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## Introduction

The purpose of this research was to characterize growth inhibition by Interleukin-4 (IL-4) in breast cancer cells and identify key signaling molecules which may be targets for future strategies to enhance the negative growth effects of IL-4. We had previously characterized IL-4 receptor expression in human breast cancer cell lines and identified an increase in apoptosis associated with IL-4-mediated growth inhibition (publication included). We considered that IL-4 may be signaling through two pathways : the IRS-1 pathway which could potentially be share with the insulin-like growth factor (IGF) mitogenic pathway, and the STAT6 transcription pathway. After inhibiting IRS-1 protein with inducible anti-sense IRS-1 fragment expression, we concluded that IRS-1 is required for IGF-I-mediated growth effects, but not IL-4-mediated growth effects (manuscript in preparation). Therefore, we examined the role of STAT6 and found that inhibition of STAT6 through expression of a dominant-negative STAT6 protein resulted in a loss of IL-4-mediated growth inhibition and apoptosis (manuscript included). Therefore, we have found that IL-4-mediated growth inhibition and induction of apoptosis in human breast cancer cells requires STAT6 and not IRS-1. Therefore, we can direct future experiments to further characterization and manipulation of STAT6 to enhance IL-4-mediated growth effects.

## Summary

One group of cytokines which may play an important role in modulating tumor growth *in vivo* is the cytokines which are secreted by tumor-infiltrating lymphocytes. Interleukin-4 (IL-4) is one such cytokine and has been reported to have an inhibitory effect on the growth of some transformed cells including renal, small cell lung, colon and breast cancer cell lines. Therefore, we examined the effects of IL-4 on human breast cancer cell lines. We found that breast cancer cell lines expressed IL-4 receptor (IL-4R) and that two lines, MCF-7 and MDA-MB-231, were inhibited by IL-4 in both monolayer and anchorage-independent growth. This inhibition was dependent upon interaction with the receptor. Importantly, IL-4 only inhibited proliferation of cells, and had no effect on basal, unstimulated cell growth. We also demonstrated that IL-4 inhibited estrogen-stimulated growth but not IGF-I-stimulated growth. Based on our previous finding that IGF-I can act to block apoptosis in breast cancer cells, we hypothesized that IL-4 was inhibiting cells growth through the induction of apoptosis. Accordingly, we show that IL-4-induced growth inhibition is associated with an increase in apoptosis. Finally, we show that IGF-I can inhibit IL-4-induced apoptosis.

We have previously reported that insulin receptor substrate-1 (IRS-1) is the predominant signaling molecule activated by IGF-I, insulin and IL-4 in MCF-7 breast cancer cells. As IGF-I can act as a proliferative and anti-apoptotic agent while IL-4 inhibits growth and induces apoptosis, it is an intriguing hypothesis that IRS-1 can act in both proliferative and apoptotic signaling pathways in human breast cancer cells. To test this hypothesis, we generated MCF-7 cells which are stably transfected with an anti-sense IRS-1 fragment under the control of a tetracycline-inducible promoter. We found that IRS-1 mRNA and protein were normal in the absence of tetracycline, but were

reduced by 40-60% in the presence of tetracycline. Using several inducible anti-sense clones and a vector alone transfected clone, we evaluated the result of IRS-1 inhibition on IGF-I and IL-4-mediated signaling and growth effects. We found that inhibition of IRS-1 resulted in less IGF-I and IL-4-induced phosphorylation of IRS-1. Interestingly, IRS-2 phosphorylation increased. Further, signaling to two down-stream targets, Erk1/Erk2 MAPK and the PI-3 kinase substrate, Akt, was reduced in the presence of anti-sense IRS-1 expression. Finally, we found that IGF-I-mediated proliferation and protection from apoptosis was impaired when IRS-1 was inhibited. In contrast, IL-4-mediated growth inhibition and induction from apoptosis was not blocked by IRS-1 inhibition. Therefore, IGF-I-mediated growth effects require IRS-1 in human breast cancer cells while IL-4-mediated growth effects are associated with, but independent of, IRS-1 activation.

In addition to IRS-1, IL-4-treatment also induces phosphorylation of the transcription factor, STAT6. Having ruled out IRS-1 as a required signaling element for IL-4 in breast cancer cells, we next determined the role of STAT-6 in IL-4-mediated growth effects. We first characterized STAT6 activation and found that STAT6 DNA binding is activated in response to IL-4 in several breast cancer cell lines. Further, STAT6 activation occurs in the absence of IRS-1, indicating that the two pathways are independent. We next created expression constructs for full-length STAT6 from a mouse cDNA (mSTAT6) as well as a truncated construct ( $\Delta$ STAT6(645)) which lacks the transactivation domain. This previously characterized mutation results in a STAT6 protein with dominant-negative activity. Accordingly, IL-4-mediated activation of a luciferase reporter construct was enhanced by transient expression of mSTAT6 and inhibited by transient expression of  $\Delta$ STAT6(645). Using a single-cell proliferation assay, we found that transient expression of  $\Delta$ STAT6(645) blocked IL-4-mediated growth inhibition and induction of apoptosis. In addition,

expression of mSTAT6 significantly enhanced the basal rate of apoptosis, with a further small increase by IL-4. Finally, we found that expression of mSTAT6 inhibits foci formation of MCF-7 cells, but expression of  $\Delta$ STAT6(645) does not. Furthermore, mSTAT6-mediated inhibition of foci formation can be rescued by addition of excess  $\Delta$ STAT6(645). These result suggest that STAT6 is required for IL-4-mediated growth inhibition and induction of apoptosis in human breast cancer cells.

# Interleukin 4 Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells<sup>1</sup>

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## ABSTRACT

Interleukin-4 (IL-4) is a pleiotropic cytokine produced by mast cells and T lymphocytes that promotes proliferation and immunoglobulin class-switching in B cells. IL-4 receptors (IL-4Rs) are also expressed by nonhematopoietic cells as well as some tumor cells. Unlike its mitogenic effect on B cells, IL-4 inhibits the growth of some cancer cells *in vitro*. In this study, we show that IL-4R is expressed by breast and ovarian cancer cell lines. Furthermore, anchorage-dependent and -independent growth of breast cancer cell lines MCF-7 and MDA-MB-231 is inhibited by IL-4 treatment, and this effect requires IL-4R. Interestingly, IL-4 only inhibited proliferating breast cancer cells and had no effect on basal, unstimulated growth. We therefore characterized the effect of IL-4 on breast cancer cell growth stimulated by either estradiol or insulin-like growth factor I (IGF-I). In both anchorage-dependent and -independent growth assays, IL-4 inhibited estradiol-stimulated growth. The antiestrogen effect of IL-4 was not due to IL-4 interference with the estrogen receptor, because IL-4 did not interfere with estrogen receptor-mediated reporter gene transactivation. In contrast, IL-4 had no effect on IGF-I-stimulated proliferation. Because IGF-I is known to inhibit programmed cell death, we examined apoptosis as a possible mechanism of IL-4 action. We established that IL-4 induced apoptosis in breast cancer cells by five independent criteria: (a) morphological indicators including pyknotic nuclei and cytoplasmic condensation; (b) DNA fragmentation; (c) the formation of DNA laddering; (d) the cleavage of poly(ADP-ribose) polymerase; and (e) the presence of cells with sub-G<sub>1</sub> DNA content. IL-4 increased the percentage of apoptotic cells in MCF-7 and MDA-MB-231 cells 6.0- and 6.7-fold over that of the control, respectively. Finally, the addition of IGF-I reversed IL-4-induced apoptosis, suggesting that the mechanism of IL-4-induced growth inhibition in human breast cancer cells is the induction of programmed cell death.

## INTRODUCTION

Breast cancer cell growth is controlled by a complex network of steroid hormones, peptide growth factors, and cytokines that induce a myriad of growth effects. The most important proliferative agents include estrogen, IGF-I,<sup>3</sup> and epidermal growth factor (1, 2). In addition, many other molecules contribute to the regulation of breast tumor growth by inducing differentiation as well as growth inhibition. One group of molecules that may be important in the regulation of breast cancer cell growth is the group of cytokines secreted by tumor-infiltrating lymphocytes. IL-4, IL-6, and IL-10 have been reported to be produced by activated T cells found to be associated with

breast tumors (3, 4). IL-4 is of particular interest, because it has been reported to be growth inhibitory to breast cancer cells *in vitro* (5-7).

IL-4 is a pleiotropic cytokine produced by mast cells and T lymphocytes and acts primarily on B cells, in which it induces proliferation and immunoglobulin class-switching (8). IL-4 acts via a multi-unit transmembrane receptor, the IL-4R (9, 10), which has also been found on a number of nonhematopoietic cells including some tumor cells (11). In contrast to its mitogenic role in B cells, IL-4 has been reported to be growth inhibitory to some cancer cells *in vitro*. Indeed, tumor cells engineered to secrete IL-4 have been shown to have decreased growth *in vitro* and *in vivo* (12-14). In addition, IL-4 has been shown to have direct antiproliferative effects on some tumor cells including renal carcinomas (15), lung carcinomas (16), gastric carcinomas (17), and breast carcinomas (5-7).

Whereas growth inhibition due to IL-4 has been reported, the role of the IL-4R and the mechanism for IL-4-induced growth inhibition have not been well characterized, particularly in breast cancer cells. Therefore, the purpose of this study was to characterize IL-4R expression and utilization by IL-4 in human breast cancer cells, to determine the effect of IL-4 on breast cancer cell growth including E<sub>2</sub>- and IGF-I-stimulated growth, and to examine apoptosis as a possible mechanism of growth inhibition. We showed by monolayer and anchorage-independent growth assays that IL-4 was growth inhibitory and acted via IL-4R expressed by breast cancer cells. In addition, we showed that IL-4 inhibited the E<sub>2</sub>-stimulated growth of MCF-7 cells in monolayer and anchorage-independent growth assays but had no effect on IGF-I-stimulated growth. Because IGF-I is known to inhibit programmed cell death, we hypothesized that IL-4 was inducing apoptosis. Accordingly, we showed that IL-4-induced growth inhibition is associated with an increase in apoptosis, and that the induction of apoptosis by IL-4 is inhibited by the addition of IGF-I.

## MATERIALS AND METHODS

### Materials

MCF-7 cells were provided by C. Kent Osborne (University of Texas Health Science Center, San Antonio, TX) and grown in IMEM (Life Technologies, Inc., Bethesda, MD) with phenol red plus 5% fetal bovine serum (Summit, Fort Collins, CO). MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in IMEM without phenol red plus 5% fetal bovine serum. MDA-10A cells were provided by V. Craig Jordan (Northwestern University Medical Center, Chicago, IL). IL-4 was a gift of Satwant Narula (Schering-Plough Research Institute, Kenilworth, NJ). IL-4R-neutralizing Ab was provided by Dr. Kathleen dePicha (Immunex, Inc., Seattle, WA). IL-4R cDNA was provided by Kevin Moore (DNAX Research Institute, Palo Alto, CA). IGF-I was obtained from GroPep (Adelaide, Australia).

### Cell Growth Assays

**Monolayer Growth.** Growth assays were performed by MTT assay as described previously (18). MCF-7 cells were plated in triplicate at a density of 18,000 cells/well, and MDA-MB-231 cells were plated in triplicate at a density of 12,000 cells/well in 24-well cell culture plates. Cells were allowed to adhere overnight and then washed once in 1 × PBS; the culture medium was replaced with SFM overnight. After treatment, 60 μl of MTT (5 mg/ml in PBS) were added to the medium for 4 h. Medium and MTT were then removed, DMSO and 2.5% DMEM were added, and the absorbance was measured at 540 nm.

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<sup>3</sup> The abbreviations used are: IGF-I, insulin-like growth factor I; IL, interleukin; IL-4R, IL-4 receptor; ER, estrogen receptor; PARP, poly(ADP-ribose) polymerase; IMEM, improved MEM; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ERE, estrogen-responsive element; SFM, serum-free medium; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; CSS, charcoal-stripped serum; Ab, antibody; E<sub>2</sub>, estradiol.

**Anchorage-independent Growth.** MCF-7 cells were plated at a density of  $1.5 \times 10^4$  cells/plate, and MDA-MB-231 cells were plated at  $7.5 \times 10^3$  cells/plate. Cultures were prepared with a base layer of IMEM supplemented with 20% FCS and containing 0.5% low-melting point agarose (Sea Plaque; FMC Bioproducts, Rockland, ME). MCF-7 and MDA-MB-231 cells were then plated over the base layer in duplicate in IMEM supplemented with 10% FCS and containing 0.5% low melting-point agarose. In addition, cultures were prepared with a base layer of IMEM supplemented with 10% CSS containing 0.5% low-melting point agarose. MCF-7 cells were then plated over the base layer in duplicate in IMEM supplemented with 5% CSS and containing 0.5% low-melting point agarose. IL-4 was added at a concentration of 10 ng/ml, and IL-4R Ab was added as indicated.  $E_2$  was added at a final concentration of  $10^{-9}$  M. Cells were allowed to grow for 7–10 days before colonies of at least 20 cells were counted.

**RNase Protection Assay**

Total RNA from breast and ovarian cancer cell lines cultured in serum-supplemented media was prepared by the guanidinium thiocyanate method (19), measured by spectrophotometry, and checked for integrity by separation on a 1% formaldehyde-agarose gel. RNase protection was performed according to our previously published method (20), and RNA loading was corrected with the ribosomal protein 36B4 (21). Briefly, 20  $\mu$ g of RNA were hybridized with radiolabeled antisense cRNAs transcribed from the IL-4R and 36B4 cDNAs. The RNase protection probe was generated by PCR from an expression vector containing the IL-4R cDNA and a COOH-terminal flag sequence (Eastman Kodak, Rochester, NY). IL-4R fragment PCR was performed using a 5' primer containing a *EcoRI* restriction site (5'-GGTAGAGGACATGCCAAAGC) approximately 400 bp upstream of the stop codon and a 3' primer containing a *XbaI* restriction site followed by the flag sequence. The resulting 400-bp fragment was subcloned into pGEM4Z by a restriction digest. pGEM4Z-IL-4R was linearized with *EcoRI*, and transcription with T7 RNA polymerase was carried out in the presence of [ $^{32}$ P]UTP to produce labeled antisense cRNA. For 36B4, a 145-bp *PstI-PstI* fragment was cloned into pGEM4Z, linearized with *EcoRI*, and transcribed with T7 RNA polymerase. After hybridization of RNA with a radiolabeled probe, single-stranded RNA was digested with RNase A, and samples were separated on 8 M urea/6% SDS-PAGE. tRNA was hybridized as a negative control. The gel was dried and exposed to X-ray film.

**Luciferase Assay**

MCF-7 cells that had been stably transfected with ERE-luciferase reporter construct were a kind gift of Dr. Michel Pons (INSERMU-58, Montpellier, France; Ref. 22). Cells were treated with  $E_2$  ( $10^{-9}$  M), IL-4 (10 ng/ml), or IGF-I (5 nM) as indicated for 48 h. Cell lysates were collected by three cycles of freeze-thawing in 1 $\times$  reporter lysis buffer (Promega, Madison, WI). Total protein was measured by Bio-Rad (Hercules, CA) assay, and luciferase activity was determined using the luciferase assay system from Promega.

**Apoptosis Assays**

For all apoptosis assays, MDA-MB-231 cells were treated in SFM alone, whereas MCF-7 cells were treated in SFM supplemented with 0.25% FCS.

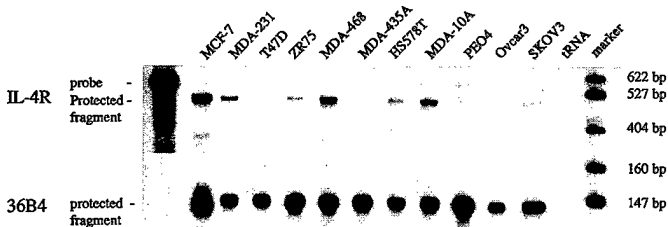


Fig. 1. RNase protection assay of IL-4R mRNA expression in breast cancer and ovarian cancer cell lines. Samples (20  $\mu$ g) of RNA from eight breast cancer and three ovarian cancer cell lines were examined for IL-4R mRNA by RNase protection assay. MDA-MB-10A is a derivative of the MDA-MB-231 cell line. tRNA was included as a negative control, and 36B4 was included as a loading control.

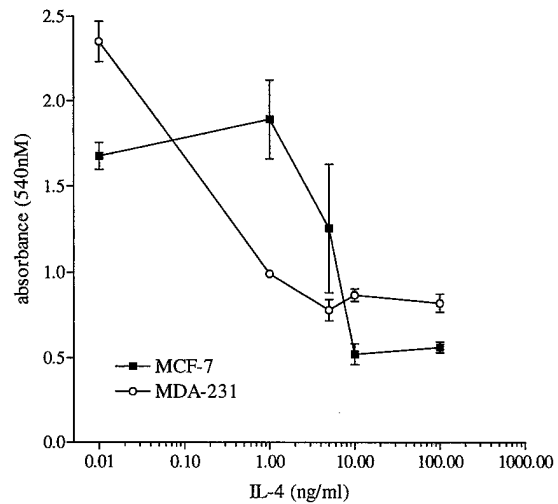


Fig. 2. Monolayer growth and dose response of MCF-7 and MDA-MB-231 cells to IL-4. MCF-7 and MDA-MB-231 cells were plated in triplicate in 24-well plates and then treated with increasing concentrations of IL-4. Growth was determined by the MTT assay after 3 days. ■, MCF-7 cells; ○, MDA-MB-231 cells. MCF-7 cells were cultured in SFM plus 0.25% serum, whereas MDA-MB-231 cells were cultured in SFM alone.

**H&E and TUNEL Staining.** Cells were plated in 8-well chamber slides (Nunc, Inc., Naperville, IL) at a density of  $7.5 \times 10^3$  cells and allowed to adhere overnight. SFM was placed on the cells overnight, and cells were then treated with IL-4 or SFM alone. IL-4 was added at 50 ng/ml for 48 h. Chambers were then removed, and slides were fixed in 70% ethanol. A TUNEL assay was performed following the manufacturer's instructions using the TACS 2tdt kit from Trevigen, Inc. (Gaithersburg, MD).

**DNA Laddering.** Cells were plated in 10-cm dishes and allowed to grow to 70% confluence. Medium was removed, cells were washed once with 1 $\times$  PBS, and SFM was added before treatment with 10 ng/ml IL-4 or 1  $\mu$ g/ml paclitaxel. After 4 days, the cells were harvested in trypsin-EDTA, washed with 1 $\times$  PBS, and then lysed in 0.15 M NaCl, 10 mM Tris-HCl (pH 7.8), 2 mM  $MgCl_2$ , 1 mM DTT, and 0.5% NP40 on ice for 40 min. Nuclei were collected by centrifugation at 1,500 rpm for 10 min and lysed in 0.35 M NaCl, 10 mM Tris-HCl, 1 mM  $MgCl_2$ , and 1 mM DTT on ice for 20 min. Nuclear lysates were extracted once with phenol-saturated chloroform, and fragmented DNA was precipitated with 0.01 M  $MgCl_2$  and 2.5 volumes of ethanol at  $-20^\circ C$  overnight. DNA was collected by centrifugation at 16,000 rpm for 30 min. DNA was resuspended in 50  $\mu$ l of Tris-EDTA with 0.1 mg/ml RNase A for 1 h at  $37^\circ C$  followed by a 1-h digestion with 1 mg/ml proteinase K. DNA was then electrophoresed in 1.5% agarose gels containing ethidium bromide.

**PARP Cleavage.** Cells were treated with 10 ng/ml IL-4 or 1  $\mu$ g/ml paclitaxel for 0, 2, and 4 days and harvested with trypsin-EDTA, pelleted, and washed with 1 $\times$  PBS. Protein from MDA-MB-231 cells was extracted using a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1% NP40, 100 mM NaCl, 100 mM sodium orthovanadate, 100  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml aprotinin, and  $10^{-7}$  M phenylmethylsulfonyl fluoride. Protein (50  $\mu$ g) was analyzed by 7.5% SDS-PAGE. After transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk Tris-buffered Saline-Tween and then immunoblotted with a 1:1000 dilution of the anti-PARP Ab (Boehringer Mannheim, Indianapolis, IN). Horseradish peroxidase-conjugated goat-anti-rabbit secondary Ab was added at a 1:2000 dilution, and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

**Flow Cytometry.** Cells were treated with 10 ng/ml IL-4 or 1  $\mu$ g/ml paclitaxel as indicated for 48 h. Cells were harvested with trypsin-EDTA and washed with 1 $\times$  PBS. Cell pellets were then resuspended in 200  $\mu$ l of 1 $\times$  PBS, and 200  $\mu$ l of ice-cold 70% ethanol were added dropwise while vortexing. Cells were fixed overnight at  $4^\circ C$ , and then 0.5 ng/ml propidium iodide and 0.5 mg/ml RNase A were added. The resulting histograms were analyzed using ModfitLT software (Verity House, Topsham, ME). Apoptotic cells were determined as the percentage of cells with a DNA content of less than 2 N.

**Statistics**

Growth in soft agar was analyzed using Student's *t* test, and *P*s were determined for a two-tailed test with 2 degrees of freedom. The results of the flow cytometric quantitation of apoptosis were also analyzed using Student's *t* test, and *P*s were determined for a two-tailed test with 4 degrees of freedom.

**RESULTS**

**IL-4 Acts via the IL-4R and Induces Growth Inhibition in Breast Cancer Cells.** IL-4 is known to act at a multiunit transmembrane receptor, IL-4R. IL-4R is composed of two subunits: (a) the ligand-binding chain, IL-4R $\alpha$ ; and (b) the  $\gamma$ -common chain (23, 24). It has been reported that IL-4R $\alpha$  is necessary for IL-4 activity but may also act independently of the  $\gamma$ -common chain (25, 26). Therefore, to determine whether IL-4 may act via the IL-4R, we examined the expression of IL-4R $\alpha$  mRNA in breast cancer cell lines using the RNase protection assay. Fig. 1 shows that IL-4R $\alpha$  mRNA was ex-

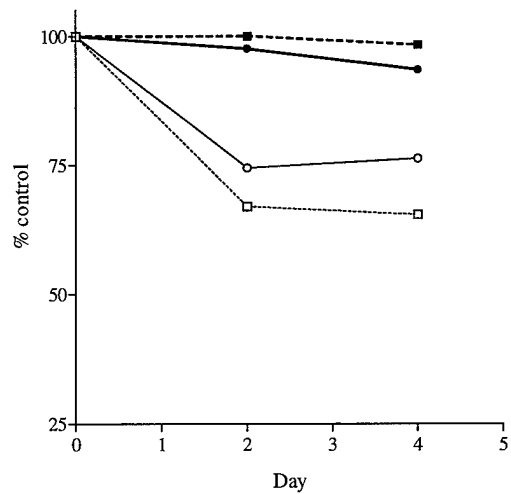
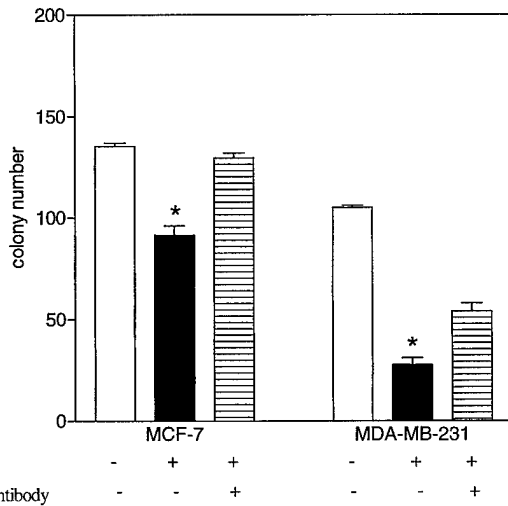


Fig. 4. IL-4 growth inhibition of MCF-7 and MDA-MB-231 cells was prevented by IL-4R-neutralizing Ab. □, MCF-7 cells treated with IL-4; ■, MCF-7 cells treated with IL-4 + IL-4R-neutralizing Ab; ○, MDA-MB-231 cells treated with IL-4; ●, MDA-MB-231 cells treated with IL-4 + IL-4R neutralizing Ab. MCF-7 and MDA-MB-231 cells were preincubated with an IL-4R-neutralizing Ab. MDA-MB-231 cells were cultured in SFM, and MCF-7 cells were cultured in SFM plus 0.25% serum. IL-4 (10 ng/ml) was added, and growth was measured by MTT assay at days 0, 2, and 4. Data shown are representative of three separate experiments.

A.



B.

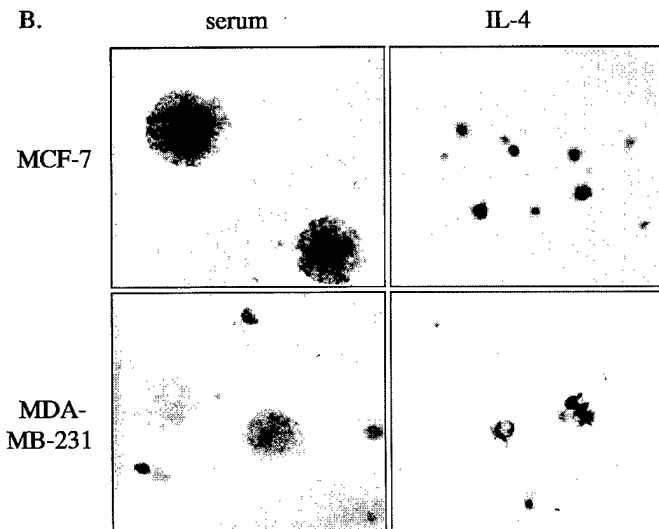


Fig. 3. Effect of IL-4 on anchorage-independent growth of MCF-7 and MDA-MB-231 cells. A, MCF-7 and MDA-MB-231 cells were plated in duplicate in soft agar containing a final concentration of 10% serum. IL-4 was added at 10 ng/ml, and IL-4R-neutralizing Ab was added at 200 and 500 ng/ml for MCF-7 and MDA-MB-231 cells, respectively. Colony formation was determined after 10 days. Data shown are representative of five separate trials. Error bars, mean  $\pm$  SE of duplicate samples. B, MCF-7 and MDA-MB-231 colonies treated with serum alone or serum plus IL-4 at  $\times 40$  magnification.

pressed by six of seven breast cancer cell lines (the MDA-10A cell line is a derivative of MDA-MB-231) and two of three ovarian cancer cell lines.

IL-4 has been reported to be growth inhibitory to some cancer cells *in vitro* (5-7, 15-17). To determine the effect of IL-4 on breast cancer cell growth, we treated MCF-7 and MDA-MB-231 cells with IL-4 in SFM over a course of 4 days. We found that IL-4 had no effect on MCF-7 cell growth when cells were cultured in SFM alone (data not shown). However, MCF-7 cells are hormone- and growth factor-dependent cells, and no basal growth was observed in SFM under our experimental conditions. The addition of 0.25% FCS resulted in proliferation. Under these conditions, IL-4 inhibited MCF-7 cell growth over a range of concentrations (Fig. 2). MDA-MB-231 cells are hormone- and growth factor-independent breast cancer cells, and proliferation was consistently observed in SFM. In these cells, IL-4 also inhibited growth over a range of concentrations (Fig. 2). Approximately 10 ng/ml IL-4 resulted in optimal inhibition in both cell lines. In addition, we examined the growth of MDA-MB-435A cells after IL-4 treatment and found that the cell line was unaffected by IL-4 (data not shown). This is consistent with the observation that MDA-MB-435A cells did not express detectable IL-4R mRNA.

We then examined the ability of IL-4 to inhibit colony formation of MCF-7 and MDA-MB-231 cells in an anchorage-independent growth assay. MCF-7 and MDA-MB-231 cells were plated in soft agar containing FCS with or without 10 ng/ml IL-4. Colony formation in both cell lines was significantly ( $P < 0.02$  and  $P < 0.001$  for MCF-7 and MDA-MB-231 cells, respectively) reduced by IL-4 (Fig. 3A). In addition to fewer colonies, IL-4 treatment also reduced the size and changed the appearance of colonies (Fig. 3B).

To determine whether the interaction of IL-4 and IL-4R was required for growth inhibition, we used a neutralizing Ab to the ligand-binding chain of the receptor (27, 28). MCF-7 and MDA-MB-231 cells were grown in monolayer culture and preincubated with Ab before IL-4 treatment. We found that IL-4-mediated growth inhibition was prevented by blocking the IL-4 ligand-binding chain with neutralizing Ab (Fig. 4). As controls, an equal concentration of a non-specific monoclonal Ab did not prevent IL-4-induced growth inhibition, and incubation with IL-4R Ab alone was not toxic to the cells

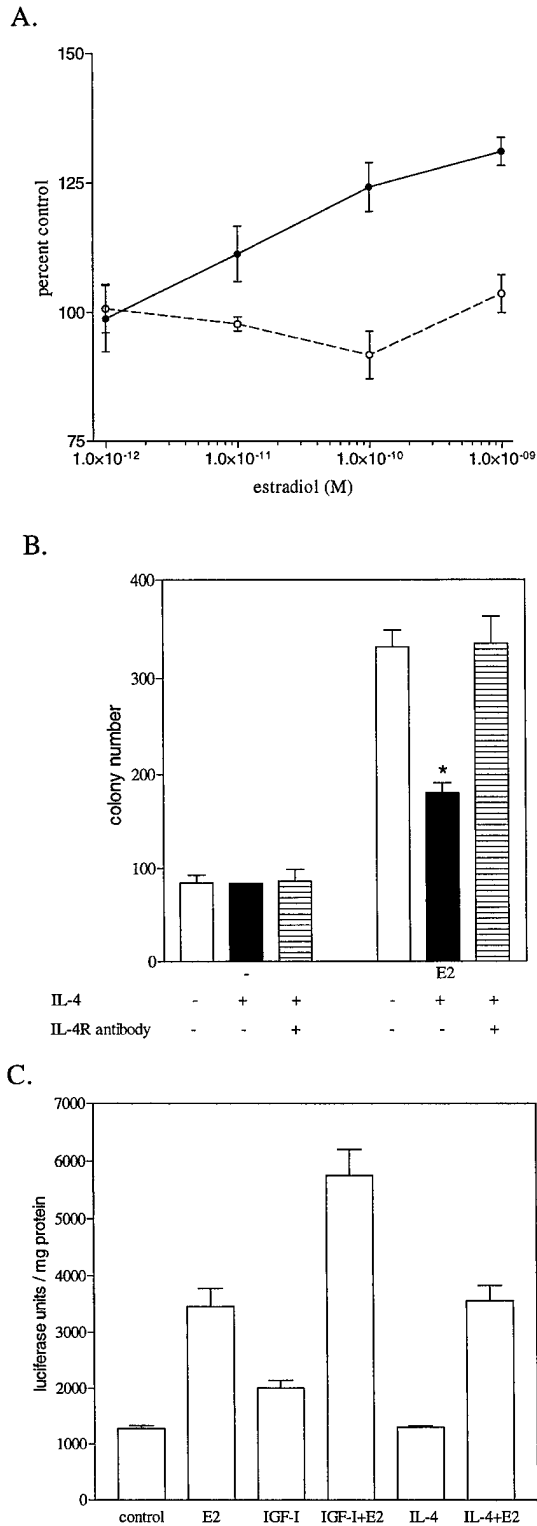


Fig. 5. Effect of IL-4 on E<sub>2</sub>-stimulated growth and ER-mediated gene transcription. A, MCF-7 cells were plated in SFM and treated with increasing concentrations of E<sub>2</sub> alone or with IL-4 (10 ng/ml), and growth was measured after 3 days by the MTT assay (●, E<sub>2</sub>; ○, E<sub>2</sub> plus IL-4). Data shown are representative of two separate experiments. Error bars, mean ± SE of quadruplicate samples. B, MCF-7 cells were plated in a final concentration of 5% CSS alone or with E<sub>2</sub> (10<sup>-9</sup> M) and treated with 10 ng/ml IL-4. IL-4R-neutralizing Ab was added at 200 ng/ml, and colony formation was determined after 10 days. Data shown are representative of four separate trials. □, CSS alone; ■, CCS plus E<sub>2</sub>; ▨, CSS plus E<sub>2</sub> and IL-4R neutralizing Ab. Error bars represent the mean ± SE of duplicate samples, asterisks indicate a statistically significant difference between IL-4 treatment and control. C, MCF-7 cells that had been stably transfected with an ERE-luciferase construct were treated with E<sub>2</sub>, IGF-I, IGF-I plus E<sub>2</sub>, IL-4, or IL-4 plus E<sub>2</sub> for 24 h. Luciferase units were measured and then normalized by the total protein. The result shown is representative of three separate trials. Error bars, mean ± SE of triplicate samples.

(data not shown). In the anchorage-independent growth assay, we again confirmed that IL-4 was acting via the IL-4R by adding neutralizing Ab to IL-4-treated cells before plating in soft agar. The addition of 200 ng/ml Ab to MCF-7 cells completely reversed the growth inhibition observed after IL-4 treatment alone, whereas 500 ng/ml Ab partially reversed the IL-4 inhibition of colony formation in MDA-MB-231 cells (Fig. 3A). Therefore, the growth-inhibitory effects of IL-4 seem to be mediated specifically through the IL-4R.

**IL-4 Inhibits E<sub>2</sub>-stimulated Growth but Does Not Affect ER-mediated Gene Transcription.** MCF-7 cells require estrogen or peptide growth factors such as IGF-I for optimal monolayer growth. Fig. 2 shows that the growth of MCF-7 cells was inhibited by IL-4 when proliferation was induced by the addition of serum. We were therefore interested in finding out whether proliferation induced by either E<sub>2</sub> or IGF-I was sensitive to growth inhibition by IL-4.

MCF-7 cells in monolayer culture were treated with increasing concentrations of E<sub>2</sub> alone or E<sub>2</sub> plus IL-4 for 3 days. IL-4 inhibited E<sub>2</sub>-stimulated growth under these conditions (Fig. 5A). We then used an anchorage-independent growth assay to confirm the IL-4-mediated inhibition of E<sub>2</sub>-stimulated growth. MCF-7 cells were plated in soft agar containing either CSS alone or CSS plus E<sub>2</sub>. As in our monolayer growth experiments, IL-4 had no effect on basal, unstimulated colony formation in CSS alone. However, in the presence of E<sub>2</sub>, IL-4 treatment significantly (*P* < 0.01) reduced the colony size and number (Fig. 5B). Again, this effect was prevented by the addition of IL-4R-neutralizing Ab.

Because E<sub>2</sub> exerts its effects through dimerization of ERs, DNA binding, and transactivation at EREs (29), we investigated the possibility that IL-4 inhibits E<sub>2</sub>-stimulated growth by disrupting ER transactivation. We treated MCF-7 cells that had been stably transfected with an ERE-luciferase reporter construct with E<sub>2</sub>, IGF-I, IGF-I plus E<sub>2</sub>, IL-4, or IL-4 plus E<sub>2</sub> for 24 h and measured luciferase activity. We have shown previously that IGF-I alone stimulates transcription, and IGF-I plus E<sub>2</sub> further enhances the transcriptional activity of the ER to levels above that of either E<sub>2</sub> or IGF-I alone (30). Therefore, IGF-I was included in this experiment as a positive control for ER transactivation. IL-4 treatment of MCF-7 cells had no effect on the E<sub>2</sub>-stimulated induction of luciferase, because there was no statistically significant difference between control and IL-4 alone or E<sub>2</sub> and IL-4 plus E<sub>2</sub> (Fig. 5C). Thus, IL-4 does not seem to exert its effects by

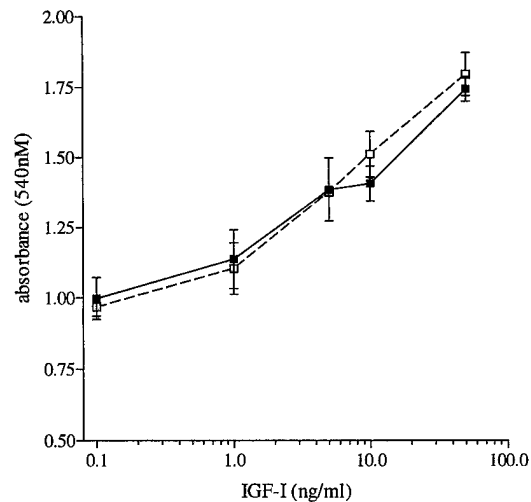


Fig. 6. IL-4 has no effect on IGF-I-stimulated growth. ■, IGF-I; □, IGF-I plus IL-4. MCF-7 cells were plated in SFM and treated with increasing concentrations of IGF-I alone or with IL-4 (10 ng/ml), and growth was measured after 2 days by the MTT assay. Error bars, mean ± SE of quadruplicate samples.

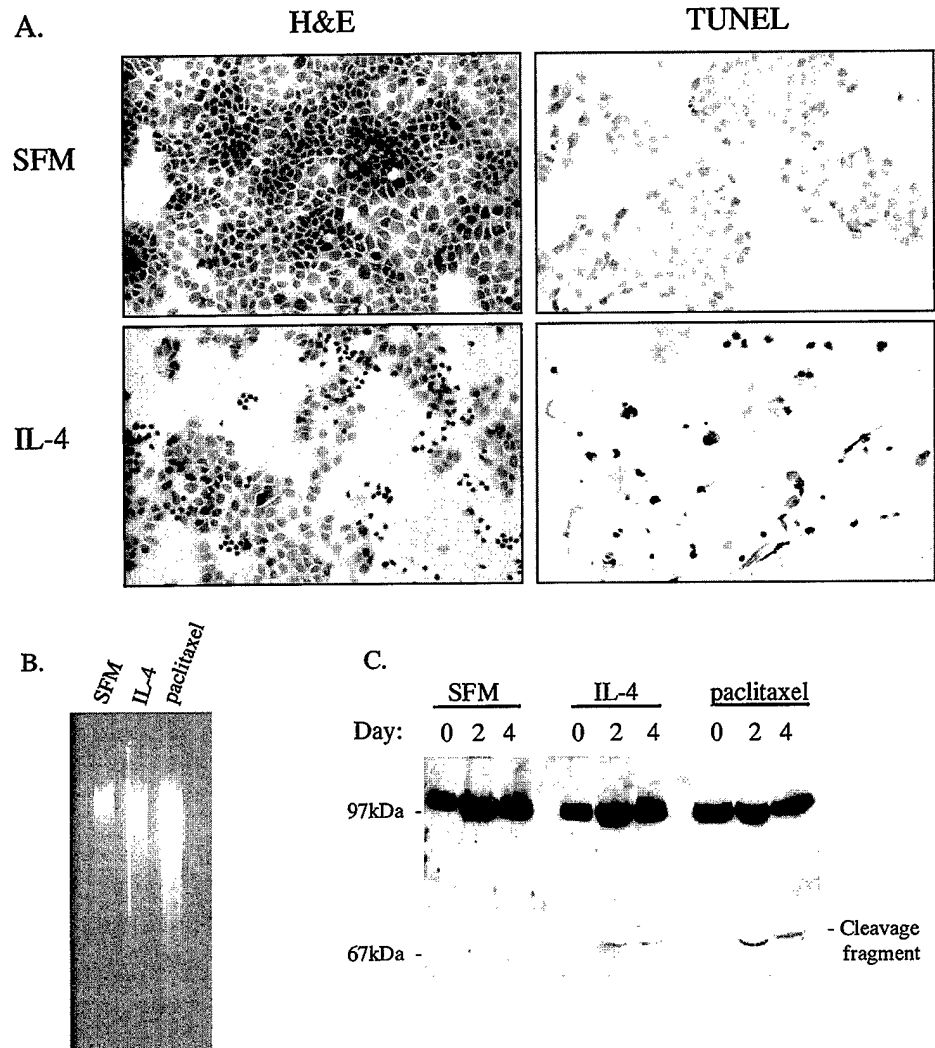


Fig. 7. H&E, TUNEL, DNA laddering, and PARP cleavage assays on IL-4-treated MCF-7 and MDA-MB-231 cells. *A*, MCF-7 cells were treated with SFM plus 0.25% serum alone or with IL-4 and grown on a chamber slide. After 48 h, the slide was fixed in 70% ethanol and stained with H&E or labeled by the TUNEL assay. *B*, MDA-MB-231 cells were treated with SFM, IL-4, or paclitaxel for 4 days. Fragmented DNA was collected and separated by agarose gel electrophoresis. *C*, MDA-MB-231 cells were treated with SFM, IL-4, or paclitaxel, and 50  $\mu$ g of total protein were separated by SDS-PAGE. Proteins were immunoblotted with an Ab against PARP. Paclitaxel-treated samples were included as a positive control for apoptosis.

disruption of the ER transactivation of EREs. It is therefore likely that IL-4 inhibits estrogen-stimulated growth via an interaction with events distal to ER activation.

Previous work has shown that E<sub>2</sub> and IGF-I stimulate MCF-7 monolayer cell growth with approximately equal effects (31, 32). Whereas IL-4 inhibited estrogen-stimulated growth, IL-4 had no effect on IGF-I-stimulated growth in monolayer culture (Fig. 6). One possible explanation for this difference is that in addition to stimulating proliferation, IGF-I has been described to induce antiapoptotic mechanisms in cancer cells (33, 34). Therefore, we considered the possibility that IL-4-induced growth inhibition was due to the induction of apoptosis.

**IL-4 Induces Apoptosis.** To determine whether IL-4 increases the rate at which the breast cancer cells undergo apoptosis, we first examined IL-4-treated MCF-7 cells and untreated controls with H&E and performed a TUNEL assay. H&E-stained IL-4-treated breast cancer cells showed morphological indicators of apoptosis including reduction of the cytoplasm and chromatin condensation. Furthermore, a greater fraction of IL-4-treated cells was positive for fragmented DNA by the TUNEL assay (Fig. 7A). IL-4-treated MDA-MB-231 cells also exhibited morphological indicators of apoptosis as well as positive staining for DNA fragmentation by the TUNEL assay (data not shown). We then confirmed that fragmented DNA detected by the TUNEL assay resulted from apoptosis. We treated MDA-MB-231 cells with IL-4, SFM, or paclitaxel for 4 days and then collected

fragmented DNA from each sample. DNA laddering was observed in the presence of paclitaxel and IL-4 but not in SFM controls (Fig. 7B). Similar results were obtained for MCF-7 cells cultured with SFM, IL-4, or paclitaxel supplemented with 0.25% serum (data not shown). Paclitaxel was included as a positive control for apoptosis (35). In addition, we determined the cleavage of the IL-1 $\beta$ -converting enzyme substrate, PARP, by Western blotting (36, 37). Immunoblotting with an Ab against the COOH-terminal portion of PARP demonstrated a *M<sub>r</sub>* 116,000 intact PARP protein as well as a *M<sub>r</sub>* 85,000 apoptosis-specific cleavage fragment in paclitaxel and IL-4-treated MDA-MB-231 cell lysates (Fig. 7C).

We then analyzed propidium iodide-stained MCF-7 and MDA-MB-231 cells by flow cytometry. This is a convenient method for quantifying apoptotic cells, because each cell can be scored for DNA content, and the percentage of apoptotic cells corresponds to the percentage of cells with a DNA content of less than 2n. MDA-MB-231 cells were treated with SFM alone, IL-4, or paclitaxel, whereas MCF-7 cells were treated with SFM, IL-4, or paclitaxel with the addition of 0.25% serum. Paclitaxel was again included in the experiment as a positive control for apoptosis. IL-4 resulted in a 6.0- and 6.7-fold increase in apoptosis over that of untreated controls in MCF-7 and MDA-MB-231 cells, respectively (Fig. 8A).

We hypothesized that if IL-4-induced growth inhibition was in fact due to apoptosis, the addition of IGF-I would rescue the cells. Specifically, we anticipated that IGF-I would rescue IL-4-induced apop-

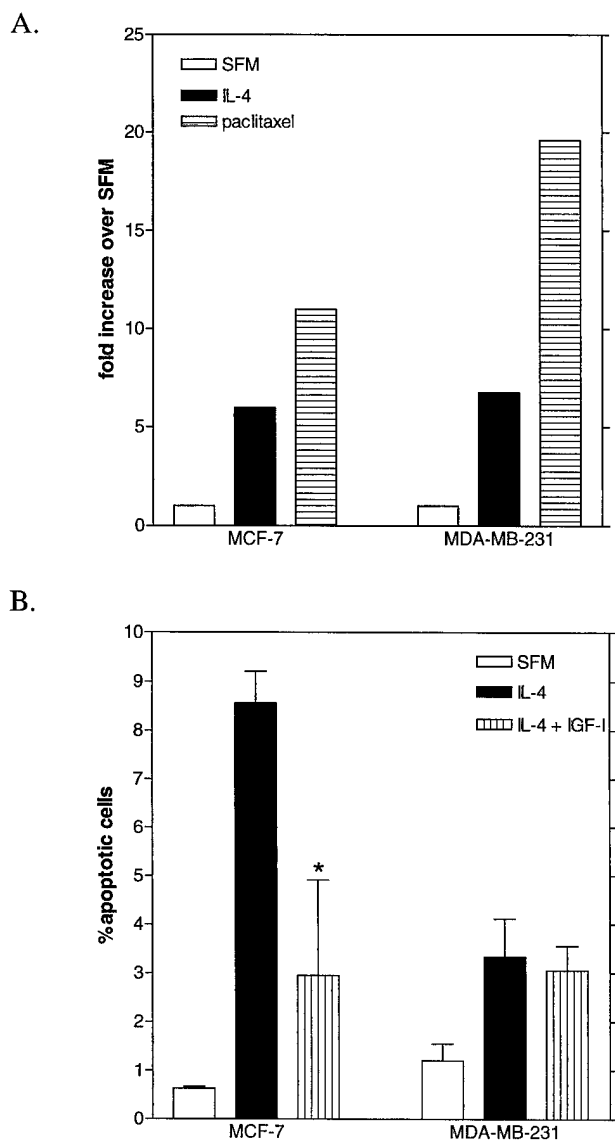


Fig. 8. IL-4-induced apoptosis quantified by flow cytometry. *A*, IL-4-treated MCF-7 and MDA-MB-231 cells were fixed in ethanol, stained with propidium iodide, and analyzed by flow cytometry. ModfitLT software was used to determine the fraction of apoptotic cells in each sample. The portion of apoptotic cells is expressed as the fold increase over SFM control samples. Paclitaxel-treated cells were included as a positive control for apoptosis. *B*, MCF-7 and MDA-MB-231 cells were treated with SFM alone, IL-4, or IL-4 plus IGF-I. Cells were fixed in ethanol, stained with propidium iodide, and analyzed by flow cytometry. ModfitLT software was again used to determine the fraction of apoptotic cells in each sample. Bars, mean of triplicate samples  $\pm$  SE. Asterisk, a statistically significant difference between IL-4 alone and IL-4 plus IGF-I.

tosis in the IGF-I-responsive cell line, MCF-7, but not in the IGF-I-nonresponsive cell line, MDA-MB-231 (38). As a result, we treated MCF-7 and MDA-MB-231 cells with IL-4 plus IGF-I and determined the percentage of apoptotic cells by flow cytometry. We found that the addition of IGF-I to MCF-7 cells partially protected the cells from apoptosis, resulting in a reduction of apoptotic cells from 8.6 to 3.1%, a statistically significant difference ( $P < 0.05$ ; Fig. 8*B*). As expected, the addition of IGF-I had no effect on IL-4-induced apoptosis in MDA-MB-231 cells.

## DISCUSSION

Whereas IL-4 has been considered as an antitumor therapy, the focus has been mainly on the ability of IL-4 to increase the host

antitumor response. This is believed to occur primarily through the up-regulation of MHC class I and II antigens by IL-4 (39, 40). Recently, however, researchers have begun to examine the role of IL-4 as a direct mediator of growth inhibition. This was demonstrated in breast cancer cells by Toi *et al.* (5) in 1992. Using human breast and colon carcinoma cell lines, Toi *et al.* demonstrated that cells bind iodinated IL-4, and that IL-4 reduces the growth of several cancer cell lines *in vitro*. Blais *et al.* (6) showed that IL-4 could inhibit the  $E_2$ -stimulated growth of MCF-7 cells.

Our results confirm these observations and show that IL-4R seems to be required for this growth inhibition. Moreover, IL-4 induces programmed cell death in breast cancer cells. We showed IL-4 induction of apoptosis in breast cancer cells through cellular morphology, TUNEL assay, DNA laddering, and PARP cleavage, and the level of apoptosis induced by IL-4 was quantitated by flow cytometry. Moreover, the effects of IL-4 are only apparent in cells that appear to be actively proliferating, suggesting that entry into the cell cycle may be required for the IL-4 induction of apoptosis.

Our data also suggest that IL-4 initiates an active response in breast cancer cells that results in cell death. We have previously shown that IGF-I, a mitogen for breast cancer cells, and IL-4 share a common signaling pathway via the insulin receptor substrate 1 molecule (41). It is curious that a cell death pathway and a cell growth pathway can both activate the same intracellular adaptor protein. Whereas the mechanism of differential biological effects through a common signaling molecule has not yet been elucidated, we find that IGF-I can protect breast cancer cells from IL-4, suggesting that the IGF-I-stimulated antiapoptotic pathway is dominant over the IL-4 apoptotic signaling pathway. There are several potential explanations for this observation: (a) IL-4 could be deficient in activating intracellular signaling pathways compared with IGF-I, and the resultant effect could be the induction of apoptosis; (b) IL-4 and IGF-I could stimulate insulin receptor substrate 1 in a temporally different pattern than IGF-I that would lead to a different time course of activation of downstream signaling molecules; and (c) a completely different set of signaling molecules could be activated by IL-4 that is responsible for its biological effects. Because Janus-activated kinase/signal transducers and activators of transcription activation by IL-4 is known to occur in lymphocytes (10), and signal transducers and activators of transcription activation has been associated with cell growth inhibition (42), this could be a potential mechanism of IL-4-induced cell growth inhibition. It is also noteworthy that  $E_2$  cannot protect the cells from IL-4-mediated cell death, suggesting that the mitogenic pathways activated by IGF-I and  $E_2$  are distinct.

In conclusion, our data suggest that IL-4 not only inhibits the growth of breast cancer cell lines but may represent a potential pathway to affect cell death in breast cancer cells. The high frequency of IL-4R expression suggests a broad application for potential therapies, and the ability of IL-4 to inhibit growth and induce apoptosis suggests a potential mechanism for such antitumor therapies. Finally, the possible connection between IL-4 signaling and IGF-I signaling in breast cancer cells suggests another avenue for further study into how breast cancer cells grow and die.

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**STAT6 mediates growth inhibition and apoptosis in human breast cancer cells.**

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running title: STAT6 is required for IL-4-induced apoptosis

**Abstract:**

In addition to acting as a hematopoietic growth factor, IL-4 also inhibits the growth of some transformed cells *in vitro* and *in vivo*. Our work has focused on identifying the signaling molecules which mediate this effect in human breast cancer cells. We have previously shown that IL-4 treatment of MCF-7 cells results in phosphorylation of the adaptor protein, IRS-1. However, IL-4-mediated growth inhibition and induction of apoptosis is unaffected by inhibition of IRS-1. Therefore, in the present study, we investigate the role of STAT6 in IL-4-mediated growth effects in human breast cancer cells. We first characterized STAT6 activation and found that STAT6 DNA binding is activated in response to IL-4 in several breast cancer cell lines. Further, STAT6 activation occurs in the absence of IRS-1, indicating that the two pathways are independent. We next created expression constructs for full-length STAT6 as well as a truncated construct ( $\Delta$ STAT6(645)) which lacks the DNA transactivation domain. This previously characterized mutation results in a STAT6 protein with dominant-negative activity. Accordingly, IL-4-mediated activation of a reporter construct was enhanced by transient expression of mSTAT6 and inhibited by transient expression of  $\Delta$ STAT6(645). Using a single-cell proliferation assay, we found that transient expression of  $\Delta$ STAT6(645) blocks IL-4-mediated growth inhibition and induction of apoptosis. In addition, expression of mSTAT6 significantly enhances the basal rate of apoptosis, with a further small increase by IL-4. Finally, we found that expression of mSTAT6 inhibits foci formation of MCF-7 cells, but expression of  $\Delta$ STAT6(645) does not. Furthermore, mSTAT6-mediated inhibition of foci formation can be rescued by addition of excess  $\Delta$ STAT6(645). These results suggest that STAT6 is required for IL-4-mediated growth inhibition and induction of apoptosis in human breast cancer cells.

## **Introduction:**

Interleukin-4 (IL-4) is a pleiotropic immunoregulatory cytokine which modulates cell surface receptor expression and proliferation of B-cells (1). IL-4 acts through a multi-unit transmembrane receptor, IL-4R, which is a type II cytokine receptor and as such lacks intrinsic kinase activity (2). Binding of IL-4 therefore activates a signal transduction cascade including the janus kinases Jak1 and Jak3 which phosphorylate the cytoplasmic domain of the receptor as well as downstream signaling molecules. Two main pathways are activated in response to IL-4, the 4PS/IRS-2 pathway and the STAT6 pathway (3). Activation of IRS-2, and IRS-1 in some cells, leads to association of the p85 subunit of PI-3 kinase and signals for mitogenesis and survival (4, 5). Phosphorylation of STAT6 leads to formation of STAT6 homodimers and subsequent translocation to the nucleus. STAT6 binds to specific sequences in the promoters of target genes and activates transcription including the IgE receptor in B-cells (6).

Interestingly, IL-4R have been identified on the surface of some solid tumors including murine sarcoma and adenocarcinoma cells (7), human renal, melanoma, ovarian (8, 9), lung (10), and breast carcinoma cells (9). In addition, Mat *et al* reported that IL-4R may be expressed by as many as 30% of primary breast tumor cells (11).

In contrast to its proliferative action in hematopoietic cells, IL-4 has been shown to have anti-tumor activity both *in vivo* and *in vitro*. Tepper *et al* found that tumor cells which were engineered to secrete IL-4 failed to form tumors when re-introduced into mice (12). Similarly, Golumbek *et al* found that when IL-4 was transfected into cells arising from a spontaneously arising murine renal carcinoma, expression of IL-4 blocked tumor formation. This observation extended to subsequent challenges with parental, non-IL-4 secreting cells (13). These results suggested that IL-4 was mediating a host-immune response. However, *in vitro* experiments also demonstrated that IL-4

directly inhibited the growth of transformed cells including breast, colon, lung, renal, and melanoma carcinoma cell lines in culture (8, 9, 14-16). In addition, we have previously published that IL-4-mediated growth inhibition of breast cancer cells is associated with an increase in apoptosis (17). However, the mechanism of IL-4-mediated growth inhibition is unknown as is the signaling pathway required for these growth effects.

Due to the observation that IL-4 treatment may inhibit tumor cell growth, key molecules in the transduction of the inhibitory signal may contribute to breast cancer therapies. Several groups including ours have shown that activation of IRS-1 is associated with IL-4-mediated growth inhibition (18, 19). In fact, we found that MCF-7 and other breast cancer cell lines express IRS-2, but activate IRS-1 in response to IL-4 (19). However, inhibition of IRS-1 mRNA and protein in MCF-7 cells did not have any effect on IL-4-mediated growth inhibition or induction of apoptosis (20). Therefore, in this study, we hypothesized that STAT6 is required for IL-4-mediated growth inhibition and induction of apoptosis. Because STAT6 activation has not been reported in conjunction with IL-4-induced growth inhibition, we first characterized STAT6 activation and DNA binding in human breast cancer cell lines. Finally, using a full-length STAT6 construct as well as a truncated STAT6 construct with dominant-negative activity, we evaluated the role of STAT6 in IL-4-mediated growth effects in MCF-7 breast cancer cells.

## **Materials and Methods:**

*Materials:* MCF-7 cells were provided by C. Kent Osborne ( San Antonio, TX) and were maintained in Improved Minimal Essential Medium (IMEM) (Gibco, Bethesda, MD) plus phenol red supplemented with 5% fetal bovine serum (Summit, Ft. Collins, CO). IL-4 was a gift from Satwant Narula (Schering-Plough Research Institute, Kenilworth, NJ). IRS-1 and IRS-2 antibodies were from ( ). STAT6 western blotting antibody was from New England Biolabs ( ) and STAT6 antibody used in immunoprecipitation and super-shifting was from Santa Cruz ( ). All other reagents were from Sigma ( ) unless otherwise noted.

*Western blots and immunoprecipitation:* Total protein was extracted using a buffer containing 50mM Tris -HCl pH 7.4, 2 mM EDTA, 1% NP-40, 100mM NaCl, 100mM Na orthovanadate, 100µg/ml leupeptin, 20µg/ml aprotinin, and  $10^{-7}$ M phenylmethylsulfonyl (PMSF). 50µg of total protein or 250µg of protein immunoprecipitated overnight with antibody followed by 4 hours incubation with Protein A agarose was separated by 8% SDS-PAGE. Following transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk-TBST (0.15 M NaCl, 0.01 Tris-HCl, pH 7.4, and 0.05% Tween 20) and then immunoblotted with 1:1000 dilution of anti-IRS-1 or IRS-2 antibody or 1:2500 dilution of STAT6. HRP-conjugated goat-anti-rabbit secondary antibody was added at 1:2000 for IRS blots and HRP-conjugated donkey-anti-mouse secondary antibody at 1:5000 was added for STAT6 immunoblotting. Proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

*Gel Shift:* Nuclear extracts were prepared by lysing cells first in a hypotonic buffer consisting of

10mM HEPES, 1.5mM MgCl<sub>2</sub>, 10mM KCl, 0.5mM DTT, 5µg/ml aprotonin, 0.5mM PMSF. Nuclear pellets were then briefly centrifuged and then resuspended in one half volume low-salt bufer (20mM HEPES, 1.5mM MgCl<sub>2</sub>, 20mM KCl, 25% glycerol, 5mM EDTA, 0.5mM DTT, 20µg/ml leupeptin, 5µg/ml aprotonin, and 0.5mM PMSF) and one half volume high salt buffer (20mM HEPES, 1.5mM MgCl<sub>2</sub>, 0.12M KCl, 25% glycerol, 5mM EDTA, 0.5mM DTT, 20µg/ml leupeptin, 5µg/ml aprotonin, and 0.5mM PMSF ) for 30 minutes on ice. Extracts were centrifuged for 30 minutes at 4 degrees and the protein concentration of the resulting extracts were determined. 10µg of nuclear proteins were then added to a reaction mixture containing 6µl buffer A (10mM HEPES, pH7.9, 1.5mM MgCl<sub>2</sub>, 0.2mM PMSF and 0.5mM DTT) containing 0.5 µg poly DI/DC, and 12µl of sample extracts to volume in buffer C (20mM HEPES, pH 7.9, 20% glycerol, 0.2mM EDTA, 100mM KCl, 0.2mM PMSF and 0.5mM DTT). 2µl labeled probe corresponding to the STAT6 binding site from the FcγR1 gene promoter (AGCTTGTATTTCCCAGAAAAGGGATC) was added at room temperature for 15 minutes. Samples were run on 4% native acrylamide gels in 0.5% Tris-Boric acid-EDTA buffer for approximately 3 hours. Gels were then dried and exposed to film overnight.

*Luciferase assays:* Cos-7 cells were plated in triplicate in DMEM + 5% FCS at 2.5x10<sup>5</sup> cells in 6-well plates and transfected the next day. Briefly, cells were washed once with PBS, and transiently cotransfected with 1 µg of each promoter construct plus 0.1 µg of pSVβ-gal using Fugene transfection reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Transfected DNA was left on cells overnight. The next morning, media were changed to control or 50ng/ml IL-4 for 24 hours. Cells were harvested and luciferase was measured using the Luciferase Assay System (Promega, Madison WI) according to the manufacturer's instructions. β-gal activity was measured as described by Rouet *et al* (21). Luciferase values were divided by the appropriate

$\beta$ -gal value to achieve relative luciferase units.

*Single cell proliferation assay:* The single cell proliferation assay used in this study is based on previously published protocols. MCF-7 cells were plated in DMEM + 5% FCS at  $2.5 \times 10^5$  cells in 6-well plates and transfected the next day. Briefly, cells were washed once with PBS, and transiently co-transfected with 5 $\mu$ g of each construct plus 0.5 $\mu$ g of pSV $\beta$ -gal using Fugene transfection reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Transfected DNA was left on cells overnight. The next day, each well was split into 2 10cm plates and allowed to adhere overnight. The next morning, media were changed to control (SFM) or 10ng/ml IL-4 for 48 hours, with no wash. Cells were then fixed in 3.7% formaldehyde for 15 at room temperature and then X-gal was added overnight. Buffer was removed and cells were washed 3 times with 1x PBS and then cells were examined for staining.

*Foci assay:* MCF-7 cells were plated at  $1.0 \times 10^6$  cells per 10cm dish and transfected the next day. 10 $\mu$ g total of DNA was transfected alone or with 1 $\mu$ g of HSV-Hagromycin plasmid. The next day, cells were transferred to 150cm dishes and allowed to adhere overnight. The following day, selection was begun using either 450ng/ml Gentamycin or 150 $\mu$ g/ml Hagromycin B. Selection was continued for 3 weeks and then resulting foci were stained with 0.1% Crystal violet.

## **Results:**

**Characterization of STAT6 activation in human breast cancer cell lines.** We first determined activation of key signaling molecules by IL-4 in human breast cancer cells. Treatment of MCF-7 cells resulted in phosphorylation of a band approximately 180kDa, detectable by anti-phosphotyrosine immunoblotting of total protein lysates (Figure 1.). As this band is the same size as the IRS proteins, we immunoprecipitated both IRS-1 and IRS-2 from lysates and then detected phosphorylation by anti-phosphotyrosine immunoblotting. We found that IRS-1 is heavily phosphorylated while IRS-2 is only minimally phosphorylated after IL-4 treatment. This is in contrast to IL-4 activation of predominantly IRS-2 in hematopoietic cells (22), but consistent with our previous finding that IRS-1 is the predominant substrate for IGF-I, insulin and IL-4 in ER positive breast cancer cells (19). In addition to the IRS proteins, we examined phosphorylation of the transcription factor, STAT6. Immunoprecipitation of STAT6 followed by anti-phosphotyrosine immunoblotting revealed IL-4-mediated activation of STAT6 (Figure 1)

As both IRS-1 and STAT6 have been reported to interact with the IL-4R $\alpha$  chain, we next determined if activation of IRS-1 and STAT6 by IL-4 were independent of one another. To do this, we took advantage of a previous observation in our laboratory that IGF-I treatment can induce degradation of IRS-1 within 24 hours (23). Therefore, we treated MCF-7 cells with SFM as a control or IGF-I for 24 hours and then treated cells with IL-4 for 10 minutes. IRS-1 protein was immunoblotted as a control and was effectively abolished by IGF-I treatment. STAT6 phosphorylation by IL-4 in the absence of IRS-1 was then determined by immunoprecipitation of STAT6 followed by anti-phosphotyrosine immunoblotting (Figure 2). Phosphorylated STAT6 was detected following IL-4 treatment in the presence or absence of IRS-1. In addition, phosphorylated STAT6 was detectable in MDA-MB-231 and MDA-MB-435A cells, neither of which phosphorylate

IRS-1 in response to IL-4 (data not shown).

**The role of STAT6 in IL-4-mediated growth effects in human breast cancer cells.** We now knew that IL-4 treatment induced phosphorylation of STAT6 in addition to IRS-1. As we have previously found that inhibition of IRS-1 protein by anti-sense expression reduced IGF-I-mediated proliferation and protection from apoptosis but had no effect on IL-4-mediated growth inhibition or induction of apoptosis (20), we hypothesized that STAT6 was mediating IL-4-induced growth effects in breast cancer cells.

To test this hypothesis, we examined IL-4-mediated STAT6 DNA binding in breast cancer cell lines. We found that in MCF-7 cells IL-4 treatment resulted in the formation of a protein-DNA complex which was super-shifted by STAT6 antibody, but not by ER antibody (Figure 3A). In Figure 3B, we then examined STAT6 DNA binding in a panel of breast cancer cell lines and found that STAT6 binding could be detected in all cells tested (albeit at a very low level in MDA-MB-435A cells) and that the approximate level of activation is consistent with our previously published result of IL-4 mRNA expression in the same cell lines (17).

In order to examine the role of STAT6 in IL-4-mediated growth effects, we utilized a full-length murine STAT6 cDNA (mSTAT6) and a truncated construct,  $\Delta$ STAT6(645), derived from deletion of amino acids 646-847. The truncation in the  $\Delta$ STAT6(645) construct has been previously characterized by Rothman *et al* (24) and removes the DNA transactivation domain from the C-terminus of the protein, resulting in a protein with dominant-negative activity. We characterized these constructs by transiently expressing them in Cos-7 cells along with an IL-4-responsive promoter element upstream of luciferase. Addition of IL-4 resulted in an almost 2-fold induction of relative luciferase activity when cells were transfected with vector alone (pCDNA). Addition of mSTAT6 enhanced IL-4-mediated induction and addition of either  $\Delta$ STAT6(645) or anti-sense mSTAT6

(asSTAT6) resulted in inhibition of IL-4-induced activity as well as basal promoter activity (Figure 4A). In figure 4B we show a dose-responsive increase in activation corresponding to increased expression of mSTAT6 as well as a dose-responsive inhibition of activity corresponding to increased expression of  $\Delta$ STAT6(645). Finally, we show that mSTAT6-mediated induction of luciferase activity is abrogated by increasing concentrations of  $\Delta$ STAT6(645).

We next determined if expression of the STAT6 constructs affected IL-4-mediated STAT6 DNA binding in MCF-7 cells. Figure 5 shows that MCF-7 cells transiently transfected with vector alone (pCDNA) respond to increasing amounts of IL-4 with increased STAT6 DNA binding. Over-expression of mSTAT6 dramatically increases the amount of IL-4-mediated STAT6 DNA binding and expression of  $\Delta$ STAT6(645) inhibits IL-4-mediated STAT6 DNA binding.

To determine the role of STAT6 in IL-4-mediated growth inhibition and apoptosis, we utilized a single-cell proliferation assay which has been previously described [ ]. We transfected MCF-7 cells with vector alone (pCDNA), mSTAT6, or  $\Delta$ STAT6(645) along with  $\beta$ -galactosidase as a marker for transfection. After treatment with IL-4 for 48 hours, the cells were stained with X-gal, and colonies of blue cells were examined for cell number. Cells transfected with vector alone had a mean of 3.5 cells per colony, roughly representing 2 doublings, when treated with SFM (control). IL-4 treatment reduced the mean number of blue cells per colony to 2.8. In contrast, there was no difference due to IL-4 in cells transfected with  $\Delta$ STAT6(645) (Figure 6A). We also examined the effect of expression of mSTAT6 and  $\Delta$ STAT6(645) on IL-4-induced apoptosis. Figure 6B shows that cells transfected with pCDNA responded to IL-4 with a 2-fold increase in apoptosis. In cells transfected with  $\Delta$ STAT6(645), however, the basal rate of apoptosis was unchanged and IL-4 failed to induce a significant increase. Finally, transfection of mSTAT6 resulted in a large increase in the basal rate of apoptosis, and IL-4 slightly enhanced the amount.

From the previous experiment, it appeared that over-expression of mSTAT6 in the absence of exogenous IL-4 was capable of inhibiting the growth of MCF-7 cells and of inducing apoptosis. Therefore, we examined the ability of mSTAT6 to inhibit the growth of MCF-7 cells in a colony-forming assay. MCF-7 cells were transfected with pCDNA, mSTAT6, or  $\Delta$ STAT6(645) and then foci were allowed to form under selection. Transfection of  $\Delta$ STAT6(645) had no effect on growth while transfection of mSTAT6 inhibited foci formation (Figure 7A). In addition, co-transfection of excess  $\Delta$ STAT6(645) rescued mSTAT6-mediated inhibition (Figure 7B).

## **Discussion:**

We have shown that IL-4 treatment results in growth inhibition and induction of apoptosis in human breast cancer cells. These growth effects are associated with phosphorylation of two potential signaling pathways, IRS-1 and STAT6. We have shown that activation of these two pathways occurs independently, consistent with published data from Kotanides *et al* and Pernis *et al* (3, 25). As our previous work to inhibit IRS-1 failed to abrogate IL-4-mediated growth effects, we considered that STAT6 may be the required signaling element. As such, we show that IL-4-mediated growth inhibition and induction of apoptosis is blocked by expression of a dominant-negative STAT6 construct. Supporting the requirement of STAT6 in IL-4-mediated growth effects is our finding that STAT6 over expression increases the basal rate of apoptosis and inhibits foci formation of MCF-7 cells. This is the first evidence showing that STAT6 is required for IL-4-mediated growth effects in breast cancer cells. Furthermore, it is the first time a potential role for STAT6 in inhibiting tumor growth has been demonstrated.

Our data demonstrates that IL-4-mediated growth inhibition and induction of apoptosis in breast cancer cells is mediated by STAT6. STAT6 is a cytoplasmic factor which upon phosphorylation dimerizes with other STAT6 molecules and then translocates to the nucleus and activates transcription at specific promoter regions. There are several places in this signaling pathway where STAT6 act to modulate cell survival. First, it is possible that STAT6 is interacting with other cytoplasmic proteins to impact growth or possibly cell death pathways. Although little is known about STAT proteins acting as cytoplasmic signal transducers, it has been reported that there is some cross-talk between STAT proteins and the PI-3 kinase pathway and the Erk1/Erk2 pathway (26-28). Unlike dimerization and transcriptional activation, interaction with these cytoplasmic pathways is associated with serine phosphorylation rather than tyrosine phosphorylation. It is also

possible that the effects exerted by STAT6 in breast cancer cells is due to transcriptional control of growth and or death regulating genes. For example, Kaplan *et al* reported that STAT6 can modulate the transcription of the cyclin-dependent kinase inhibitor, p27 (29). Finally, STAT6 action may be due to the specific nature of STAT6-mediated control of gene expression. Unlike other transcription factors, STAT6-mediated induction of transcription is associated with class-switch recombination (30, 31). This form of recombination involves introduction of double-strand breaks in the DNA followed by recombination of immunoglobulin receptor-coding sequences mediated by switch recombinases and potentially other proteins such as LR1 (32). It is possible that components of this pathway are not expressed by breast cancer cells, resulting in STAT6-mediated transcription coupled to DNA double strand breaks but without resolution of the recombination event, resulting in programmed cell death.

Our data suggests that STAT6 over expression alone may be capable of inducing apoptosis, which is a novel result. In the single cell proliferation assay, we observed a significant increase in the basal level of apoptosis when mSTAT6 was expressed, and a small increase in the presence of IL-4. It would seem that STAT6 in the absence of additional exogenous IL-4 is capable of inducing apoptosis. There are several explanations for this result. First, transient expression of mSTAT6 in MCF-7 cells resulted in a dramatic increase in sensitivity to IL-4-mediated STAT6 DNA binding (compare 1ng/ml IL-4 in the pCDNA transfected lane with the 1ng/ml IL-4 in the mSTAT6 transfected lane). However, there was no binding of STAT6 in the absence of IL-4, indicating that over expression alone is not sufficient for activation of STAT6 DNA binding and that MCF-7 cells likely do not produce IL-4, consistent with previously published finding (16). However, the dramatic increase in sensitivity may explain the inhibition of foci formation due to STAT6 over expression observed in Figure 7.

Due to toxicity observed in phase I and II trials for IL-4 (33, 34), IL-4 may never be a feasible therapeutic agent, despite its anti-tumor capabilities *in vitro* and in *in vivo* animal models. Therefore, the signaling pathway required for IL-4-mediated growth effects may be a source of novel therapeutic targets. As such, the significance of STAT6-mediated growth inhibition and induction of apoptosis in human breast cancer cells remains to be seen. It will be very important to define what target genes are activated in response to STAT6. As with many important signaling molecules in breast cancer cells, including IGF-IR and IRS-1 (35, 36), it will be very interesting to determine if there is prognostic significance associated with STAT6 expression in human breast tumors. These are important areas of future investigation, possibly yielding new therapeutic insights for regulating the growth of breast and potentially other cancers.

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## **Figure Legends:**

**Figure 1. IL-4 treatment results in phosphorylation of IRS-1, IRS-2, and STAT6 in MCF-7 breast cancer cells.** MCF-7 cells were treated with IL-4 (10ng/ml) for 10 minutes. IRS-1, IRS-2 and STAT6 were immunoprecipitated from total protein lysates, separated by SDS-PAGE and then immunoblotted with anti-phosphotyrosine antibody. Total cell lysates were also immunoblotted with antibodies to IRS-1, IRS-2 and STAT6. Data shown is representative of 3 independent experiments.

**Figure 2. STAT6 is activated by IL-4 in the absence of IRS-1.** MCF-7 cells were treated with SFM as a control or IGF-I to degrade IRS-1 for 24 hours. Cells were then treated with IL-4 (10ng/ml) for 10 minutes and phosphorylated STAT6 was detected by immunoprecipitation followed by anti-phosphotyrosine immunoblotting. Data shown is representative of 3 independent experiments.

**Figure 3. STAT6 DNA binding is activated by IL-4 in breast cancer cell lines.** A) MCF-7 cell nuclear extracts were incubated with labeled probe corresponding to the STAT6 binding site in the Fcγ1 gene promoter. Extracts were also super-shifted with STAT6 antibody or an irrelevant antibody, ER. B) A panel of breast cancer cell lines were treated with IL-4 (10ng/ml) for 30 minutes. Nuclear extracts were isolated and 10μg of protein was incubated labeled probe. Data shown is representative of 3 independent experiments.

**Figure 4. IL-4-mediated transcriptional activation of a reporter construct is enhanced by expression of STAT6 and inhibited by expression of ΔSTAT6(645).** A) Cos-7 cells were transfected in triplicate with vector alone (pCDNA), mSTAT6, ΔSTAT6(645), or anti-sense

mSTAT6 (asSTAT6) and then treated with SFM (control) or IL-4 (50ng/ml). Luciferase activity was determined as was  $\beta$ -gal as a transfection control. Results are expressed as relative luciferase which was determined as luciferase divided by  $\beta$ -gal. B) Increasing amounts of mSTAT6,  $\Delta$ STAT6(645), or STAT6 +  $\Delta$ STAT6(645) were transfected into Cos-7 cells. Luciferase was measured and normalized by  $\beta$ -gal. Data shown is representative of 3 independent experiments.

**Figure 5. STAT6 over-expression and STAT6(640) dominant-negative activity can be observed in MCF-7 cells by gel-shift.** MCF-7 cells transiently transfected with 10 $\mu$ g pCDNA, mSTAT6, or  $\Delta$ STAT6(645) were treated with increasing amounts of IL-4. Nuclear extracts were prepared and then 10 $\mu$ g protein extracts were incubated with labeled probe. Data shown is representative of 2 independent experiments.

**Figure 6. IL-4-mediated growth inhibition and apoptosis is enhanced by expression of mSTAT6 and blocked by expression of  $\Delta$ STAT6(645).** A) MCF-7 cells were transfected with pCDNA or  $\Delta$ STAT6(645) and  $\beta$ -gal, as a transfection marker. Cells were treated with IL-4 for 48 hours, fixed with 3.7% formaldehyde, and then stained with X-gal. Blue cells in colonies of two or more were counted as a measure of proliferation of transfected cells. B) Cells were treated as described in (A) and the percentage of blue, apoptotic cells was determined by morphology. Data shown is the combined result of 3 independent experiments.

**Figure 7. STAT6 over-expression inhibits foci formation of MCF-7 cells.** A) MCF-7 cells were transfected with pCDNA, mSTAT6, or  $\Delta$ STAT6(645) and then selected with neomycin for 3 weeks. Resulting foci were stained with 0.1% crystal violet. B) MCF-7 cells were transfected with mSTAT6

and increasing ratio of  $\Delta$ STAT6(645) and co-transfected with a plasmid containing hagromycin resistance gene. Cells were selected in hagromycin B and resulting foci stained with crystal violet. Quantitation of each plate is shown as a bar graph.

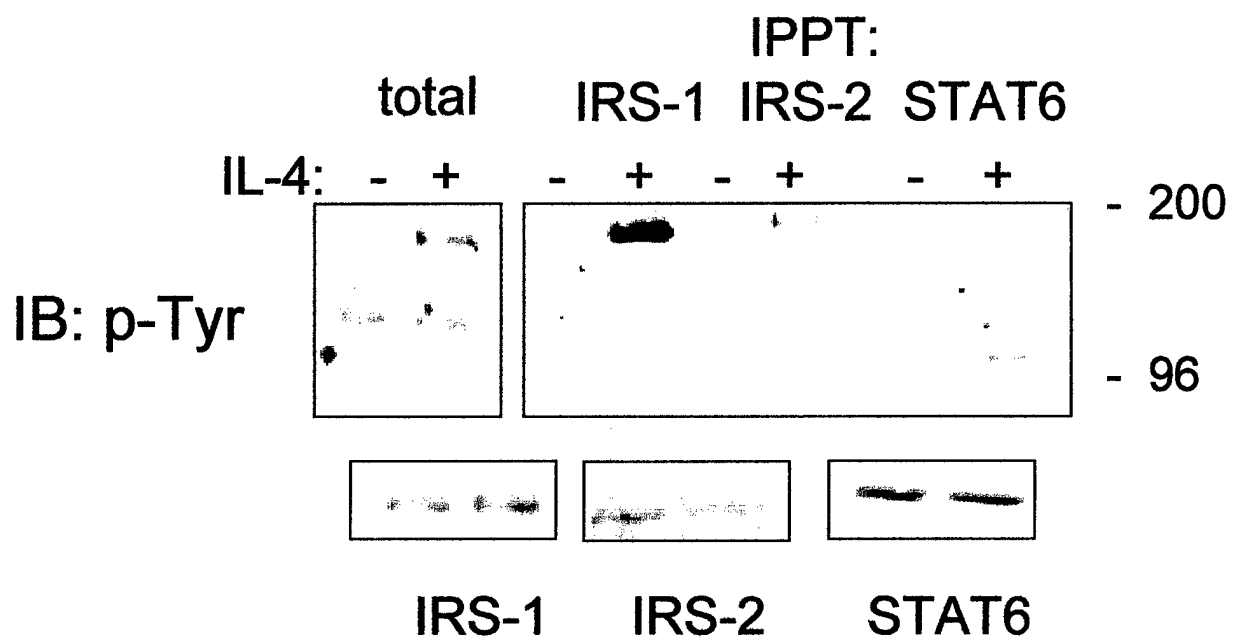


Figure 1.

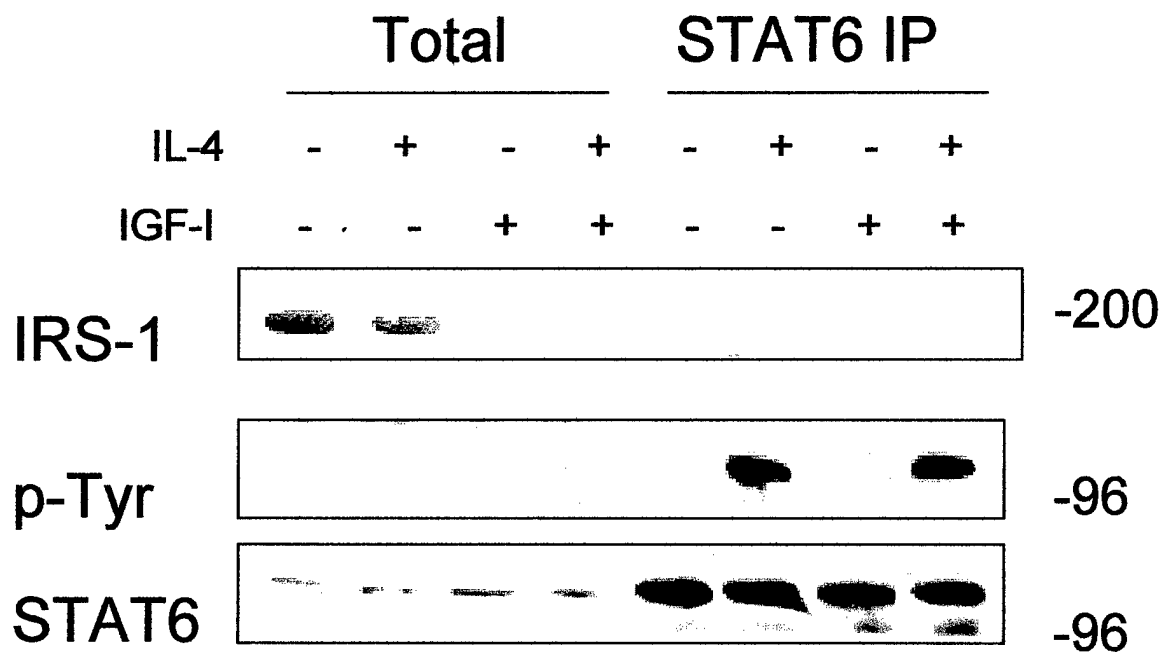
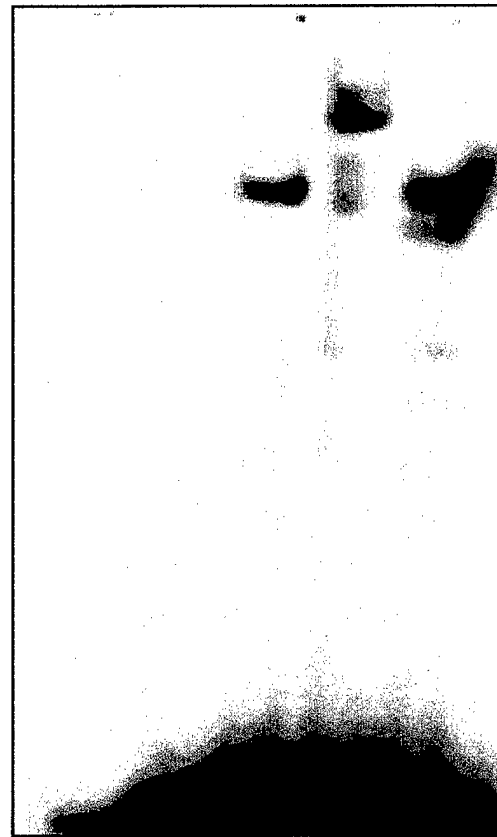


Figure 2.

**A.**

1 2 3 4 5

- 1 - probe
- 2 - untreated
- 3 - IL-4
- 4 - IL-4 plus STAT6 ab
- 5 - IL-4 plus ER ab



**B.**

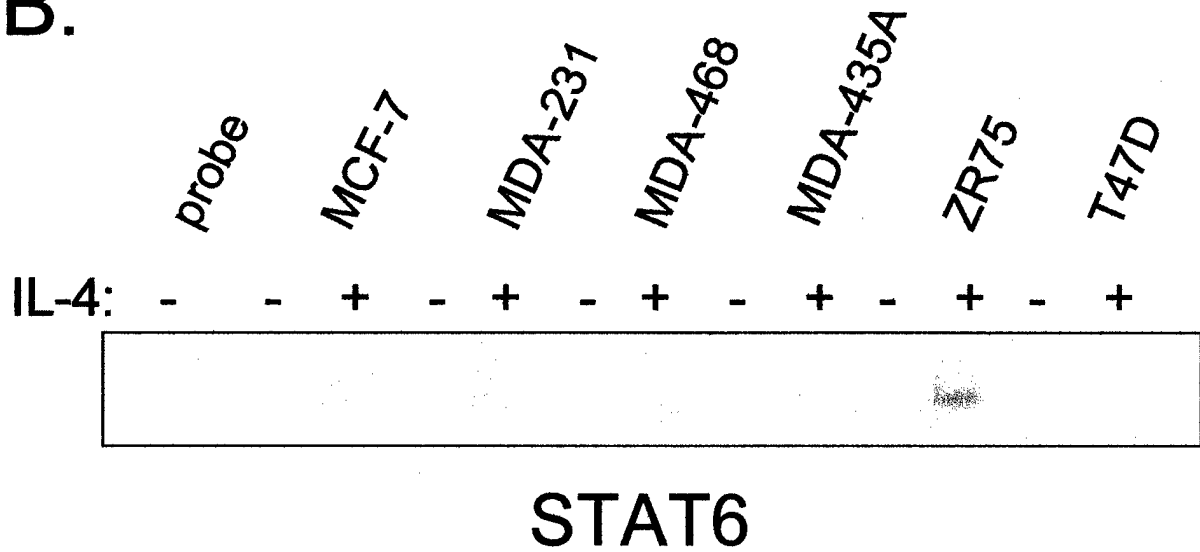
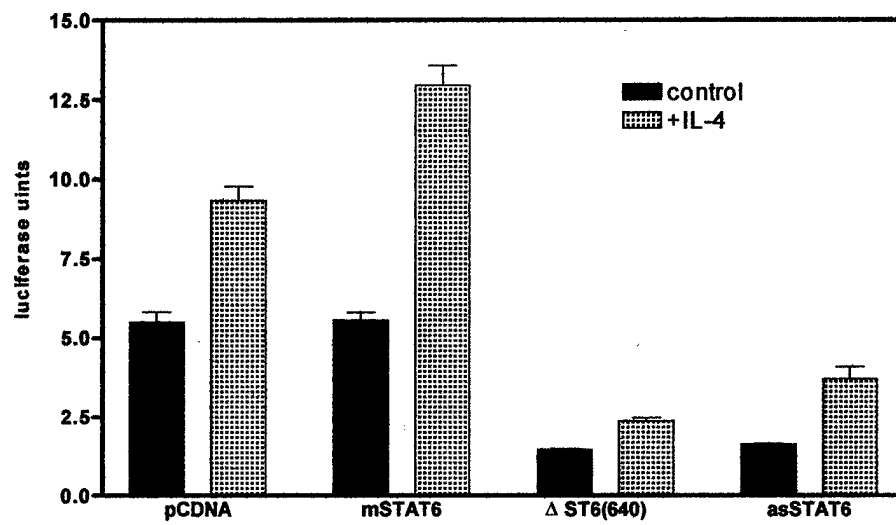


Figure 3.

A.



B.

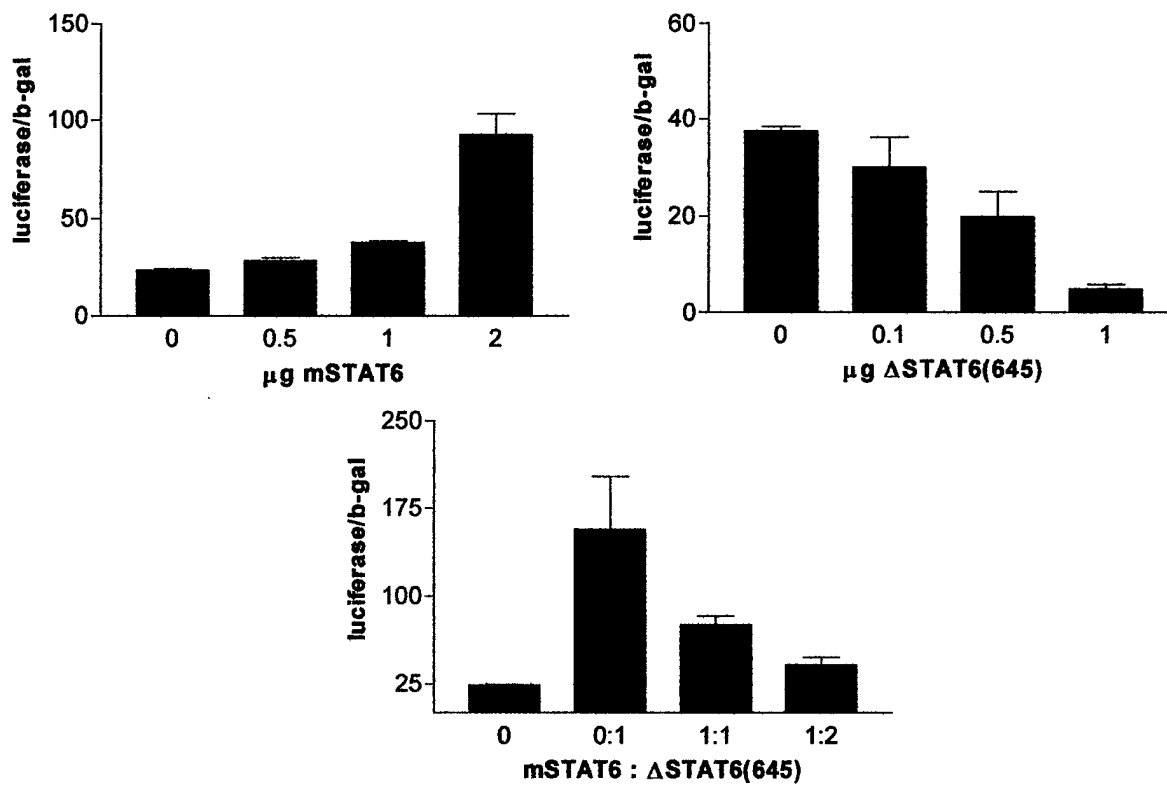


Figure 4.

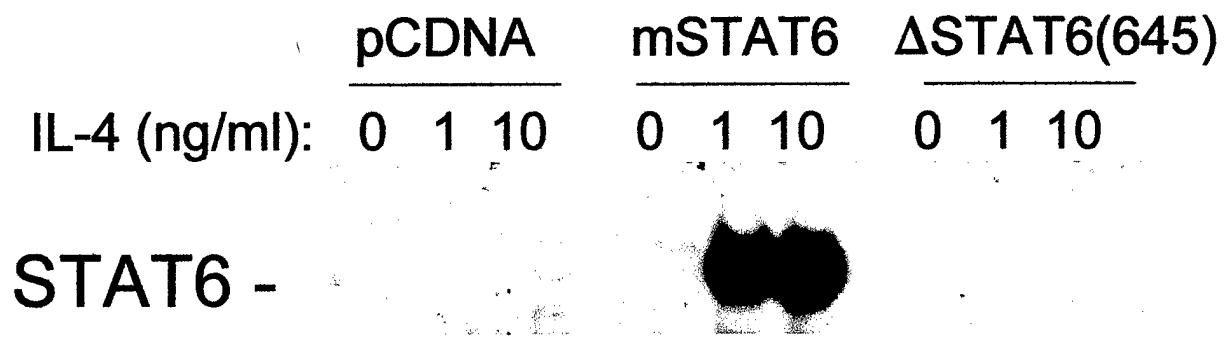
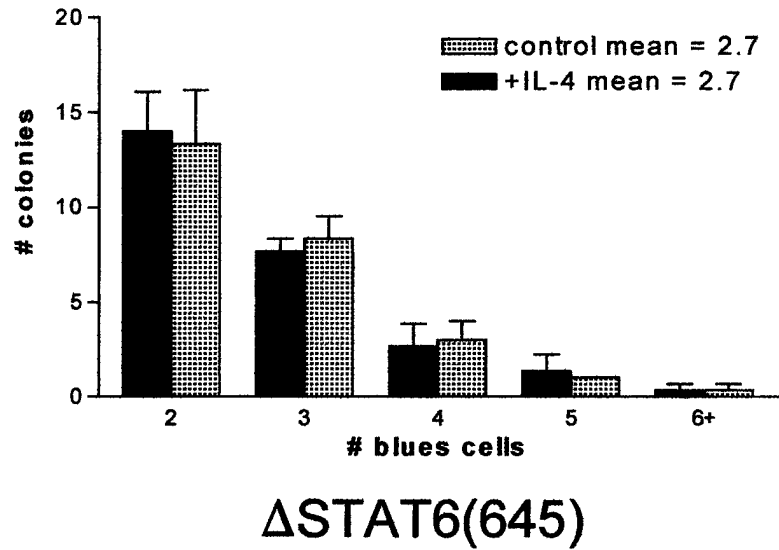
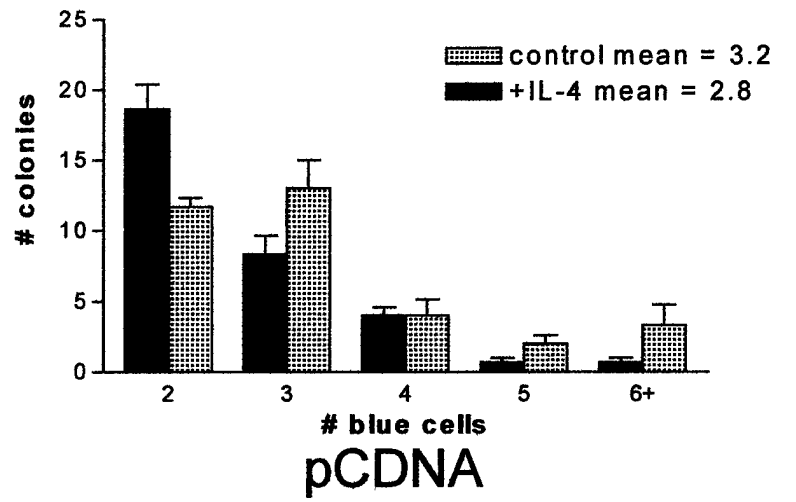


Figure 5.

A.



B.

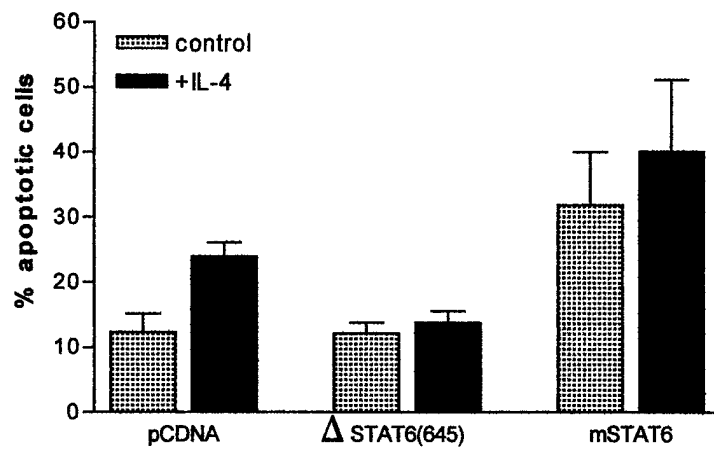
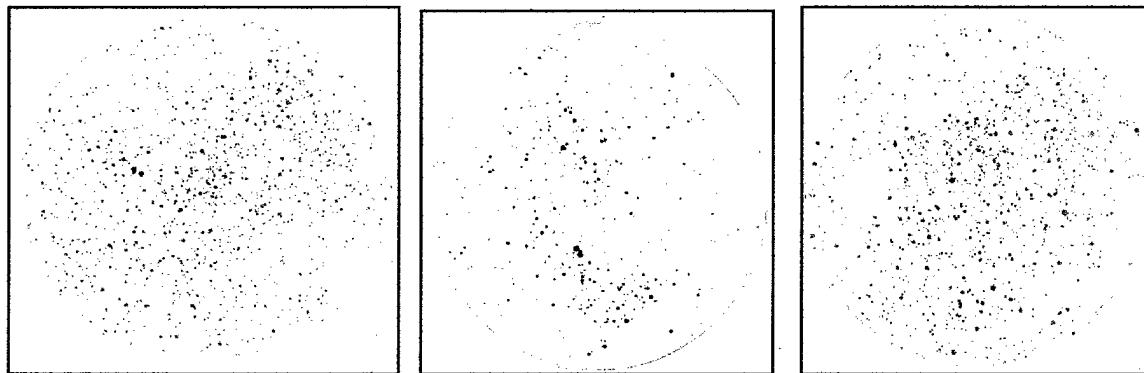


Figure 6.

**A.**

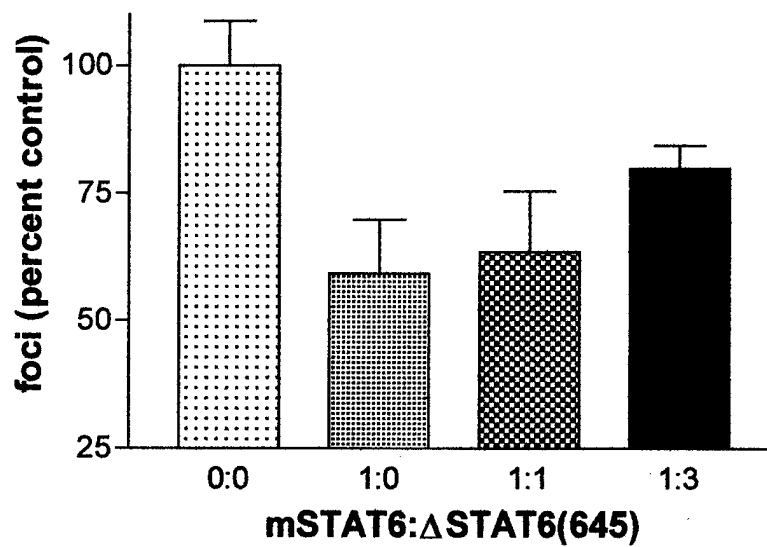


**pCDNA3.1**

**mSTAT6**

**ΔSTAT6(645)**

**B.**



**Figure 7.**