of the early steps in oncogene-dependent carcinogenesis in vivo will have to prove the relevance of the concept of premature senescence as a cellular safeguard against malignant transformation. Apart from this issue, another important inference from our data is the apparent preservation of such an antiproliferative program in carcinoma cells. Targeted therapies for breast cancer will have to deal with the fact that a variety of stimuli, including the activation of oncogenes, may trigger premature senescence instead of cell death. The significance of the senescence program in clinical breast cancer has recently been analyzed by SA-β-gal staining (40). Chemotherapy-induced senescence seems to be a specific response of tumor cells in more than 40% of patients. In addition, spontaneous senescence was detected in a small number of tumors. SA-β-gal expression was associated with low P53 staining, suggesting the lack of mutant P53, whereas staining for the CDK P16 was increased in SA-β-gal positive breast carcinoma cells.

The data presented pinpoint the CDK inhibitor P21 as the key mediator of ERBB2-driven premature senescence in breast carcinoma cells. Associated with both transient and permanent forms of growth arrest, P21 induction is a well-known phenomenon in the course of premature senescence in mammalian cells. Physiologically, P21-mediated transient growth arrest may prevent damaged cells to enter into mitosis to avoid the consequential mitotic catastrophe. But P21 induction also occurs at the onset of permanent growth arrest in primary and tumor cells, provoked by DNA damage or the introduction of oncogenic RAS (reviewed in refs. 16–18). The P53 tumor suppressor protein is an important regulator of P21 (41, 42). However, we do not observe any increase of P53 protein in the course of premature senescence (data not shown), suggesting that P21 induction observed in response to hypermitogenic ERBB2 signaling is mediated by P53-independent mechanisms, as described for other cellular settings (43, 44). Instead, P21 induction and associated

Figure 7. Oncogenic ERBB2 signaling causes growth arrest, premature senescence and P21 upregulation in murine embryonic fibroblasts. A, MEFs were transduced with recombinant retroviruses carrying either the NeuT cDNA (NeuT) or no insert (Vector) and the influence of NeuT signaling on MEF proliferation was analyzed. The proliferative response of MEFs infected with different titers of retrovirus (MOI >10, MOI = 1) is depicted. Relative cell numbers at indicated time points were determined in triplicate. Bars, SD. The immunoblot inserted (right) shows the expression level of NeuT in MEFs at day 6, as well as actin as loading control. Kinetics and strength of the antiproliferative effect provoked by NeuT were similar to the effects of oncogenic HRAS-V12 (data not shown). B, SA-β-gal staining at day 6 (MOI >10) shows an increase in number of senescent cells (●) in the population of NeuT-expressing MEFs when compared with mock-infected cells. Relative numbers of stained cells were determined in independent countings. Bars, SD. Similar results were obtained with MEFs infected at MOI = 1. C, Coimmunofluorescence staining of NeuT-transduced (bottom) and mock-infected (top) MEFs (MOI >10) with P21- and ERBB2-specific antibodies at day 6. A strong increase of P21 expression was observed as a consequence of NeuT-expression. The merged photographs of FITC (NeuT-specific), Cy5 (P21-specific), and DAPI (nuclei) emissions clearly show the presence of nuclear as well as cytoplasmic P21 signals specifically in NeuT-expressing cells (similar results were obtained with MEFs infected at MOI = 1).
phenotypic alterations are reversed by specific inhibition of the P38 subfamily of MAPKs. These results are in line with recent observations characterizing the P38 signal transduction cascade as the common senescence pathway (45).

The P38 MAPK pathway mediates responses to environmental stress, including DNA-damaging agents such as UV and γ-irradiation (46). P38 also mediates premature senescence elicited by hypermitogenic RAS (27). The signaling involves MEK, which activates the ERK1/2 MAPKs and subsequently stimulates the activity of P38-activating kinases MKK3 and MKK6. Constitutive activation of P38 by active MKK3 or MKK6 is sufficient to induce premature senescence. In addition, RAS fails to provoke senescence when P38 activity is inhibited, supporting an essential role of P38 in the induction of the senescence response. In analogy to the work of Wang and coworkers (27), we show that inhibition of the P38 MAPK pathway can completely abolish the premature senescence response. Identifying P21 as the major effector of associated cell cycle effects, we thus implicate hypermitogenic ERBB2 signaling in the stimulation of an MKK3/6-P38-P21 signaling sequence leading to growth arrest and premature senescence.

Up-regulation of P21 has been previously linked to ERBB2 signaling (38, 47). Recent clinical data corroborates the importance of these findings, showing a strong positive correlation of ERBB2 and P21 staining in a larger series of patients with breast cancer (48). In contrast to our results, however, P21 was predominantly located in the cytoplasmic compartment of tumor cells, both in vitro and in vivo, and only cytoplasmic staining of P21 proved to be an independent predictor of poor outcome in clinical breast cancer. By analyzing cell lines constitutively overexpressing ERBB2, Zhou and coworkers have clearly shown that activation of AKT kinase may be responsible for mislocalization of P21 protein to the cytoplasm and disruption of its potent growth-inhibiting activity (38). Tetracycline-controlled ERBB2 signaling in our cell lines, on the other hand, causes growth arrest by inducing P21 protein that accumulates in the nucleus. Taking both observations together, it is tempting to speculate that subcellular redistribution of P21 by AKT may thus be an essential secondary step to escape premature senescence in the course of ERBB2-driven tumorigenesis. In keeping with this hypothesis are clinical data correlating overexpression of ERBB2 and activation of AKT in breast cancer (49). Constitutive ERBB2 overexpression in vitro inevitably selects for additional changes to escape the primary fail-safe mechanism and may thus explain why the induction of premature senescence has not been recognized yet.

In essence, we propose a multistep model for the process of malignant transformation by ERBB2 wherein initial P21 induction provokes premature senescence as an inherent anticarcinogenic program. Cooperative secondary lesions then either inactivate P21 or target downstream effectors of senescence. In view of the strong positive correlation of ERBB2 and P21 expression in clinical breast cancer, further dissection of the molecular pathways leading to P21 induction in the course of ERBB2-induced premature senescence is indicated. One prediction of our hypothesis is the emergence of actively proliferating cells after long-term ERBB2 induction. The identification of putative secondary lesions enabling tumor cells to escape premature senescence will help to disclose the relevant steps resulting in uncontrolled proliferation and tumorigenesis. Our results imply that in addition to existing molecular therapies directly tackling the ERBB2 molecule (50), restoration of the premature senescence response in ERBB2-overexpressing tumors by specifically targeting secondary lesions [as proposed in a recent review (51) describing the potential of P21-targeting therapies], like the cytoplasmic mislocalization of the P21 protein by AKT, may offer additional therapeutic options in which direct inhibition of ERBB2 fails.

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