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cFos is critical for MCF-7 breast cancer cell growth

Chunhua Lu[1], Qiang Shen[1], Elizabeth DuPré[1], Heetae Kim[1], Susan Hilsenbeck[1] and Powel H Brown*,[1]

[1]*Departments of Medicine and Molecular and Cellular Biology, Baylor College of Medicine, Breast Center, One Baylor Plaza MS600, Houston, TX 77030, USA*

The activating protein-1 (AP-1) transcription factor is a converging point of multiple signal transduction pathways in many cells. We have previously demonstrated that overexpressing Tam67, a dominant-negative (DN) form of cJun, blocks AP-1 activity and inhibits breast cancer cell growth. We hypothesized that Tam67 forms dimers with other AP-1 proteins to suppress the growth of breast cancer cells. In the present study, we used immunoprecipitation-Western blotting to demonstrate that Tam67 binds all Jun and Fos proteins in breast cancer cells. In addition, we used two variants of the Tam67 mutant to investigate whether Jun or Fos protein was required for breast cancer cell growth. We created a Tam/Fos mutant in which the cJun dimerization domain was replaced by the cFos dimerization domain, and a Tam/Squelcher mutant in which the cJun dimerization domain was deleted. We then isolated MCF-7 cell lines that stably expressed these cJun-DN mutants under the control of an inducible promoter. Using AP-1-dependent reporter assays, we observed that Tam67 and Tam/Fos mutants inhibited AP-1 transcriptional activity, while the Tam/Squelcher mutant did not. We then determined whether Tam/Fos or Tam/Squelcher inhibited breast cell growth as well as Tam67. We found that while Tam67 repressed cell growth, neither Tam/Fos nor Tam/Squelcher mutant affected cell growth. These results indicate that Tam67 likely inactivates Fos family member proteins to suppress breast cancer cell growth. Finally, we performed antisense experiments to knock down the expression of individual family members (cJun or cFos). Our results demonstrated that antisense cFos inhibited breast cancer cell proliferation and colony formation, while antisense cJun did not. These results suggest that Tam67 suppresses breast cancer cell growth by interacting with Fos family members, specifically with cFos, to produce an inactive AP-1 complex.
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Introduction

The activating protein-1 (AP-1) transcription factor is a key component of many signal transduction pathways. The AP-1 transcription factor is a dimeric complex consisting of homodimer of Jun proteins (cJun, JunB, or JunD) or heterodimer of Jun and Fos proteins (cFos, FosB, Fra-1, or Fra-2). These heterodimer or homodimer complexes regulate the expression of AP-1 target genes by binding to the AP-1 site (the phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element) within gene promoters. Other factors such as the Jun dimerization partners (JDP1 and JDP2), the activating transcription factor (ATF)/CRE-binding protein families, Maf proteins, and the neural retina-specific gene product Nrl may also interact with AP-1 proteins and regulate the transcriptional-activating activity of AP-1 complexes (Vogt and Bos, 1990; Angel and Karin, 1991; Aronheim *et al*., 1997; Karin *et al*., 1997; Piu *et al*., 2001). Although the AP-1 factor was identified 15 years ago, the mechanism by which AP-1 and its components control cancer cell proliferation remains unclear. Several mitogenic signaling cascades converge upon the AP-1 factor, making it a potential target for inhibiting breast cell proliferation. Extracellular stimuli such as peptide growth factors and steroid hormones lead to the activation of AP-1 signaling and cell proliferation (Chen *et al*., 1996; Webb *et al*., 1999; Lin *et al*., 2000).

Differential expression and activation of individual Jun and Fos family members allow the AP-1 factor to control a wide variety of cellular functions. AP-1 has been implicated in many different biological processes including cell differentiation, proliferation, and apoptosis (Holt *et al*., 1986; Szabo *et al*., 1991; Brown *et al*., 1993, 1994; Rodgers *et al*., 1994; Ham *et al*., 1995). Extensive analyses of mice and cultured cells have shown that such functional diversity is achieved through the formation of various dimers with different combinations of Jun and Fos proteins.

In breast cancer cells, the AP-1 proteins have been identified as important regulators of growth and invasion. AP-1 proteins are differentially expressed in human breast tumors, and AP-1 activity is modulated by many critical growth factors and hormones such as EGF, IGFs, estrogen, and retinoids (Schule *et al*., 1991; Chen *et al*., 1996; Webb *et al*., 1999; Lin *et al*., 2000). Elevated levels of cJun and phospho-cJun in breast cancer tissue are associated with low estrogen receptor

*Correspondence: PH Brown; E-mail: pbrown@breastcenter.tmc.edu
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(ER) expression and tamoxifen resistance (Smith *et al.*, 1999; Schiff *et al.*, 2000). cJun overexpression in MCF-7 breast cancer cells also produces an invasive and hormone-resistant phenotype (Smith *et al.*, 1999). Our previous studies have shown that a specific AP-1 inhibitor, the cJun-dominant negative mutant (cJun-DN), Tam67, blocks AP-1 activity and inhibits breast cancer cell growth *in vitro* and *in vivo* (Brown *et al.*, 1994; Ludes-Meyers *et al.*, 2001; Liu *et al.*, 2002). We also demonstrated that Tam67 causes cell cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity (Liu *et al.*, 2004).

In the present study, we developed three MCF-7 cell lines that express inducible AP-1 mutants (Tam67, Tam/Fos, and Tam/Squelcher) to address the role of Jun and Fos proteins in regulating breast cell growth. Tam67 forms dimer with Jun, Fos, and other cJun-interacting proteins. Tam/Fos is a Tam67 mutant in which the cJun dimerization domain has been replaced by the cFos dimerization domain; thus, it can only heterodimerize with Jun family members to form Tam/Fos : Jun complexes. Tam/Fos lacks the ability to dimerize with cFos or other Fos family member proteins. Tam/Squelcher is a Tam67 mutant that has no dimerization domain, and therefore does not dimerize with any AP-1 family members. We observed that Tam67 inhibited breast cancer cell growth, while Tam/Fos and Tam/Squelcher did not. These results suggest that Fos family members may be inactivated by Tam67. We therefore investigated the role of specific Jun and Fos family members in regulating breast cancer cell growth by performing antisense experiments. Our results demonstrate that cFos is a critical regulator of breast cancer cell growth. These studies will enhance our current understanding of how AP-1 factors function in breast cancer cells and provide a firm rationale for developing selective AP-1 inhibitors for the treatment and prevention of breast cancer.

Results

Construction of cJun mutants

We developed three cJun mutants by mutating or deleting the functional domains of cJun as shown in Figure 1. These mutants include Tam67 (Jun Δ3–122), Tam/Fos, and Tam/Squelcher. Tam67 is a cJun-DN mutant that can dimerize with Jun, Fos family members, and other interacting proteins (such as ATF proteins). Tam/Fos is a Tam67 mutant in which the cJun dimerization domain has been replaced by the cFos dimerization domain; thus, Tam/Fos is only able to form dimers with Jun family members (no Tam67 : Fos dimers will be formed). Tam/Squelcher is a Tam67 mutant that lacks the cJun dimerization domain and hence lacks the ability to dimerize with any AP-1 family members.

Expression of cJun mutants in MCF-7 cells

Four MCF-7 cell lines (MCF-7 Tet-off Tam67, MCF-7 Tet-off Tam/Fos, MCF-7 Tet-off Tam/Squelcher, and

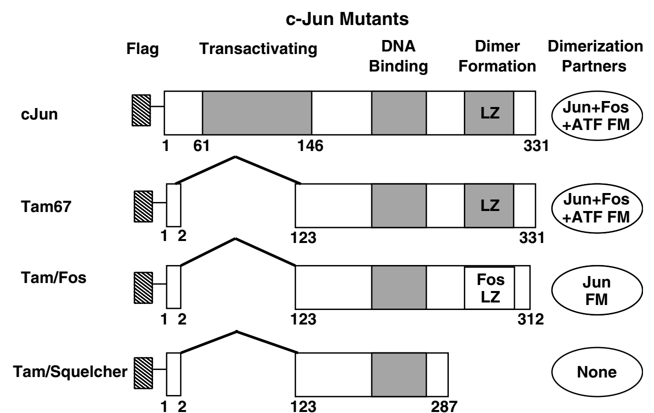


Figure 1 Schematic map of cJun-DN mutants. Tam67 is a cJun-DN mutant in which cJun transactivation domain was deleted. The leucine zipper domain of Tam67 was replaced with that from cFos to produce Tam/Fos. The leucine zipper domain of Tam67 was deleted to produce Tam/Squelcher

MCF-7 Tet-off vector cells) were generated as described in Materials and methods. Doxycycline (DOX) was removed to induce the expression of cJun mutants, and total protein lysates were prepared 3–7 days after DOX removal. As shown in Figure 2a, each of Tam67 mutants was expressed within 3–5 days after DOX removal. In Figure 2b and c, we show that Tam67 was able to interact with all Jun and Fos proteins using immunoprecipitation (IP)-Western blotting techniques. In Figure 2b, the Tam67 was immunoprecipitated using an anti-Flag antibody, and the resulting precipitated proteins were run on a polyacrylamide gel and screened for specific Jun and Fos proteins. All Jun and Fos proteins were co-immunoprecipitated with Tam67, indicating that Tam67 can bind cJun, JunB, JunD, cFos, FosB, Fra-1, and Fra-2 (Figure 2b). In Figure 2c, the total proteins were immunoprecipitated with individual Jun and Fos antibodies, and the resulting immunoprecipitated proteins were Western blotted using the anti-Flag antibody to determine if Tam67 was present within the protein complexes. Consistent with Figure 2b, Tam67 was shown to be co-immunoprecipitated with each Jun and Fos family members (Figure 2c). Thus, the cJun-DN mutant Tam67 is able to dimerize with all Jun and Fos proteins present in MCF-7 cells.

cJun mutants Tam67 and Tam/Fos suppress basal and heregulin (HRG)-induced AP-1 activity

Tam67 has previously been shown to inhibit AP-1 activity in several different cell lines (Brown *et al.*, 1994; Ludes-Meyers *et al.*, 2001; Liu *et al.*, 2002). In this study, we investigated whether the other cJun mutants, Tam/Fos and TAM/Squelcher, affect AP-1 activity. The MCF-7 Tam/Fos (clones #28 and #73), Tam/Squelcher (clones #25 and #44), and vector-transfected cells (clone #1) were cultured in the presence or absence of DOX to block or induce the expression of Tam/Fos or Tam/Squelcher. As shown in Figure 3, Tam67 and Tam/Fos

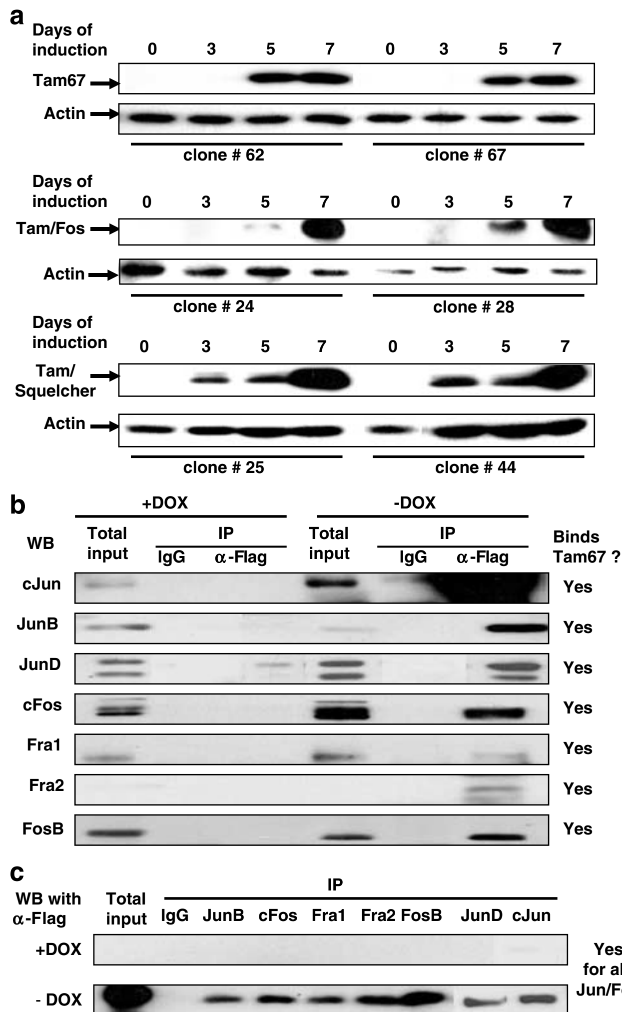


Figure 2 Expression of cJun mutants in MCF-7 Tet-off cells. (**a**) The MCF-7 Tet-off Tam67, MCF-7 Tet-off Tam/Fos, and MCF-7 Tet-off Tam/Squelcher cells were cultured in the medium without DOX for 7 days, and the protein expression was measured by Western blotting. Actin was used as a loading control. (**b**) IP-Western blot to demonstrate that Tam67 was capable of forming dimers with other Jun and Fos proteins. (**c**) IP-Western blot to demonstrate that all Jun and Fos family proteins were able to form dimers with Tam67

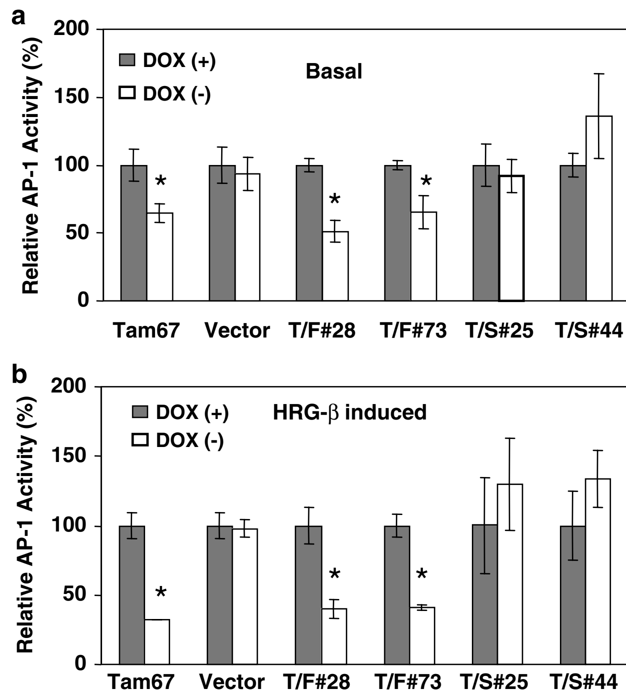


Figure 3 Effect of cJun mutants on AP-1 transcriptional activity. The MCF-7 Tet-off vector, MCF-7 Tet-off Tam67, MCF-7 Tet-off Tam/Fos, and MCF-7 Tet-off Tam/Squelcher cells were cultured in the medium with or without DOX for 7 days to block or induce the expression of cJun mutants. The basal- (**a**) and HRG-β- (**b**) induced AP-1 activities were measured by luciferase assays. *Statistical significance ($P<0.01$)

repressed the basal level of AP-1 activity (Figure 3a) and also inhibited AP-1 activity induced by HRG (Figure 3b) and TPA (data not shown). Tam/Squelcher did not affect basal or induced AP-1 activity (Figure 3a and b).

Effect of cJun mutants on breast cancer cell growth

In previous studies, we demonstrated that Tam67 inhibits breast cancer cell growth (Ludes-Meyers *et al*., 2001; Liu *et al*., 2002). For the present study, we wanted to identify the dimer partners of Tam67 that when inactivated by Tam67 lead to growth suppression. The MCF-7 Tet-off Tam/Fos (clones #28 and #24), Tam/Squelcher (clones #25 and #44), and vector cells (clone #1) were cultured in the presence or absence of DOX for 7 days to suppress or induce the expression of Tam/Fos or Tam/Squelcher. Cell proliferation was then measured. As shown in Figure 4, Tam67 suppressed breast cancer cell growth in the absence of DOX. However, while Tam/Fos inhibited AP-1 activity (as defined by activity of collagenase reporter construct and shown in Figure 3), it did not suppress cell growth. This result suggests that suppression of AP-1 transcriptional-activating activity alone is not sufficient to suppress breast cancer growth. Tam/Squelcher (which failed to suppress AP-1 transcriptional-activating activity) also did not inhibit breast cancer cell growth. These results suggest that Tam67 must dimerize with factors other than Jun family members to inhibit breast cancer cell growth. Thus, we hypothesized that Tam67 inactivates Fos to suppress cell growth.

Antisense cJun and cFos inhibit AP-1 activity

To determine whether cFos is required for breast cancer growth, we used antisense cDNA to inhibit cJun and cFos expression in the breast cancer cells. MCF-7 cells were transfected with antisense cJun, antisense cFos, or pcDNA3.1 vector. We first verified that cJun and cFos protein expression was suppressed by antisense cJun or cFos cDNA. As shown in Figure 5, antisense constructs effectively reduced cJun or cFos protein expression. We then performed luciferase reporter assays using an

AP-1-dependent reporter construct to determine the effect of antisense cJun and antisense cFos on AP-1 activity. As shown in Figure 6, we observed that both antisense cJun and antisense cFos reduced AP-1 transcriptional-activating activity.

Antisense cFos suppresses breast cancer cell growth

We next used a single-cell proliferation assay previously described by us (Ludes-Meyers *et al.*, 2001) to investigate the importance of cJun and cFos on the growth of breast cancer cells. In this assay, shown schematically in Figure 7a, cells were transfected with a β-galactosidase plasmid along with Tam67, antisense cJun, antisense cFos constructs, or vector. The transfected cells were then split and plated as single cells that were then allowed to grow for three to five doublings to form colonies. The cells were then stained with X-gal to identify transfected cells (cells positive for β-galactosidase, stained blue). Cells stained blue are progeny from one transfected cell. The number of β-galactosidase-positive progeny cells in each stained colony was then counted to assay the growth of the single transfected cells. The numbers of progeny cells (number of blue cells in a colony) were then plotted as a histogram. This assay allowed us to determine the effect of knocking down cJun or cFos expression on the growth of individual transfected cells.

Our results showed that after 3 days, Tam67 dramatically reduced the number of cells that grew from a single transfected cell as compared to vector-transfected cells. This degree of growth suppression was similar to that seen when antisense cFos was transfected. Alternatively, the colony distribution of vector control and antisense cJun showed similar patterns (Figure 7b). Thus, cFos knockdown with antisense cDNA significantly suppressed the growth of breast cancer cells ($P<0.0001$).

We next confirmed the results of our single-cell growth assay by performing a colony formation assay in cells stably transfected with antisense cJun and antisense cFos cDNA. In these experiments, we stably transfected cJun, and cFos antisense cDNAs into MCF-7 cells, and measured the colony formation of the resulting clones. As shown in Figure 7c, the vector-transfected cells produced 476 ± 65 colonies, cells transfected with cJun antisense cDNA formed 386 ± 75 colonies, and cFos antisense-transfected cells formed 304 ± 14 colonies. Thus, antisense cFos significantly reduced the ability of MCF-7 cells to form colonies, compared to the vector control ($P<0.01$). These results are consistent with our results from the single-cell proliferation assay (shown in Figure 7b), and demonstrate that cFos is critical for breast cancer cell growth. From these results, we conclude that cFos is an important regulator of MCF-7 breast cancer cell proliferation that may play a critical role in the regulation of human breast cancers.

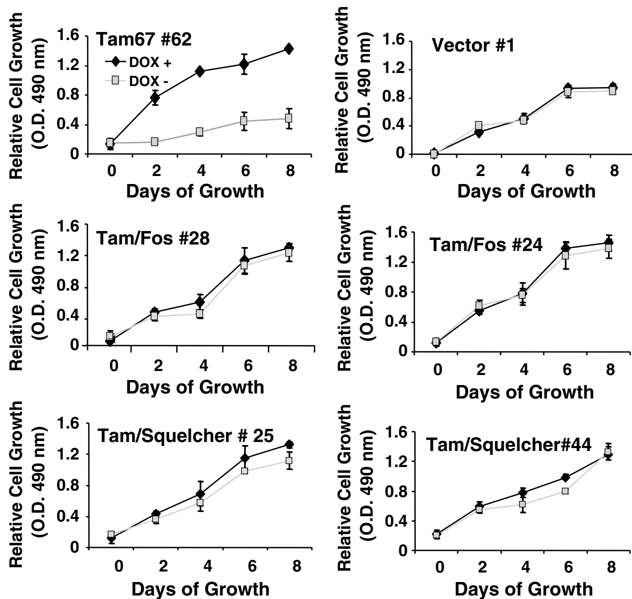


Figure 4 Effect of cJun mutants on MCF-7 cell proliferation. The MCF-7 Tet-off vector, MCF-7 Tet-off Tam67, MCF-7 Tet-off Tam/Fos, and MCF-7 Tet-off Tam/Squelcher cells were cultured in the medium with or without DOX for 7 days to block or induce the expression of cJun mutants. Cell proliferation was measured over the next 8 days using MTS assay

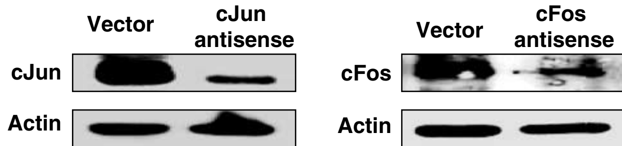


Figure 5 Expression of cJun and cFos after antisense cDNA treatment. The MCF-7 cells were transfected with pcDNA3.1, pcNDA3.1 antisense cJun, or antisense cFos. After 2 weeks of selection, the whole-cell lysates from transfected cells were used to measure cJun and cFos protein expression by Western blotting

Discussion

We have previously shown that the cJun-DN mutant Tam67 blocks AP-1 transcriptional activity and inhibits

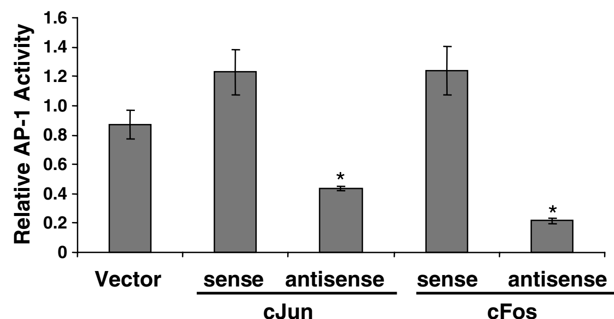
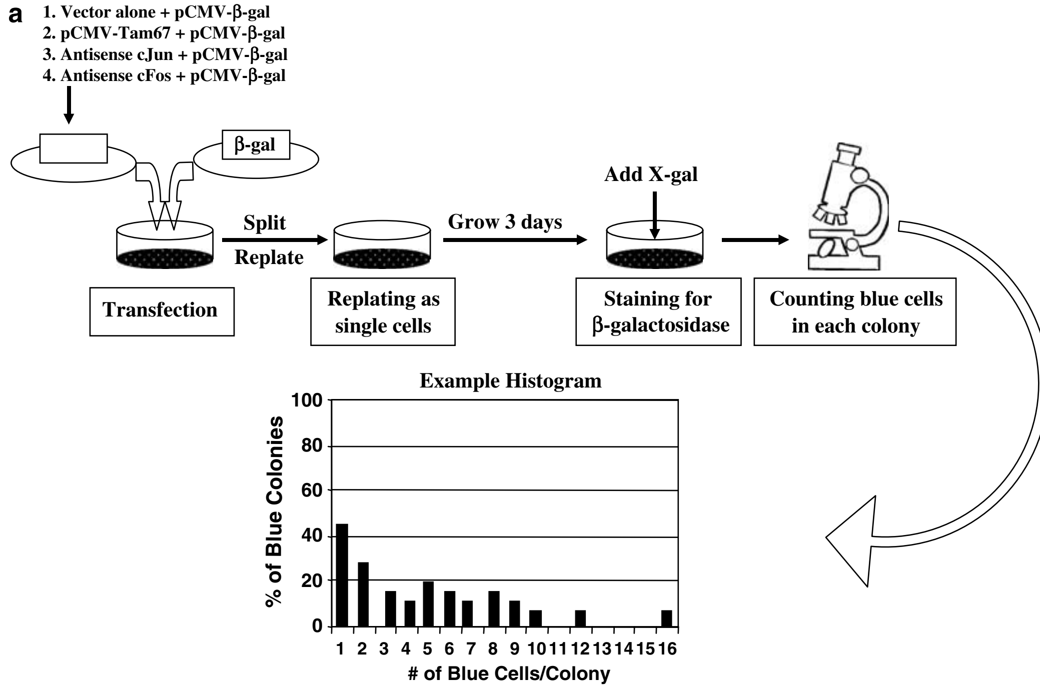
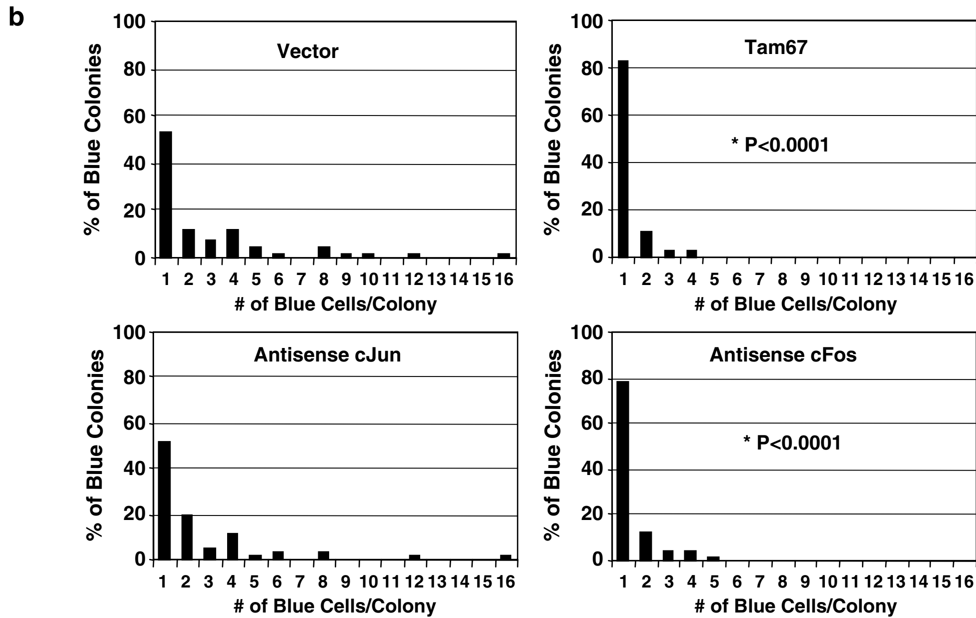
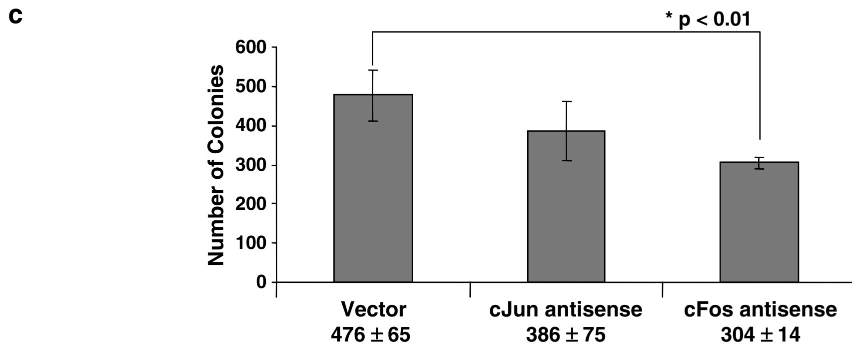


Figure 6 Effect of antisense cJun and cFos cDNA on AP-1 activity. The MCF-7 cells were transfected with pcDNA3.1 alone or pcDNA3.1 antisense cJun or antisense cFos. After 48 h, the cells were lysed and AP-1 activity was measured by luciferase assays. *Statistical significance ($P<0.005$) between the effect of vector and antisense cJun or cFos


a
1. Vector alone + pCMV-β-gal
2. pCMV-Tam67 + pCMV-β-gal
3. Antisense cJun + pCMV-β-gal
4. Antisense cFos + pCMV-β-gal
β-gal
Split
Replate
Grow 3 days
Add X-gal
Transfection
Replating as single cells
Staining for β-galactosidase
Counting blue cells in each colony
Example Histogram
% of Blue Colonies
# of Blue Cells/Colony


b
Vector
Tam67
* P<0.0001
Antisense cJun
Antisense cFos
* P<0.0001
% of Blue Colonies
# of Blue Cells/Colony


c
* p < 0.01
Number of Colonies
Vector
476 ± 65
cJun antisense
386 ± 75
cFos antisense
304 ± 14

breast cancer cell growth *in vitro* and *in vivo* (Brown *et al*., 1994, 1996; Ludes-Meyers *et al*., 2001; Liu *et al*., 2002). This cJun mutant dimerizes with Jun or Fos proteins to inhibit AP-1 activity and suppress the expression of AP-1-regulated genes involved in cell growth (Brown *et al*., 1994, 1996; Hennigan and Stambrook, 2001). The mechanism by which AP-1 blockade suppresses breast cell growth is not entirely clear, and is the focus of this study. We used three cJun mutants, and antisense cDNA for cJun and cFos to elucidate the underlying mechanism by which Tam67 suppresses cell growth. Since Tam67 is able to interact with Jun, Fos, and other factors, we diminished the binding capacity of Tam67 by replacing the dimerization domain with that from cFos. This produces a cJun mutant that only forms Tam : Jun family dimers. We also created the Tam/Squelcher mutant that is unable to bind any AP-1 proteins. We then tested whether these mutants could suppress the growth of MCF-7 breast cancer cells as effectively as Tam67 does. We found that the Tam/Fos mutant, which only forms complexes with Jun proteins, did not suppress MCF-7 cell growth, despite being able to decrease basal and induced AP-1 transcriptional activity. These results suggest that cFos or other Fos family member proteins must be inactivated by Tam67 to suppress cell proliferation. Our results also show that molecules that suppress AP-1 activity as measured by a collagenase reporter assay do not necessarily suppress breast cancer cell growth. This result suggests that it will be more relevant to screen growth-suppressive anti-AP-1 agents using AP-1-dependent promoters that are more directly related to growth. Such AP-1-dependent, growth-regulatory genes include cyclin D1 and c-Myc. We have recently demonstrated that these important growth-regulatory genes are critically regulated by AP-1 in breast cancer cells (DeNardo *et al*., 2005).

To further investigate the role of Fos family members in regulating growth, we used antisense strategies to knockdown cJun or cFos mRNA and protein. We observed that cFos antisense suppresses breast cancer cell growth, while antisense cJun does not. From these studies, we conclude that cFos is a critical mediator of AP-1-regulated breast cancer cell growth.

AP-1 transcription factors are involved in cellular proliferation (Holt *et al*., 1986), differentiation (Szabo *et al*., 1991), apoptosis (Ham *et al*., 1995), oncogene-induced transformation (Brown *et al*., 1993, 1994), invasion (Angel *et al*., 1987; McDonnell *et al*., 1990; Mackay *et al*., 1992; Matrisian, 1994), and several other cellular functions (Chiu *et al*., 1989; Schutte *et al*., 1989; Yoshioka *et al*., 1995). Jun and Fos members are differentially expressed in human breast cancers (Bamberger *et al*., 1999), and AP-1 is activated by important growth factors including EGF, IGFs, and estrogen (Chen *et al*., 1996; Ludes-Meyers *et al*., 2001; Liu *et al*., 2002). Elevated levels of cJun and phospho-cJun in human breast cancers are associated with low ER expression (Tang *et al*., 1997; Bamberger *et al*., 1999; Gee *et al*., 2000) and with tamoxifen resistance (Daschner *et al*., 1999; Johnston *et al*., 1999; Schiff *et al*., 2000; Liu *et al*., 2004). In addition, cJun overexpression in MCF-7 cells produces a tumorigenic-, invasive-, and hormone-resistant phenotype (Smith *et al*., 1999; Gee *et al*., 2000; Schiff *et al*., 2000). All of these previous studies suggest that AP-1 plays a critical role in the breast cell growth and transformation.

As a member of the AP-1 transcription factor complex, cFos is an essential modulator of cell proliferation, differentiation, and transformation (Preston *et al*., 1996). Depending on the cell type and environment, cFos can function either as a transcriptional activator or as a transcriptional repressor. Transition between these functions is regulated through post-translational modifications of the C-terminal region of the protein, possibly by phosphorylation of the serine residues (Barber and Verma, 1987). Earlier studies demonstrated that antisense RNA or Fos-specific antibodies that block cFos expression inhibited fibroblasts proliferation (Holt *et al*., 1986; Nishikura and Murray, 1987; Riabowol *et al*., 1988). Our current results in breast cells are consistent with these previous studies. The specific role of different Fos family proteins is still undefined, although each of the four Fos proteins may be required for cellular proliferation (Piechaczyk and Blanchard, 1994). In breast cancer, increased cFos protein expression was associated with poor prognosis (Bland *et al*., 1995). Knockdown of cFos expression was able to prolong survival and inhibit the proliferation and invasiveness of breast cancer xenografts (Robinson-Benion *et al*., 1994; Arteaga and Holt, 1996). Furthermore, Gee *et al*. (1995) have observed a significant association between elevated Fos protein expression and increased proliferation, *de novo* endocrine insensitivity, and a poor prognosis in clinical breast cancers. In addition to its role in normal development and cellular growth, the cFos protein is associated with apoptotic

Figure 7 Cell proliferation assays of MCF-7 breast cancer cells. (**a**) Schematic diagram of the single-cell proliferation assay. MCF-7 cells were cotransfected with pCMV-β-Gal (0.2 μg) and either pcDNA3.1 vector (2 μg), pcDNA3.1 Tam67 (2 μg), pcDNA3.1 antisense cJun cDNA (2 μg), or pcDNA3.1 antisense cFos cDNA (2 μg). After 3 days of growth (approximately three doublings), the transfected cells were identified by staining *in situ* with X-gal for β-galactosidase activity, and the number of transfected cells per colony was counted. The results are shown as histograms of one to 16 stained cells per colony. Colonies with same numbers of blue cells are shown as a percentage of the total stained colonies. (**b**) Antisense cFos inhibits single-cell proliferation in MCF-7 cells. MCF-7 cells were treated as described above. The statistical significance of these results was analysed using Wilcoxon's rank-sum test. The *P*-values from Wilcoxon's rank-sum test are shown. (**c**) Antisense cFos cDNA reduces colony formation in MCF-7 cells. MCF-7 cells were transfected with pcDNA3.1 alone or pcNDA3.1 antisense cJun or antisense cFos. After 2 weeks of selection with G418, survived colonies were stained with crystal violet and counted. The statistical significance of these results was analysed using two-sample *t*-tests

cell death in antiproliferative conditions, and in response to cellular injury (Preston *et al*., 1996). However, the proapoptotic role of cFos is not evident in *c-fos* knockout mice (Gajate *et al*., 1996; Roffler-Tarlov *et al*., 1996). Our studies support these data by indicating that cFos is involved in mammary cell proliferation and transformation. It appears that cFos is an important activator of the AP-1-regulated genes involved in growth.

While our results show that cFos is a critical AP-1 protein involved in regulating breast cell proliferation, other AP-1 family members within the mammary glands may regulate breast cell proliferation as well. Other factors such as FosB, Fra-1, Fra-2, or ATF proteins (all of which can dimerize with cJun) may also be important for breast cell growth. We are currently investigating the role of those AP-1-related proteins on breast cell growth.

In our previous studies, we have seen that Tam67 effectively inhibited the growth of normal, immortal breast cells, and ER-positive breast cancer cells, but not of ER-negative breast cancer cells (Ludes-Meyers *et al*., 2001). These previous results suggest that some breast cancer cells do not require AP-1 for their growth. It will be interesting to explore whether cFos has a similar role in these breast cancer cells. Such differential sensitivity of these breast cells to AP-1 blockade indicates that cFos is a critical factor in only a subset of breast cancers.

Based on our results, we propose that suppression of AP-1 activity by Tam67 leads to cFos inactivation, which in turn causes suppression of breast cancer growth by modulating AP-1-dependent genes. We are currently attempting to identify the critical growth-regulatory genes that are affected by cFos inactivation. Such studies will lead to a better understanding of how AP-1 controls proliferation, and may ultimately uncover new targets for the treatment and prevention of breast cancer.

Materials and methods

Plasmids

The Tam67, Tam/Fos, and Tam/Squelcher genes were constructed using the polymerase chain reaction(PCR) as described previously (Alani *et al*., 1991; Brown *et al*., 1994, 1996). The correct sequence of these fusion genes was confirmed by sequencing using an automated DNA sequencer. All cJun mutants were cloned into pUHD 10-3 5′ Flag vector for further use.

Cell culture, transfection, and cell lines established

The generation of MCF-7 Tet-off Tam67 clones has been described previously (Ludes-Meyers *et al*., 2001). The cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, NY, USA), with 100 μg/ml of geneticin and 100 μg/ml hygromycin in the presence of DOX. The MCF-7 Tet-off parental cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, NY, USA) with 100 μg/ml of geneticin. The cells were transfected with Tam67, Tam/Fos, Tam/Squelcher, or vector alone using Fugene 6 reagent (Roche, Indianapolis, ID, USA) according to the manufacturer's recommendations. Stable clones were isolated after selection in hygromycin.

Luciferase assay to measure AP-1 activity

AP-1 transcriptional activity in cells was measured using the Dual-Luciferase™ Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. The cells were cotransfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter that contains a single AP-1 binding site (TGAG/CTCA) and pRL-TK, a *Renilla* construct for normalizing transfection efficiency. To determine the AP-1 activity stimulated by heregulin-beta (HRG-β), the cells were treated with HRG-β (10 ng/ml, R&D System, Minneapolis, MO, USA) or DMSO, respectively, for 6 h before harvest. Transfected cells were lysed 36 h after transfection and luciferase activity was measured with equal amounts of cell extract using a microplate luminometer (Labsystems, Helsinki, Finland) and normalized to *Renilla* activity.

Single-cell proliferation assay

This assay was performed as described previously (Ludes-Meyers *et al*., 2001). Briefly, MCF-7 cells were cotransfected with 0.2 μg of pCMV-β-Gal and 2 μg of either pcDNA3.1 vector, pcDNA3.1 TAM67, pcDNA3.1 antisense cJun cDNA, or pcDNA3.1 antisense cFos cDNA. At 12 h after transfection, the cells were trypsinized to make single-cell suspensions, and split into 100 mm dishes. After 3 days, colonies of cells were fixed and stained with X-gal to detect cells expressing β-galactosidase *in situ*. Colonies containing stained, blue cells were visualized under a light microscope and scored for the total number of stained colonies and the number of blue cells per colony. The cells in these blue colonies received DNA and arose from a single transfected cell. Therefore, transfection efficiency in these counted blue colonies is 100%. The number of blue cells per colony is a function of the growth rate of a single transfected cell. The results were then plotted as the % colonies that showed one to 16 stained cells per colony. Transfected genes that cause decreased growth reduce the number of blue cells within individual colonies.

Colony formation assay

This assay was performed as described previously (Ludes-Meyers *et al*., 2001). Briefly, 2×10^5 cells were cotransfected in a six-well plate with 2 μg of either pcDNA3.1 empty vector, pcDNA3.1 antisense cJun, or pcDNA3.1 antisense cFos. At 12 h after transfection, the cells were split and maintained in 100 mm dishes. At 24 h after transfection, G418 (Invitrogen) was added to make a final concentration of 800 μg/ml. After 2 weeks of selection in G418, resistant colonies were stained with crystal violet and counted under a light microscope. All experiments were performed in triplicate and the mean numbers of G418-resistant colonies were calculated.

Cell proliferation assay of stably transfected Tet-off cell lines

The CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) (MTS) assay; Promega, Madison, WI, USA) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. Approximately 1000 cells were seeded in a 96-well plate. A solution containing a 20 : 1 ratio of MTS and PMS (phenazine methosulfate) was added to the cells for 2 h at

37°C and absorption at 550 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption ± s.e.

Western blot assay

Cells were transfected with antisense cDNA for cJun, cFos, or vector alone. After 36 h, whole-cell lysates were extracted. Then, equal amounts of total protein were electrophoresed on a 10% acrylamide denaturing gel and transferred onto a nitrocellulose membrane. The cJun or cFos expression was detected by using the following antibodies: rabbit polyclonal antibody specific for cJun (cat.#: PC06, Oncogene Science, Cambridge, MA, USA; 1 : 200); mouse monoclonal antibody specific for cFos (cat.#: sc-7202, Santa Cruz, CA, USA; 1 : 200).

IP-Western blot

MCF-7 Tet-off TAM67 cells were maintained as described above in the Cell Culture section. Induction of TAM67 was obtained by withdrawal of DOX for 3–5 days. Then, total cell lysates were collected. Lysates with equal amounts of total protein were precleared with 50 μl of protein G-agarose for 30 min at 4°C and then incubated overnight with 1 μg of antibodies against individual Jun and Fos proteins. All antibodies were procured from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Protein G-agarose was added for another 4 h and the beads were pelleted and washed three times with PBS. Bound proteins were eluted in SDS sample buffer, subjected to SDS–PAGE, and analysed by regular Western blot as described above.

Statistical analysis

The results of colony formation assays were expressed as mean number of colonies ± s.e. Statistical significance was determined using the Student's *t*-test. Single-cell proliferation assays produced results that showed a distribution of cells per colony. For this assay, Wilcoxon's rank-sum tests were used to compare distributions between antisense cJun- and vector-transfected cells, or antisense cFos- and vector-transfected cells.

Abbreviations

ATF, activating transcription factor; AP-1, activating protein-1; Tam67, cJun dominant-negative mutant; DOX, doxycycline; HRG-β, heregulin-beta; TPA, phorbol 12-*O*-tetradecanoate-13-acetate.

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