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Several tumor suppressor genes (TSGs) have been cloned and found to be mutated in a variety of cancers, including breast cancer. However, few breast cancer-specific TSGs are known. The purposes of this proposal are to (1) clone novel TSGs specific to human breast cancer; (2) examine the alteration of these TSGs in primary breast tumors; and (3) identify their characteristics, regulation and function. We are utilizing the tetracycline (tet) regulable system. We have constructed a cDNA library from normal human breast epithelia and cloned this cDNA library into a vector that is negatively regulated by tet repressor (tetR) and simultaneously expresses enhanced green fluorescence. These vectors were then co-transfected into LCC6, 231, and MCF-7 cells that have the capability to express tetR. Upon withdrawal of tet, the repressed expression of the cDNA of interest is released, and the cDNA is expressed. Using a novel dye that was retained in nonproliferating cells, we were able to identify growth inhibited clones which were then sorted by Flow Cytometry. This functional screen has provided the basis for identifying TSGs which are expressed in the growth inhibited cells. Using PCR, we have obtained the insert sequences. We will now characterize these genes and begin to assess their function and expression in primary breast carcinomas.

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Proposal Title: ***Tumor Suppressor Genes in Breast Cancer***

(4) Table of Contents

(5) Introduction

Breast cancer is one of the most common malignancies of women in the United States. Most molecular genetic abnormalities contributing to breast cancer susceptibility remain unknown. Recent studies have revealed genomic changes in breast cancer, including amplification of proto-oncogenes, such as c-myc, c-erbB2, int-2, bcl-1, PRAD-1, EMS, EGF receptor (c-erbB 1), IGF-1 receptor, flg and bek (1,2) and intragenic mutations or suppressed expression in tumor suppressor genes (TSGs) including p53, Rb and p33 (3-5). TSGs constitute a relatively new class of genes and has been implicated in regulation of cell proliferation, cell cycle progression, apoptosis induction, and DNA repair and recombination. Many TSGs have been cloned from humans and found to be mutated in

variety of human cancers (3-13). The study of TSGs should not only speed up basic cancer research, but it may also aid in the early diagnosis, prognostication, and treatment of human malignancies. Loss of heterozygosity (LOH), which is usually considered the hallmark of TSGs, has been observed in at least 15 out of the 23 pairs of chromosomes in human tumors. This result suggests that there may be numerous TSGs. However, only two genes specifically related to breast cancer have so far been cloned (BRCA-1) (14-16) or mapped to a specific chromosomal segment (BRCA-2) (17, 18); moreover the prevalence of intragenic somatic mutations in BRCA-1 is not very high in sporadic breast tumors (less than 10% of cases). Therefore, it seems likely that the cloning of new tumor suppressor genes of specific importance in breast cancer will be important and promising task for future research into this common disease.

The current proposal focuses on the isolation and characterization of novel TSGs in human breast cancer. A human epithelial eukaryotic cDNA expression library has now been constructed and transfected into the human breast cancer cell lines including MCF-7, MDA-MB-231 and MDA435/LCC6. Gene(s) inhibiting the growth of breast cancer cells and MCF-7 cells were considered candidate TSGs for breast cancer. These will be cloned and the full length cDNA sequence obtained. Expression of cloned genes will first be investigated in RNA populations derived from two immortalized "normal" human breast epithelial cell lines (A1N4 and MCF-10A), and in MCF-7 cells growth arrested either by antiestrogen-treatment or estrogen withdrawal. This approach provides a rapid and sensitive functional screen for growth inhibition-related activities using renewable resources, and is particularly important should a significant number of unique cDNAs be isolated. Subsequently, the expression of clones exhibiting an appropriate pattern of expression will be investigated in a series of RNA populations isolated from primary breast tumors. Once we have identified the most promising candidates, we will further screen genomic DNA from cell lines and primary breast tumors for somatic alterations, including deletion, mutation, and change in expression level. In the longer term, the most promising cDNAs will be studied to establish their characteristics and regulation. Putative TSGs that are growth-suppressive and specifically altered in breast cancers may be useful tools for the early diagnosis, prognostication, and eventual treatment of human breast cancers.

(6) Body of Report

A. Brief statement of ideas and reasoning

Tumor suppressor genes (TSGs) function in normal tissues by regulating the growth of normal cells. Mutations, deletions, or other modes of inactivation of TSGs should contribute to uncontrolled growth and malignant transformation of normal cells. Many TSGs have been cloned from humans and found mutated in variety of human cancers,

including breast cancer (3-18). Many human chromosomes show high rates of loss of heterozygosity (LOH) in breast cancer; however, very few breast cancer-specific TSGs, such as BRCA-1, have actually been cloned (14). Moreover, the mutation rate of BRCA-1 in primary human breast tumors is less than 10% (15). Therefore, additional specific tumor suppressor genes for breast cancer are likely to exist. A cDNA expression library made from mRNA of normal human mammary glands should contain potential TSGs for human breast cancer, and thus can reasonably be used to isolate TSG(s) specific to breast cancer that inhibit the growth of breast cancer cells. **We propose a functional screen for the discovery of TSGs, which dramatically decreases the time to isolation and *a priori* demonstrates the function of novel TSGs. In addition, by using a cDNA expression library from normal human breast epithelia, transfected into breast cancer cells, we hope to clone TSG(s) that are specific to breast cancer.**

B. Hypotheses/Purposes

We hypothesize that:

1) Normal human mammary gland epithelia should contain all normally expressed potential TSGs for breast cancer.

2) TSGs play an important role in growth regulation of breast cancer cells in culture.

3) TSGs contribute significantly to the carcinogenic process in a significant portion of breast cancers.

The purpose of this proposal is to clone TSG(s) specific to breast cancer, examine their alteration in primary breast tumors, identify their characteristics, and ultimately study their regulation and function.

C. Technical Objectives

1). To clone novel TSGs for human breast cancer from a cDNA expression library made from normal human mammary gland epithelia.

2). To characterize the cloned TSGs by sequence homology analysis and study their functional effect on *in vitro* tumorigenesis for the most promising candidates.

3). In the long term: to study the regulation of cloned TSGs by finding their promoter regions and regulatory elements.

D. Experimental Methods, Assumptions and Procedures

Outline and rationale for approach

A major problem in the identification of growth inhibitory genes in a functional assay is that it is the non-proliferating (suppressed) cells that are the cells containing the genes of interest. We have constructed a novel approach that we believe is optimized for the specific purpose of identifying growth suppressor genes. Thus, we have utilized the tetracycline repressor (tetR)-based gene expression system. We have directionally cloned the cDNA library (see below) into an expression vector placing each cDNAs under the control of the tetracycline resistance operon that is regulated by tetR (19-22). These vectors were also able to express enhanced green fluorescence protein (EGFP) reporter by which the expression of genes of interest were monitored indirectly (22). These plasmids were co-transfected with the puromycin resistant plasmid into MCF-7 cells, MDA435/LCC6 cells and MDA-MB-231 cells already transfected with a plasmid expressing both the tetR repressor and the G418 resistance marker (MCF-$7^{tetR+neoR}$, $LCC6^{tetR+neoR}$, $231^{tetR+neoR}$). Upon withdrawal of doxycycline, the tetR/VP16 binds and activates transcription of the cDNA (19, 21). Double resistant and EGFP expressing cells were selected and expression of the gene of interest studied in the presence of increasing concentrations of doxycycline.

While we have a method to regulate genes expression, we also have an approach for enriching bulk transfected cell populations for growth inhibited cells. We have used our adaptation of the dye enrichment method of Maines et al. (23). The dye, PKH26-GL, (Sigma Chemical Co. St Louis, MO) (24) is non-toxic and specifically retained in non-proliferating cells. Since Flow Cytometry can be used to visually sort cells retaining dye (cells are maintained in the absence of tet), and there is a state-of-the-art Flow Cytometry Core Facility at the Lombardi Cancer Center, we were able to rapidly enrich the population for the growth inhibited cells, including cells that are completely growth arrested, sorting for the most red fluorescent cells (23,24). Thus, following the 24 hr recovery period immediately post-transfection, the cells were selected with puromycin, the resistance marker coexpressed in the plasmids containing the cDNA library. The concentration of puromycin was 1 μg/ml.

Surviving cell populations were stained with PKH26-GL and grown, now in the absence of tet, for the equivalent of several generations as described by Maines *et al.* (23). The estimated generation time for non-inhibited MCF-7 cells is 24-36 hrs and LCC6 and 231 18-24 hrs (25). Subsequently, single cells were aseptically sorted by Flow Cytometry (double sort - red for growth inhibition, green for gene expression) into the wells of 96-well plates. This provided individual cell clones expressing putative growth inhibitory genes. Cell clones containing growth suppressing cDNAs were then rapidly expanded by adding doxycyclineto block the putative TSG expression and release its growth suppression. The putative growth inhibitory genes were analyzed by PCR, subcloning and sequencing. Growth suppressor activity can be further confirmed, in a functional assay,

by following experiments: (1) cloning putative tumor suppressing genes into expression vector; (2) transiently transfecting MCF-7 cells, LCC6 cells, and 231 cells with these vectors containing the genes interested; (3) observing cell growth by cell-cycle analysis using Flow Cytometry. RNA containing the expressed putative suppressor genes can be obtained, by introducing different concentrations of doxycyclineto the culture medium to establish a tet-based dose response relationship for cell proliferation.

The Mentor (Dr. Clarke) also has generated and characterized MCF-7 cells that do not require estrogen for growth *in vitro* or *in vivo* (MCF7/MIII; MCF7/LCC1) but exhibit an antiestrogen-induced growth suppression that is reversed by estrogen (26-28) or are antiestrogen resistant (MCF7/LCC2; MCF7/LCC9) (29). By using both MCF7/LCC1$^{tetR+neoR}$ (grow without estrogen but respond to estrogen) and MCF-7$^{tetR+neoR}$ cells (require estrogen to grow), we can distinguish between genes that merely suppress growth (*i.e.,* MCF7/ LCC1$^{tetR+neoR}$ cells are growth inhibited regardless of the presence of estrogen) and those that suppress estrogen-induced proliferation (*i.e.,* both MCF7/ LCC1$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ growth inhibition is reversed by estrogen). ***The use of these additional cell lines would likely constitute an alternate/additional approach, since the work with existing tetR+neoR cells is sufficient for the initial time period of this application.***

There are several significant advantages to this novel approach:

(1) Growth inhibition will be apparent only upon removal of tet, and this will reduce the background due to insertional mutagenesis, which could randomly produce slowly proliferating/growth inhibited cells independent of the inserted cDNA.
(2) We can identify genes that completely suppress proliferation, as well as genes that merely reduce the rate of proliferation.
(3) We can rapidly identify, in a functional assay, genes that specifically inhibit estrogen-regulated growth.

Construction of a cDNA expression library from normal human breast epithelia

To successfully isolate mRNA from normal human breast tissues, there are three important concerns: 1) effective disruption of tissue and denaturation of nucleoprotein complexes, 2) inactivation of RNase activity, and 3) purification of mRNA away from contaminating DNA and protein. Thus, we used the PolyA TractR system 1000 (Promega Corp. Cat.#Z5410), since this procedure yields an essentially pure fraction of mature mRNA without extractions or precipitations. This method combines guanidine thiocyanate (GTC) and β-Mercaptoethanol to inactivate RNase. Then GTC is associated with SDS to disrupt nucleoproteins and allows for hybridization between the poly(A) sequence of mRNAs and a synthetic biotinylated oligo(dT)probe. The biotinylated oligo(dT):mRNA hybrids were captured with Streptavidin Paramagnetic Particles (SA-

PMPs). The particles were washed at high stringency and purified mRNA was eluted by the addition of nuclease-free deionized water (30).

To construct a cDNA library, we used The CapFinderTM PCR cDNA Library Construction Kit (CLONTECH Laboratories, Inc., Cat.#K1051-1). This is a novel, PCR-based method for making high-quality libraries from a small quantity of RNA. This technique also utilizes the unique CapSwitchTM oligonucleotide in the first-strand synthesis, followed by long distance PCR amplification to produce high yields of full-length, double-strand (ds) cDNA (31-33). Therefore, we performed the reverse transcription (RT) to transcribe 100ng poly A+ mRNA into single-strand (ss) DNA by using reverse transcriptase, a modified oligo (dT) primer (CDS/3' PCR primer) (32) and a CapSwitch oligonucleotide. The CDS/3' PCR primer was used to prime the first-strand reaction. The CapSwitch oligonucleotide was used as a short, extended template at the 5' end for the RT. When the RT reached the 5' end of the mRNA, the enzyme switched templates and continued replicating to the end of the CapSwitch oligonucleotide. The resulting full-length ss cDNA contained the complete 5' end of the mRNA and the sequence complementary to the CapSwitch oligonucleotide, which then served as a PCR priming site (CapSwitch anchor). The PCR was performed by directly using the CapSwitch anchor. In this reaction, only those oligo (dT)-primed ss cDNA having a CapSwitch anchor sequence at the 5' end served as templates and was amplified using the 3' and 5' PCR primers and Advantage KlenTaq Polymerase (33). This selective amplification did not allow incomplete cDNAs and cDNA transcribed from polyA-RNA to be amplified, therefore eliminating library contamination by genomic and polyA-RNA.

The ds cDNAs were ligated using T4 ligase to specific-adaptors which contains a pre-existing EcoRI "sticky end" and phosphorylated blunt end for the efficient ligation to the blunt-ended cDNA. This ligation eliminated the need to methylate and EcoRI-digest the cDNA, and left the internal EcoRI sites intact. Following adaptor ligation, the ds cDNAs were phosphorylated at the EcoRI sites and size-fractionated using column to remove small (0.5kb) cDNA fragments and non-cDNA contaminants (0.1kb) (unincorporated primers and unligated adaptors). The resulting cDNA was then cloned into λgt11, which is an EcoRI-digested and phosphorylated phage vector.

We quantified the three test ligations, compared the titers and determined the optimal ratio of vector to cDNA insert. Phage packaging reaction was performed according to the λ-DNA *in vitro* packing module instructions (Amersham LIFE SCIENCE, Cat.#RPN1717). In this procedure, we used cell extracts derived from two induced lysogens whose prophages carry different, but complementing, mutations in the genes required for assembly of mature phage particles. Subsequently, we mixed these cell extracts together with λ-DNA, the DNA was packaged into infectious phage particles and then introduced into *Ecoli* host cells by infection processes. From the five ligations combined, we obtained the unamplified library which contains 1×10^6 independent

clones. This library was then amplified, and the titer of amplified library was determined to be 1 x 10^{10} pfu/ml.

Conversion of a λ phage library into a Plasmid cDNA expression library

To effectively generate a plasmid cDNA expression library, there are two important issues to be addressed: 1) an optimal plasmid that allows simultaneous expression of both the gene of interest and a selective marker, 2) a plasmid that contains the tetracyclin-regulated expression systems. Therefore, we used pBI-EGFP (CLONTECH Laboratories, Inc., Cat#6154-1) which is a responsive plasmid that can coexpress a gene of interest and enhanced green fluorescent protein (EGFP) from a bidirectional tetracyclin-responsive promoter (21,22). This pBI-EGFP Tet vector contains the bidirectional promoter that is responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On Systems, respectively. Furthermore, EGFP is a unique protein that is the brightest known GFP variant and can be expressed in mammalian cells. Importantly, the expression of EGFP can be visually monitored using fluorescence microscopy as well as Flow Cytometry. Thus, the EGFP reporter gene system provided a very convenient way to detect gene expression and localize the fusion protein within the cells without a specific assay. Consequently, by adapting this excellent system, the time applied on selection of growth inhibited cell lines has been significantly reduced and the quality of plasmid cDNA library transfection has also been greatly improved.

The established λ phage cDNA library was used to construct a plasmid cDNA expression library. The cDNAs were obtained by digesting λ phage cDNA library with the EcoRI restriction enzyme. These cDNAs were then trimmed using Klenow enzymatic reaction to generate blunt-ends suitable for subsequent ligations. Meanwhile, pBI-EGFP was linearized by Pvu II restriction enzyme digestion and dephosphorylated by calf intestinal alkaline phosphatase reaction, respectively. It is important to mention that the quantity and the high quality of the ligations were required to successfully reconstruct this pBI-EGFP cDNA library. Thus, the blunt-end ligations we have performed in this experiment were extremely difficult. In order to accomplish this task, one of the approaches we took was to use the Takara ligase kit (Takara Shuzo Co., Ltd., Cat#6021) which has been shown to be very effective in blunt end ligation (34). While it took considerable effort, we also were able to optimize the ligation conditions. Consequently, the ligation efficiency was greatly improved, and the blunt-end ligations were successfully carried out by integrating cDNAs into linearized pBI-EGFP with the assistance of Takara ligase. pBI-EGFP vectors containing the cDNA library were then introduced into *E. coli* host cells by transformation. With the twenty combined ligations, an unamplified pBI-EGFP cDNA expression library was finally generated, which contains 1 x 10^6 independent clones. This library was further amplified to be approximately 1 x 10^{10} pfu/ml. The cDNA library was then purified and ready for transfection.

Establishment of stable LCC6$^{tetR+neoR}$ and 231$^{tetR+neoR}$ cell lines

(1) MDA435/LCC6 cells and MDA-MB-231 cells were co-transfected with plasmid expressing the tetR repressor and the G418 resistance marker as described previously (19, 21).

(2) Transfected MDA435/LCC6 and MDA-MB-231 cells were treated with G418 (400μg/ml).

(3) Surviving LCC6 and 231 cells were transfected with pBI-EGFP and regulated with the addition of doxycycline. The selection of Lcc6$^{tetR+neoR}$ and 231$^{tetR+neoR}$ cell lines was performed using fluorescence microscopy.

(4) The selected stable LCC6$^{tetR+neoR}$ and 231$^{tetR+neoR}$ cell lines were further confirmed by transfecting with a plasmid that contains a doxycycline regulable element and expresses luciferase (Promega, Cat# 1500) and assaying the enzymatic activity. Clones with the lowest background and highest induction of expression were selected.

Transfection of LCC6$^{tetR+neoR}$, 231$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ cells

To obtain the optimal transfection, there are several basic elements involved: 1) highly efficient transfection reagent, 2) ratio of transfection reagent to plasmid DNA, 3) the quality of plasmid DNA, 4) quantity of transfection complex, and 5) optimal cell confluency, 6) appropriate transfection time (30). Therefore, we chose to use SuperFect transfection reagent (QIAGEN, Cat#301305) which is a specifically designed polycation and showed higher transfection efficiencies than those obtained with many liposome transfection reagents. The ratio of SuperFect and cDNA plasmid was optimized to 4μl of SuperFect per 1μg of DNA according to our several carefully designed tests. Finally, the stable transfection condition was establised by transfecting Lcc6$^{tetR+neoR}$, 231$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ cells in 30% confluency in flask-75 with a complex of 40μg of DNA and 160μl of SuperFect for a 95-minute-incubation period. This resulted in maximum transfection efficiency (about 40-50%) and minimum cytotoxic effects. Transfection efficiency was further verified by transfecting LCC6$^{tetR+neoR}$, 231$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ cells with a plasmid expressing β-galactosidase, which was assayed colorimetrically using the substrate *O*-nitrophenyl-β-D-galactopyranoside.

Identification of cells containing growth inhibitory genes

To help identify growth inhibited cells, cells were labeled with PKH26-GL (Sigma Chemical Co. St Louis, MO, Cat# 17621), a non-toxic, red fluorescent cell linker that is incorporated into the cell membrane by selective partitioning (24). Importantly, it is retained in non-proliferating cells. The appearance of labeled cells varies from bright and uniform labeling to punctuate and patch appearance. However, one problem is an over-

labeling that can result in loss of membrane integrity and cell recovery. Thus, it was essential to determine the best ratio of dye to cell. In our experiment, the optimal concentration of dye and cell was determined to be approximately 2 x 10^{-6} M PKH26-GL and 1 x10^{7} cells/ml. The PKH26-GL labeled LCC6, 231, and MCF-7cells were then checked for their recovery, viability, and their fluorescence intensity using fluorescence microscopy and Flow Cytometry.

To enrich bulk transfected cell populations for growth inhibited cells, a systemic approach has been developed in this study. MCF-7 cells already transfected with a plasmid expressing both the tetR repressor and the G418 resistance marker were initially treated with G418. Surviving cell populations were then stained with PKH26-GL and grown for 24-36 hours. These PKH26-GL labeled MCF-7 cells were subsequently transfected with pBI-EGFP cDNA expression library using SuperFect and optimal transfection conditions as described previously. pBI-EGFP cDNA vectors have each of the transfected cDNAs expressed under the control of the tetracycline resistance operon that is negatively regulated by doxycycline. This system also enabled us to establish the double stable cell lines.

Cells transfected with pBI-EGFP cDNA library were grown, in the absence of tet, for the equivalent of several generations and then selected for growth suppressed clones. The identification of those cell clones that contained growth inhibiting cDNAs was performed by selecting cells that concurrently exhibit EGFP expression and strong PKH26-GL labeling. Therefore, single EGFP expressing (green) and PKH26-GL (red) double-labeled cells were aseptically sorted by FACS into the wells of 96-well plates after 96-120 hours of transfection. To date, we have single-cell sorted fourteen 96-well plates, which provided a significant number of individual cell clones expressing growth inhibitory genes. Immediately after sorting, doxycyclinewas added to the culture medium at a concentration of 1μg/ml, and cell clones containing growth suppressing cDNAs were rapidly expanded by releasing the growth suppression.

Cloning growth suppressing genes

To clone growth-inhibiting genes, identified cell clones containing growth suppressing genes were quickly enriched from single cell to multiple cells with the addition of tet. Of the utmost importance to succeed this experiment is to avoid the contamination during cell population enrichment. Single cells were first sorted into the medium containing doxycyclineat the concntration of 1μg/ml and 1x Penicillin Streptomycin (GibcoBRL, Cat# 5140-122). Twenty-four hours after sorting, the medium was changed with the addition of anti-fungal and yeast agents, Amphotericin B (Sigma, Cat# A-2940) and Nystatin (Sigma, Cat# N-4014) with the concentrations commercially suggested. The next day, the medium was changed back with only 1μg/ml of doxycyclineand 1 x Penicillin Streptomycin. Since then, the medium was replaced almost every 24 hours, but every 5 days added with Amphotericin B and Nystatin. During this selection process,

cells were gradually transferred from 96-well plates into 48-well plates, 24-well plates, 12-well plated, 6-well plates, T-25 flasks and T-75 flasks. The further selection was performed with G418 (400μg/ml). The selected cell clones were stored in -70°C and then liquid nitrogen.

The putative growth inhibiting genes were identified by PCR with either TaqBead™ Hot Start Polymerase (Promega, Cat#M5661), or Expand™ High Fidelity PCR System (Boehringer Mannheim, Cat#1 732 650) using the genomic DNAs as templates, which were extracted from the cell clones containing growth suppressing genes. The PCR primers were designed containing partial sequences of pBI-EGFP, one pair # 426 (5'-GTACCCGGGTCGAGTAGGCGTGTA-3') and # 650 (5'-GGTCCCCAAACTCACCCTGAAGT-3'), and another pair # 426 and # 657 (CAATCAAGGGTCCCCAAACTCACC-3'), according to primer design programs (DNAStar). Several PCR products were found and ranged from 600 bp to 2 kb. The PCR segments were cut out from the gels and purified. The purified cDNAs were then re-amplified with the same primers. The obtained cDNA products were sequenced either by direct sequencing or subcloning the cDNAs into the vector and then sequencing.

E. Results and Discussion

For cDNA library construction we utilized a strategy similar to that already used to generate a normal human esophageal epithelia cDNA library. Normal human breast tissues were collected from reduction mammoplasties performed at Georgetown University Hospital. These specimens were immediately snap frozen in liquid nitrogen. Based on histological analysis and our prior experience, we obtained several specimens each of which contains sufficient glandular epithelium to yield several micrograms of mRNA. Thus, we can generate more than one library if necessary. The quality of mRNA was verified by formaldehyde-agarose gel electrophoresis and RT-PCR using a probe for human GADPH.

A λ phage cDNA library was constructed. The quality of the cDNA library was checked by PCR for the size of inserts (Figs. 1 and 2) and by endonuclease restriction enzyme analysis (Fig. 3). Size-fractionation of synthesized cDNAs showed the peak size to lie in the range of 600 to 2,000 bp. Using PCR and endonuclease restriction enzyme digestion, we determined the average length of our cDNAs to be approximately 1.3 kb.

The λ phage cDNA library was used to obtain cDNAs that were then cloned into the pBI-EGFP tet responsive plasmids. The quality of this pBI-EGFP cDNA expression library cloning was controlled by verifying the ligation efficiency and showing that the cDNA sizes still ranged from 600 to 2,000 bp. Subsequently, LCC6$^{tetR+neoR}$, 231$^{tetR+neoR}$, and MCF-7$^{tetR+neoR}$ cells were stained with PKH26-GL red fluorescent cell linker and transfected with pBI-EGFP cDNA expression library. The transfection efficiency was

checked by parallel transfections performed with a pBI-EGFP absent cDNA and a plasmid expressing β-galactosidase, respectively.

Transfection of MCF-7 cells: The MCF-7$^{tetR+neoR}$ cells have already been generated in the Mentor's laboratory for other studies not related in this application. We utilized a previously cloned MCF-7 cell population (MCF-7 clone#2) that exhibits a total dependence upon estrogen for growth both in vivo and in vitro (Table 1). These cells were used for the initial transfection.

Cell Line	Treatment	G0/G1	δG0/G1	G2/M	δG2/M	S	δS
MCF-7	Control:1 nM E2	31%	-	27%	-	42%	-
	Vehicle	89%	+58%	4%	-23%	7%	-35%

Table 1: Representative analyses of E2-withdrawal on cell cycle distribution in MCF-7 cells as determined by Flow Cytometry. The % change in each phase (d) is indicated, where % distribution in control treatment = 100%.

For the selection of cell populations containing growth suppressing genes, cell clones stained with PKH26-GL and transfected with the pBI-EGFP cDNA expression library were grown, in the absence of tet, for the equivalent of several generations. Growth suppressed cell clones were then able to be identified by those cells that, simultaneously, expressed enhanced green fluorescent protein and had brightest PKH26-GL labeling (Fig. 4). Consequently, single green and red fluorescence expressing cell clones containing putative growth inhibiting genes were aseptically sorted by FACS into 96-well plates. The sorted cell clones were subsequently grown in the presence of tetracyclin in order to expand cell populations by the mechanism of releasing growth inhibition.

For the cloning of putative growth inhibiting genes, the specific genes in the vector pBI-EGFP which were inserted into the genomic DNAs were amplified by PCR using either TaqBead™ Hot Start Polymerase, or Expand™ High Fidelity PCR System with the primers designed containing partial sequences of pBI-EGFP. Several PCR products from 600 bp to 2 kb have been found, sequenced and analyzed. Among them, one putative gene was sequenced (Fig. 5) and further studied, and no homologues were found to match it through the available gene banks. Therefore, this gene seems highly possible to be new member in tumor suppressor gene family. More importantly, this putative gene would be new candidate of tumor suppressor genes specifically for human breast cancer.

F. Progress on the Statement of Work

In the past months, we have successfully followed our previous plans as described below:

Technical Objective 1: Identify putative TSG(s)

Task 1: Months 1-6: Construct and Characterize cDNA library.Task 1: Months 1-6: Construct and Characterize cDNA library.

Task 2: Months 6-8: Re-construct plasmid cDNA library and Transfect MCF-$7^{tetR+neoR}$ cells, $LCC6^{tetR+neoR}$cells, and $231^{tetR+neoR}$ cells.

Task 3/4: Months 8-24: Identify cells containing growth inhibitory genes and Clone TSG(s).

Technical Objective 2: Characterize putative TSG(s)

Task 5: Months 24-36: cDNA sequencing and sequence analysis.

Task 6: Months 30-48*: Screen tumors for mutations in putative TSG(s).

*We anticipate that completion of these studies will take longer than the three year period. However, it is likely we have sufficient data to enable the Fellow to apply for additional funding.

We should mention that up-to-now, we have not had any special problems in accomplishing any of our tasks. In the near future (next few months), we will continually focus our attention on DNA sequencing, sequence analysis and will start functional assays to transfect these putative TSGs into human breast cancer cell lines. We will also screen tumors for mutations in putative TSG(s), as indicated in the work statement.

We have successfully constructed and performed our initial characterization of the first library from a reduction mammoplasty (normal breast tissue). We also have obtained clones of MCF-7 cells MDA435/LCC6 cells, and MDA-MB-231 cells, transfected with the tetR vector. With the cloning of pBI-EGFP cDNA expression library, the initial transfection of MCF-$7^{tetR+neoR}$ cells **MDA435/$LCC6^{tetR+neoR}$ cells and MDA-MB-$231^{tetR+neoR}$** ***cells*** *were successfully completed. Our accomplishments of identifying cell clones containing putative growth inhibitory genes have provided a solid basis. Current DNA sequencing and analysis have lead us so close to the final cloning TSGs as described in original application.*

In this last year, Dr. Pu gave birth. Nevertheless, we are on track with regard to the original time frame. As indicated in the Statement of Work, we expected to have identified cells containing putative growth inhibitory genes by the end of month 24. We

have now reached this point. We believe this to be highly encouraging, and supportive of the dedication and abilities of the Fellow, the nature of the training environment, and our ability to perform the remainder of the proposed studies within the anticipated time frame.

Other Training

In addition to the training obtained in the laboratory, Dr. Pu has been participating in several other research activities within The Lombardi Cancer Center. Dr. Pu has been attending the regular center-wide research Journal Club, and is required to present 1-2 times per year. She also has been attending and participating in the center-wide Research Data Meetings, at which she also is required to present 1-2 times per year. Dr. Pu continues to interact with the other scientists within Dr. Clarke's laboratory and to work and consult with other investigators within the Cancer Center. Dr. Pu will be expected to attend the AACR and USAMRMC meetings next year and to submit abstracts for her data. She also may attend other meetings as necessary or appropriate.

Future Plans

Each transfected clone will initially be examined for the integrity and copy number of transfected cDNAs by Southern analysis (there may be more than one plasmid in some transfectants), and the appropriate mRNA expression by Northern analyses. The Northern analysis will provide critical information on the size of the expressed transcript(s). Since we have used a regulable promoter approach, we will perform dose response analyses with increasing concentrations of the regulating agent (i.e., tet). This will enable us to assess the potency of the gene, i.e., what level of expression is associated with a corresponding level of growth inhibition. The effects of doxycyclineon expression of mRNA from the repressed promoter will be monitored by Northern analysis. Controls will consist of parallel cultures of non-transfected cells and cells transformed with the tetR operator expression vectors without the cDNA inserts and that are treated with tet.

We cannot exclude the possibility that the level of expression required for growth suppression is below the limit of detection by Northern. When this occurs we will use RNase protection or semiquantitative PCR to detect product. We will use primers from the portion of the regulable promoter sequence that is transcribed in the final product, and a site internal to the inserted cDNA sequence. This also will enable us to distinguish those products amplified from newly transcribed RNA from those derived from the endogenous gene.

7. Conclusions

This is a postdoctoral fellowship application by an individual who previously worked in another field, and was not the original recipient of the award. Despite having lost considerable time for reasons unrelated to the project, we are on time. This reflects strongly on the dedication, commitment and skill of the Fellow and we are confident that she will be able to obtain the necessary training and expertise in the technical skills to complete each remaining aspect of the proposed study.

We have now successfully constructed the cDNA library and performed our initial characterization of the first library from a reduction mammoplasty (normal breast tissue). We also have obtained two clones of MCF-7 cells transfected with the tetR vector. With the cloning of pBI-EGFP cDNA expression library, we have successfully transfected MCF-7$^{tetR+neoR}$ cells, LCC6$^{tetR+neoR}$cells, and 231$^{tetR+neoR}$ cells with pBI-EGFP cDNAs. We have identified cell clones contained putative growth inhibitory genes and sequenced some of candidates for putative growth inhibitory genes, providing a basis for the final cloning of TSGs as described in our original application. Furthermore, her participation in local and national meetings permits a further level of training and exposure to breast cancer research. We have also presented this work at the AACR meeting in 1999. Thus, we believe that we are making excellent progress towards the successful accomplishment of the aims and goals of the original application.

Publications.

Pu, L.P., Skaar, T.C., Gu, Z.P., Leonessa, F. **& Clarke, R.** "A novel selection system for identifying growth suppressed human breast cells." *Proc Am Assoc Cancer Res* 40: 32, 1999.

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9. Appendices

Figures and figure legends.

Figure 1. PCR screening inserts in λgt11 cDNA library. Lambda DNA was prepared by picking up a plaque with a micropipette and transferring into deionized H_2O. PCR was performed basically according to CLONTECH=s LD-Insert Screening Amplimer Sets (CLONTECH Laboratories, Inc., Cat # PT1579-1). This PCR showed different size of inserts. Lanes 1-4 used 5% DMSO, and lanes 5-7 used 10% DMSO. Note the arrow that the size of insert (lane 5) is approximately 1 kb.

Figure 2. PCR screening the size of inserts in λgt11 cDNA library. Lambda DNA was prepared from Midi-Prep DNA extraction. This PCR showed the peak size to lie in the range of 600 to 2,000 bp.

Figure 3. Endonuclease restriction enzyme checking the average length of inserts in λgt11 cDNA library. This reaction showed the average length of inserts of cDNAs to be approximately 1.3 kb.

Figure 4. Selection of cell populations containing growth suppressing genes. Cell clones stained with PKH26-GL and transfected with pBI-EGFP cDNA expression library were grown, in the absence of tet, identified by cells that, simultaneously, expressed enhanced green fluorescent protein (green) and had brightest PKH26-GL labeling (red) and then sorted by FACS into 96-well plates. Cells (square 2) were selected as green and red double fluorescence expressing cell. However, cells were considered as either single red (square 1) and green (square 4) or no (square 3) fluorescence labeling.

Figure 5. DNA sequence of one of putative tumor suppressor genes. This sequence was obtained from one of identified cell clones containing putative growth inhibiting genes, amplified by PCR, and directly sequenced. This putative TSG contains about 700bp and seems to be new and has no homologues to other known TSGs and genes.

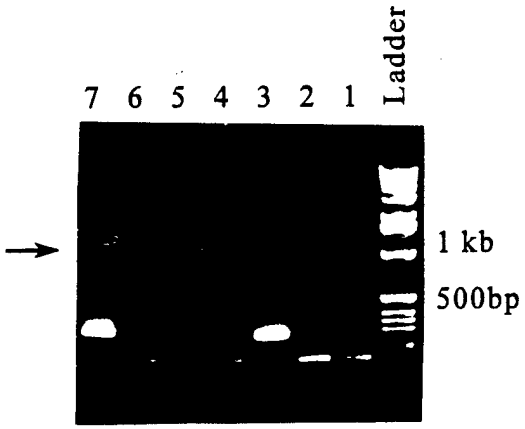


Figure 1.

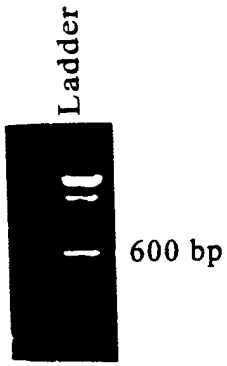


Figure 2.

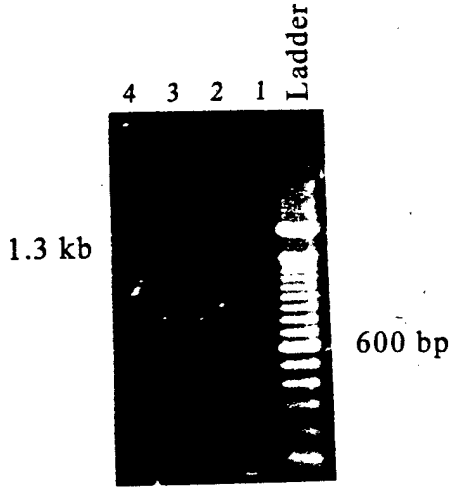
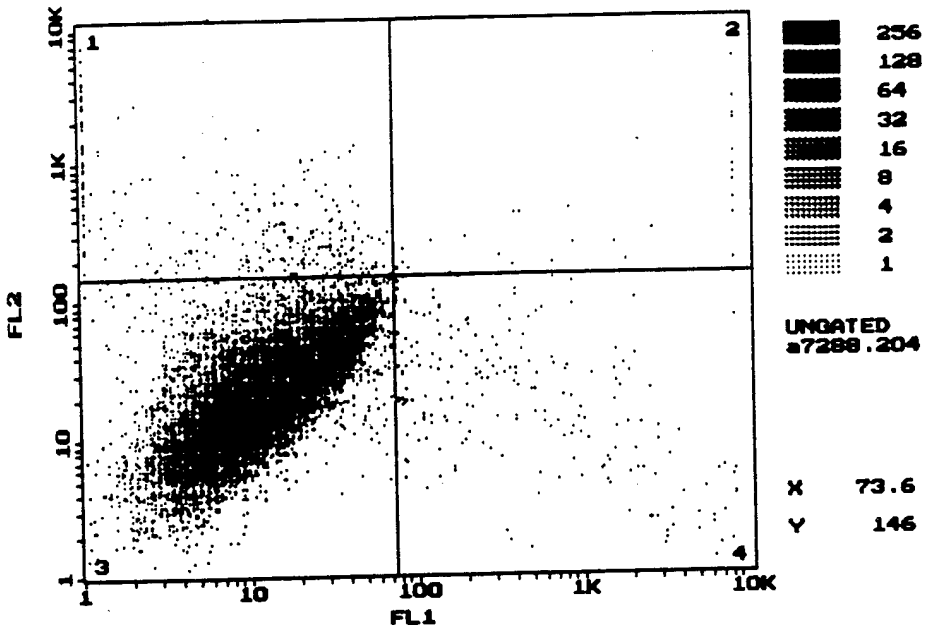


Figure 3.

Figure 4.



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1	307	3.07	33.2	146	2	9.39	347
2	29	0.29	73.6	146	1	1065	387
3	9445	94.45	7.03	10.5	10	10.8	19.3
4	219	2.19	8347	3.41	2	481	15.0

ABI PRISM

Model 377
Version 2.1.1

28 -PU7-

PU7-
Lane 28

Signal G:852 A:1015 C:753 T:398
DT {dR Set Any-Primer}
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Points 901 to 8268 Base 1: 901

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Thu, May 27, 1999 12:49 PM
Spacing: 9.94 ABI100

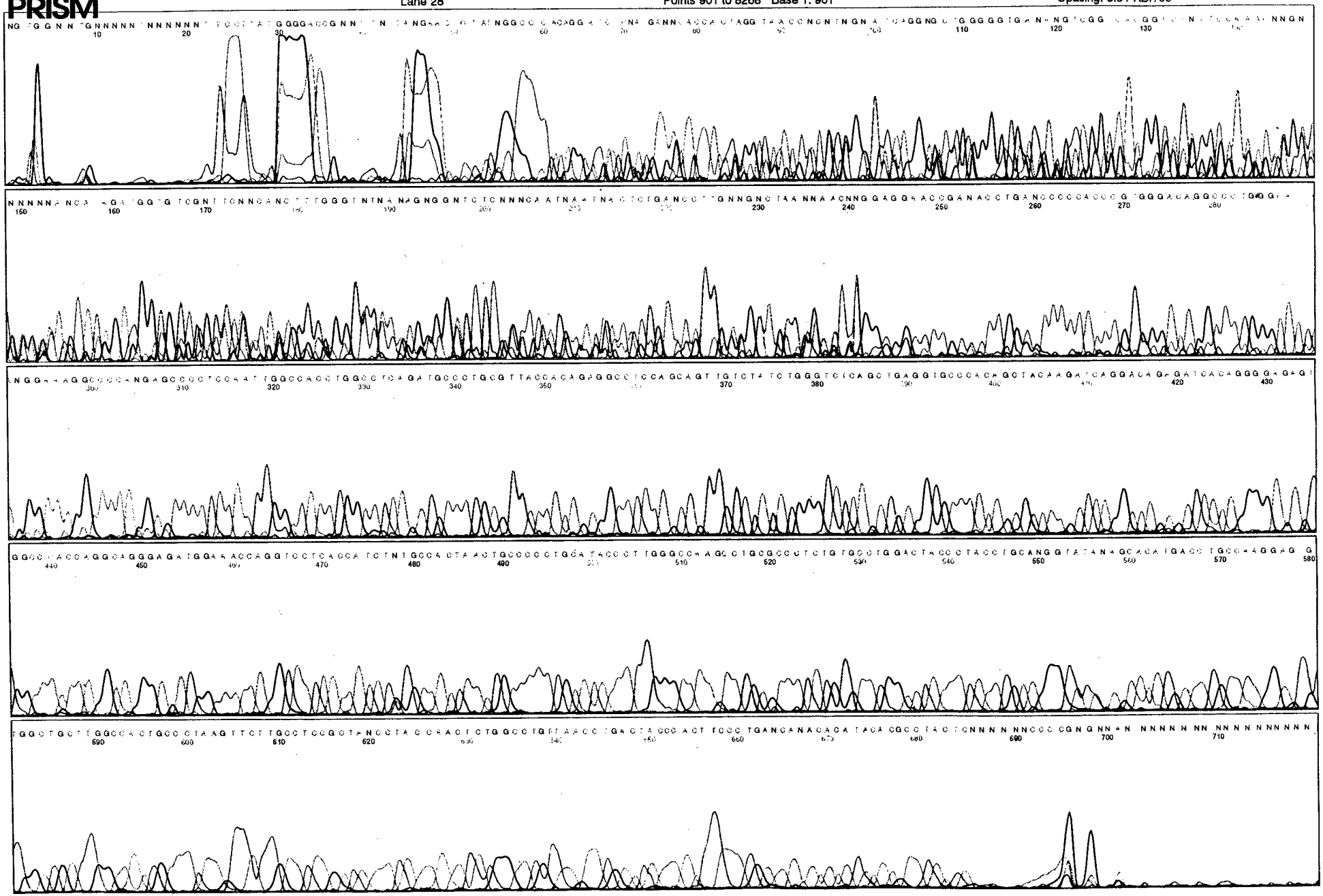

Figure 5