



AP-1 blockade inhibits the growth of normal and malignant breast cells

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We have previously demonstrated that basal AP-1 transcriptional activity is high in normal human mammary epithelial cells, intermediate in immortal breast cells, and relatively low in breast cancer cells. In this study we investigated whether differences in AP-1 transcriptional activity reflect differences in breast cells' dependence on AP-1 for proliferation. The cJun dominant negative, TAM-67, was used to determine the effect of AP-1 blockade on the growth of normal, immortal and malignant breast cells. We first showed that TAM-67 inhibits AP-1 activity in normal and malignant breast cells. We then determined whether this AP-1 inhibitor affected colony forming efficiency of the immortalized and malignant breast cells. The AP-1 inhibitor reduced colony formation of immortal breast cells by over 50% (by 58% in 184B5 cells and 62% in MCF10A cells), and reduced colony formation in the breast cancer cell line MCF7 by 43%, but did not reduce colony formation in the other breast cancer cell lines (T47D, MDA MB231 and MDA MB 435). We also determined the effect of AP-1 blockade on the growth of normal breast cells using a single cell proliferation assay. Using this assay, the growth of normal breast cells was extremely sensitive to AP-1 blockade, while immortal breast cells were moderately sensitive. We next directly tested the effect of TAM-67 expression on the growth of MCF7 breast cancer cells, using cells stably transfected with TAM-67 under the control of a doxycycline-inducible promoter. Upon induction, TAM-67 was expressed and AP-1 activity was inhibited in these cells. We then measured the growth of these cells in the presence or absence of TAM-67. The results of these studies show that the growth of MCF7 cells was suppressed by the AP-1 inhibitor, TAM-67. These results demonstrate that normal and immortalized breast cells, and some breast cancer cells (such as MCF7), require AP-1 to transduce proliferative signals, while other breast cancer cells (such as T47D, MDA MB 231 and MDA MB 435) do not. These studies suggest that the AP-1 transcription factor is a potential target for future

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Introduction

Breast cancer is one of the most common malignancies in women, and the leading cause of death for women between the ages of 40 and 55 in the United States (Landis *et al.*, 1999). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor 'initiation' and 'promotion' events (Bishop, 1987). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer 'initiation' events (Malkin *et al.*, 1990; Tripathy and Benz, 1993). However, the molecular mechanism of breast tumor 'promotion' is poorly defined. In model systems (Berenblum and Shubik, 1947), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis (Harris *et al.*, 1993).

Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGF- α , and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs that inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) and drugs that block growth factor receptors (such as antibodies specific for the Her2/neu receptor) are now being used to

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treat or prevent breast cancer. However, inhibition of individual signal transduction pathways may be only partially effective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, inhibitors of these transcription factors may be more potent inhibitors of breast cell growth.

A key family of transcription factors transducing multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (Baselga and Mendelsohn, 1994). Thus, AP-1 is a central component of many signal transduction pathways in many different cell types.

We have previously shown that normal breast cells have high basal levels of AP-1, while breast cancer cells have low basal levels (Chen *et al.*, 1996). We hypothesized that the high basal level of AP-1 present in normal breast cells is required to support the growth of normal cells and that these cells would be more dependent on AP-1 for their growth than breast cancer cells. To investigate the role of AP-1 in controlling breast cell growth we have used normal breast cells, immortal breast cells, and breast cancer cells. We determined the effect of AP-1 blockade on the growth of these different breast cells using the cJun dominant-negative mutant, TAM-67. These studies demonstrate that the growth of normal and immortal human mammary epithelial cells, which have the highest basal AP-1 transcriptional activity, is suppressed by AP-1 blockade. Therefore, normal and immortal breast cells require AP-1 for their growth. Breast cancer cells have lower basal AP-1 transcriptional activity are less sensitive to AP-1 blockade. Of the breast cancer cells tested, MCF7 cells were the most sensitive to the growth suppressive effects of the AP-1 inhibitor. The other breast cancer cells tested, T47D, MDA MB 435, and MDA MB 231, were resistant to the growth suppressive effects of TAM-67. These results demonstrate that the growth of normal breast cells and some breast cancer cells is inhibited by AP-1 blockade, and suggest that AP-1 is a promising target for agents for the prevention or treatment of breast cancer.

Results

Expression of the cJun dominant-negative mutant, TAM-67, in breast cells

To determine whether AP-1 transcription factor complexes are involved in controlling breast cell growth we have investigated the effect of inhibiting AP-1 activity on breast cell proliferation. To block AP-1 activity we used the cJun dominant-negative mutant, TAM-67. TAM-67 is a mutant form of cJun in which the transactivation domain has been deleted leaving the DNA binding and the leucine zipper domains intact (Figure 1a). We and others have used TAM-67 to investigate the role of AP-1 in cell transformation, cell differentiation, and apoptosis in many different cell types (Brown *et al.*, 1993; Chen *et al.*, 1996; Domann *et al.*, 1994; Dong *et al.*, 1994; Ham *et al.*, 1995; Petrak *et al.*, 1994).

Expression of the TAM-67 protein in different breast cells was determined by immunoblot analysis of whole cell extracts of breast cells co-transfected with 0.5 μ g of the pCMV-TAM-67 expression vector. TAM-67 protein was detected using antibodies directed against the DNA binding domain of the human cJun protein (Figure 1b). Similar levels of TAM-67 protein were observed in all cell lines, with MDA MB 435 cells showing the lowest level of expression. We then

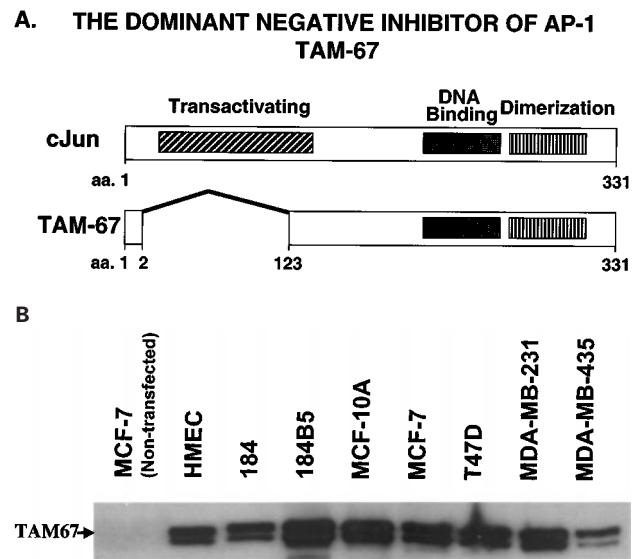


Figure 1 TAM-67, a dominant negative mutant of cJun. (a) Schematic representations of the protein functional domains in cJun and the cJun mutant, TAM-67. Three functional domains of cJun are shown: the transactivation domain, the DNA binding domain, and the dimerization domain. TAM-67 has most of the transactivation domain deleted. (b) Transient protein expression of TAM-67 in different breast cells. TAM-67 protein expression was determined 36 h after transfection of the different cell lines with 2.5 μ g of the TAM-67 expression vector, pCMV-TAM-67. TAM-67 is distinguished from endogenous cJun, because TAM-67 protein is a cJun deletion mutant, which migrates faster than endogenous cJun in the gel. TAM-67 protein was measured in whole cell extracts using Western blot analysis as described in Materials and methods

measured the ability of the TAM-67 protein to inhibit AP-1 activity in these breast cells as shown in Figure 2.

TAM-67 inhibits AP-1 activity in normal and malignant breast cells

To demonstrate that TAM-67 inhibits AP-1 activity in breast cells, we co-transfected the luciferase reporter construct, Col-Z-Luc, with increasing amounts of the TAM-67 expression plasmid, pCMV-TAM-67. The results of these experiments are shown in Figure 2a,b. We observed that the high basal AP-1 activity present in normal and immortal breast epithelial cell is significantly inhibited by TAM-67 expression (Figure 2a). Because basal AP-1 activity is relatively low in breast cancer cells (Chen *et al.*, 1996), we measured the effect of TAM-67 expression on TPA-induced AP-1

transcriptional activity in the breast cancer cells (Figure 2b). Increasing amounts of pCMV-TAM-67 resulted in inhibition of TPA-induced AP-1 activity in all breast cancer cell lines examined. In addition, we have previously observed that TAM-67 inhibits serum-stimulated AP-1 activity in breast cancer cells (Chen *et al.*, 1996). These results demonstrate that TAM-67 inhibits AP-1 transcriptional activating activity in all of the different breast cells.

TAM-67 inhibits colony formation of immortal breast cells and some breast cancer cells

We next investigated whether inhibition of AP-1 transcriptional activity affects breast cell proliferation using a colony forming assay. This assay has been extensively used to demonstrate the effects of tumor suppressors and oncogenes on cell growth. As described in Materials and methods, breast cells were co-transfected with pZeoSV and either pCMV vector or pCMV-TAM-67. The pZeoSV plasmid contains a zeocin resistance gene allowing selection of transfected cells. We have measured the effect of TAM-67 on colony formation in immortal and malignant breast cells. Normal mammary epithelial cells could not be analysed because these cells undergo a finite number of doublings and did not form zeocin-resistant colonies. The colony forming results for all cell lines tested are shown in Figure 3. Colony formation of immortal breast cells was reduced when these cells were co-transfected with pCMV-TAM-67 compared to cells co-transfected with pCMV (Figure 3a). The number of colonies was reduced by 62% and by 58% for MCF10A and 184B5, respectively. Colony formation of the MCF7 breast cancer cell line was also reduced (by 43%; see Figure 3b). TAM-67 did not significantly reduce the colony formation in the other breast cancer cell lines. Colony formation of T47D breast cancer cells was not reduced and in fact was significantly increased. These results suggest that TAM-67 is stimulating growth in these cells, either by interacting with a negative growth regulator in T47D cells, or alternatively by activating transcription through interactions with co-activators present in T47D cells.

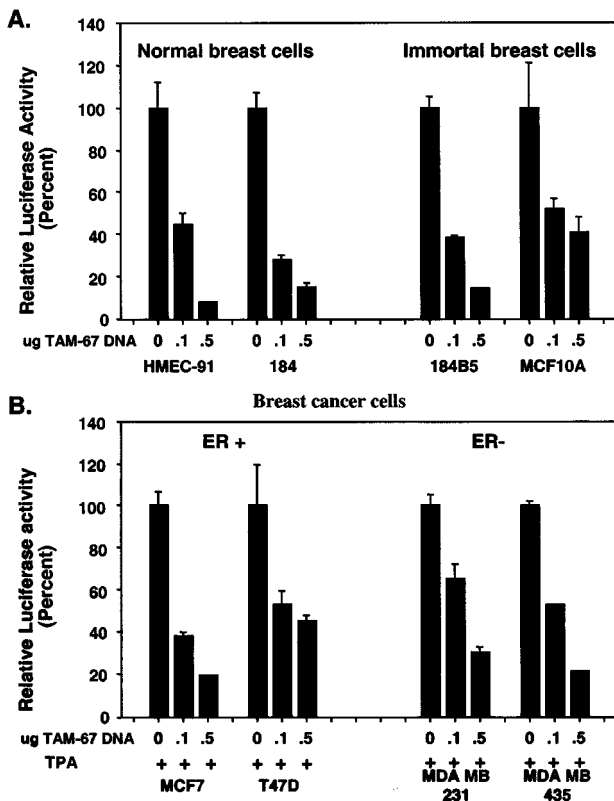


Figure 2 TAM-67 inhibition of Col-Z-Luc activity in all the breast cell lines. (a) TAM-67 inhibition of basal Col-Z-Luc promoter activity in normal and immortal human breast cells. Cells were transfected with 1 μ g of Col-Z-Luc reporter plasmid plus increasing amounts (0, 0.1 or 0.5 μ g) of the expression plasmid pCMV-TAM-67. pCMV (empty vector) was included (0.5, 0.1 or 0 μ g) to maintain equal amounts of DNA in each transfection. Transfection procedures were performed as described in Materials and methods. Cells were lysed and luciferase activity was determined 36 h after transfection. (b) TAM-67 inhibition of TPA induced Col-Z-Luc promoter activity in breast cancer cells. Cells were transfected with 1 μ g of Col-Z-Luc reporter plasmid plus increasing amounts pCMV-TAM-67 as described in (a). Induction of AP-1 activity was done 36 h post-transfection by treating the cells with TPA (0.1 nM) for 4–6 h. AP-1 activity was measured as described in Materials and methods

TAM-67 inhibits normal human mammary epithelial cell growth

To investigate the effect of AP-1 blockade on the growth of normal human mammary epithelial cells we used a second assay, the single cell proliferation assay (SCPA) previously described by Timchenko *et al.* (1996). We used this assay to analyse normal and immortal breast cell growth in the presence of TAM-67. The cells were co-transfected with 5 μ g of the expression vector pCMV (empty vector) or pCMV-TAM-67 plus 0.5 μ g of pCMV- β -gal. After allowing recovery from the transfection the cells were plated at low cell densities and cultured to allow single cells to grow into small colonies ranging from 1–20 cells. The

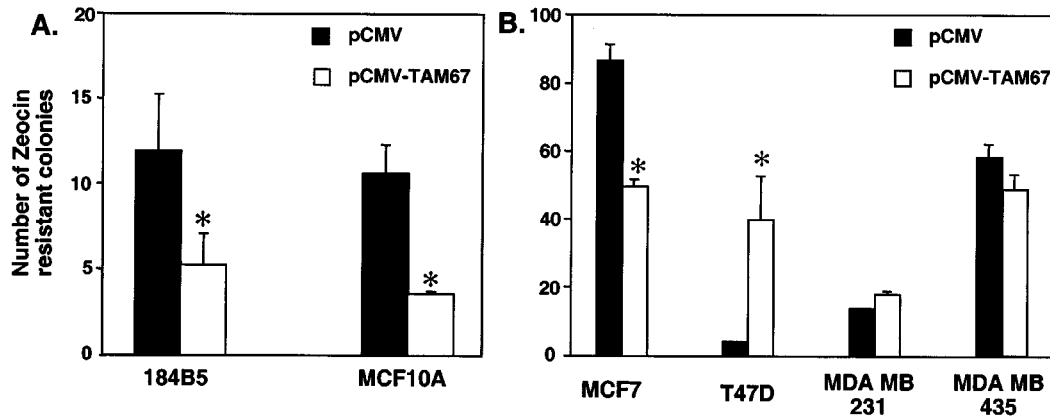


Figure 3 Colony forming efficiency of TAM-67 transfected breast cells. Colony formation of immortalized (a) and malignant (b) breast cells. Immortalized (a) and malignant (b) breast cells were transfected with pSVZeo (0.5 μ g) and either pCMV (5 μ g) or pCMV-TAM67 (5 μ g). Transfections were performed as described in Materials and methods. After 2 weeks of selection Zeocin resistant colonies were stained with crystal violet and counted. The data shows the average number of Zeocin resistant colonies from experiments done in triplicate with error bars representing the s.e.m. The names of the different cell lines analysed are given on the x-axis. **P*-value < 0.05

cells were then fixed and stained *in situ* for β -galactosidase activity and transfected cells were identified as blue cells by light microscopy. The number of transfected cells observed per colony were scored and presented as a histogram of the percentage of colonies having 1, 2, 3, or more transfected cells per colony. An example of a blue colony obtained by transfection of breast cells with pCMV- β -gal is shown in Figure 4a.

Results from these experiments showing the number of cells present in blue colonies in normal and immortal cells transfected with either pCMV or pCMV-TAM-67 is shown in Figure 4b. The upper panels show the histogram obtained when pCMV is transfected into the cells, while the lower panels show the histogram obtained when pCMV-TAM-67 is transfected into the cells. Note that TAM-67 reduces the number of blue cells per colony in both normal cells (HMEC and 184 cells) and immortal breast cells (184B5 and MCF10A). These results demonstrate that the growth of normal and immortal human mammary epithelial cells is inhibited by expression of TAM-67. Also note that the mean number of blue cells per colony is reduced more in normal breast cells (HMEC and 184 cells) than in the immortalized cells (184B5 and MCF10A) (Figure 4b). This result suggests that normal human mammary epithelial cells are more sensitive to the AP-1 inhibitor than are the immortalized breast cells.

Isolation of breast cancer clones expressing TAM-67 under the control of an inducible promoter

To directly investigate whether AP-1 blockade inhibits the growth of breast cancer cells, we created MCF7 cell lines that express TAM-67 under the control of an inducible promoter. The Tet-off system was used for creation of inducible MCF7-TAM-67 and MCF7-

vector cell lines. MCF7 tTA cells were transfected with either the empty vector, or with the expression plasmid containing the flag-tagged TAM67 cDNA inserted downstream of a tetracycline-responsive transcriptional promoter as described in Figure 5a. This plasmid was cotransfected with a plasmid carrying the hygromycin resistance gene, allowing for selection of transfected cells. Hygromycin-resistant colonies were then selected under conditions that repress expression of the TAM-67 cDNA. The cells were then screened for inducible TAM-67 protein expression by immunoblotting with anti-cJun antibodies. We used two MCF7 Tet-off TAM-67 clones for further study. Figure 5b shows the inducible expression of these two clones (MCF-7 #62 and MCF7 #67) found to express TAM-67. These clones do not express TAM-67 in the presence of doxycycline, but do express high levels of TAM-67 protein when doxycycline is removed from the media (Figure 5b).

The functional activity of TAM-67 in these MCF7 clones was determined by analysing inhibition of basal and TPA-induced AP-1 transactivating activity (Figure 5c,d). Cells were transfected with the AP-1 reporter plasmid, and then split 1:2. Half the cells were cultured in medium containing doxycycline while the other half of the cells were grown in medium without doxycycline. After induction, the cells were harvested for measuring basal AP-1 activity, or were treated with TPA for 4–6 h and then harvested to measure TPA-induced AP-1 activity. Figure 5c shows the results of transcriptional activation assays of basal AP-1 activity in the presence of doxycycline (TAM-67 not expressed), or in the absence of doxycycline (TAM-67 expressed). Removal of doxycycline did not affect basal AP-1 activity in vector-transfected clones (Clones #1 and #7), but did significantly reduce basal AP-1 activity in both the TAM-67 expressing clones (Clones #62 and #67) (Figure 5c).

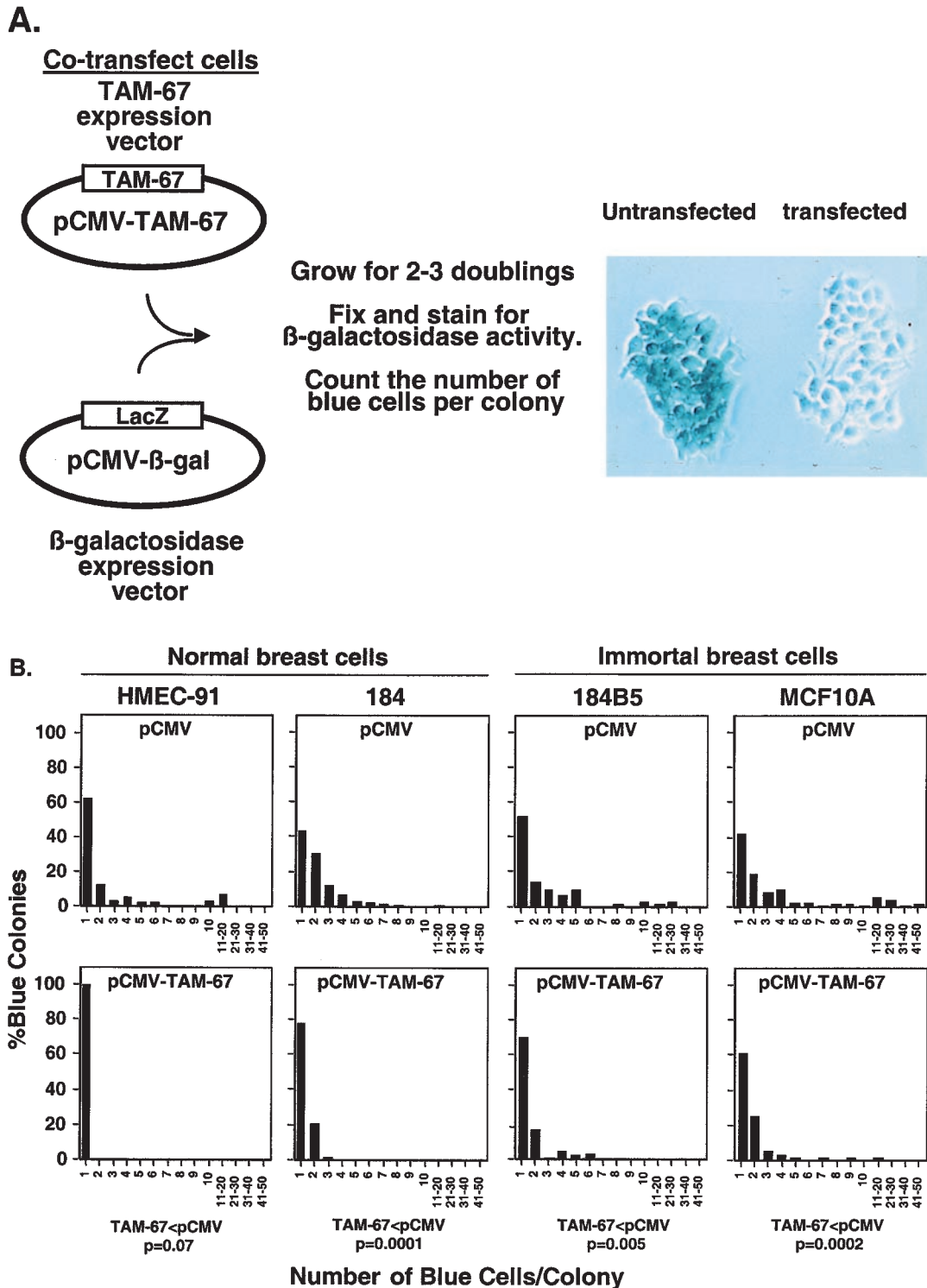


Figure 4 Single cell proliferation assay of normal and immortal breast cells. (a) Schematic diagram of the single cell proliferation assay. MCF7 cells were co-transfected with pCMV (5 μg) and pCMV-β-gal (0.5 μg) and stained with X-gal. A transfected colony (blue cells) and an untransfected colony (white cells) are shown. (b) Single cell proliferation assay of normal and immortal breast cells. The indicated breast cells were transfected with pCMV-β-gal (0.5 μg) and either pCMV (5 μg) or pCMV-TAM-67 (5 μg). After approximately three doublings, the transfected cells were identified by staining *in situ* for β-galactosidase activity and the number of transfected cells per colony were counted. The results are shown as histograms of 1–20 cells per colony and were analysed using the Wilcoxon rank sums test as described in the Statistical Analysis section of Materials and methods. *P* values from the Wilcoxon rank sums test are shown

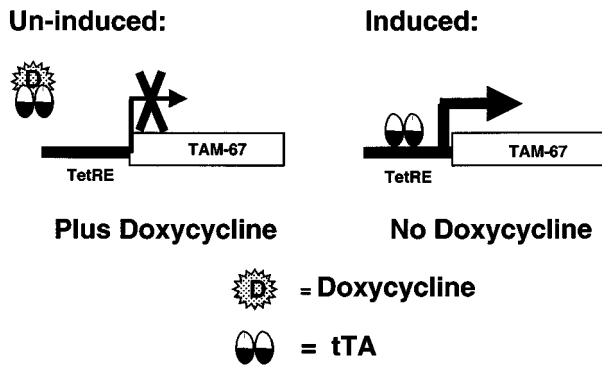
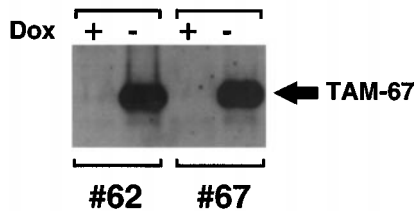
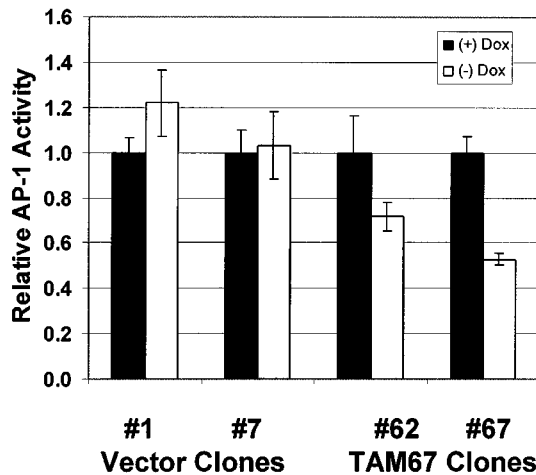
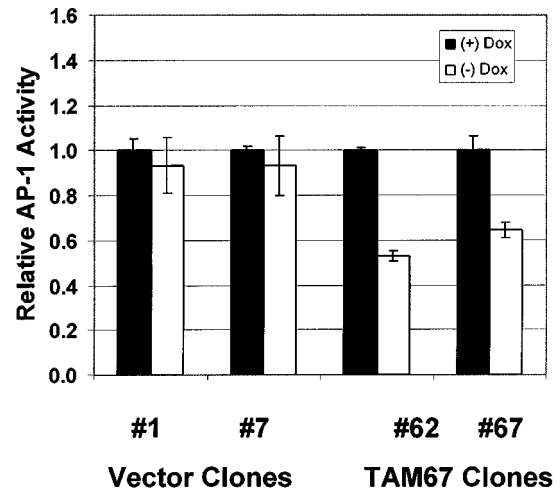
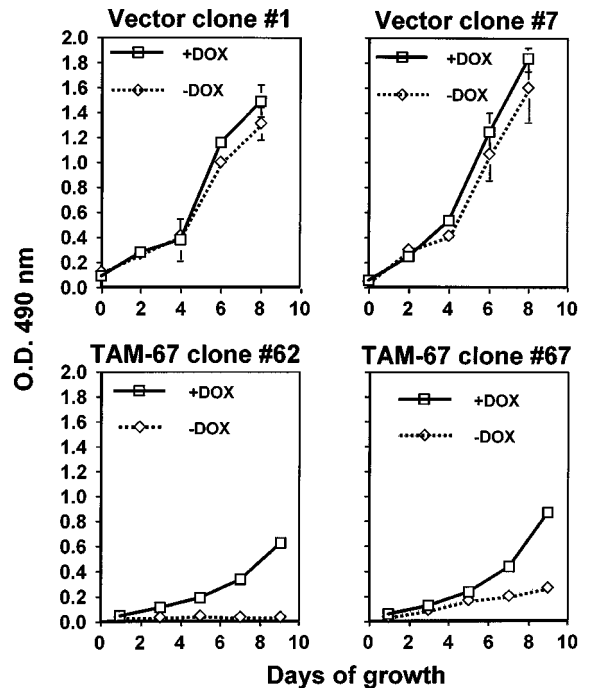
A. Tet-Off System:

B. MCF-7 TAM-67 Tet-off Clones:

C. BASAL AP-1 ACTIVITY

D. TPA-INDUCED AP-1 ACTIVITY

E. MCF7 tet-off clones


Figure 5 Induction of TAM-67 protein in MCF7 cells. (a) The Tet-off inducible protein expression system is shown. The Tet-off system utilizes the tetracycline dependent transcriptional repression activity of the tTA protein. The TAM-67 cDNA was cloned downstream of a tTA repressible CMV promoter element (TetRE) in a plasmid having a hygromycin-selectable marker. With the Tet-off system transfected cells are selected for hygromycin-resistance in the presence of doxycycline to maintain repression of TAM-67 expression and protein expression is induced by removal of doxycycline. (b) TAM67 protein expression in MCF7 Tet-off TAM-67 clones. Immunodetection of induced TAM-67 protein expression in MCF7 Tet-off cells. Total cellular protein was extracted 48 h after growing the cells in the presence (+) or absence (-) of doxycycline, and equal amounts of protein was analysed for TAM-67 expression using anti-cJun antibody. The TAM-67 protein band is indicated with an arrow. (c) Inhibition of basal AP-1 activity in MCF7 Tet-off TAM-67 clones. Basal AP-1 activity was determined by measuring the effect of TAM-67 protein induction on the activity of the transfected AP-1-dependent luciferase reporter plasmid, Col-Z-Luc. Cells were transfected with 1 μ g of Col-Z-Luc (and 0.5 μ g of CMV- β Gal plasmid to measure transfection efficiency) for 12 h and split into two plates, with or without doxycycline. After 36 h of incubation, the cells were harvested to measure basal AP-1 activity. Cell extracts were prepared, and luciferase and β -galactosidase activity were measured as described in Materials and methods. Each transfection was done in triplicate, and the luciferase results were normalized for differences in transfection efficiency using the β -galactosidase results. The data are presented as the luciferase activity in the presence of TAM-67 relative to the luciferase activity in the absence of TAM-67 for each MCF7 clone. Mean values are shown and error bars show standard deviation from the mean. (d) Inhibition of TPA-induced AP-1 activity in MCF7 Tet-off TAM-67 Clones. The effect of TAM-67 on TPA-induced AP-1 activity was determined by measuring AP-1 activity in the presence or absence of doxycycline in clones transfected with the AP-1 dependent luciferase reporter plasmid, Col-Z-Luc. The cells were transfected with 1 μ g of Col-Z-Luc Luc (and 0.5 μ g of CMV- β Gal plasmid to measure transfection efficiency) for 12 h and split into two plates, with or without doxycycline. After 36 h of incubation the cells were treated

Figure 5d shows the results of experiments measuring TPA-induced AP-1 activity in MCF7 cells. When the cells were grown in the presence of doxycycline (uninduced conditions), AP-1 activity was induced more than fivefold with TPA treatment (data not shown). TPA-induced AP-1 activity was not affected by removal of doxycycline in the MCF7 vector clones (Clones #1 and #7, Figure 5d). In contrast, when TAM-67 transfected cells were grown in the absence of doxycycline (TAM-67 induced), TPA-induced AP-1 activity was reduced in both MCF7-TAM67 clones (Clones #62, and #67, Figure 5d). These results demonstrate that TAM-67 protein inhibits both basal and TPA-induced AP-1 activity in these inducible MCF7 breast cancer cell lines.

We next directly measured the growth of the MCF7 cells under conditions in which TAM-67 was either repressed or induced (Figures 5e). As shown in Figure 5e, the growth of both of the MCF7 Tet-off TAM-67 clones was inhibited when doxycycline was removed from the media. The growth of two independently isolated vector-transfected clones was not inhibited by withdrawal of doxycycline. These results, along with the results of studies of transiently-transfected cells, shown in Figure 3, demonstrate that the growth of MCF7 cells is inhibited by the expression of the AP-1 inhibitor, TAM-67.

Discussion

The above results show that the growth of normal and immortal human mammary epithelial cells and MCF7 breast cancer cells is inhibited by AP-1 blockade. We have previously shown that normal human breast cells express high basal levels of AP-1 activity and that breast cancer cells express lower levels of AP-1 activity (Smith *et al.*, 1997). The studies reported here suggest that the high levels of AP-1 activity in normal and malignant breast cells reflect these cells' dependence on AP-1 for their growth. In addition, the present results show that some breast cancer cells, but not all, also depend on AP-1 for the growth.

The present results demonstrating that premalignant breast cells depend on AP-1 to transduce mitogenic signals is consistent with previous reports demonstrating that normal human mammary epithelial cells require peptide growth factors to support their growth. Stampfer and Yaswen (1992) and Zajchowski *et al.* (1988) have demonstrated that peptide growth factors are required for the *in vitro* growth of normal human mammary epithelial cells. In addition, Stampfer *et al.*

(1993) have shown that 184 normal human mammary epithelial cells, and the immortalized derivative 184B5, are both dependent on TGF α for continued growth demonstrating that normal breast cells require peptide growth factors to sustain their growth. The present results extend this observation to show that the activity of the AP-1 transcription factor, a downstream transducer of these peptide growth factors, is critical for the growth of normal and immortal breast cells.

Our results also demonstrate that certain breast cancer cells are less dependent on AP-1 for their growth than are normal breast cells. The breast cancer cell line most sensitive to AP-1 inhibition was MCF7. The other cancer cell lines studied (T47D, MDA MB 231 and MDA MB 435) were resistant to AP-1 blockade. These results suggest that at least for some breast cancer cell lines (T47D, MDA MB 231 and MDA MB 435), activation of AP-1-dependent pathways may not be essential for their growth. The breast cancer cell line T47D was observed to have an increased colony forming efficiency when AP-1 activity was blocked. This observation indicates that AP-1 activity may be involved in negative growth regulation of this breast cancer cell line. Studies are ongoing to investigate the role of AP-1 in negative growth regulation of T47D breast cancer cells.

We are currently investigating the mechanism by which AP-1 blockade leads to growth suppression of breast cells. Depending on cell type, AP-1 transcription factors transduce mitogenic signals from peptide growth factors, or stress signals through the SAPK or JNK pathways (reviewed in Karin *et al.*, 1997). Thus, AP-1 blockade could inhibit growth either by blocking cell cycle progression, or by inducing apoptosis. Preliminary results from our laboratory suggest that TAM-67 inhibits cell proliferation in MCF7 cells by suppressing entry into the S phase of the cell cycle, without inducing apoptosis (data not shown). This interference with S phase entry is consistent with previous studies of the role of AP-1 in rat fibroblasts. Kovary and Bravo (1991) have previously shown in fibroblasts that microinjection of antibodies specific for Jun or Fos proteins inhibits cell cycle progression and entry into S phase. Based on our preliminary studies, we predict that AP-1 blockade induced by the cJun dominant-negative, TAM-67, also blocks cell cycle progression and entry into S phase in human breast cells.

It is possible that breast cancer cells that have genetic alterations in tumor suppressor genes, such as p53 or Rb mutations, or overexpression of oncogenes, such as *c-erbB2/her2/neu*, *c-myc*, or cyclin D, no longer

with TPA for 4–6 h, after which time cell extracts were made and luciferase and β -galactosidase activity was measured. Each transfection was done in triplicate. The data are presented as luciferase activity in the presence of TAM-67 relative to luciferase activity in the absence of TAM-67 for each clone. Mean values are shown and error bars show standard deviation from the mean. (e) Effect of induced TAM-67 expression on the growth of MCF7 and MDA MB 435 breast cancer cells. Growth of MCF7 Tet-off vector and TAM-67 clones was determined in the presence (squares) and absence (diamonds) of doxycycline. Cell growth was measured using the MTS assay as described in Materials and methods. Each point represents the mean of three independent cultures, and error bars show standard deviation from the mean

require mitogenic signals that are normally transduced by AP-1. The observation that normal human breast cells, which lack these genetic alterations, require AP-1 activity for their growth is consistent with this hypothesis. Most of the breast cancer cells studied here have known genetic alterations (Bonsing *et al.*, 1997; Katayose *et al.*, 1995; Lesoon-Wood *et al.*, 1995). T47D, MDA MB 231 and MDA MB 435 have known p53 mutations, while MCF7 breast cancer cells, 184 normal breast cells, and the immortalized cells have been shown to express normal p53 (Lehman *et al.*, 1993). These genetic alterations may disrupt the normal mitogenic signal transduction pathway at a step distal to AP-1, and thus render these transformed cells more resistant to AP-1 inhibitors.

Recent studies of the expression of Jun and Fos family members in human breast tumors also suggest that these proteins have variable expression in human breast tumors. Bamberger *et al.* (1999) investigated the expression of members of the AP-1 family in 53 breast tumors. They observed relatively uniform expression of cJun, JunB, cFos, and Fra2 and variable levels of JunD and FosB. In this study, the authors noted that the expression of FosB correlated with ER-positivity and a well-differentiated phenotype, while expression of Fra1 showed a strong negative correlation with FosB expression, ER receptor positivity, and the differentiation status. Thus, tumors expressing FosB (the well differentiated, ER-positive tumors) would be expected to be sensitive to AP-1 inhibitors, such as TAM-67. Fra-1 has been shown to inhibit AP-1 transcription factor activity (Yoshioka *et al.*, 1995), and thus tumors expressing Fra-1 may be resistant to AP-1 inhibitors. Our present results support this hypothesis: MDA MB 231 cells, which express high levels of Fra-1, are resistant to the anti-proliferative effects of TAM-67 (see Figure 3), while MCF7 cells, which express FosB, but do not express Fra-1 (Chen *et al.*, 1996, and unpublished observation), are sensitive to TAM-67.

More recent studies performed by Gee *et al.* (2000) examined the activation of the AP-1 transcription factor complex in human breast tumors. They measured the expression of phospho-cJun in 78 primary breast tumors. These authors found that phospho-cJun expression was associated with expression of peptide growth factors and their receptors (TGF α and EGFR), as well as with expression of the phosphorylated form of the activating kinase of cJun, JNK. They observed correlations between high phospho-cJun expression and decreased overall survival and presence of distant metastasis. In addition, estrogen receptor-positive tumors that expressed high phospho-cJun developed progressive disease more rapidly than did tumors expressing low phospho-cJun. These clinical data suggest that in a subset of breast tumors, the AP-1 transcription factor is activated, and that this transcription factor is likely transducing growth factor signals *in vivo*. These clinical observations also provide rationale to target this transcription factor for the prevention and treatment of breast cancer.

The present results and those of Gee *et al.* (2000) and Bamberger *et al.* (1999) demonstrate that the AP-1 transcription factor is an important mitogenic signaling complex for normal and malignant breast cancer cells. These studies suggest that this transcription factor complex could be targeted for the development of future therapeutic agents. Thus, agents that inhibit AP-1 or that block AP-1 activation, such as inhibitors of Jun N-terminal kinases, may be useful agents for the prevention or treatment of breast cancer.

Materials and methods

Primary cell cultures and cell lines

The human mammary epithelial cells and cell lines used in these studies are described in previous studies by Smith *et al.* (1997). Cells used include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9–10); normal 184 cells provided by Dr Martha Stampfer (Stampfer and Yaswen, 1992); nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells 184A1 and 184B5 (Stampfer and Bartley, 1985), a nontumorigenic spontaneously immortalized HMEC cell line MCF10A (from Dr J Russo); and cancer cells: MCF7, a human breast adenocarcinoma cell line provided by Dr Ken Cowan, and T47D, MDA MB 231, and MDA MB 435 (from ATCC). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA, USA) for normal HMECs 184, 184A1, and 184B5 (Stampfer *et al.*, 1980; Stampfer and Bartley, 1985) DME/F-12 with 5% horse serum and supplements for MCF10A (Ciardiello *et al.*, 1990; Soule *et al.*, 1990) and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for the breast cancer cell lines. MCF-7 tTA cells were purchased from Clontech.

Transfection of breast cells

The breast cells 184, HMEC, 184B5, MDA MB 231, MCF7, and T47D were transfected using Eugene 6 reagent (Boehringer–Mannheim); MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western analysis

Whole cell protein extracts normalized were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA, USA). Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase assay to measure AP-1 activity

AP-1 transcriptional activating activity in cells was measured using the enhanced luciferase assay (Tropix) as previously described (Chen *et al.*, 1996). The cells were transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter

which contains a single AP-1 binding site (TGAG/CTCA) between nucleotides -73 and -63. Transfected cells were lysed 36 h after transfection and luciferase activity was measured with equal amounts of cell extract. The cells were also transfected with a CMV- β Gal plasmid and β -galactosidase activity was measured to normalize the luciferase results for transfection efficiency as previously described (Chen *et al.*, 1996).

Cell growth assays

Colony formation assay Two $\times 10^5$ cells were co-transfected in 35 mm wells with 0.5 μ g pZeoSV (Invitrogen), and 5 μ g of either pCMV (empty vector) or pCMV-TAM-67. Twelve hours after transfection the cells from each 35 mm well were split into four 35 mm wells. Twenty-four hours after the Transfection, Zeocin (Invitrogen) was added to a final concentration of 400 μ g/ml. All cells were found to be killed at this concentration if not transfected with the Zeocin resistance plasmid. After 2 weeks of selection in Zeocin, resistant colonies were stained with crystal violet and counted.

Single cell proliferation assay Cells were co-transfected as described for the colony forming efficiency assay with 0.5 μ g of pCMV- β -gal and 5 μ g of either pCMV (empty vector) or of pCMV-TAM-67. Twelve hours after transfection the cells were trypsinized and replated as single at cells densities of 0.2 to 1.0×10^5 in 100 mm plates. After approximately three doublings, colonies of cells were fixed and stained with X-Gal to detect cells expressing β -galactosidase *in situ*. Colonies containing blue cells were visualized by light microscopy and scored for the number of blue cells per colony. The cells in these blue colonies all received DNA and arose from a single transfected cell. Therefore, transfection efficiency in these counted blue colonies is 100%.

Cell proliferation assay of stably transfected Tet-off cell lines The CellTiter 96TM Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. One thousand to 2000 cells were seeded in a 96 well plate in media containing

doxycycline, and the next day doxycycline was removed in half the samples to induce the transfected gene (MCF7 tTA-vector or -TAM67 lines). During the experiment the medium was replaced every other day. A solution containing a 20:1 ratio of MTS and PMS was added to the cells for 2 h at 37°C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption \pm standard error.

Statistical analyses

Colony formation assays result in counts of numbers of colonies. After log-transformation, as indicated by Box-Cox analysis (Box and Cox, 1962), TAM-67 transfected cells were compared to vector alone using two-sample *t*-tests. Single cell proliferation assays results in a distribution of cells per colony for each cell type. Wilcoxon rank sum tests were used to compare distributions between TAM-67 and vector transfected cells.

Abbreviations

AP-1: Activating Protein-1; ER: Estrogen receptor; HMECs: Human mammary epithelial cells; IGF: insulin-like growth factor; SCPA: Single cell proliferation assay; SEM: standard error of the mean; TGF α : transforming growth factor alpha; TPA; 12-O-tetradecanoylphorbol-13-acetate; X-Gal: 5-Bromo-4-Chloro-3-Indoyl- β -galactopyranoside.

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