

AD _____

GRANT NUMBER DAMD17-94-J-4349

TITLE: Use of Cytokines to Prevent Breast Cancer Growth and Progression

PRINCIPAL INVESTIGATOR: Philip M. Grimley, M.D.

CONTRACTING ORGANIZATION: Henry M. Jackson Foundation for the
Advancement of Military Medicine
Rockville, Maryland 20852

REPORT DATE: August 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19970523 136

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 1996	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 95 - 31 Jul 96)	
4. TITLE AND SUBTITLE Use of Cytokines to Prevent Breast Cancer Growth and Progression		5. FUNDING NUMBERS DAMD17-94-J-4349	
6. AUTHOR(S) Phillip M. Grimley, M.D.		8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Henry M. Jackson Foundation For the Advancement of Military Medicine Rockville, Maryland 20852			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Goals of this project include: (1) characterization of a novel transcriptional knock out (TKO) factor which negatively regulates signal activation by type I interferons (IFN- α/β) in breast cancer cells; and (2) analysis of signal interactions of IFN- α/β and prolactin (PRL) in breast cancer cells. (1) Improved purification of TKO from suspension cultures of human cancer cells has been achieved: a single functionally active polypeptide of ~20 kDa has been isolated. Approximately 300 ng of TKO is obtained per batch of ~70 x 10 ¹⁰ cells, and samples are in process for proteolytic digestion and mass spectrometric analysis. (2) PRL interactions with IFN- α/β were explored in differentiated (T47D, MCF-7) and undifferentiated (BT-20) cells. IFN- α/β predictably activated Jak1 and Tyk2 protein tyrosine kinases (pTK) involved in Stat2-Stat1 phosphorylations. Whereas PRL typically activates Jak2, it also activated Jak1 and Tyk2. While differential regulation of pTK activity may be one control mechanism influencing PRL gene transactivation signals; there nevertheless was no evidence of antagonism between IFN- α/β and PRL at the level of Stat2-Stat1 activation. Indeed, additive Stat activation was found in T47D and MCF-7 cells. While PRL invariably activated Stat1, activation of Stat3 and Stat5 proved selective. Current work is probing the mechanisms and consequences of such signal diversity.			
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 18	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		16. PRICE CODE	
18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		20. LIMITATION OF ABSTRACT Unlimited	
19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

 Where copyrighted material is quoted, permission has been obtained to use such material.

 Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

 Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

 In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Phil M. J... 11-15-76
PH - Signature Date

TABLE OF CONTENTS

TITLE	PAGE
Front Cover	1
SF 298, Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	7
Conclusions	14
References	15

(5) INTRODUCTION

Treatment decisions for patients with disseminated breast cancer can pose a difficult problem for oncologists due to the morbidity associated with conventional genotoxic agents which are mutagenic and increase the risk for subsequent development of leukemia¹. This can be particularly onerous when a stage II breast cancer is negative for estrogen receptors and the patient is under age 50 years. Interferons (IFNs) are natural cytokines with antiproliferative properties. Type I interferons (IFN- α/β) can block entry of cancer cells into S-phase of the cell-division cycle² and act to cause cell differentiation³⁻⁵. Potential benefits of IFN antiproliferative therapy include an avoidance of permanent damage to normal non-proliferating tissues, mitigation of cytotoxic side-effects and elimination of the hazard of delayed carcinogenicity. IFN- α has been tested repeatedly in preclinical models⁶⁻⁸ and in phase I/II clinical trials^{9, 10}; and it is currently included as adjuvant therapy in some treatment protocols¹¹.

The current project encompasses two major goals: (1) to purify and characterize a novel transcriptional knock out (TKO) polypeptide which we have identified in breast cancer cells and which competitively interrupts the ISGF3 signaling pathway for IFN- α/β ; and (2) to explore and define the molecular interactions of IFN- α/β , which act as growth inhibitors, with prolactin (PRL) which is as a potential growth promoter for breast cancer^{12, 13}.

(1) **TKO:** Expression of genes leading to the principal biologic actions of IFN- α/β , including antiproliferative activity, depends upon the transcriptional function of cytoplasmic Stat proteins which serve in a dual capacity as signal transducers and activators of transcription¹⁴⁻¹⁸. In the case of type I interferons, signal transduction is initiated by dimerization and transphosphorylations of type I IFN receptors^{15, 17}. This signal initiation is intimately associated with autophosphorylation and transphosphorylations of reciprocally interdependent Janus tyrosine kinases Jak1 and Tyk2^{14, 17}. Associated tyrosine transphosphorylations of Stat proteins predispose to the formation of Stat homodimers or heterodimers which translocate to the nucleus, bind to highly conserved promoter regions upstream of IFN stimulated genes, and enhance or initiate transcription^{15, 16}. Relevant nucleotide elements within upstream promoter regions include IR¹⁸,¹⁹ and Fcy GAS²⁰ which bind Stat1 dimers and an interferon sensitive response element (ISRE) which uniquely binds a multimeric complex (ISGF3)^{14, 16, 17}. The ISGF3 holocomplex incorporates Stat2, Stat1 and a 48kDa DNA-binding protein (ISGF3 γ) in the *myb* family^{14, 16, 17, 21}. The ISRE acts as a positive transcriptional regulator in a large proportion of known interferon responsive genes¹⁶. The intranuclear translocation of ISGF3 and maintenance of gene transactivation by IFN has been directly correlated with the Stat phosphorylation status²². Activation of Stat1 by a IFN- α/β depends upon the activation of Stat2²³. IFN- α/β can not generate a Stat1 signal in mutant cells which lack either Stat2 or the transphosphorylating Janus kinases Jak1 or Tyk2²³⁻²⁵. *In vivo*, deficiencies of Stat1 tyrosine phosphorylation have been linked to a loss of IFN stimulated antimicrobial defenses^{26, 27}; and a physiologic specificity of Stat1 proteins in the biologic responses to IFN in mice was clearly shown by targeted disruption of Stat1 gene function²⁷.

In work preliminary to this project, we showed that the signal transduction pathway for IFN- α can be selectively interrupted by a negative regulator of transcriptional activation detected

in the cytoplasm of malignant cells²⁸. This negative regulator was ultimately identified as a polypeptide with a molecular weight of ca. 20 kDa and is designated as *transcriptional knock out* factor or *TKO*. The mechanism of TKO negative regulatory action was explored and related to inhibition of DNA binding by the 48 kDa subunit in the ISGF3 transcriptional holocomplex. The 48 kDa polypeptide is structurally homologous to a family of IFN regulatory factors (IRF) which include a DNA-binding tumor suppressor protein IRF-1¹⁸, an oncoprotein IRF-2, and a gene repressor ICSBP²¹.

TKO activity is detected in breast and cervical cancer cell extracts by means of electrophoretic mobility gel shift assays (EMSA) using specific oligonucleotide probes to represent the ISRE²⁸. Although both the Stat2-Stat1 heterodimer and the 48 kDa polypeptide of ISGF3 each individually are capable of binding to an ISRE nucleotide sequence, the binding affinity is ~25-fold increased when subunits are combined in the ISGF3 holocomplex. By actively competing with the 48 kDa polypeptide (ISGF3 γ), TKO inhibits binding to a ³²P-oligonucleotide ISRE probe. In EMSA the TKO inhibition results in absence of a band shift or a mobility "supershift". TKO inhibitory activity also can be detected with a probe for the IR promoter region of the IRF-1 gene by EMSA²⁸. Using EMSA, significant TKO activity thus was found in cytoplasmic extracts from two lines of human cells originating from malignant breast lesions (Fig. 2 in Progress Report of 1995).

(2) Interferon-prolactin interactions: In addition to the interferons and interleukin cytokines, certain polypeptide hormones or growth factors, including erythropoietin, growth hormones, and prolactin (PRL) similarly activate gene expression through the Janus kinase / Stat pathway^{14, 29-31}. PRL is a principal mammatrophic growth factor³² and 40-70% of human tumor biopsies are positive for PRL receptors³³. Intriguingly, PRL can serve either as a mammary epithelium growth promoter^{12, 32}, or as a cytostatic differentiation agent^{34, 35}. Many tumor-derived cell lines express increased levels of PRL receptors and can proliferate in response to PRL *in vitro* (14-18)³⁶⁻³⁸. Although PRL normally originates from pituitary gland acidophil cells, recent work indicates that it can be endogenously produced in human breast cancers¹³. Thus endogenous PRL could be exercising some autocrine or paracrine role in modulating the antiproliferative response of breast cancer cells to IFN- α/β therapy. In present work we have explored the interactions of IFN- α/β and of PRL in three breast cancer cell lines representing differentiated (T47D, MCF-7) and undifferentiated (BT-20) phenotypes.

(6) BODY:

TKO purification and mass spectrometric analysis: A cervical cancer cell line, C33a has proven optimal for isolation of TKO, since it can be grown rapidly in mass suspension cultures. Using an extraction procedure refined during this project, we were able to obtain ~300 ng of highly purified product from each batch of ~70 liters of C33a cells grown in multi-liter spinner cultures at a density of $\sim 1 \times 10^6$ cells/ml. Purification of protein with TKO activity was refined under the direction of collaborator E. Petricoin. Cells were dounce homogenized in Buffer A (20 mM KCl; 10 mM HEPES @ pH7; 5 mM dithiothreitol) with 20% glycerol and 0.1% NP40. Cytoplasmic extract is obtained by centrifugation of the whole cell lysate at $\sim 8,000g$ for 30 min. The supernatant is loaded over a heparin-sepharose resin. The flow-through and two column washes are collected and pooled, then loaded onto a hydroxylapatite column. The active fraction elutes in a 0.2-0.4 M sodium phosphate gradient. It is brought to 35% ammonium sulfate final concentration and spun at $8,000g$ for 30 minutes. A detergent layer from the top of the tube is collected and further purified by loading onto a phenyl-Sepharose hydrophobic interaction column equilibrated with 35% ammonium sulfate/Buffer A without detergent. The flow-through material is collected and applied to a T-butyl hydrophobic interaction C column at 35% ammonium sulfate. This further depletes non-specific proteins before final concentration on an hydroxylapatite column. Functional quality of product was verified by EMSA using recombinant 48 kDa protein (p48): TKO inhibits binding of the p48 to a ^{32}P -oligonucleotide representing the ISRE sequence of the interferon stimulated gene ISG54K. Specific activity, in EMSA assay, ultimately was increased $> 10^4$ X (based upon relative protein concentrations in crude and purified extracts). An active TKO fraction similarly was purified from extracts of ZR75-1 breast cancer cells. The TKO polypeptide was resolved by two-dimensional gel electrophoresis (2D-gel). Fig. 3 in the previous Progress Report compared the electrophoretic mobility of TKO polypeptide(s) in the ZR-75-1 breast cancer cells and C33a cells in two dimensional gel electrophoretograms (2D-gels). The resolved spot with specific TKO activity was identified by a procedure which depleted TKO from the column purified extracts. Product was exposed to a synthetic N-terminal fragment (125 AA) of the 48 kDa ligand prior to the 2D-gel procedure. This significantly diminished silver or Coomassie blue staining intensity of the assumed TKO spots as compared to other gel zones. By way of confirmation, EMSA showed that the supernatant had retained the competitive inhibitory activity for DNA binding by ISGF3. Thus far, we have made 8 successful batches, out of a total of 12 attempts during which culture conditions were optimized. During these efforts we overcame a number of technical problems which led to dissipation of useful product in terminal stages of isolation.

Maximum column chromatographic and two dimensional electrophoretic purification of the TKO polypeptide has been combined with an improved immunoblot transfer to facilitate direct spectrometric analysis. Initial purified samples were sent to the laboratories of Dr. Donald Hunt at the University of Virginia Department of Chemistry or Dr. Lois Epstein at the University of California in San Francisco. Those efforts were not successful due either to quantitative sample insufficiency, a qualitative problem (amino-terminal blocked), equipment technical problems or personnel transfers in the collaborating facilities. A collaboration was therefore developed with the laboratory of Dr. Ruedi Aebersold in the Department of Microbiology at the University of Washington in Seattle. This laboratory is proficient in

analyses of minimal samples using the techniques tryptic digestion and electrospray ionization mass spectrometry^{39, 40}. The method of transfer of the resolved TKO from 2D-gels has proven critical. Dr. Aebersold recommended staining of the 2D-gel with amido black to identify the TKO prior to transfer to nitrocellulose membrane. The procedure previously used unstained gels and transfer to PVDF membranes. The nitrocellulose-transferred TKO was washed in HPLC grade water, and wet frozen at -70°C for shipping in dry ice.

Successful and reproducible analysis of two separate batch samples was finally achieved by Dr. Steve Gygi in the Aebersold laboratory. Dr. Gygi was able to analyze amino acid sequences of nine main peptide fragments obtained by tryptic digestion. Material analyzed in each of these runs was from 2D-gel spots of functional TKO as indicated by an arrow in Fig. 3 of the 1995 Annual Progress Report. Analysis of the amino acid sequences has shown a region rich in hydrophobic amino acids consistent with a membrane or protein binding function. Based upon the sequence data we synthesized oligopeptides representing two highly charged hydrophilic fragments including the C-terminal fragment of the putative TKO molecule. Rabbits have been immunized and immune sera with polyclonal antibodies soon will be available to begin experimental testing of utility for functional inactivation of native TKO and detection of denatured TKO by immunoprecipitation and Western blotting. Depending upon the antibody quality, antisera could be used to assist in purification of native protein for whole protein mouse immunizations and monoclonal antibody production. We plan to generate monospecific antibodies by mouse intranodal injections; since microgram amounts of purified protein may be needed for this approach to succeed. Immune sera will be screened in EMSA for functional neutralization or supershift activities using recombinant p48 protein. Antibodies also will be tested in immunofluorescence or immunoperoxidase procedures with TKO positive cells from the ZR75-1 breast cancer line⁴¹. Tests with samples of other breast cancer cells lines, frozen biopsy tissues or samples of deparaffinized surgical biopsies, or aspiration cytology samples will follow.

Since TKO can block transcriptional activation of IRF-1 it can impede growth regulation and apoptosis. Identification of TKO in human breast cancers therefore has significant potential both as a therapeutic variable and prognostic indicator. Tissue identification will depend upon the technical capacity to screen patient samples. While TKO function could in principle be tested directly in EMSA assays of fresh tissue, this approach would be practically constrained by uncertainties in sampling of early lesions and diagnostic imperatives restricting the volume of tissue available for destructive analysis. Therefore, our ultimate goal is to develop means of TKO polypeptide or mRNA identification in tissue sections. In principle, the latter could be achieved with an anti-sense riboprobe based upon a cDNA sequence.

Analyses of interaction of PRL and IFN- α on Jak/Stat signaling in human breast cancer cells: Three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) were tested for Stat activation by IFN- α/β or PRL. Results indicated that signalling might be cell dependent both at the levels of Janus kinase and Stat activation. IFN- α/β had an expected effect in activating Jak1 and Tyk2 protein kinases involved in Stat2-Stat1 tyrosine phosphorylations, similar to results with other cell types. Results with PRL differed. In previous work with lysates from pre-T, pre-B or myeloid cell lines it was shown that Jak2 is the kinase primarily involved in transphosphorylation of Stat1 after PRL treatment (Fig. 1, panel A). In the breast

cancer cells a novel observation was that PRL also activated Jak1 and Tyk2. Results are compared in Fig. 1, panel B and indicate differences in intensity of response. Thus, pleiotropic regulation at the level of Jak activation may be one control mechanism governing the outcome of PRL receptor activation. Preliminary characterization of the effect of PRL on Jak1, Jak2 and Tyk2 in T47D cells showed that the three enzymes had comparable activation kinetics, peaking at 2.5-5 min (Fig. 2, panel A). Concentration responses also were similar, with EC_{50} -values of 3-10 nM PRL (Fig. 2, panel B).

PRL also activated Stat1 in each cell line (Fig. 3). This probably reflected the common Jak2 activation, since this particular kinase has been proposed to transphosphorylate Stat1 directly¹⁷ and was the only invariably activated kinase in all three cell lines. The activation of Stat3 and Stat5 proved to be more cell dependent (Fig. 4). The activation of Stat5b shown in fig. 4 is of particular interest, since it has been shown to be involved in sustained proliferation of erythroid progenitors⁴². Since Stat proteins bind with partially overlapping specificity to regulatory regions of genes, current work aims to further identify and elucidate mechanisms and consequences of such signal diversity. Despite differences in the growth regulatory actions of IFN- α/β and PRL, no evidence of antagonism between IFN- α/β and PRL thus far has been detected at the level of the Stat2-Stat1 activation which is essential to the biological action of IFN- α/β . Indeed Fig. 3 shows an additive effect on Stat1-Stat2 tyrosine phosphorylation in T47D cells treated with IFN- α +PRL and MCF-7 cells treated with IFN- β +PRL for 15 min. We note that in highly differentiated MCF-7 cells, PRL, but not IFN- α , induced early Stat1 tyrosine phosphorylation as determined by immunoprecipitation with anti-Stat1 and phosphotyrosine immunoblotting; whereas, in well differentiated T47D cells PRL and IFN- α/β each induced comparable Stat1 tyrosine phosphorylation. In estrogen-receptor-negative and morphologically less differentiated BT-20 cells, IFN- α induced marked phosphorylation of Stat1 and Stat2, whereas PRL had only a minimal effect on Stat1. Interestingly, pretreatment of T47D or MCF-7 cells with IFN- γ for 24 hr served to enhance the PRL effect on phosphorylation of Stat1, possibly due to stimulation of Jak2 phosphorylation.

Based on knowledge from other related cytokine receptors and previous observation of selective Jak2 activation by PRL (see Fig. 1), it will now be of major significance to determine how PRL receptors couple to so many different Jak proteins in the same cell and to formulate the basis for differential activation of Stats. The prevailing view has been that individual receptor proteins have strong preference towards one Jak enzyme⁴³. The common roles of Jak and Stat signaling elements in the signal transduction pathways of PRL and IFN- α/β and their biologic impact at the level of malignant cell proliferation needs further investigation. Future work must involve EMSA quantitation of Stat functional capacity by EMSA with [³²P]-oligonucleotide probes representing the ISRE or the gamma response region (GRR) enhancer elements of genes stimulated by IFN- α (ISRE and GRR) or PRL (GRR only). Proliferation status of the cells will be being monitored by cell cycle analyses, including flow cytometric analyses and immunoblot analyses of Rb phosphorylation, cyclin E and cyclin-dependent CDK or CDC kinase activities.

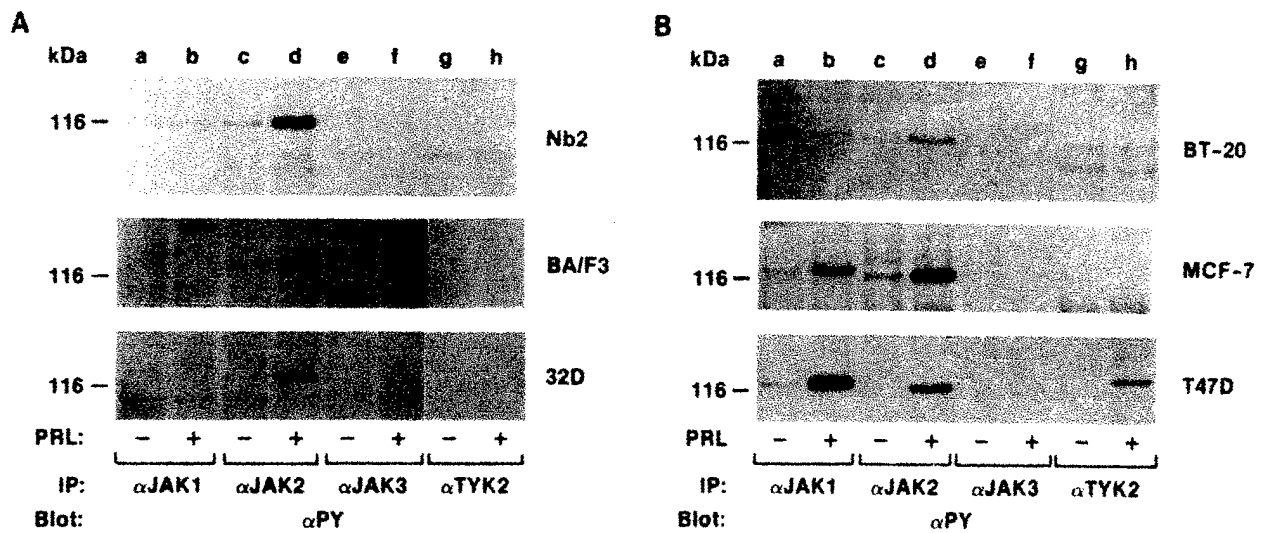


Fig. 1 PRL induces tyrosine phosphorylation of multiple Jak tyrosine kinases in a cell-dependent manner: Antiphosphotyrosine (α PY) immunoblots of immunoprecipitated (IP) Jak proteins (α Jak1, α Jak2, α Jak3 and α Tyk2) from lysates of pre-T lymphoid Nb2 cells, pro-B lymphoid BA/F3-PRLR cells (BA/F3), and myeloid 32D-PRLR cells (32D) (Panel A), and mammary epithelial cell lines BT-20, MCF-7 and T47D (Panel B). The cells had been incubated with (+) or without (-) 100 nM PRL for 5 min at 37° C. The 116 kDa molecular weight markers are indicated.

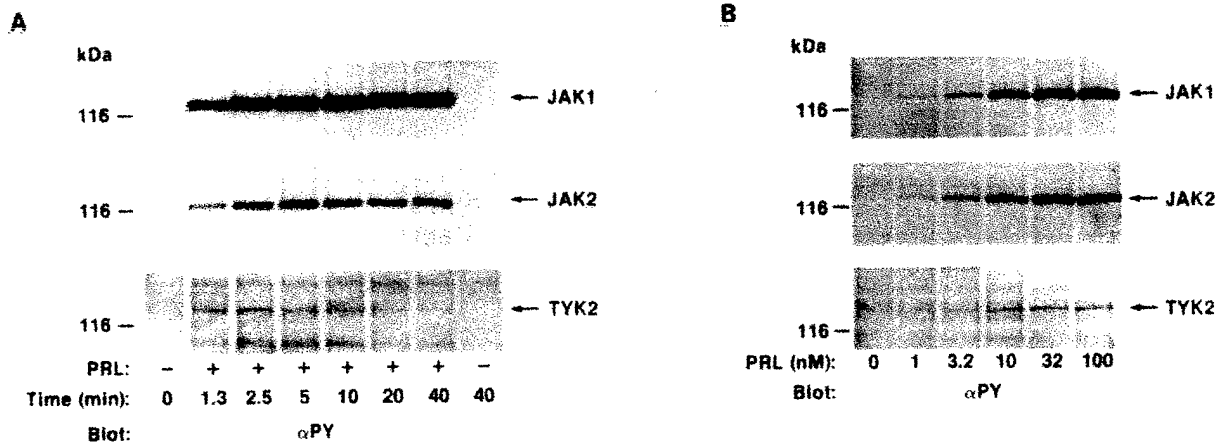


Fig. 2 PRL-induces tyrosine phosphorylation of Jak1, Jak2 and Tyk2 in T47D cells with similar time-kinetics and dose-responses: Antiphosphotyrosine immunoblots of immunoprecipitated Jak1, Jak2 and Tyk2 from lysates of T47D cells. The cells had been incubated with 100 nM PRL for varying times up to 40 min at 37 °C (Panel A), or with varying concentrations of PRL up to 100 nM for 5 min (Panel B). Molecular weight markers are indicated.

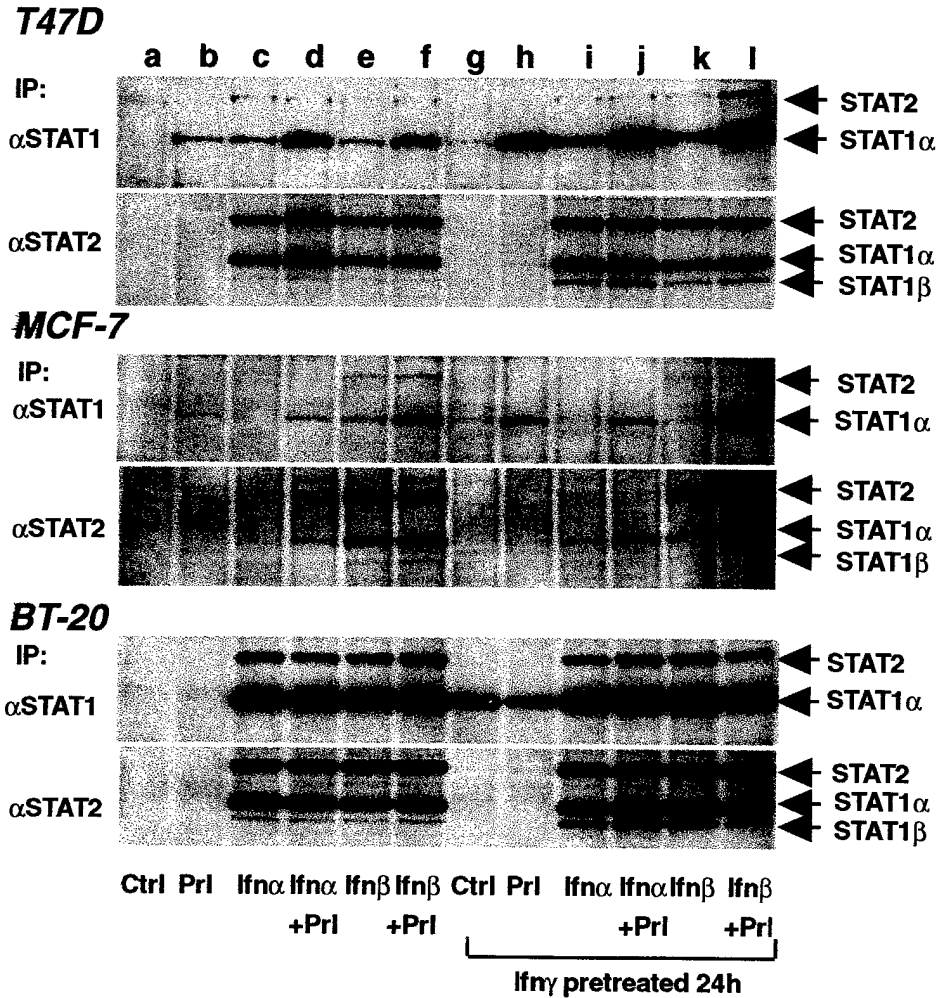


Fig. 3 Prolactin and interferon- α/β induced tyrosine phosphorylation of Stat1 and Stat2 transcription factors in human breast cancer cells: Effect of pretreatment with interferon- γ . Antiphosphotyrosine immunoblots of Stat1 and Stat2 immunoprecipitated (IP) from lysates of T47D cells (top panel), MCF-7 cells (middle panel) and BT-20 cells (lower panel). The cells had been preincubated for 24 h in serum-free medium in the absence or presence of human IFN- γ (10 ng/ml), before being stimulated for 15 min at 37 °C with medium (Ctrl), PRL (100 nM), IFN- α (1000 U/ml), IFN- β (100 U/ml), PRL+IFN- α , or PRL+IFN- β as indicated. Note that IFN- α/β and PRL-induced Stat1 tyrosine phosphorylation in general was additive. Furthermore, IFN- γ pretreatment augmented IFN- α/β induced tyrosine phosphorylation of Stat1 β , particularly in T47D and BT-20 cells.

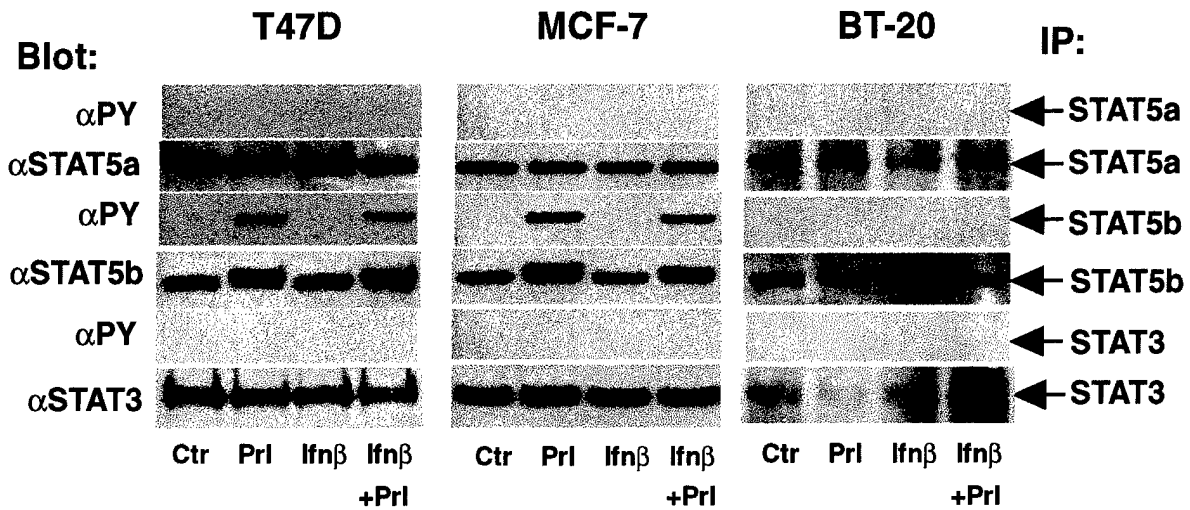


Fig. 4. Prolactin and interferon- β induced tyrosine phosphorylation of Stat5a, Stat5b and Stat3 transcription factors in human breast cancer cells. Antiphosphotyrosine (α PY) immunoblots and corresponding anti-Stat immunoblots of Stat5a, Stat5b and Stat3 immunoprecipitated (IP) from lysates of T47D cells (left panel), MCF-7 cells (middle panel) and BT-20 cells (right panel). The cells had been preincubated for 24 h in serum-free medium before being stimulated for 15 min at 37 °C with medium (Ctrl), prolactin (Prl; 100 nM), IFN- β (1000 U/ml), or PRL plus IFN- β as indicated. Note that PRL activates Stat5b, but not Stat5a in T47D and MCF-7 cells. IFN- β did not stimulate tyrosine phosphorylation of either Stat5a/b or Stat3 in these cell lines.

(7) CONCLUSIONS:

Repeated analyses have confirmed that a cytoplasmic protein, which is a negative regulator of transcriptional activation by IFN- α/β , human breast cancer cells, corresponds to a ~20 kDa polypeptide purified from human cervical carcinoma cells and designated *TKO* (transcriptional knock-out factor). Chromatographic purification of the material has been refined and western transfer from 2D gels improved to maximize retrieval of protein for mass spectrometric analysis. Peptide analysis and amino acid sequence analysis of proteolytic fragments from the highly purified material has been frustratingly delayed by technical problems or personnel transfers. Work now is in actual progress at the laboratory of Dr. Ruedi Aebersold, at the University of Washington in Seattle where there is unparalleled expertise in mass spectrometric technology. We can now realistically expect that it will be feasible to generate and pilot test monoclonal or polyclonal antibodies per original statement of work.

Tests of PRL signaling in three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) have indicated that Stat1 activation following PRL treatment is cell-dependent: differences in Janus kinase activities and Stat phosphorylations were observed in cells from well differentiated, estrogen-receptor-positive malignancies and cells from a poorly differentiated malignancy. PRL can differentially activate at least three distinct Jak tyrosine kinases in different human breast cancer cell lines, including Jak1, Jak2 and Tyk2. Initial findings support an hypothesis that changes in cytokine or growth factor signalling may regulate or reflect neoplastic evolution. Cell dependent differences may pertain to individualization of patient treatment protocols. Further work will continue to analyze differences in pathways of transcriptional activation by Stat3 and Stat5 and involve assessment of Stat DNA binding functions in EMSA using oligonucleotide probes representing the promoter elements of major interest to the antiproliferative pathway. These will include an IR sequence to represent the IRF-1 promoter critically implicated in IFN- α/β biologic activities.

(8) REFERENCES:

1. Curtis RE; Boice JD Jr; Stovall M; Bernstein L; et al. Risk of leukemia after chemotherapy and radiation treatment for breast cancer *N Engl J Med* 326:1745-51, 1992
2. Kumar R; Korutla L; Zhang K Cell cycle-dependent modulation of alpha-interferon-inducible gene expression and activation of signaling components in Daudi cells. *J Biol Chem* 14:25437-41, 1994
3. Exley, R., Gordon, J., Nathan, P., Walker, L., and Clemens, M. J. Anti-proliferative effects of interferons on Daudi Burkitt lymphoma cells: induction of cell differentiation and loss of response to autocrine growth factors. *Int J Cancer* 40: 53-7, 1987.
4. Van den Berg HW; Leahey WJ; Lynch M; Clarke R; Nelson J Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitizes them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 55:255-7, 1987
5. Genot, E., Sarfati, M., Sigaux, F., Petit-Koskas, E., Billard, C., Mathiot, C., Falcoff, E., Delespesse, G., and Kolb, J. - P. Effect of interferon-Alpha on the expression and release of the CD2 molecule in hairy cell leukemia. *Blood* 74: 2455-2463, 1989.
6. Borden EC; Balkwill FR; Preclinical and clinical studies of interferons and interferon inducers in breast cancer. *Cancer* 53(3 Suppl):783-9, 1984
7. Balkwill FR; Mowshowitz S; Seilman SS; Moodie EM; et. al. Positive interactions between interferon and chemotherapy due to direct tumor action rather than effects on host drug-metabolizing enzymes. *Cancer Res* 44:5249-55, 1984
8. Balkwill F.R., Moodie E.M. Positive interactions between human interferon and cyclophosphamide or adriamycin in a human tumor model system. *Cancer Res* 44:904-8, 1984
9. Hansen R.M., Borden E.C. Current status of interferons in the treatment of cancer. *Oncology (Huntington)* 6:19-24, 1992
10. Antimi M., Majoli L., Minelli M., Stampino C.G., et. al. A phase I safety study of doxifluridine and interferon-alpha-2a in patients with advanced neoplastic disease. *Tumori* 79:336-9, 1993
11. Buzdar, A.U., Hortobagyi G.N., Kau S.W., Smith T.L., et. al. Adjuvant therapy with escalating doses of doxorubicin and cyclophosphamide with or without leukocyte alpha-interferon for stage II or III breast cancer. *J Clin Oncol* 10:1540-6, 1992
12. Bernstein L, Ross RK; Endogenous hormones and breast cancer risk. *Epidemiol Rev* 15: 48-65, 1993

13. Ginsburg E., Vonderhaar B.K. Prolactin synthesis and secretion by human breast cancer cells. *Cancer Research* 55; 2591-2595, 1995
14. Darnell J.E., Kerr I.M., Stark G.R. Jak-Stat pathways and transcriptional activation in response to IFNs and other extracellular signalling proteins. *Science* 264; 1415-1421, 1994
15. Ihle, J. N. Stats: Signal transducers and activators of transcription *Cell* 84:331-334, 1996
16. David, M. Transcription factors in interferon signaling *Pharm. Ther.* 65:149-161, 1995
17. Finbloom D.S., Larner A.C. Regulation of the Jak Stat signalling pathway. *Cell Signal* 7:739-745, 1995
18. Li X., Leung S., Qureshi S., Darnell J.E., Jr., and Stark, G.R. Formation of Stat1-Stat2 heterodimers and their role in the activation of *IRF-1* gene transcription by Interferon- α . *J. Biol. Chem* 271:5790-94, 1996
19. Tanaka N; Kawakami T; Taniguchi T; Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. *Mol Cell Biol* 13:4531-8, 1993
20. Pearse, R. N., Feinman, R., Shuai, K., Darnell, J. E., Jr, and Ravetch, J. V. Interferon gamma-induced transcription of the high-affinity Fc receptor for IgG requires assembly of a complex that includes the 91-kDa subunit of transcription factor ISGF3. *Proc Natl Acad Sci U S A*. 90: 4314-8, 1993.
21. Veals, S. A., Santa Maria, T. and Levy, D. E. Two Domains of ISGF3 γ that Mediate protein-DNA and protein-protein interactions during transcription factor assembly contribute to DNA-binding specificity. *Mol. Cellular Biol.* 13: 196-206, 1993.
22. David, M., Grimley, P. M., Finbloom, D. S., and Larner, A. C. A nuclear tyrosine phosphatase downregulates interferon-induced gene expression. *Mol Cell Biol.* 13: 7515-21, 1993.
23. Qureshi S.A., Leung S., Kerr I.M., Stark G.R., Darnell J.E. Jr. Function of Stat2 protein in transcriptional activation by alpha interferon *Mol Cell Biol* 16:288-93, 1996
24. Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. *Cell.* 70: 313-22, 1992.
25. Muller M., Briscoe J., Laxton C., Guschin, D., et al. The protein tyrosine kinase Jak1 complements defects in interferon- α/β and - γ signal transduction. *Nature* 366:129-135, 1993

26. Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and Schreiber, R. D. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell*. 84: 431-42, 1996.
27. Durbins J.E., Hackenmiller, R, Simon, M.C., Levy D.E. Targeted disruption of the mouse *Stat1* gene results in compromised innate immunity to viral disease *Cell* 84:443-459, 1996
28. Petricoin, E. 3rd, David, M., Fang, H., Grimley, P., Lerner, A. C., and Vande Pol, S. Human cancer cell lines express a negative transcriptional regulator of the interferon regulatory factor family of DNA binding proteins. *Mol Cell Biol*. 14: 1477-86, 1994.
29. Sadowski, H. B., Shuai, K., Darnell, J.E., Jr., and Gilman, M. Z. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors *Science* 261: 1739-44, 1993.
30. Rui H., Kirken R.A., Farrar W.L. Activation of receptor-associated tyrosine kinase Jak2 by prolactin. *J Biol Chem*, 269; 5364-5368, 1994
31. David M., Petricoin E.F. 3rd, Igarashi K., Feldman G.M., et. al. Prolactin activates the interferon-regulated p91 transcription factor and the Jak2 kinase by tyrosine phosphorylation. *Proc Natl Acad Sci USA* 91:7174-8, 1994
32. Welsh CW, Nagasawa H; Prolactin and murine mammary tumorigenesis: a review. *Cancer Res* 37: 951-63, 1977
33. Bonneterre J., Peyrat J.P., Beuscart R., Demaille A. Biological and clinical aspects of prolactin receptors (PRL-R) in human breast cancer. *J Steroid Biochem Mol Biol* 37; 977-981, 1990.
34. Shiu R.P., Paterson J.A., Alteration of cell shape, adhesion, and lipid accumulation in human breast cancer cells (T-47D) by human prolactin and growth hormone. *Cancer Res* 44: 1178-1186, 1984
35. Taverna D., Antoniotti S., Maggiora P., Dtai C. et. al. ErbB-2 expression in estrogen-receptor-positive breast tumor cells is regulated by growth-modulatory reagents. *Int J Cancer* 56; 522-8, 1994
36. Foxwell, B.M.J., Barrett, K., Feldmann, M. Cytokine receptors: structure and signal transduction. *Clin Exp Immunol* 90:161-9, 1992
37. O'Neal,KD; Yu-Lee,L-y. The proline-rich motif (PRM): A novel feature of the cytokine/hematopoietin receptor superfamily. *Lymphokine and Cytokine Research* 12: 309-312, 1994

38. Kelly, P.A., Djiane, J., Postel-Vinay, M.C., Ederly, M.: The prolactin/growth hormone receptor family. *Endocr. Rev.* 12: 235-251, 1991.
39. Aebersold R. Mass spectrometry of proteins and peptides in biotechnology *Curr Opin Biotechnol* 4:412-9, 1993
40. Figeys D., van Oostveen I., Ducret A., Aebersold R. Protein identification by capillary zone electrophoresis/microelectrospray ionization-tandem mass spectrometry at the subfemtomole level. *Anal Chem* 68:1822-8, 1996
41. Engel LW; Young NA; Tralka TS; Lippman ME; et. al. Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res* 38:3352-64, 1978
42. Mellitzer, G, Wessely, O, Decker, T., Meikne, A., Hayman, M.J., and Hartmut, B. Activation of Stat 5b in erythroid progenitors correlates with the ability of ErbB to induce sustained cell proliferation. *Proc. Nat. Acad. Sci. USA* 93:9600-05, 1996
43. Stahl, N; Yancopoulos, GD (1993): The alphas, betas, and kinases of cytokine receptor complexes. *Cell*. 74, 587-590.