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13. ABSTRACT ***(Maximum 200 Words)***

Mac25 is the first member of the IGFBP-rP family (IGFBP-rP1). IGFBP-rP1 is expressed in normal human mammary epithelial cells (HMECs) and upregulated in senescent HMECs. IGFBP-rP1 is not expressed in twelve/sixteen breast cancer cell lines and its expression is absent in estrogen receptor positive (ER^+) breast cancer cell lines.

IGFBP-rP1 may regulate HMEC growth. We tested the hypothesis that IGFBP-rP1 suppresses growth in ER^+ breast cancer cells by transducing the cDNA into normal and human breast tumor cultures. IGFBP-rP1 negatively regulates growth in ER^+ breast cancer cells. Growth inhibition is independent of apoptotic regulatory pathways. We observed an initial change in cell morphology to a senescent phenotype. IGFBP-rP1 may inhibit growth through senescence-associated pathways. IGFBP-rP1 secreted by ER^+ breast cancer cells exhibit two higher molecular weight proteins compared to protein secreted by ER^- breast cancer cells. We were unable to determine the binding properties of IGFBP-rP1 by growth assays in presence of IGF-I, IGF-II, R^3-IGF-I, and R^6-IGF-II.

We have detected cell-specific and gender-specific differences of IGFBP-rP1 mRNA expression in male and female C57BL6 mice suggesting that IGFBP-rP1 may be hormonally regulated. We would like to determine regulation of IGFBP-rP1 at the transcriptional level by characterization of the promoter and have isolated two potential clones from a genomic library screen that may contain the 5'end of IGFBP-rP1 and a portion of the promoter sequence.

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Karen Wisshel 08/17/2000

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Table of Contents

INTRODUCTION

The IGF system plays an important role in growth of mammary epithelial cells (*1*), and imbalances in this system may contribute to breast carcinogenesis (*2, 3*). Members of the IGF system include growth factors, regulatory binding proteins, and receptors. Studies show that in addition to regulating interaction between ligand and receptor, the IGF binding proteins (IGFBPs) are capable of IGF-independent growth regulating activity (reviewed in (*4-6*)). IGFBP-rP1 is a member of the IGFBP-related protein (IGFBP-rPs) subfamily of IGFBPs and exhibits low affinity for IGFs (reviewed in (*7, 8*)). IGFBP-rP1 mRNA is upregulated 3- to 8-fold in senescent HMECs and upregulated by all-trans-retinoic acid and the synthetic retinoid, fenretinide, in normal, proliferating HMECs (*9*). IGFBP-rP1 mRNA is downregulated in primary prostate cancer (*10*) and detected in differentiating granulosa cells which eventually enter replicative senescence (*11*). Full-length IGFBP-rP1 inhibited prostate tumor formation in nude mice via apoptosis (*12*). Truncated IGFBP-rP1, lacking the IGF binding domain, inhibited growth of human Saos II cells (*13*). Collectively, these studies suggest that IGFBP-rP1 may play a tumor suppressor role in human breast cancer and possess IGF-independent functions. To test these hypotheses, we transduced full-length IGFBP-rP1 into MCF-7 breast cancer cells and assayed their growth.

BODY

Aim 1: Experiments to address the potential of IGFBP-rP1 for modulating cell growth of ER+ breast cancer cells.

IGFBP-rP1 cDNA, previously cloned from a normal HMEC lambda Zap cDNA library, was ligated into the pBluescriptIIKS at the BamHI site, and the insert verified by DNA sequence analysis. Initial analysis indicated that the cDNA construct was missing one nucleotide when compare with the sequence submitted to Genbank. Further sequence analysis of cDNA products isolated by RT-PCR using two normal and three tumor cell lines revealed the cDNA construct to contain the same sequence. It is likely the Genbank construct had a sequencing error or the cell line in which it was cloned contains a mutated IGFBP-rP1. The verified IGFBP-rP1 insert was ligated into the BamHI site in the mammalian retroviral-derived expression vector conferring neomycin resistance, LXSN. Two packaging cell lines were used to generate virus for the transduction of selected breast cancer cell lines. MCF-7 breast cancer cells, lacking endogenous IGFBP-rP1 expression, were transduced with virus to generate two pooled cell lines and two clonal cell lines of LXSN and of LIGFBP-rP1SN-tranduced cells for a total of eight cell lines.

In order to determine the potential growth inhibitory properties of IGFBP-rP1, we transduced MCF-7 cells with IGFBP-rP1 cDNA and assessed their proliferative capacity. We observed a marked reduction in cell numbers over seven days in the IGFBP-rP1 transduced cell lines. Nuclear fragmentation, a morphological marker of apoptosis excluded programmed cell death as a possible mechanism. A change in morphology to a senescent phenotype along with a two-fold increase in senescence-associated β-galactosidase activity suggests that IGFBP-rP1 expression may confer a senescent phenotype. Further analysis by a Hoechst-BrdU cell proliferation assay confirms that a greater number of cells from the IGFBP-rP1-transduced cell lines are present in the initial

G_0/G_1 phase of the cell cycle, suggesting these cells are senescent or undergoing terminal differentiation. The results from these studies are described in detail in the attached manuscript in preparation for submission to *Endocrinology* (Appendix A). Analysis of cell cycle regulatory genes reveals that p21 mRNA levels are increased in the presence of IGFBP-rP1. Total Rb protein and p53 mRNA appeared to be decreased in the IGFBP-rP1 transduced cells, however, further analysis by densitometry indicate the levels may not be significant (Figures 1 & 2)

We transduced BT474 breast cancer cells in order to determine if IGFBP-rP1 exhibits the ability to suppress growth proliferation in another ER-positive/IGFBP-rP1-negative breast cancer cell line. In three independent seven-day growth curves using LXSN- and IGFBP-rP1-transduced BT474 pooled cultures, IGFBP-rP1 inhibited cell growth between 15% and 54% (Figure 3).

We have determined the possible mechanism of proliferation inhibition IGFBP-rP1 in MCF-7 breast cancer cells and would like to determine its role in senescence of normal HMECs. In order to distinguish if IGFBP-rP1 induces senescence or if it is upregulated as a result of senescence, we transduced normal HMECs (AG11132) at an early passage with LXSN (control) and IGFBP-rP1 retrovirus. To date, our results have been inconclusive in the determination of the role of IGFBP-rP1 in early senescence.

Aim 2: Experiments for promoter studies: library screening and clone analysis.

IGFBP-rP1 functions as a growth inhibitor, as presented in Aim 1. The purpose of Aim 2, isolating and analyzing the promoter, is to understand how IGFBP-rP1 gene expression is regulated in normal HMECs and breast cancer cells.

Previous studies demonstrated upregulation of IGFBP-rP1 mRNA in the presence of retinoic acid in normal human mammary epithelial cells (HMECs) and in senescent HMECs (*9*). IGFBP-rP1 is homologous to prostacyclin-stimulating factor (PSF) from which a partial promoter sequence has been isolated. The PSF promoter does not contain a TATA box, has many G/C rich regions, and contains a consensus sequence for Sp1 binding (*14*). We performed a visual comparison of the reported PSF sequence with known retinoic acid response elements (RAREs) composed of two specific sequences having 1-5 miscellaneous nucleotides in between them. We found a potential RARE in the PSF promoter sequence containing 6 miscellaneous nucleotides located between the two specific sequences, suggesting a novel RARE (Figure 4). Our goal was to verify the RARE to determine if it is a novel response element and locate potential enhancer sites.

Ten clones were isolated from a human genomic library enriched for chromosome 4. The clones were analyzed by restriction digest, DNA-DNA hybridization of dot blots, PCR, and sequence analysis. Southern hybridization using a 42 base pair probe composed of IGFBP-rP1 5'sequence, including the ATG translation start site, demonstrated strong hybridization signal over two clones and weaker signal over three clones, indicating five of the ten clones may contain upstream sequence to the IGFBP-rP1 translation start site and include promoter region (Figure 5).

We have not completed Aim 2 due to time constraints and prioritization of tasks. It was not feasible to accomplish this aim in addition to the other three aims within the given time frame. However, the work accomplished will provide a sound basis for future studies. Future studies would include sequence analysis of the genomic clones to identify transcription start sites and motifs for transcription factor binding elements. Once a

promoter region has been successfully isolated, cloning the sequence into a promoterless Luciferase reporter vector would allow us to test the entire promoter region versus truncated region to determine the minimal sequence necessary for activity. We would also test for response to potential regulators such as retinoic acid and estrogen.

Aim 3: Experiments to determine binding characteristics of IGFBP-rP1 using purified IGFBP-rP1 protein and a specific antibody.

IGFBP-rP1-transduced ER-positive MCF-7 breast cancer cells secreted IGFBP-rP1 as 33 and 34 kDa proteins, compared to the 31 kDa protein detected from ER-negative Hs578T breast cancer cells (positive control). We also detected the higher molecular weight IGFBP-rP1 protein when we transduced another ER-positive breast cancer cell line, BT474. There are several possible explanations for the observed increase in protein size. One possibility is that the protein is post-translationally modified in ER-positive breast cancer cells. Predicted motifs for potential modification include: 4 casein kinase phosphorylation sites, 4 protein kinase C phosphorylation sites, 9 myristoylation sites, and one N-glycosylation sites. We have excluded N-glycosylation as one potential modification because endogeneous IGFBP-rP1 expressed by Hs478T breast cancer cells is composed of a 27 kDa core protein and 4 kDa of N-linked sugars (*15*). Myristoylation was also eliminated as a possibility because all known myristoylated proteins are modified at a site immediately adjacent to the amino terminal (*16-18*) and no such consensus myristoylation sites are found in IGFBP-rP1. The small increase in molecular weight suggests possible protein phosphorylation. Phosphorylated IGFBP-1 and IGFBP-3 regulate the growth potentiating activities of these proteins (*19*). The same regulation may occur with IGFBP-rP1 protein. To test this hypothesis, we phosphatase-treated conditioned medium containing secreted, recombinant IGFBP-rP1 protein from the transduced MCF-7 breast cancer cells with calf intestinal phosphatase. Following immunoblotting the signal from phosphatase-treated IGFBP-rP1 of 33 and 34 kDa was diminished, but we were unable to detect the emergence of a lower molecular weight protein as would have been predicted (Figure 6).

The secreted higher molecular weight IGFBP-rP1 protein may be partially processed in ER-positive breast cancer cells. Examples of proteins (all peptide hormone precursors) that are only partially processed by tumor cells include ACTH, calcitonin, and somatostatin (*20, 21*). Significant levels of these prohormones or processing intermediates have been detected using tumor cell cultures (Roger Birnbaum, unpublished data).

We have studied the response of IGFBP-rP1-transduced cells to various growth factors in hopes of determining a potential ligand(s) regulated by IGFBP-rP1. We were unable to distinguish differences in cell growth response over five days between IGFBP-rP1- and LXSN-transduced MCF-7 cells using following growth factors in serum-free medium supplemented with BSA: EGF, insulin, IGF-I, IGF-II, R^3-IGF-I, and R^6-IGF-II.

IGFBP-rP1 has been referred to as a follistatin-like gene, suggesting that it may share functional properties with members of the follistatin family (*13*). A recent publication has demonstrated full-length IGFBP-rP1 protein binds to activin A (*22*). Our data, presented in Aim 1 and the attached manuscript (Appendix A), suggests that IGFBP-rP1 may function to inhibit growth by enhancing interaction between the activin A ligand and its receptor.

Aim 4 (Addition): Experiments to determine if tissue specificity is gender dependent for RNA expression/protein localization of IGFBP-rP1 in mice.

In order to determine if IGFBP-rP1 was differentially expressed in tissues under hormonal influence, Northern analysis and RNA *in situ* hybridization was used to compare expression levels in various tissues of male and female mice (Tables I and II).

Northern analysis studies revealed the 1.5 kb mouse transcript to be highly expressed (+++) in kidney, strongly expressed (++) in lung, and expressed (+) in brain, heart, liver skeletal muscle, mammary tissue, uterus, and ovaries of C57BL6 mice. We found a second mRNA transcript of 3.1 kb to be expressed in kidney, heart, lung, uterus, ovaries, and mammary tissue (Figure 7). Quantitation by densitometry (NIH Image 1.60) revealed that IGFBP-rP1 mRNA expression was higher in the heart and kidney of female mice versus male mice, and that male mouse spleen had higher expression compared to female mouse spleen suggestive of gender-specific hormonal regulation (Figure 8).

We had difficulty detecting IGFBP-rP1 mRNA expression in brain by Northern analysis. Use of RNA *in situ* hybridization techniques allowed us to test this complex organ by localizing signal to specific regions within the brain. We observed specific IGFBP-rP1 expression by RNA *in situ* hybridization in the hippocampus, cerebellum, and the cortex. In these regions of the brain, hybridization is occurring over the granule cells. In the cerebellum, hybridization is seen in the granular and Purkinje layers. Both cell types are projection neurons. The granule cells in this region are cerebellar interneurons. The Purkinje cells are inhibitory projection neurons and are part of one of the strongest exitatory connections in the central nervous system.

Cell specific RNA hybridization was observed *in situ* over ductal epithelial cells of mammary tissue and uterus, epithelial cells lining the lumen of the uterus, and the entire ovary suggesting that IGFBP-rP1 may play a role in reproductive tissues.

Data obtained from RNA *in situ* hybridization conflicts with that obtained from Northern analysis regarding expression in mouse spleen. We observed strong RNA *in situ* signal over the white pulp region of female spleen, but no signal in male (Table II). However, Northern analysis reveals higher expression in male, using the same tissue tested by RNA *in situ* hybridization (Figures 7 and 8). The ethidium bromide staining and 36B4 probing of the northern blot do not indicate mRNA degradation. Therefore, it is likely that the signal observed by RNA *in situ* hybridization in female mouse spleen may be due to non-specific hybridization to some other RNA present that is gender specific.

Our data suggests that although IGFBP-rP1 is expressed ubiquitously in mouse and the gene may be under hormonal or cell-specific regulation in tissues such as kidney, heart, uterus, mammary tissue, and ovaries. These findings place more emphasis on the importance of isolating and characterizing the promoter region of IGFBP-rP1 to understand the growth regulatory role of IGFBP-rP1.

KEY RESEARCH ACCOMPLISHMENTS:

Aim 1. IGFBP-rP1 inhibits growth in breast epithelial cells.

- New sequence for IGFBP-rP1 identified.
- MCF-7 breast cancer cells transduced with IGFBP-rP1 were analyzed for cell growth curves, senescence-associated beta-galactosidase activity, flow cytometry, and nuclear fragmentation analyses. BT474 cells were subjected to cell growth curves.
- Growth curves show that growth is suppressed in the presence of IGFBP-rP1 in both MCF-7 and BT474 breast cancer cells.
- Senescence-associated β-galactosidase activity is increased in the IGFBP-rP1-transduced MCF-7 cells. Levels of apoptosis are unaltered according to nuclear fragmentation assay results. It is likely that IGFBP-rP1 is using a senescent or senescent-like pathway to inhibit growth. This is further supported by the alteration in morphology of the rP1-transduced MCF-7 which have a large, flattened appearance with a large cytoplasmic to nuclear ratio, characteristic of senescent cells.
- BrdU/Hoechst assay for cell proliferation demonstrates a higher number of IGFBP-rP1 transduced cells in initial G0/G1 compared to empty-vector (LXSN) control cell.
- Upregulation of p21 mRNA observed in IGFBP-rP1 transduced cells.
- No changes found in protein expression of cyclin D and p16 IGFBP-rP1 transduced cells.
- Total retinoblastoma protein may be reduced in IGFBP-rP1 transduced cells.
- Response to various growth factors have not yielded conclusive results.
- Transduced AG11132 (normal HMECs) with empty-vector and IGFBP-rP1 retrovirus.

Aim 2. Experiments for promoter studies: library screening and clone analysis.

- Two clones isolated from genomic library screen enriched for chromosome 4 contain 42 base pairs located at the 5' end of IGFBP-rP1 and potentially the region upstream to the transcription start site. Another three clones may also contain this region.

Aim 3. IGFBP-rP1 is post-translationally modified in ER+ breast cancer cells.

- IGFBP-rP1 protein was immunodetected by Western analysis and has been successfully immunoprecipitated with anti-rP1 Ab.
- Higher MW bands are observed in IGFBP-rP1-transduced MCF-7 and BT474 breast cancer cells.
- Phosphorylation studies suggest IGFBP-rP1 may be post-translationally modified in MCF-7 cells. Treatment with calf intestinal phosphatase results in decreased signal of the upper molecular weight proteins, but no presence of a lower 31 kDa protein.

Aim 4 (Addition): Experiments to determine if tissue specificity is gender dependent for RNA expression/protein localization of IGFBP-rP1 in mice.

- Tissue distribution: IGFBP-rP1 RNA expressed in all tissues tested.
- Higher expression in female kidney and liver when compared to male.
- Cell specific hybridization found in brain: pyramidal cells of cortex Purkinje and granule cells of the cerebellum, and granule cells of the hippocampus.
- Female cell specific expression observed in epithelial cells that line the lumen of the uterine horn and ductal epithelium, throughout ovary, and in ductal epithelium of mammary gland.

REPORTABLE OUTCOMES

Poster Presentation
Mac25 (IGFBP-7) Expression in Mouse. H-M.P. Wilson and K.L. Swisshelm. 4th International Symposium on Insulin-like Growth Factors, Tokyo Japan, October 21-24, 1997. *(I was awarded a travel grant)*

IGFBP-rP1 inhibits growth and induces a senescent phenotype in MCF-7 breast cancer cells. H-M.P.Wilson, R.S.Birnbaum, M.Poot, K.Swisshelm. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta GA, USA, June 8-12, 2000.

Oral Presentation
Introduction of IGFBP-rP1/mac25 into MCF-7 breast cancer cells induces growth inhibition and senescent morphology. H-M.P.Wilson, R.S.Birnbaum, M.Poot, and K.Swisshelm. 81st Annual Meeting of The Endocrine Society, San Diego CA, USA, June 12-15, 1999. *(I was selected and awarded a travel grant to present)*

Papers
H-M.P.Wilson, R.S.Birnbaum, M.Poot, and K.Swisshelm. Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) inhibits proliferation in MCF-7 breast cancer cells. *(in preparation)*

K.L.Haugk, H-M.P.Wilson, K.Swisshelm, and L.S.Quinn. Insulin-like growth factor (IGF)-binding protein-related protein-1: an autocrine/paracrine factor that inhibits skeletal myoblast differentiation but permits proliferation in response to IGF. Endocrinology, 141(1):100-110 (2000).

CONCLUSIONS

In this report, we demonstrate a cell proliferation inhibitory role of IGFBP-rP1 in the ER-positive/IGFBP-rP1-negative MCF-7 breast cancer cell line. Unaltered levels of apoptosis, yet increased senescence-associated β-galactosidase activity suggests a link to cellular senescence or a senescence-associated pathway. Further evidence of a senescence-related mechanism is provided by an observed increase in expression of p21 mRNA and the number of non-cycling cells in the IGFBP-rP1-transduced MCF-7 cell lines. These results indicate the inhibitory role of IGFBP-rP1 in MCF-7 breast cancer cells is via a senescent-associated pathway, independent from apoptosis and the IGF system.

IGFBP-rP1 protein is expressed at a higher molecular weight in two ER-positive breast cancer cell lines (MCF-7 and BT474) when compared to the endogenous protein secreted by ER-negative Hs578T breast cancer cells. We suggest phosphorylation as a potential modification of IGFBP-rP1 based on our studies by Western analysis using calf intestinal phosphatase-treated IGFBP-rP1.

Studies of IGFBP-rP1 expression patterns in mouse tissue indicate that although there may be gender specific expression of IGFBP-rP1, suggestive of hormonal regulation, cell-specific expression may play a more important role in IGFBP-rP1 function, as supported by initial research done to isolated and identify this protein. IGFBP-rP1 has been isolated from various cell types under various names descriptive of its functional role. Mac25/IGFBP-7/IGFBP-rP1 was first identified using a subtraction library created from normal leptomeningeal cells and a meningioma cell line (*23*). It was subsequently isolated by differential display between normal and senescent HMECs and shown to contain homology with members of the IGFBP family (*9*). Prostacyclin-stimulating factor (PSF) is secreted from human diploid fibroblasts and was isolated by its functional activity. Tumor-derived adhesion factor (TAF), isolated from human bladder carcinoma, demonstrated cell adhesion activity in human umbilical vein endothelial cells (*24*).

We have determined the inhibitory role of IGFBP-rP1 in MCF-7 breast cancer cells and have supported this role in experiments that demonstrate proliferation inhibition using another ER-positive/IGFBP-rP1-negative breast cancer cell line, BT474 cells. Now that we have elucidated a possible functional role of IGFBP-rP1 in ER-positive human breast cancer, determination of IGFBP-rP1 gene regulation (Aim2) becomes central to bridging the functional role of IGFBP-rP1 and controlling its expression as a potential therapeutic agent in breast cancer. We have visually identified a potential RARE in the known promoter sequence of PSF. These studies suggest that IGFBP-rP1 may be regulated through retinoids. Current studies in our laboratory focus on understanding the role retinoids play in breast cancer and their potential role as therapeutic agents. IGFBP-rP1, shown to be upregulated in HMECs when cultured in the presence of all-trans-retinoic acid and synthetic retinoid fenretinide retinoic acid (*9*), may function downstream of retinoids by carrying out growth suppression of breast tumor cell lines in response to retinoids.

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Material and Methods

Aim 1: presented in attached manuscript (Appendix A) and below.

Western Analysis

For analysis of cell cycle regulating protein expression, cells were plated at 50,000 cells per 60-mm tissue culture dish in complete αMEM. On day 7, cells were washed twice with PBS, scraped in lysis buffer (1% SDS, 50mM PBS, 1mM PMSF, 1 μg/ml leupeptin), drawn through a 25 gauge needle 10x, and microcentrifuged at the highest setting for 5 minutes, RT. The non-viscous layer (top) was collected, protein content was analyzed by Bradford assay, and 5 μg of whole cell extract was separated on a 12% polyacrylamide gel with a 4% polyacrylamide stacking gel. Protein was transferred onto Immuno-Blot PVDF membrane (Bio-Rad) at 30 V overnight in transfer buffer at 4°C (39mM glycine, 48mM Tris, 0.04% (w/v) SDS, 20% MeOH). The membrane was incubated with 10% hydrogen peroxide for 10 min and blocked 1 h in 5% blotting-grade nonfat dry milk in 0.1% Tween-20/Phosphate Buffered Saline (PBS-T). The membrane was cut into horizontal strips and incubated 1 h at RT with the following antibodies, corresponding to protein size: anti-Rb (1:4000 in 2.5% milk in PBS-T, Pharmingen), anti-p16 (1:3000 in 1%BSA/0.5% milk in TTBS, Pharmingen), cyclin D (1:1000 in 2.5% milk in PBS-T, Upstate Biotechnology, Inc., Lake Placid, NY), and ERK1/ERK2 (1:40,000 in 5% milk in PBS-T, gift from E. Krebs, Univ. of WA). Blots were washed in PBS-T and incubated 1 h with the following secondary Ig horseradish peroxidase linked whole antibodies corresponding to host of primary antibody: anti-mouse (1:20,000 in 2.5% milk in PBS-T, Pierce, Rockford, IL) and anti-rabbit (1:2000 in 2.5% milk in PBS-T, Amersham Life Science). Protein was detected with Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL) and CL-Xposure Film (Pierce).

Aim 2

Purification of lambda DNA

Lambda DNA clones were amplified by large scale phage lysates following the protocol described (*25*). The amplified λ DNA was isolated using a Lambda DNA purification kit (Qiagen).

Southern analysis of λ DNA clones

Lambda DNA was run on a 0.8% agarose gel. The gel was denatured in 0.5N NaOH, 1M NaCl, rinsed with distilled H_2O, and neutralized with 1.5M Tris, pH 7.4, 3M NaCl. The λ DNA fragments were transferred to Zeta Blot membrane (Bio-Rad, Ca) by capillary transfer. The blot was hybridized with ^{32}P-labeled probe made from 42bp at the 5' end of IGFBP-rP1 cDNA.

Aim 3

Phosphorylation Studies

Hs578T and MCF-7 breast cancer cells were grown to 90% confluency and incubated in SFM overnight (24 hours). 750μl of conditioned medium was collected and incubated with 20μM ethanolamine and 4μl calf intestinal phosphatase (Roche Molecular Biochemicals) at 37°C for 1 hour, concentrated onto nitrocellulose, eluted into 1X sample

buffer, separated by PAGE, and transferred to PVDF membrane. IGFBP-rP1 protein was immunodetected using anti-hrIGFBP-rP1 (*26*) at 1:2500 in 1% BSA/0.5% milk in TTBS overnight at 4°C. Blots were washed in TTBS, incubated with 1:3000 Donkey Anti-Rabbit Ig horseradish peroxidase linked whole antibody (Amersham Life Science). IGFBP-rP1 protein was detected with Supersignal Chemiluninescent Substrate (Pierce, Rockford, IL) and CL-Xposure Film (Pierce).

Aim 4

Northern Analysis

Tissue Collection. Tissue was collected from female, pregnant and virgin, and male C57BL6 mice, snap frozen in liquid nitrogen. RNA was isolated with Ultraspec II (Biotecx, Houston, TX). 10μg of RNA was analyzed by standard Northern blot analysis using Zeta Probe membranes (Biorad).

Hybridization. Blots were probed with ^{32}P-labeled IGFBP-rP1 cDNA probe overnight at 42°C and washed at 1XSSC at 60°C. Quantitation of the autoradiographic signal was obtained by scanning with an Agfa, Arcus II scanner and Adobe Photoshop 3.0 and 4.0, NIH Image 1.6, and Excel 5.0.

In Situ Hybridization

Tissue Collection. Tissue was collected from ICR (outbred) and C57BL6 (inbred) mouse strains and frozen in O.C.T. freezing medium in isopentane chilled in liquid nitrogen. Samples were stored at –70°C and thawed to –20°C before and during cutting on a cryostat. Six micron sections were transferred to SuperFrost Plus microslides (VWR) and stored at –20°C.

Preparation of DNA for generation of ^{33}P-labeled RNA. The pBluescriptIIKS-IGFBP-rP1 full length construct was digested with SmaI (antisense) and MspI (sense) in separate reactions and purified by pheno/chloroform extraction and ethanol precipitation.

^{33}P-labeled riboprobes. RNA was transcribed from 1μg of DNA, transcription buffer, 10mM each of ATP, GTP, and CTP, α 33P-UTP, and Rnase Inhibitor. T7 polymerase yielded at 600 nucleotide antisense probe and T3 RNA polymerase a 200 nucleotide sense probe (reagents from Boehringer Mannheim).

Preparation of tissues on slides. Slides were air dried at room temperature and fixed in 4% paraformaldehyde in phosphate buffer, pH 7.4. The tissues were dehydrated, delipidated, rehydrated and prehybridized with hybridization buffer for two hours at 50°C, then washed twice in PBS, pH 7.4 and hybridized with the riboprobe overnight at 50°C. Sildes were rinsed in 4X SSC, washed in 2X SSC, and incubated 30 minutes with RNAse A at 37°C. Slides were rinsed with PBS, pH 7.4 and washed with 2X SSC, 50% formamide and 1X SSC, 50% formamide for 30 minutes each at 60°C, followed by 30 minutes in 1X SSC at 37°C. The slides were dehydrated, allowed to air dry two hours, and dipped in emulsion (Kodak NTB-3). Slides were developed with D19 and Fixer (Kodak, USA) at 1, 2, and 4-5 week intervals. Slides were stained with H & E and visualized with a Hamamatsu C58-10 video camera using Adobe Photoshop 3.0.

FIGURE LEGENDS
Figures on pages 20-27

Figure 1. *Altered expression of regulating cell cycle genes is observed in IGFBP-rP1 transduced MCF-7 cells. Lanes 1-4:* LXSN-transduced MCF-7 cells (*Lane 1*-T1, pooled; *2*-T2, pooled; *3*-cl.2; *4*-cl.6). *Lanes 5-8:* IGFBP-rP1-transduced MCF-7 cells (*Lane 5*-T1, pooled; *6*-T2, pooled; *7*-cl.9; *8*-cl.10). *Lane 9:* MDA-MB-435 breast cancer cells (positive control for p16 protein expression). A) Northern blot demonstrating p53 and p21 mRNA expression. B) Western blot exhibiting total Rb, cyclin D, p16, and ERK1/ERK2 (control for loading and transfer) protein expression. Panels demonstrating total Rb and 42 kDa ERK1/ERK2 expression are from the same immunoblot. Cyclin D and p16 expression panels come from a second immunoblot. The longer exposure of ERK1/ERK2 is taken from a third immunoblot to demonstrate both bands are detected when exposed to film for a greater length of time. Whole cell extract used for all three immunoblots came from the same sample source. Each immunoblot was verified with ERK1/ERK2 for even loading and transfer of protein.

Figure 2. *Verification of changes in expression of regulating cell cycle genes by densitometry using NIH Image 1.60.* A) Standardized p53 mRNA signal using 36B4 loading/transfer control signal. B) Standardized p21 mRNA signal using 36B4 loading/transfer control signal. C) Total Rb protein signal standardized with ERK1/ERK2 signal. White bars indicate MCF-7 cell lines transduced with empty-vector (LXSN) control. Black bars represent IGFBP-rP1-transduced MCF-7 cell lines.

Figure 3. *Cell proliferation may be inhibited in IGFBP-rP1-transduced BT474 breast cancer cells.* Cells were plated at 50,000 cells per 60-mm tissue culture dish in complete αMEM medium and counted on day 7. Each bar represents one experiment done in duplicate. The error bars represent the deviation from the mean.

Figure 4. *A potential RARE is identified in PSF promoter sequence.* Sequence of PSF promoter region identified by Mizushima et al. (*14*). Bars over nucleotides represent potential RAR binding sites.

Figure 5. *Five potential candidate clones isolated that may contain promoter region.* A. Diagram of 42 basepair probe located at 5' end of IGFBP-rP1. B. Southern blot analysis demonstrates strong hybridization signal in clones 4 and 7 and weak signal in clones 5, 6, and 8.

Figure 6. *Phosphatase-treated IGFBP-rP1 protein signal is decreased.* Hs578T and MCF-7 cells were grown, conditioned medium collected and treated with calf intestinal phosphatase as described in Material and Methods. Hs578T and LXSN-transduced MCF-7 breast cancer cells were used as controls. Under various testing conditions, IGFBP-rP1 protein signal was decreased in the presence of phosphatase (denoted by arrows).

Figure 7. *IGFBP-rP1 mRNA expression is detected in all mouse tissue examined.* IGFBP-rP1 is expression as a 1.5 kb transcript in tissues examined. A second transcript of 3.1kb is expressed in kidney (+++), heart (+), and lung (+), suggesting a splice variant of the gene. This Northern is representative of expression patterns observed in mice examined in Table I.

Figure 8. *IGFBP-rP1 mRNA expression is higher in female kidney and heart.* Analysis of mRNA signal from male and female C57BL6 mice (one of each gender) was performed using NIH Image 1.60. Higher expression of IGFBP-rP1 mRNA was observed in female kidney and heart. IGFBP-rP1 mRNA expression was higher in male spleen.

STATEMENT OF WORK

Technical Objective 1
Test the potential of IGFBP-rP1 function.

Task 1: (Months 1-4) Prepare plasmids for transfection. Ligate full-length IGFBP-rP1 cDNA into retroviral vector. *Completed*

Task 2: (Months 5-12) Transfection of packaging cell lines and transduction of breast cancer cell lines, select clones and begin to test growth parameters. *Completed*

Task 3: (Months 15-24) Complete growth studies, demonstrating that IGFBP-rP1 inhibits growth in breast cancer cells. *Completed – paper for resubmission, oral presentation given*

Task 4: (Months 24-36) Test for mechanism behind growth suppression in transduced-breast cancer cell lines (apoptosis vs. senescence, IGF-independent). *Completed*

Task 5: (Months 36-40) Test the function of IGFBP-rP1 in normal human mammary epithelial cells and analyze transduced-AG11132 normal cells for alterations in growth and markers of senescence. *In Progress*

Task 6: (Months 43-48) Identify alterations in cell cycle and/or cell cycle regulatory genes. *Completed*

Technical Objective 2
Characterize the mac25 promoter.

Task 1: (Months 4-7) Finish screening genomic libraries for IGFBP-rP1 promoter. *Completed*

Task 2: (Months 12-15, 24-32) Restriction and sequence analysis of promoter clones. *In Progress*

Technical Objective 3
Determine protein binding characteristics of mac25.

Task 1: (Months 1-12) Test antiserum generated mac25 peptide by Elisa. Have access to an antibody generated in another lab that has been successfully used in our lab. *Completed*

Task 2: (Months 12-28) Perform immunoblots for normal HMECs and breast tumor cell lines. *Completed*

Task 3: (Months 15-24) Determine ligand interaction with IGFBP-rP1. *Completed*

Task 4: (Months 28-36) Determine modification of higher molecular weight IGFBP-rP1 proteins secreted by transduced-ER+ breast cancer cells. *In Progress*

Technical Objective 4 (Additional)
Determine IGFBP-rP1 tissue specificity of RNA.

Task 1: (Months 5-12) Test mRNA expression localization in various mouse tissue. *Completed – abstract presented*

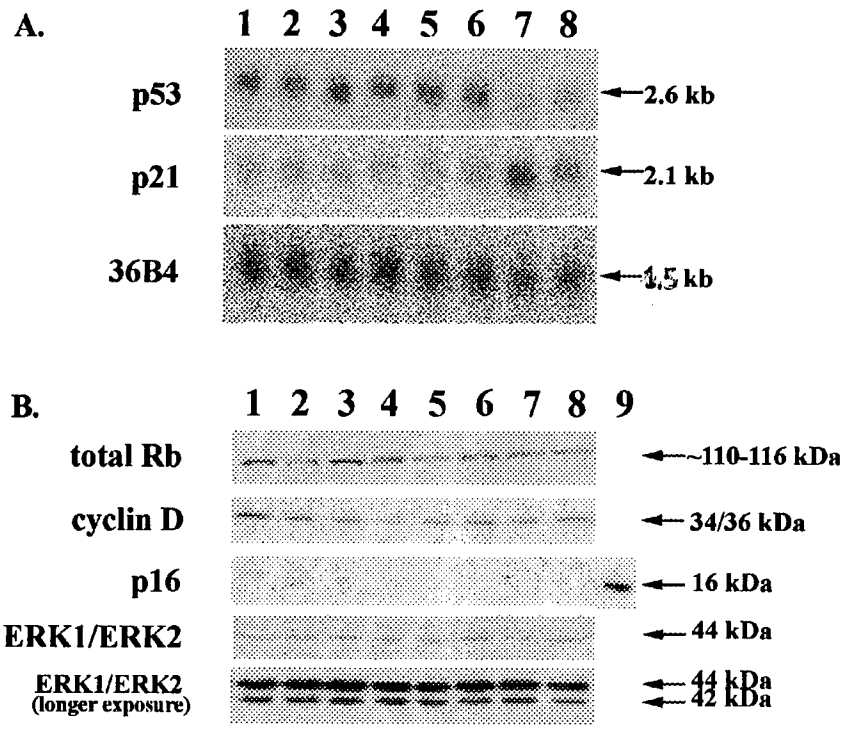
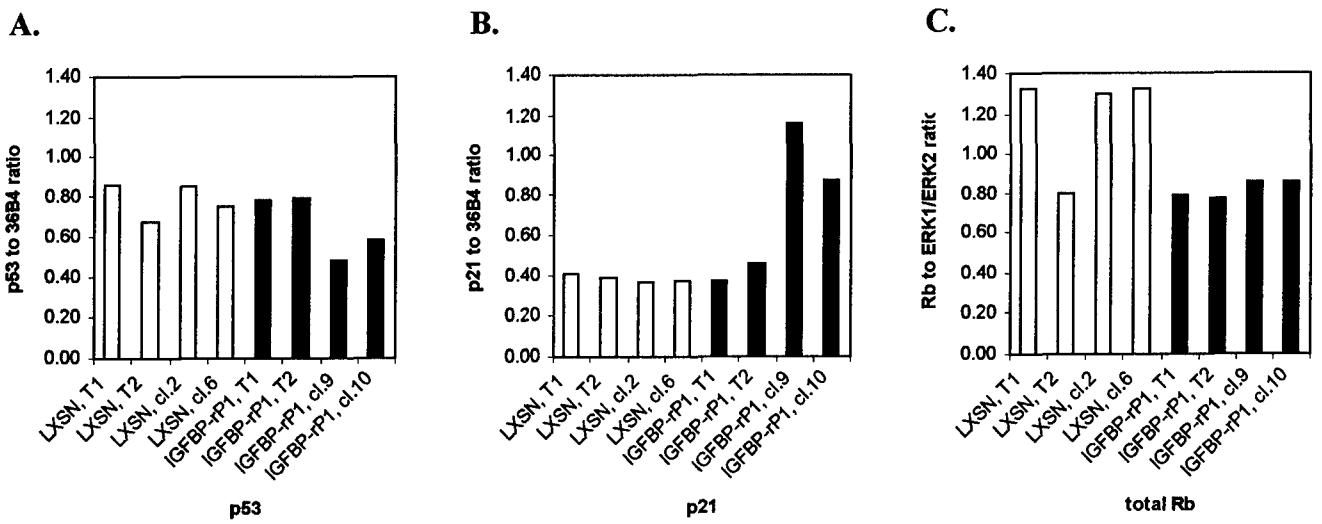

A.
1 2 3 4 5 6 7 8
p53
2.6 kb
p21
2.1 kb
36B4
1.5 kb
B.
1 2 3 4 5 6 7 8 9
total Rb
~110-116 kDa
cyclin D
34/36 kDa
p16
16 kDa
ERK1/ERK2
44 kDa
ERK1/ERK2
(longer exposure)
44 kDa
42 kDa

Figure 1

Figure 2


A.
B.
C.
p53 to 36B4 ratio
p21 to 36B4 ratio
Rb to ERK1/ERK2 ratio
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
LXSN, T1
LXSN, T2
LXSN, cl.2
LXSN, cl.6
IGFBP-rP1, T1
IGFBP-rP1, T2
IGFBP-rP1, cl.9
IGFBP-rP1, cl.10
p53
p21
total Rb

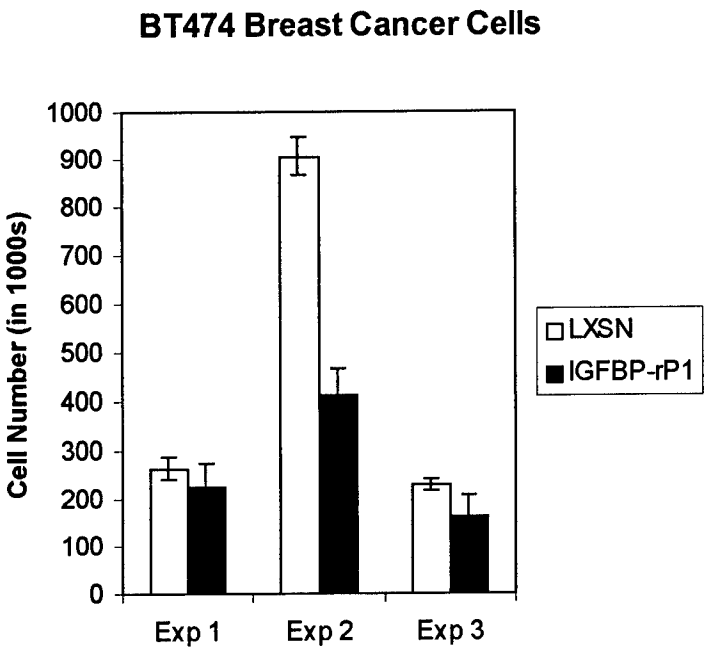

BT474 Breast Cancer Cells
Cell Number (in 1000s)
1000
900
800
700
600
500
400
300
200
100
0
Exp 1
Exp 2
Exp 3
LXSN
IGFBP-rP1

Figure 3

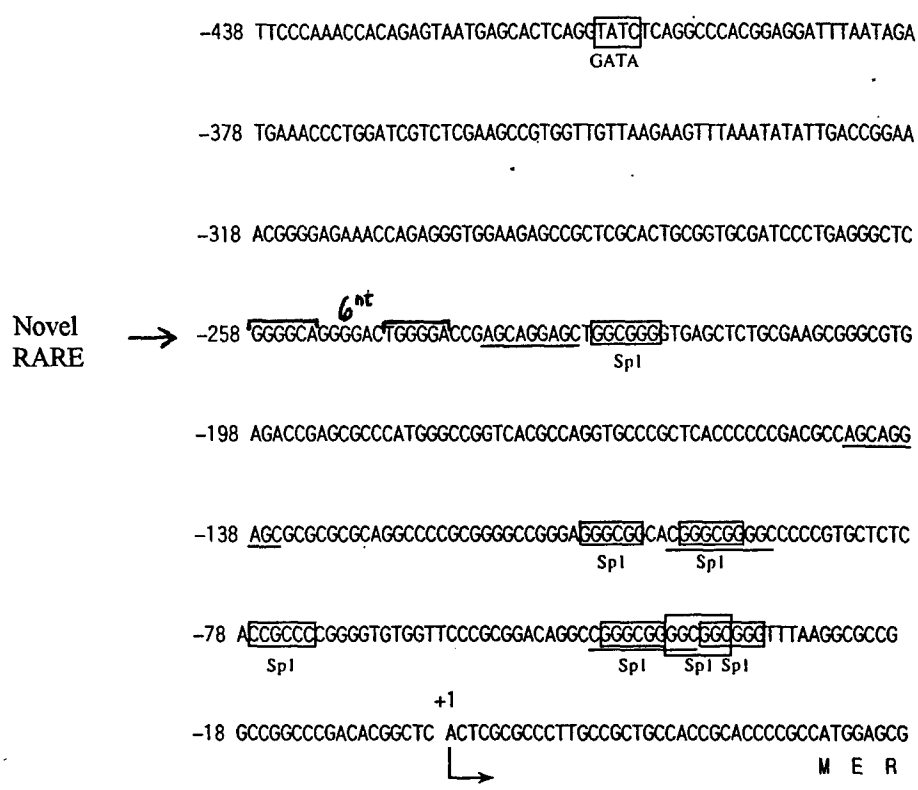
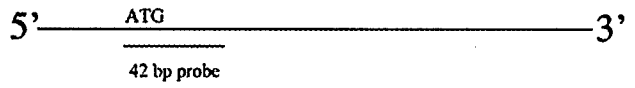


Fig. 5. **Nucleotide sequence of the 5′-flanking region of the human PSF gene.** Adenosine, which is the major transcription start site, is marked by an arrow and is numbered +1. Putative Sp1 and GATA sites are boxed. Repeated sequences are underlined.

Reprinted from:
Mizushima et al. (1996) Isolation and characterization of the human chromosomal gene for prostacyclin-stimulating factor. *J Biochem* (Tokyo) **120**, 929-933.

Figure 4

A. IGFBP-rP1 fragment used as probe.



B.

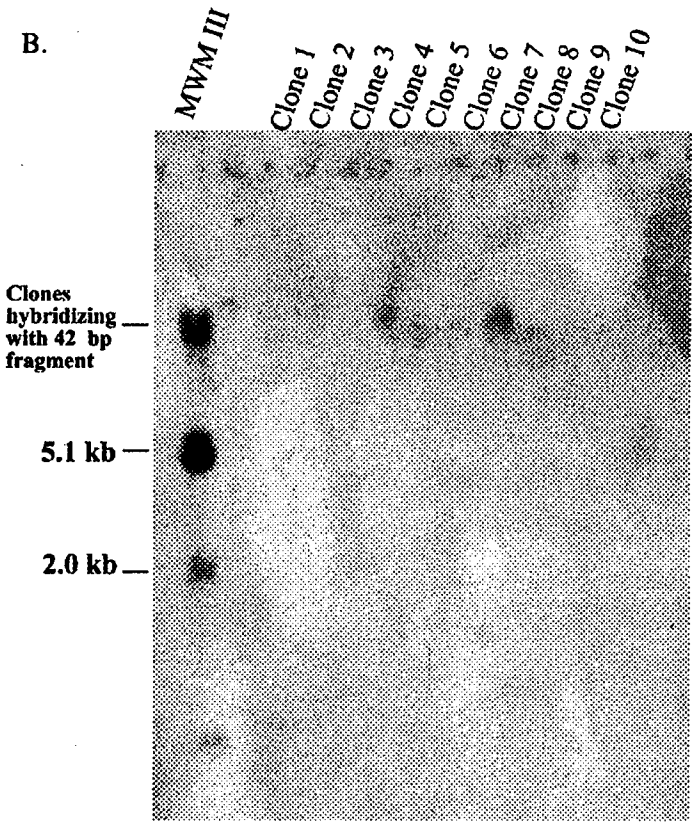


Figure 5

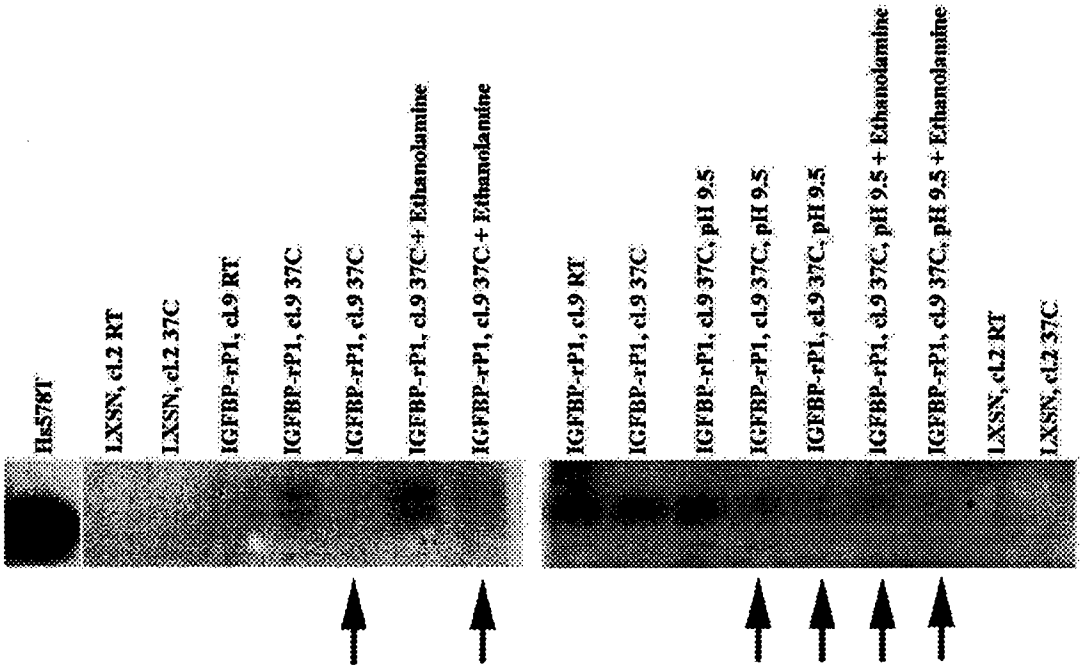

Hs578T
LXSN, cl.2 RT
LXSN, cl.2 37C
IGFBP-rP1, cl.9 RT
IGFBP-rP1, cl.9 37C
IGFBP-rP1, cl.9 37C
IGFBP-rP1, cl.9 37C + Ethanolamine
IGFBP-rP1, cl.9 37C + Ethanolamine
IGFBP-rP1, cl.9 RT
IGFBP-rP1, cl.9 37C
IGFBP-rP1, cl.9 37C, pH 9.5
IGFBP-rP1, cl.9 37C, pH 9.5
IGFBP-rP1, cl.9 37C, pH 9.5
IGFBP-rP1, cl.9 37C, pH 9.5 + Ethanolamine
IGFBP-rP1, cl.9 37C, pH 9.5 + Ethanolamine
LXSN, cl.2 RT
LXSN, cl.2 37C

Figure 6

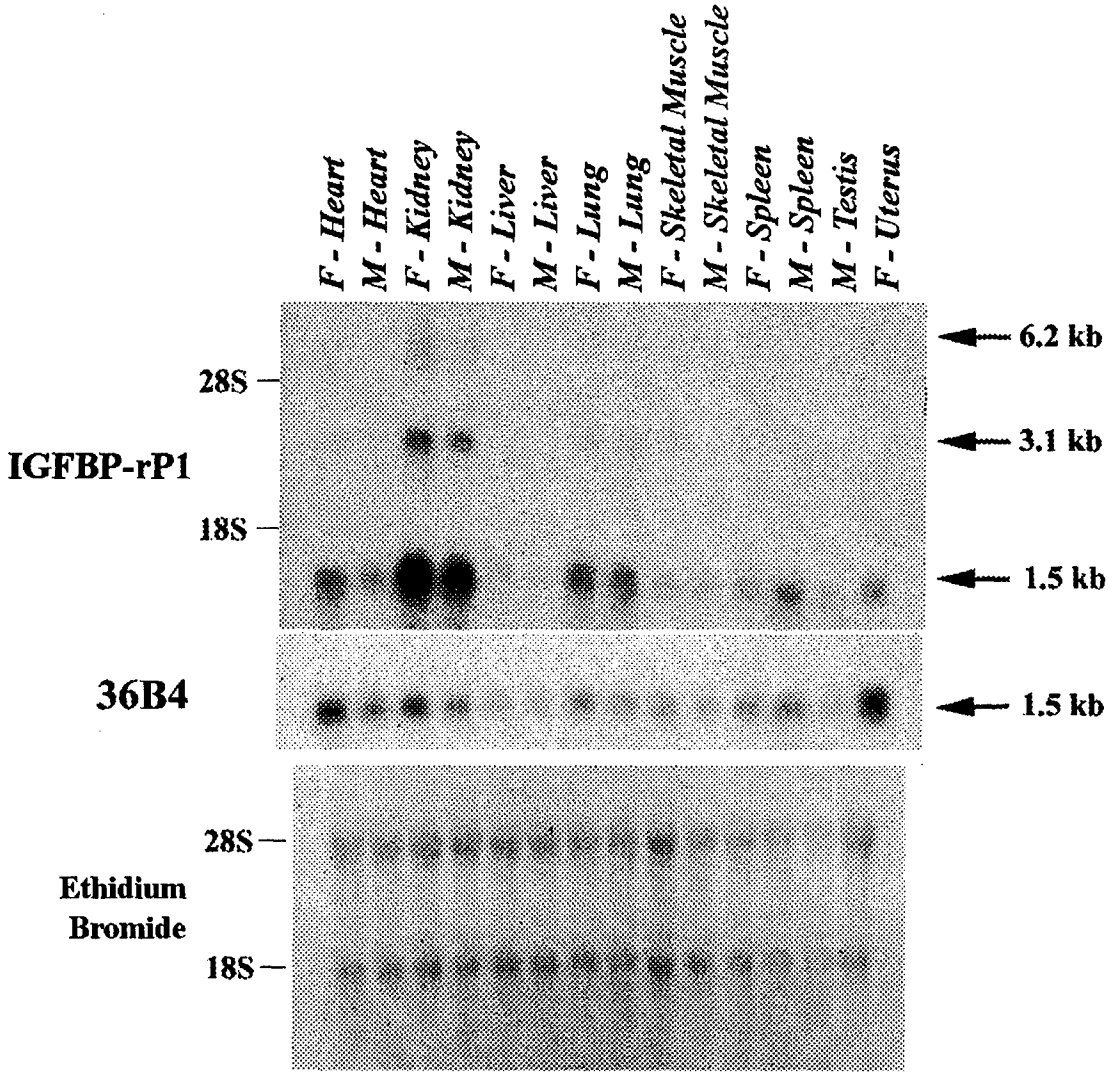

F - Heart
M - Heart
F - Kidney
M - Kidney
F - Liver
M - Liver
F - Lung
M - Lung
F - Skeletal Muscle
M - Skeletal Muscle
F - Spleen
M - Spleen
M - Testis
F - Uterus
28S
IGFBP-rP1
18S
6.2 kb
3.1 kb
1.5 kb
36B4
1.5 kb
28S
Ethidium Bromide
18S

Figure 7

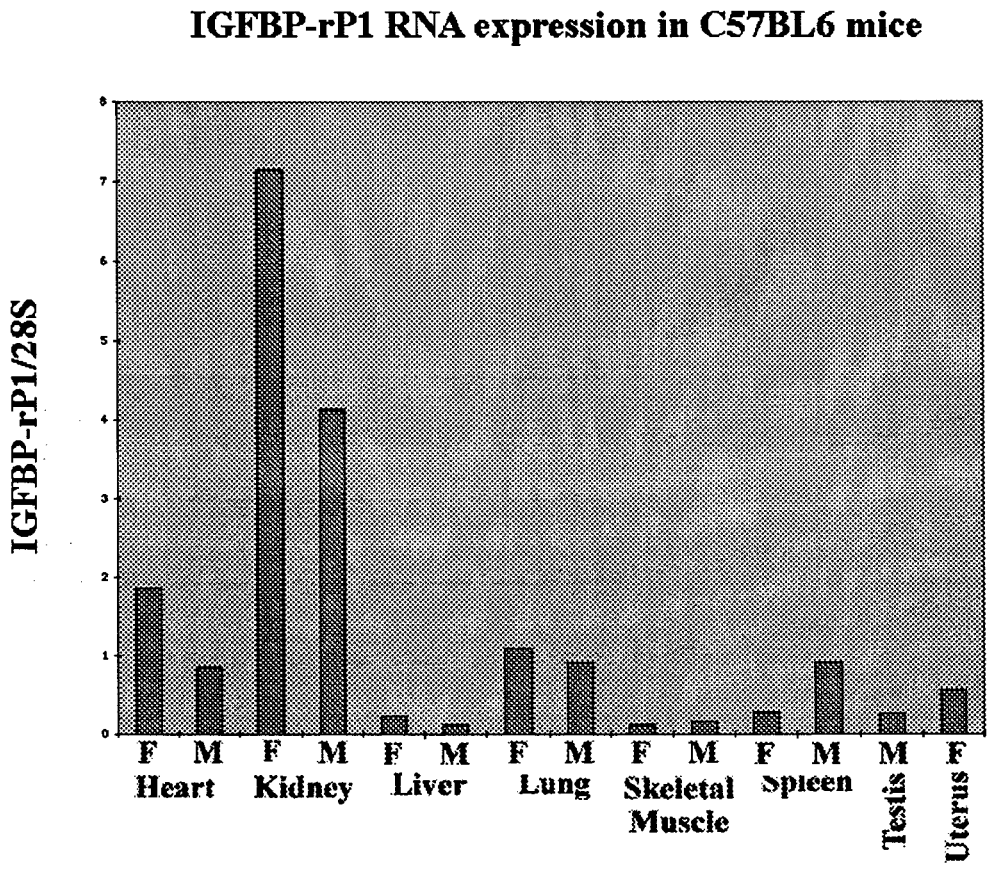

IGFBP-rP1 RNA expression in C57BL6 mice
IGFBP-rP1/28S
0
1
2
3
4
5
6
7
F M F M F M F M F M F M F M F
Heart
Kidney
Liver
Lung
Skeletal Muscle
Spleen
Testis
Uterus

Figure 8

Table I. Northern Analysis: IGFBP-rP1 expression in mouse tissue

Tissue	*total # of mice*	*Male*	*Female*	*Pregnant Female*	*Strain*	*IGFBP-rP1 expression*	*Transcript size(s)*
Brain	23	18	2	3	BDF1, C57BL6	+	1.1kb
Heart	13	5	5	3	C57BL6	++	1.1kb, 3.1kb
Kidney	25	17	5	3	BDF1, C57BL6	+++	1.1kb, 3.1kb
Liver	24	18	3	3	BDF1, C57BL6	+	1.1kb
Lung	5	1	3	1	C57BL6	++	1.1kb, 3.1kb
Mammary Tissue	3	-	2	1	C57BL6	++	1.1kb, 3.1kb
Ovaries	7	-	4	3	C57BL6	++	1.1kb, 3.1kb
Skeletal Muscle	7	3	3	1	C57BL6	+	1.1kb
Spleen	2	1	1	-	C57BL6	+	1.1kb
Testis	1	1	-	-	C57BL6	+	1.1kb
Uterus	8	-	2	1	C57BL6	++	1.1kb, 3.1kb

Table II. mRNA *in situ* hybridization: IGFBP-rP1 expression in mouse tissue

Tissue	*total # of mice*	*Male*	*Female*	*Pregnant Female*	*Strain*	*Signal Localization*	*Gender Specificity*
Aorta	2	1	1		C57BL6	smooth muscle cells	no
Brain	4	1	2	1	C57BL6, ICR	hippocampus cerebellum - granule cells	no
Heart	4	2	2		C57BL6, ICR	sparsely scattered cells of unknown origin	detected in female only
Kidney	4	2	2		C57BL6, ICR	throughout	no
Liver	4	2	2		C57BL6, ICR	throughout - Kupffer cells?	no
Lung	2	1	1		C57BL6	septal cells	no
Mammary Tissue	2	1	1		C57BL6	ductal epithelium	-
Ovaries	2	-	2		C57BL6, ICR	throughout	-
Skeletal Muscle	2	1	1		C57BL6	satellite cell?	no
Spleen	2	1	1		C57BL6	white pulp - lymphocytes	detected in female only
Testis	2	2			C57BL6		-
Uterus	1	-	1		C57BL6	lumen of uterine horn, ductal epithelium	-

BIBLIOGRAPHY

Abstracts

Mac25 (IGFBP-7) Expression in Mouse. H-M.P. Wilson and K.L. Swisshelm. 4th International Symposium on Insulin-like Growth Factors, Tokyo Japan, October 21-24, 1997.

IGFBP-rP1 inhibits growth and induces a senescent phenotype in MCF-7 breast cancer cells. H-M.P.Wilson, R.S.Birnbaum, M.Poot, K.Swisshelm. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta GA, USA, June 8-12, 2000.

Introduction of IGFBP-rP1/mac25 into MCF-7 breast cancer cells induces growth inhibition and senescent morphology. H-M.P.Wilson, R.S.Birnbaum, M.Poot, and K.Swisshelm. 81st Annual Meeting of The Endocrine Society, San Diego CA, USA, June 12-15, 1999.

Journal Publication

K.L.Haugk, H-M.P.Wilson, K.Swisshelm, and L.S.Quinn. Insulin-like growth factor (IGF)-binding protein-related protein-1: an autocrine/paracrine factor that inhibits skeletal myoblast differentiation but permits proliferation in response to IGF. Endocrinology, 141(1):100-110 (2000).

Insulin-like Growth Factor Binding Protein-Related Protein 1 (IGFBP-rP1) Inhibits Proliferation in MCF-7 Breast Cancer Cells (< 150 characters, currently 156, including spaces)

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Running Title: IGFBP-rP1 inhibits proliferation

Manuscript information: 17 pages; 3 figures, 4 tables

Key Words: IGFs (insulin-like growth factors), Insulin-like growth factor binding proteins (IGFBPs), IGFBP related-protein 1 (IGFBP-rP1), breast cancer, proliferation, apoptosis, senescence

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Abstract

Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a member of the IGFBP-related proteins (IGFBP-rPs), a subgroup in the IGFBP family classified by their low binding affinity for IGFs. Upregulation of IGFBP-rP1 mRNA in normal senescent human mammary epithelial cells (HMECs) suggests that the protein may possess growth inhibitory function. In order to assess growth-inhibiting properties of IGFBP-rP1 in human breast cancer, we retrovirally introduced IGFBP-rP1 cDNA into the IGFBP-rP1-deficient MCF-7 breast cancer cell line. An average of 39% and 74% reduction in cell numbers was observed after seven-day growth in IGFBP-rP1-transduced pooled and clonal populations, respectively. Nuclear fragmentation assays excluded apoptosis as a possible mechanism used by IGFBP-rP1. The number of cells containing senescence-associated β-galactosidase activity was doubled in IGFBP-rP1-transduced MCF-7 cells. The number of non-cycling cells arrested in G0/G1 was increased in IGFBP-rP1-transduced MCF-7 cells. We suggest that IGFBP-rP1 may inhibit proliferation in MCF-7 breast cancer cells via a senescence-associated pathway.

Introduction

Replicative senescence is a process that limits the capacity for cell division (reviewed in (*1, 2*)). Senescent cells arrest in G_1 stage of cell cycle and can remain metabolically active for months or even years, though they are incapable of DNA synthesis (*3, 4*). Because cellular senescence results in suppressed growth and eventual cell death, it is an optimal system to study as a model for tumor suppression.

IGFBPs have been linked to senescence. Increasing IGFBP-3 levels, associated with inhibition of cell proliferation, are observed in conditioned medium of human diploid fibroblasts with increasing donor age, *in vitro* senescence, and increasing confluency (*5-8*). Recent studies indicate tissue-specific regulation of IGFBP expression in senescent cells. IGFBP-2 expression is upregulated in senescent human retinal pigment epithelial cells (*9*), but decreases with age in cerebral spinal fluid (*10*). Proteolytic degradation of 24 kDa-34 kDa IGFBPs is increased in senescent cells which may decrease the ability of these proteins to present IGFs to the IGF receptors on the cell surface (*6*).

IGFBPs and IGFBP-rPs are potential positive/negative mediators of IGF or IGF-like peptide signaling (*11*), although IGF-independent actions have been documented ((*12, 13*) and reviewed in (*14*)). IGFBP-rPs are members of the insulin-like growth factor binding protein (IGFBP) superfamily. IGFBP-rP1 (mac25/TAF/PSF/IGFBP-7) shares approximately 30% homology at the amino acid level with the IGFBPs (*15, 16*) and exhibits low affinity for the known IGFBP ligands, IGF-1 and IGF-2 (*17*).

Expression levels of IGFBPs in breast cancer appears to be related with estrogen receptor status (*18-20*). IGFBP-1 (exclusively, but not all), -3, and -6 are found in ER-negative breast cancer cell lines (*19, 21*). Whereas IGFBP-2, -4, -5, -6 and low levels of IGFBP-3 are found in ER-positive cells (*19, 22*). An inverse correlation exists between IGFBP-rP1 mRNA expression and estrogen receptor status in human breast cancer cells (*23, 24*).

IGFBP-rP1 mRNA expression is three to eight-fold higher in senescent versus proliferating normal human mammary epithelial cells (HMECs) prompting us to speculate that IGFBP-rP1 may possess tumor suppressing capabilities (*24*). A potential tumor suppressor role is supported by data revealing down-regulated IGFBP-rP1 protein in primary prostate cancer *versus* normal prostate stroma and glandular epithelium (*25*). Moreover, IGFBP-rP1 inhibits growth of immortalized or malignant human prostate epithelial cells in soft agar and tumor formation in nude mice (*26*). Induction of IGFBP-rP1 mRNA is detected in differentiating granulosa cells which eventually enter replicative senescence (*27*). Truncated *murine* mac25, which lacks the IGF binding domain, attenuated clonal growth of human Saos II cells and suggests that the physiologic role of IGFBP-rP1 may be independent of IGF signaling (*28*). Collectively, these previous findings led us to hypothesize that over-expression of IGFBP-rP1 may suppress the proliferative potential of ER-positive, IGFBP-rP1-negative breast cancer cells. To test this hypothesis, we stably introduced the IGFBP-rP1 cDNA into the human breast carcinoma cell line MCF-7 and assessed their growth.

Material and Methods

Cell Culture

The PE501 and PA317 virus packaging cell lines were cultured in αMEM (Gibco BRL) supplemented with 10% fetal bovine serum. Hs578T and MCF-7 cells (American Type Culture Collection, Manassas, VA) were cultured in αMEM supplemented as previously described (*29*) and referred to as complete αMEM. Serum-free medium (SFM) consisted of αMEM supplemented with 10mM HEPES, 1mM sodium pyruvate, 1x non-essential amino acids, and 0.05% BSA (Sigma, St. Louis, MO).

Generation of IGFBP-rP1 retroviral vector constructs and transduced MCF-7 cells

IGFBP-rP1 cDNA was ligated into the BamHI site of the pLXSN plasmid (*30, 31*). The PE501 (ecotropic) and PA317 (amphotropic) retroviral-packaging cell lines were used to generate virus as described by Miller *et al.* (*31*). MCF-7 cells were transduced by LXSN or L*IGFBP-rP1*SN virus in the presence of 4 μg/ml Polybrene (Sigma). Infected cells were selected in media containing 1mg/ml G 418 (Calbiochem), and maintained in 0.75 mg/ml G 418. When cell clones had grown to about 50 cells, they were trypsinized and transferred to 24-well plates.

Northern analyses

MCF-7 cells were grown to 90% confluency on 100-mm dishes, and RNA was isolated using the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX). Total RNA (10 μg) was separated on formaldehyde-agarose gels and transferred to Zetaprobe membrane (Bio-Rad, Hercules, CA) using standard techniques

(*29*). Full-length IGFBP-rP1 cDNA, p53 (gift from J. Gudas), p21 (ATCC), and 36B4 probes were labeled with ^{32}P α-dCTP using the Random Primed DNA Labeling Kit (Boehringer Mannheim). Human acid ribosomal phosphoprotein PO with estradiol-independent mRNA expression (36B4) was used as a control for loading and transfer (*32*). Final wash was done at: 1XSSC/0.1% SDS, 65°C for IGFBP-rP1, p21, and 36B4; 1XSSC/0.1% SDS, 55°C for p53.

Western blotting

For immunodetection of secreted IGFBP-rP1 protein, cells were plated at $5x10^5$ cells per 35-mm tissue culture dish in complete αMEM. On day 5, cells were washed once with PBS and fed 2 ml SFM. Twenty-four hours later (day 6), 750μl of conditioned medium was collected and immediately concentrated onto nitrocellulose membrane (*33*). The concentrated protein was eluted by boiling in 25μl of 1x sample buffer (0.5M Tris, 10% glycerol, 8M urea, and 2% SDS). The proteins were separated on a 15% polyacrylamide gel with a 4% polyacrylamide stacking gel. Proteins were transferred onto Immuno-Blot PVDF membrane (Bio-Rad) at 100 V for 1 h in transfer buffer (39mM glycine, 48mM Tris, 0.04% (w/v) SDS, 20% MeOH). The membrane was incubated with 10% hydrogen peroxide for 10 minutes, blocked 1 h in 1% BSA/0.5% milk (Bio-Rad) in 0.05% Tween-20/Tris Buffered Saline (TTBS), and incubated overnight at 4°C with anti-hrIGFBP-rP1 (*34*) at 1:2500 in blocking solution. Blots were washed in TTBS and incubated for 3 h at RT with donkey anti-rabbit Ig horseradish peroxidase-linked whole antibody (Amersham Life Science) at 1:3000 in blocking solution. The IGFBP-rP1 protein was detected with SuperSignal Chemiluminescent Substrate (Pierce, Rockford,

IL) and CL-Xposure Film (Pierce).

Cell number assay

Parental and retrovirally transduced MCF-7 cell lines (described above) were plated on 60-mm tissue culture plates at $5x10^4$ cells/plate and grown in complete αMEM. Duplicate plates for each time point were trypsinized and cells counted on days 1, 2, 3, 5, and 7 using a hemocytometer.

Nuclear fragmentation assay as a parameter for apoptosis

Cells were plated, described above, in complete αMEM. After seven days, cells were harvested with by trypsinization. To avoid loss of non-adherent cells, the conditioned medium, PBS wash, cells and trypsin, and the complete αMEM used to rinse the plates were combined and pelleted by centrifugation. Cells were resuspended and fixed in methanol:acetic acid (3:1) and placed at -20°C for a minimum of 24 hours. Cells were applied to ethanol-cleaned glass slides and stained with 20 μM Hoechst 33258 (Sigma) in PBS for 30 min. Nuclei were analyzed on a Zeiss fluorescent microscope using standard DAPI excitation and emission (480 nM) filters. Approximately 500 cells per cell line were analyzed in each experiment. The percentage of cells with fragmented nuclei was determined from two independent experiments.

Senescence-associated β-galactosidase activity

Cell lines were plated at a density of $2.5x10^4$ cells per 35-mm tissue culture dish in duplicates and grown for 3 days in complete αMEM. On day 3, cells were washed

twice with PBS, fixed with 2% formaldehyde, 0.2% glutaradehyde in PBS, rinsed twice with PBS, and stained (*35*). A solution containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) was overlaid onto cells and incubated at 37°C overnight (*35*). The cells were rinsed twice with H_2O the following day. Approximately 500 cells were counted from each plate and percent of cells exhibiting blue stain, indicative of senescence-associated β-galactosidase activity, were scored.

BrdU/Hoechst assay for cell proliferation

Cells were plated at a density of $5x10^4$ cells per well on a six-well plate. 1X BrdU (100 μM; Sigma) in αMEM complete medium was added the following day. Cells were incubated in the presence of BrdU for 92 hours. Cells were harvested by trypsinization, pelleted, and resuspended in 0.5ml αMEM complete containing 10%DMSO. Cells were stored at -20°C until analyzed. Prior to flow cytometry analysis, samples were thawed, pelleted, and resuspended in PBS buffer containing Hoechst 33258 (1.2μg/ml) and analyzed using standard procedures previously described (*36, 37*).

Statistical analysis

For all experimental data, mean, standard deviation, and standard error were calculated, and significance of difference was determined using a two-sided Student's T-test.

Results

Introduction and expression of IGFBP-rP1 in MCF-7 breast cancer cells

MCF-7 breast cancer cells were retrovirally transduced with LXSN (empty-vector control) and L*IGFBP-rP1*SN (IGFBP-rP1 gene cloned into X site of LXSN vector) (Fig. 1). The following cell lines were generated from MCF-7 breast cancer cells and are used in the experiments described in this report: parental (non-transduced control); two lines each of LXSN and LIGFBP-rP1 mass-transduced cells and selected in G418 for two or more passages (pooled cultures, T1 and T2); two clonal cell lines each of LXSN (cl.2 & cl.6) and L*IGFBP-rP1*SN (cl.9 & cl.10), collected from the second passage following transduction.

Transduced cells were assessed for mRNA expression by Northern blot analysis (Fig. 2A). We detected endogenous IGFBP-rP1 expression in Hs578T breast cancer cells (control) and not in the parental MCF-7 cells as previously reported (*23, 24*), nor in the pooled and clonal LXSN-transduced MCF-7 cells. The IGFBP-rP1 transduced MCF-7 cells exhibited a 4.2 kb transcript corresponding to the predicted transcript size within the viral 5' LTR (Fig. 1B and 2A).

Endogenous IGFBP-rP1 is detected as a 31 kDa protein in ER-negative Hs578T cells (control). IGFBP-rP1 protein was not detected by Western immunoblot analysis of concentrated conditioned medium from either parental or empty-vector (LXSN) transduced MCF-7 cells (Fig. 2B). Secreted IGFBP-rP1 protein with molecular weights of 33 and 34 kDa was observed in conditioned medium from IGFBP-rP1-transduced MCF-7 cells (Fig. 2B). All three molecular weights (31, 33, and 34 kDa) of IGFBP-rP1 protein were detected from IGFBP-rP1-transduced MCF-7, cl.10.

To test for possible effects of transduction on cell growth, cell numbers after seven day growth were compared between the parental and two pooled LXSN-transduced cell lines (Fig. 3A & 3B). We observed no significant difference in cell proliferation and concluded that introduction of vector alone has no effect on cell growth in MCF-7 cells.

IGFBP-rP1 suppresses growth in MCF-7 Cells

We assessed the effect of IGFBP-rP1 on cell growth by determining cumulative cell numbers of empty-vector- and IGFBP-rP1-transduced MCF-7 pooled and clonal cell lines on days 1, 2, 3, 5, and 7 (Table I). In the pooled cultures, IGFBP-rP1-transduced cells show a 39% reduction ($p=0.007$) of cells compared to LXSN-transduced cells on day seven. A more pronounced difference in cell growth was detected in the IGFBP-rP1 clonal cell lines, which exhibited a 74% reduction ($p<0.001$) in cumulative cell numbers on day seven. The kinetics indicate a reduction of the growth rate is responsible for the reduced cell numbers.

Nuclear fragmentation is not altered in the presence of IGFBP-rP1

To assess if cell death is involved in the lower rate of cell growth observed in IGFBP-rP1-transduced cells, we examined cells for fragmented nuclei, the morphologic marker of apoptosis. Using the nuclear dye Hoechst 33258, fragmented nuclei were scored and the percentage of apoptotic cells in the total population was calculated. We detect no significant difference in the number of apoptotic cells in IGFBP-rP1 transduced MCF-7 cells versus empty-vector and non-transduced parental controls after seven days in culture indicating that IGFBP-rP1 inhibits proliferation independent of apoptosis

(Table II).

Senescence-associated β-galactosidase activity is increased by IGFBP-rP1

In initial observations of the IGFBP-rP1-transduced MCF-7 breast cancer cells, we observed a change in morphology reminiscent of senescent cells (Fig. 3). The IGFBP-rP1 transduced cells were larger and flattened with larger cytoplasmic nuclear ratio than the LXSN-transduced (control) and parental (non-transduced) MCF-7 cells. These observed morphological changes have been reported in senescent cells (*38, 39*). Therefore, we tested the transduced cells for senescence-associated (SA) β-galactosidase activity at pH 6 (*35*), a marker of senescence. SA β-galactosidase activity is increased 2-fold in IGFBP-rP1-transduced compared to LXSN-transduced MCF-7 breast cancer cells (Table III). The observed increase SA-β-galactosidase activity and change in morphology to a senescent phenotype indicates IGFBP-rP1 inhibitory function is linked to replicative senescence.

Higher G_0/G_1 cell fraction in IGFBP-rP1-transduced cell cultures

Cell proliferation in transduced cell lines was determined using BrdU-Hoechst assay (*36*). This assay identifies the fraction of non-cycling and cycling cells. Our results show a larger population of non-cycling cells in the IGFBP-rP1-transduced cell lines, implying that these cells are undergoing senescence or terminal differentiation (Table IV).

Discussion

The purpose of this study was to determine if IGFBP-rP1, a gene upregulated during senescence of normal HMECs, could inhibit growth of human breast cancer cells. We hypothesized that IGFBP-rP1 may function as a tumor suppressor by a senescence-associated pathway. MCF-7 breast cancer cells, a cell line lacking endogeneous IGFBP-rP1 expression, was selected and transduced with the IGFBP-rP1 cDNA. Subsequent expression of IGFBP-rP1 corresponded with a marked reduction in cell growth over a seven-day period and an accompanying change in morphology and increase in SA-β-galactosidase activity. BrdU/Hoechst flow cytometry revealed an increase in the number of cells accumulated at G_0/G_1 in IGFBP-rP1 transduced cultures. Programmed cell death was excluded as a possible explanation as no changes in apoptotic cell number were detected. Our studies point to a senescence- or differentiation-associated pathway as the mechanism for IGFBP-rP1 growth inhibition.

The negative effect of IGFBP-rP1 on cell proliferation is likely to be more dramatic than what we have presented in this paper. This bias is a result of collecting and analyzing only proliferating clones and pooled populations to study the antiproliferative effects of IGFBP-rP1. It is likely that cells undergoing senescence were selected out either by passaging or due to an increased sensitivity to G 418. Of approximately $1.67x10^5$cells plated on 60-mm dishes and transduced with IGFBP-rP1, only nine viable clones were detected and selected for transfer to a 24-well plate. The resulting two clonal cell lines (cl.1 and cl.2) did not survive passage from the 24-well plate to a larger dish and IGFBP-rP1 transduction was repeated. The two clonal cell lines (cl.9 and cl.10) used in this study were the first colonies to survive transfer from the 24-well plate and

subsequently proliferate in order to establish a cell line for study (clones 3-8 did not survive). The LXSN-transduced MCF-7 cells produced colonies too numerous to count. Of the subsequent 23 colonies transferred to a 24-well plate, two of the twelve surviving LXSN clonal cell lines were arbitrarily selected for study (cl.2 and cl.6). The selected IGFBP-rP1-transduced clones may be at the threshold between senescence and proliferation. The pooled population represents a variety of clones with varying sensitivity to G 418 and IGFBP-rP1. Cells with higher growth rates and less sensitivity to G 418 and IGFBP-rP1 are inadvertently selected in pooled cultures with each passage explaining why we consistently see more dramatic results in the clonal versus pooled IGFBP-rP1-transduced cell lines. For this reason, we did not use cell lines after ten passages following retroviral transduction in our studies.

It has been previously speculated that IGFBP-rP1 may elicit growth inhibition independent of IGF binding (*28, 40*); however, the mechanism by which this occurs has not been elucidated. Studies of IGFBP-3 and its ability to induce apoptosis independent of IGF in the MCF-7 human breast cancer cell line suggested that this may be a likely mechanism for IGFBP-rP1 (*41*). Further support to this hypothesis was provided by the demonstration of IGFBP-rP1 to inhibit growth of the malignant human prostate epithelial cell subline M12 via apoptosis and is associated with an altered morphology (*26*). Non-utilization of an apoptotic pathway by IGFBP-rP1 indicates that IGFBP-rP1 involves a distinct pathway of replicative homeostasis from IGFBP-3, which induces apoptosis in both ER-negative (Hs578T) and ER–positive (MCF-7) breast cancer cells (*41, 42*). The apoptosis-independent inhibitory function of IGFBP-rP1 observed in breast cancer cells versus its apoptosis-inducing role in human prostate cancer cells may be related to

estrogen receptor status. ER-α is frequently expressed in breast cancer cells, while ER-β may be playing a significant role in prostate cancer [as reviewed in (*43*) and (*44*)]. Differential expression of ER status may contribute to altered expression of genes involved in cell cycle regulation so that one pathway is preferentially activated, such as senescence over programmed cell death.

IGFBP-rP1 has been referred to as a follistatin-like gene and recently reported to bind to activin A using cell lysates and conditioned medium from pCMV-mac25-GFP transfected Saos-2 cells (*28, 45*). Expression of the full length IGFBP-rP1 cDNA in the Saos-2 cells corresponded with an increase in G_1 (*45*), similar to our findings. The activin receptor gene is expressed in MCF-7 cells (*46*). Expression of activin A is also present in MCF-7 cells, although in minute quantity (*47*). Activin A inhibits growth of MCF-7 cells in a dose dependent manner (at 2 ng/ml) (*46*). This inhibition was accompanied by withdrawal from the cell cycle at G_1 and an increase in cell size and ruffling of cell edges. The altered morphology is also similar to our observations in IGFBP-rP1 transduced MCF-7 cells. The similarity in results from these previous studies and our data suggest that IGFBP-rP1 may inhibit growth via activin receptor activation.

Further analysis by immunocytochemistry localized activin A to the cytoplasm of MCF-7 cells suggesting that activin A may have an autocrine/paracrine function (*47*). Picogram quantities of activin A in cystic fluid and serum (*48*) versus the nanogram quantities required to exert the reported effects above of activin A in MCF-7 cells suggests that IGFBP-rP1 may enhance interaction between secreted activin A and the activin receptor. Endogenous IGFBP-rP1 may be acting upstream of activin A and may not inhibit growth in ER-negative cells due to a reported loss of sensitivity to activin A

(*49*). Therefore, a relationship may exist between the lack of IGFBP-rP1 expression in ER-positive breast cancer cells, activin A insensitivity in ER-negative breast cancer cells, and regulation of cell proliferation. Our observations in IGFBP-rP1-transduced MCF-7 cells share the following characteristics with activin receptor activation leading to senescence/differentiation: corresponding increase of cells in G_0/G_1 and an altered morphology. We suggest that IGFBP-rP1 inhibits proliferation of MCF-7 cells via regulation of activin A.

Expression of IGFBP-rP1 in normal breast epithelial cells (*24*) may function in conjunction with activin A to regulate cell proliferation. Increased expression of IGFBP-rP1 as cells enter senescence may serve to facilitate interaction between activin A and the activin type II receptor to suppress growth. An important question that remains to be answered is whether IGFBP-rP1 is acting as an effector or enhancer of senescence.

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Figure Legends

FIG. 1. Analysis of IGFBP-rP1 expression in transduced MCF-7 breast cancer cells. (A) Northern blot analysis of total mRNA. Endogenous IGFBP-rP1 transcript is detected at 1.5 kb in Hs578T breast cancer cells (control). Retrovirally-generated IGFBP-rP1 transcript is detected at 4.2 kb in pooled, transduction 1 and 2 (T1 & T2), and clonal (cl.9 & cl.10) IGFBP-rP1-transduced MCF-7 cells. (B) Western blot analysis of conditioned media. A 31 kDa protein is detected in medium from Hs578T breast cancer cells (control). Immunoreactive proteins of 33 and 34 kDa are detected in medium from two pooled (T1 & T2) and one clonal (cl.9) IGFBP-rP1-transduced MCF-7 cell lines. Medium from one IGFBP-rP1-transduced MCF-7 clonal cell line (cl.10) exhibited all three molecular weights (31, 33, and 34 kDa).

FIG. 2. Parental and LXSN-transduced MCF-7 breast cancer cells have similar cumulative cell numbers. There was no difference in cumulative cell numbers between parental MCF-7 cell line and LXSN T1 (A) or LXSN T2 (B) ($p=0.93$ and $p=0.68$, respectively).

FIG. 3. Morphology is altered in IGFBP-rP1-transduced MCF-7 breast cancer cells. A. 200X, LXSN-transduced cells exhibit morphology characteristic of the non-transduced parental MCF line. Cells are tightly packed and have a cobblestone appearance. B./C. 200X, IGFBP-rP1-transduced cells contain more vacuoles (V) and are multinucleated (MN). Cell surfaces not in contact with other cells exhibit ruffling (R) at

the edges and more shrunken cells (S) are present.

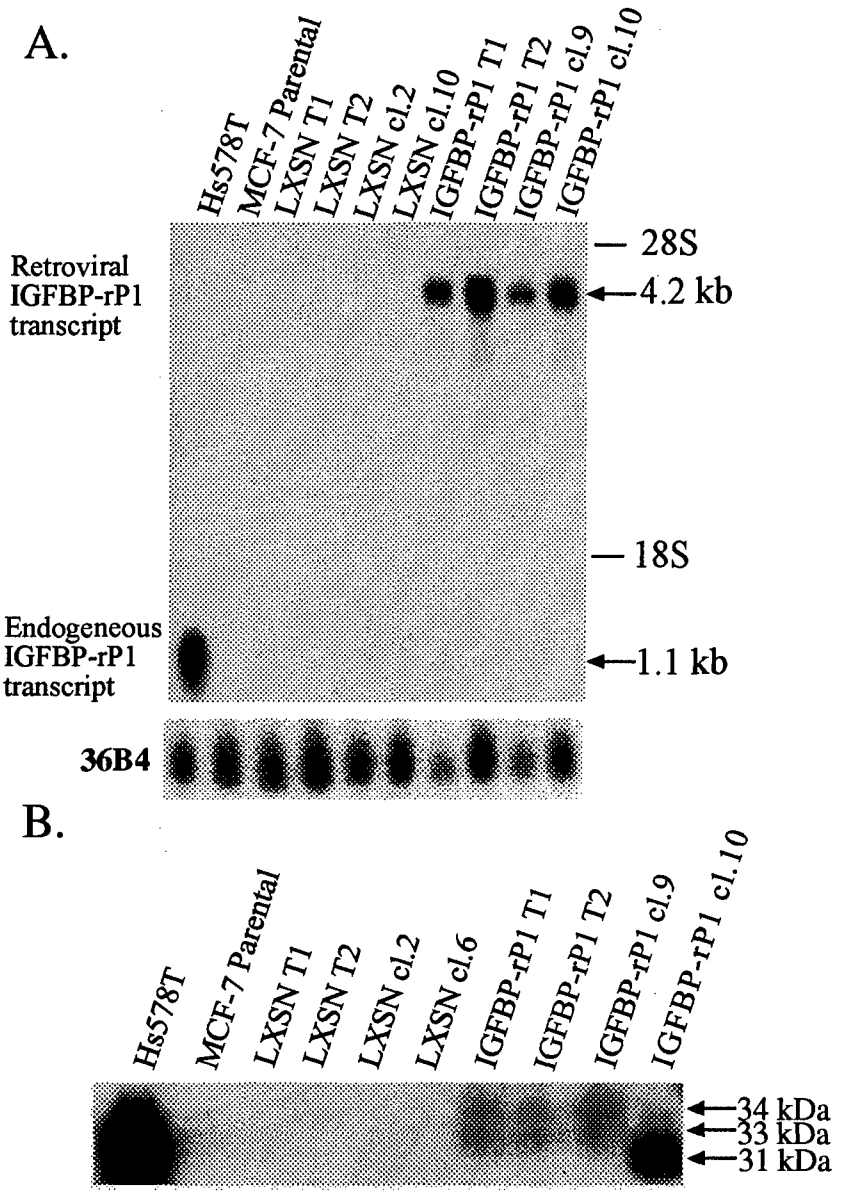
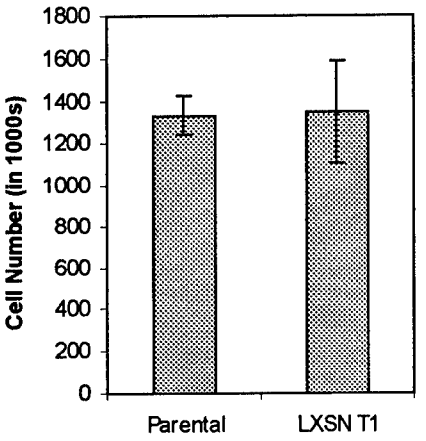

A.
Hs578T
MCF-7 Parental
LXSN T1
LXSN T2
LXSN cl.2
LXSN cl.10
IGFBP-rP1 T1
IGFBP-rP1 T2
IGFBP-rP1 cl.9
IGFBP-rP1 cl.10
Retroviral IGFBP-rP1 transcript
28S
4.2 kb
18S
Endogeneous IGFBP-rP1 transcript
1.1 kb
36B4
B.
Hs578T
MCF-7 Parental
LXSN T1
LXSN T2
LXSN cl.2
LXSN cl.6
IGFBP-rP1 T1
IGFBP-rP1 T2
IGFBP-rP1 cl.9
IGFBP-rP1 cl.10
34 kDa
33 kDa
31 kDa

Figure 1

A.


1800
1600
1400
1200
1000
800
600
400
200
0
Cell Number (in 1000s)
Parental
LXSN T1

B.

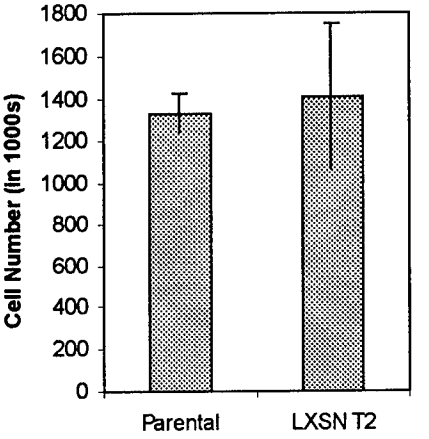

1800
1600
1400
1200
1000
800
600
400
200
0
Cell Number (in 1000s)
Parental
LXSN T2

Figure 2

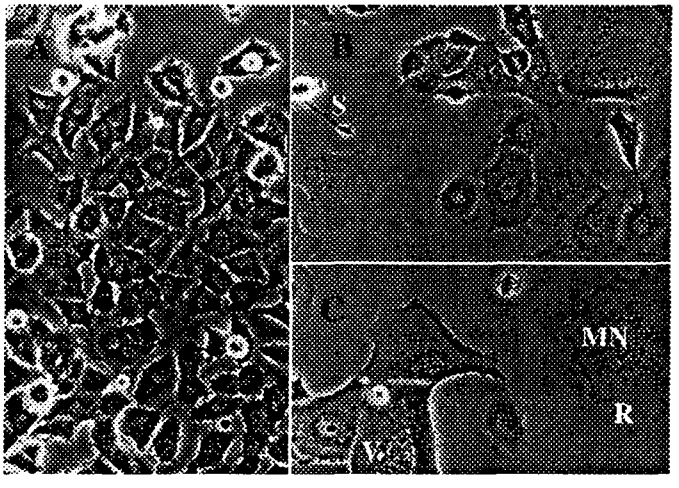


Figure 3

TABLE I. Cell numbers are reduced in IGFBP-rP1-transduced MCF-7 cells over seven days

	Pooled Cultures			***Clonal Cultures***		
day	***LXSN****	***IGFBP-rP1****	*Percent Difference IGFBP-rP1 vs. LXSN*	***LXSN******	***IGFBP-rP1******	*Percent Difference IGFBP-rP1 vs. LXSN*
0	50	50		50	50	
1	67.5 +/-16.5	50.5 +/-1.9	**25.2%**	90.6 +/-12.3	62.3 +/-11.0	**31.2%**
2	98.5 +/-13.7	69.5 +/-7.2	**29.4%**	156 +/-46.9	96.3 +/-15.4	**38.2%**
3	161 +/-35.2	111 +/-11.6	**31.1%**	269 +/-100	123 +/-26.2	**54.3%**
5	435 +/-161	250 +/-26.1	**42.5%**	589 +/-179	172 +/-42.1	**70.8%**
7	964 +/-88.3	588 +/-52.0	**39.0%**	1092 +/-318	284 +/-105	**74.0%**

All values represent cell number in 1000s. Aliquots of cells were plated on 60-mm dishes and counted after 7 days of growth as described in Material and Methods. *Values represent the mean number of cells counted from four independent experiments (in duplicates), +/- the standard error. **Values represent the mean number of cells counted from five independent experiments (in duplicates), +/- the standard error.

TABLE II. Apoptotic cell numbers are unchanged in IGFBP-rP1 transduced MCF-7 cells

	Non-transduced	***Pooled Cultures***		***Clonal Cultures***	
	parental^	**LXSN***	**IGFBP-rP1***	**LXSN***	**IGFBP-rP1***
Apoptosis	**0.9%** +/-0.4	**0.8%** +/-0.3	**0.6%** +/-0.1	**0.6%** +/-0.2	**0.7%** +/-0.2

All values represent percent of cell population. Aliquots of cells were plated at 50,000 cells per 60-mm dish and assessed for presence of fragmented nuclear DNA, a morphological marker of apoptosis, after seven day growth. No significant difference in percent of apoptotic cells was detected in any of the cell lines examined. ^ From two independent experiments (in duplicate), +/- the standard error. *From four independent experiments (in duplicate), +/- the standard error.

TABLE III. Increased activity of a senescence-associated protein is observed in IGFBP-rP1 transduced MCF-7 cells

	Non-transduced	***Pooled Cultures***		***Clonal Cultures***	
	parental^	**LXSN***	**IGFBP-rP1***	**LXSN***	**IGFBP-rP1***
SA-beta-gal Activity	**18.2%** +/-3.8	**34.0%** +/-4.6	**68.7%** +/-4.7	**41.8%** +/-9.9	**82.9%** +/-5.1

All values represent percent of cell population. Cells were plated at 25,000 cells per 35-mm dish and examined on day 3 for the presence of senescence-associated beta-galactosidase activity. Significant changes were observed ($p<0.0001$). ^ From two independent experiments (in duplicate), +/- the standard error. *From four independent experiments (in duplicate), +/- the standard error.

TABLE IV. Higher percentage Go/G1 cells in IGFBP rP1 transduced MCF-7 cell cultures

	*Pooled Culture**	*Clonal Cultures*
Exp.	***IGFBP-rP1/LXSN ratio***	***IGFBP-rP1/LXSN ratio***
1	**1.59**	**3.02**
2	**1.99**	**3.41**
3	**1.53**	**2.08**

Ratio of non-cycling cells: IGFBP-rP1- versus LXSN-transduced MCF-7 cell in intial G0/G1 from the total population. Aliquots of cells were plated at 50,000 cells per well on 6-well plates. BrdU was added 24 h after plating and cells were incubated an addition 92 hours before collection of cells. *Values represent T2 pooled culture only.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be Downgraded to Unlimited Distribution

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