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

A genetic system has been established for the isolation of candidate tumor suppressor genes in MCF7 breast cancer cells. This system includes the IPTG-inducible episomal vector, pEpiLac, which combines the advantages of inducible expression systems and extrachromosomal replication systems. A cDNA library from normal human breast epithelial cells was constructed in the pEpiLac vector system, and transfected into a cell line (LAP5) derived from the human breast cancer MCF7 cells which allows the inducible expression of the library DNA. After several rounds of selection, 12 sequences that confer growth inhibitory effects of on LAP5 cells were isolated. Sequence analysis revealed that some of these sequences are fragments of known genes, while others represent novel gene sequences with no known functions. One of them, termed TGIF1, represents a novel gene and has been further characterized. Additional rounds of selection are on-going to identify more growth inhibitory sequences that may control breast cancer cell growth.

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INTRODUCTION

Breast cancer is the most common malignancy observed among women in the Western hemisphere. It is estimated that one out of every nine women in the United States will develop the disease, underscoring an urgent need to develop novel therapeutic agents. Many lines of evidence support the notion that breast cancers develop in part due to the loss of functional tumor suppressors, and that the introduction of a functional tumor suppressor gene can inhibit the proliferation of breast cancer cells. Identification of such genes can lead to potential methods of treatment, including targeted gene therapy. We have developed a novel approach, called SETGAP (selectable expression of transient growth-arrest phenotype)(Pestov and Lau, 1994; Pestov et al., 1998), to isolate cDNAs of genes from normal human breast cells that can suppress the growth of breast cancer cells. This approach allows the identification of growth suppressors of breast cancer cells using a direct functional assay from a cDNA library. No specific information on the genes to be isolated is necessary.

RESULTS

The underlying hypothesis of this project is that there exists previously unknown tumor growth suppressor genes that may be useful in the treatment of breast cancer and in the understanding of breast cancer etiology. A novel genetic selection procedure, SETGAP, has been developed to identify and isolate gene transcripts that are capable of inhibiting cell growth in culture. This approach may lead to the identification of previously unknown tumor suppressors, which may in turn open new doors for potential gene therapy and rational drug design. In an attempt to identify novel suppressors of breast cancer cell growth, we have applied the SETGAP selection to isolate gene transcripts present in normal human breast epithelium that are capable of inhibiting the growth MCF7 breast cancer cells in culture.

In last year's report, we have described the construction of a novel set of inducible episomal vectors that facilitate the selection process. We have also established an MCF7-derived cell system that allows the inducible expression of foreign genes with this vector system. Briefly, we have altered a key component of the SETGAP selection -- the IPTG-inducible expression vector pX11, a plasmid containing 15 copies of *lac* repressor binding sites in front of the MMTV basal promoter. We constructed two episomal vectors (pEpiLac) with different mutlicloning sites based on pX11, placing the IPTG-inducible cassette in an episomal vector that allows extra-chromosomal replication in primate cells (Yates et al., 1985). These episomal vectors have several advantages over other vectors, including high efficiency of stable transfection, high level expression of transfected genes, easy recovery of transfected sequences, and low complexity of transfected sequences in a given cell when a population of DNA sequences is introduced (Deiss et al., 1995). In addition, we also established the LAP5 cell line, which was created by introducing the LAP267 transactivator (Baim et al., 1991) into MCF7 cells. Inducible expression of foreign genes in LAP5 cells is efficient. For example, expression of the luciferase reporter gene is induced 300 fold by IPTG when delivered via the pEpiLac vector in LAP5 cells.

Since the pEpiLac vectors efficiently express growth inhibitory genes in LAP5 cells and can be easily recovered using Hirt's extraction method, they have been adapted in the SETGAP procedure outlined in Fig. 1. Briefly, the procedure requires several steps: 1. A cDNA library is constructed by cloning cDNAs, in the sense orientation, into the multiple cloning sites in the pEpiLac vectors. 2. LAP5 cells are transfected with the library DNAs and selected against hygromycin, resistence to which is conferred by the episomal vector. 3. Hygromycin-resistent cells are treated with IPTG to induce the expression of exogenous genes. 4. Proliferating cells are killed by BrdU/Hoechst dye/light selection, whereas, growth arrested cells will survive. 5. Growth arrested cells are rescued by the removal of IPTG inducer. 6. Episomal DNA are extracted from surviving cells, amplified in bacteria and applied to the next round of selection, or subjected to DNA sequence analysis.

Selection of growth inhibitory sequences from a human breast epithelial cell cDNA library. We have constructed a cDNA library in pEpiLac1 using mRNAs from normal human mammary gland cells using standard techniques. LAP5 cells were transfected with the library DNAs and about 6,000 hygromycin-resistant clones were obtained. These cells were then subjected to SETGAP selected. After one round of selection, extra-chromosomal DNAs were isolated from

cells that survived the SETGAP selection and amplified in bacteria. The resulting DNA was applied to the second round of selection. The growth inhibitory effect of the library DNA was obvious after the second round of selectio (Fig. 2). Expression $p27^{KIP1}$, a well-characterized growth inhibitor, gave rise to a similar number colonies that survived the SETGAP selection as the library DNA. By contrast, the parental LAP5 cells showed no such surviving cells following the selection. These results indicated that the pool of cDNAs from normal human epithelium contain sequences that can inhibit MCF7 cell growth.

Episomal DNA was harvested from the pool of LAP5 cells that survived the second round of SETGAP selection, and re-transfected into LAP5 cells for another round of selection. After the third round of selection, cells that survived the selection were grown to form colonies and 36 individual cell clones were picked and episomal DNA from these cells harvested. DNA from each individually picked LAP5 cell colony was amplified in bacteria. The bacterial transformation also served to purify the harvested episomal DNA into a single DNA species. DNA from each representative bacterial transformant was harvested, and again transfected into LAP5 cells to determine whether these individual DNA species can confer growth inhibition. After testing the 36 individually isolated clones, we identified 12 individual sequences that exhibit a growth arrest phenotype in the SETGAP assay. These sequences were named TGIF (Tumor growth inhibitory fragment) 1-12.

Sequence analysis of *TGIFs*. The cDNA sequences of TGIF 1-12 were isolated and subjected to DNA sequence analysis. The sequences obtained were compared to those in the GenBank database. Results are summarized in Table 1. Some of these cDNAs correspond to known genes, whereas others correspond to novel genes or uncharacterized EST sequences. It is interesting to note that many of these sequences represent partial cDNAs rather than full-length cDNAs. This suggests that the partial cDNAs may encode dominant negative variants of normally expressed genes and interfere with the function of the full-length proteins. It is possible that these dominant negative variants may cause a more powerful growth arrest phenotype than other gene products that might be growth inhibitory; this may explain why in a genetic selection, the predominant products were partial cDNAs.

Analysis of *TGIF1*. Among the 12 TGIF sequences isolated thus far, TGIF1 appeared promising since its expression confers a powerful growth inhibitory effect when expressed. Moreover, TGIF1 represents a novel gene sequence and characterization of this gene may lead to interesting new insights into the roles it may play in growth control. The growth inhibitory effects of TGIF1 is illustrated in Fig. 3. In a SETGAP selection assay, expression of TGIF1 gave rise to a similar number of surviving cells as the expression of $p27^{KIP1}$, a known potent growth inhibitor. Thus, TGIF1 is a novel sequence that is able to confer reversible growth inhibition in MCF7-derived breast cancer cells.

Fig. 4 shows a growth curve of LAP5 cells transfected the TGIF1 containing vector. In the presence of the IPTG inducer, expression of TGIF1 results in a severe growth retardation when compared to the same cells without IPTG induction. By contrast, LAP5 cells transfected with the pEpiLac vector containing the luciferase gene grew faster either in the presence or absence of IPTG.

The 1.4 kb cDNA insert of the TGIF1 clone was completely sequenced and compared to sequences in GenBank. TGIF1 corresponds a fragment of an uncharacterized gene with no known function (sequence KIAA0692; Ishikawa et al., 1998). The full-length 4.5 kb TGIF1 cDNA was also isolated from a human placental cDNA library and the sequence determined. The predicted open reading frame is shown in Fig. 5. Analysis of the encoded protein sequence did not yield any significant sequence homology to known proteins, and no functional domain that might indicate activity has been identified.

Expression of *TGIF1*. The expression of *TGIF1* in several human cell lines and in human tissues samples was examined by Northern blot analysis. These cell lines include three normal human mammary gland cell lines (MCF10A, MCF12A, and Hst578Bst), four human breast cancer cell lines (MCF7, Hst578T, CAMA1, and Hut292DM) and one human lung cancer cell line (A549). Two distinct transcripts of ~4.5 kb and 3.5 kb were detected using ^{32}P-labeled *TGIF1* anti-sense RNA probes, indicating the possibility of differential splicing or alternative transcription start sites to produce the two transcripts (Fig. 6A). Although the expression levels vary among these cell lines, no specific correlation between gene expression and tumorigenesis can be identified. Northern blot analysis was also used to examine the expression of *TGIF1* in various human tissues (Fig. 6B). *TGIF1* expression was easily detectable in cells from human heart, placenta, skeletal muscle, pancreas and kidney cells (Fig. 6B).

Further SETGAP selections. Although the SETGAP selection we have conducted thus far has identified a number of cDNAs that confer growth inhibition in MCF7 cells, we have not yet identify a full-length cDNA sequence that represent a growth inhibitory gene expressed in normal human cells. This may be due to the fact that only a small fraction of a complete cDNA library has been screened. We are now in the process of conducting further SETGAP selections in order to identify other sequences that may correspond to a growth inhibitory gene expressed in normal cells. Another round of SETGAP selection has been started, and transfection of the LAP5 cells with library DNA yielded 8,000 hygromycin-resistant clones. SETGAP selection of these clones are now on-going.

Progress with respect to the Statement of Work

The technical objectives originally proposed that cover the first 2 years of work are as follows:

Task 1:	Months 1-4:	Preparation of subtracted breast epithelial cell cDNA library
Task 2:	Months 5-7:	Cloning of cDNA library into MCF-7 cells expressing LAP267; titrate conditions for genetic selection
Task 3:	Months 8-11:	SETGAP selection of growth-inhibitory sequences in MCF-7 cells; first round selection
Task 4:	Months 11-14:	Second round selection of growth inhibitory cDNAs
Task 5:	Months 15-18:	Isolate individual cDNAs and test individual clones for growth-inhibition
Task 6:	Months 18-20:	Isolation of full-length cDNAs
Task 7:	Months 21-22:	Preparation of deletions of cDNAs for sequence analysis
Task 8:	Months 22-24:	Sequence analysis of cDNA clones

Our work is on course as anticipated in the original proposal. We have constructed a set of episomal inducible vectors, established the MCF7-derived LAP5 cell system that would support the inducible system, and conducted SETGAP selection to isolate growth inhibitory sequences. 12 individual sequences have been identified and sequenced. One of these, TGIF1, has been examined to further detail, and the complete cDNA was isolated. We have also began another round of SETGAP selection to identify new growth inhibitory sequences.

EXPERIMENTAL METHODS AND PROCEDURES

Plasmid constructs pLac vectors were derived from pX12 (Pestov and Lau, unpublished data) through two modifications. The multiple cloning site in pX12 was reconstructed with a pair of oligonucleotides containing three rare-cutting restriction enzyme sites, Fse I, Sfi I and Not I (Fig.1). Two other rare-cutting restriction enzyme sites (Sgf I and Srf I) were inserted into the Afl III site in the plasmid backbone. Proper sequences were confirmed through DNA sequencing. pEpiLac episomal vectors were constructed from pLac vectors and the episomal vector pREP4 (Invitrogene Corporation, Carlsbad, CA). pLac vectors were double digested with Afl III and Sac I; the cohesive ends were filled-in using Klenow fragments. pREP4 was digested with Sal I and the cohesive ends were also filled-in. The large fragment from pREP4 was ligated to the small fragment from pLac. The proper orientation was confirmed through physical mapping. pX6LAP267 is the auto-regulatory LAP267 expressing vector (Pestov et al., 1998). pWLneo consistently expresses the neomycin-resistant gene (Stratagene, La Jolla, CA). pHyg is the construct which consistently expresses the hygromycin-resistant gene (Sugden et al., 1985).

Cell culture MCF7 cells and MCF7/LAP5 cells were maintained, at 37°C and 5% CO_2, in modified Eagle's medium containing non-essential amino acids (Gibco BRL, Gaitherburg, MD), plus 1 mM of MEM sodium pyruvate (Gibco BRL), 1 mM of glutamine (Gibco BRL), 10% of fetal bovine serum (FBS, Intergen Company, Purchase, NY) and 10 μg/ml of bovine insulin (Gibco BRL). Cells were refreshed every 2-3 days. Where indicated, hygromycin (Boehringer Mannheim Corporation, Indianapolis, IN) or G418 (Gibco BRL) were added to mediums to the final concentrations of 75 μg/ml or 600 μg/ml respectively.

MCF7/LAP5 cells, called LAP5 cells in short, were derived from MCF7 cells by the cotransfection of pX6LAP267 (5 μg per 100 mm plate) and pWLneo (0.5 μg) using the calcium-phosphate precipitation technique. Two days after transfection, cells were replated into five 100 mm plates and selected against G418. The expression of LAP267 in individual clonal lines, including LAP5 cell line, was demonstrated by the IPTG-dependent expression of the luciferase reporter gene in transient transfection assays.

For serum stimulation, MCF7 cells were plated in 100 mm tissue culture dishes (1×10^6 cells each) in whole mediums and cultured for two days. Then, the cells were washed twice with PBS and cultured in phenol red free MEM (Gibco BRL) plus 1 mM of MEM sodium pyruvate (Gibco BRL), 1 mM of glutamine (Gibco BRL) and 0.1% FBS (Intergen Company); no insulin was added. Three days later, cells were changed back into whole mediums containing 10% FBS and incubated for indicated times. For UV irradiation, MCF7 cells, with mediums and covers of culture plates removed, were exposed to UV light in the Stratalinker (Stratagene) at 100 mJ/cm^2 and cultured in fresh mediums for indicated times.

Transfection For electroporation, 2×10^7 cells (in 0.8 ml of PBS, pH7.35) and 20 μg of DNA were incubated at RT for 10 min, and electrically pulsed, in a 0.4 cm cuvette at 960 μFD and 330 volts. After 5 min at RT, cells were cultured in fresh medium. For calcium phosphate precipitation, 7.5 μg of DNA and 5×10^5 cells were used for each 60 mm plate. The cells were exposed to DNA-calcium phosphate precipitates for 4-8 h and glycerol-shocked (15% glycerol in

20 mM HEPES-K^+, pH7.2) at RT for 1 min. Glycerol was removed by washing cells twice with PBS and cells were cultured in fresh medium and IPTG was added for 30-36 h where indicated.

cDNA library construction Human mammary gland polyA$^+$ RNA (Clontech Laboratories, Palo Alto, CA) were utilized to synthesize cDNAs using the SuperScript system (Gibco BRL) with one modification to the manufacturer's guide. Double-stranded cDNAs were ligated to Fse I adaptors. The resulted cDNAs were cloned between Fse I and Not I sites in the multiple cloning site of pEpiLac1. Approximately 4 X 10^5 bacterial colonies were obtained after electroporation of DH10B competent cells with the ligation mixture. Colonies were pooled together, amplified in 2X YT medium at 30°C for 4 h. Plasmid DNAs were extracted using standard techniques. The library contains cDNA inserts averaging about 1 kb.

DNA extraction Episomal DNAs were isolated from LAP5 cells using Hirt's extraction method (Anant and Subramanian, 1992) with some modifications. Briefly, 1-2 X 10^6 cells were harvested in TEN buffer (40 mM Tris.HCl, pH7.5, 150 mM NaCl, and 1 mM EDTA), washed once with cold PBS, and then resuspended in 0.36 ml of ice cold TE buffer (10 mM Tris-HCl, pH 7.2, and 10 mM EDTA). Into the cell suspension, add 10% SDS to the final concentration of 1% and gently invert the tube several times. Incubate the sample at RT for 10-20 min, add 1/5 volume (80 µl) of 5M NaCl and mix the solution gently. Incubate at 4°C for more than 8 h or overnight. Centrifuge the samples at 14,000 rpm for at least 30 min at 4°C. Collect the supernatants, extract once each with phenol, phenol:chloroform, and chloroform:IAA(24:1). Precipitate the episomal DNAs with 0.3 M of sodium acetate and 2-3 vol of absolute alcohol, and dissolve the DNA pellet in water.

SETGAP selection The previously described SETGAP procedure (Pestov and Lau, 1994) was improved by the adaption of the IPTG-inducible episomal expression system into LAP5 cells. Briefly, 1 X 10^5 cells per 100 mm plate were cultured for 40-48 h in mediums containing none or 1 mM of IPTG (Sigma, Chemical Co., St. Louis, MO). Then BrdU (Sigma) was added to the final concentration of 15 µM in fresh medium. 40-48 h later, Hoechst 33342 (Calbiochem-Novabiochem Corporation, San Diego, CA) was added to 2.0 µg/ml. After 3 h incubation, tissue culture dishes were placed on a sheet of clear glass 5 cm directly above a 15 W fluorescent bulb (daylight type, Sylvania Electric Products, Fall River, MA), covered with aluminum foil, and irradiated from beneath for 30 min. Wash cells twice with PBS and culture in fresh medium for 3 days followed by 3 week incubation in hygromycin-containing mediums. Colonies were stained with 1% crystal violet (Sigma) in 20% ethanol.

RNA extraction and Northern blot analysis Total RNAs were isolated using the TriZol reagent (Gibco BRL) according to the manufacturer's guidance, and dissolved in 100% formamide (Fluka, Milwaukee, WI) to protect RNAs from degradation. RNAs were separated on 1% formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham Life Science, Little Chalfont, Buckinghamshire, England). To generate hybridization probes, pLac3 constructs containing indicated cDNA sequences were linerized with Sgf I digestion. Linerized DNAs were used to transcribe anti-sense RNA probes with the SP6 polymerase Strip-EZ kit (Ambion, Inc., Austin, TX) and ^{32}P-UTP (NEN, New England Nuclear, Boston, MA). Nylon membranes were incubated with the probe-labeling reaction mixture in the Zip-Hyb solution

(Ambion, Inc.) at 65°C for 2 h, washed 2 X 15 min in 0.2 X SSC/0.2% SDS at RT, and 3 X 15 min in 0.1 X SSC/0.1% SDS at 80-85°C. After being rinsed in 2 X SSC buffer, the membranes were exposed to PhosphoImager screen (Molecular Dynamics, Sunnyvale, CA). After analyzing the data, membranes were striped with reagents in the Strip-EZ kit (Ambion) and re-probed, if necessary.

PCR amplification PCR amplifications were performed with Pfu (Stratagene, La Jolla, CA) or Taq (Fisher, Pittsburgh, PA) DNA polymerases using M5 and P3 primers (Pestov and Lau, 1994). The reactions started with incubation at 94°C for 2 min. The cycle number was 25. For each cycle, DNA templates were denatured at 94°C for 1 min, annealed to primers at 55°C (for Taq) or 50°C(for Pfu) for 1 min, and amplified at 72°C for 3 min. Following the 25 cycles of amplification was the incubation at 72°C for 10 min.

Sequence analysis Nested 5' and 3' exonuclease Bal31 deletions of the cDNAs were cloned in the pGEM2 vector. DNA sequences were determined using Sequenase 2.0 (USB, Cleveland, OH) and EXCEL (Epicentre Technologies, Madison, WI) kits following manufacturers' instructions.

CONCLUSIONS

A genetic system was established for isolating candidate tumor suppressor genes based on their growth inhibition phenotype. The IPTG-inducible episomal vectors, pEpiLac, were constructed. These vectors combined the advantages of inducible expression systems and extrachromosomal replication systems. The LAP5 cell line was derived from human breast cancer MCF7 cells by the introduction of the LAP267 transactivator. pEpiLac vectors expressed exogenous genes more efficiently than non-episomal vectors. Taking advantages of pEpiLac vectors and LAP5 cells, the SETGAP protocol is improved to make it easier to recover and identify exogenous growth arresting sequences.

The human breast cancer MCF7 cell line is one of the best-studied model systems in cancer research. Tightly controlled inducible gene expression will provide a mechanism for the study of tumor suppressor genes or cytotoxic genes. Based on the IPTG-dependent release of the *lac* repressor from the *lac* operator, Lee and colleagues tried to generate an IPTG-inducible expression system in MCF7 cells (Lee et al., 1997). But, the IPTG-induction of exogenous gene expression was not high; only a few fold of induction was reached (Lee et al., 1997). In this study, using the IPTG-dependent transactivator LAP267, we established the LAP5 clonal line from MCF7 cells. Exogenous genes can be overexpressed several hundred fold higher after IPTG stimulation.

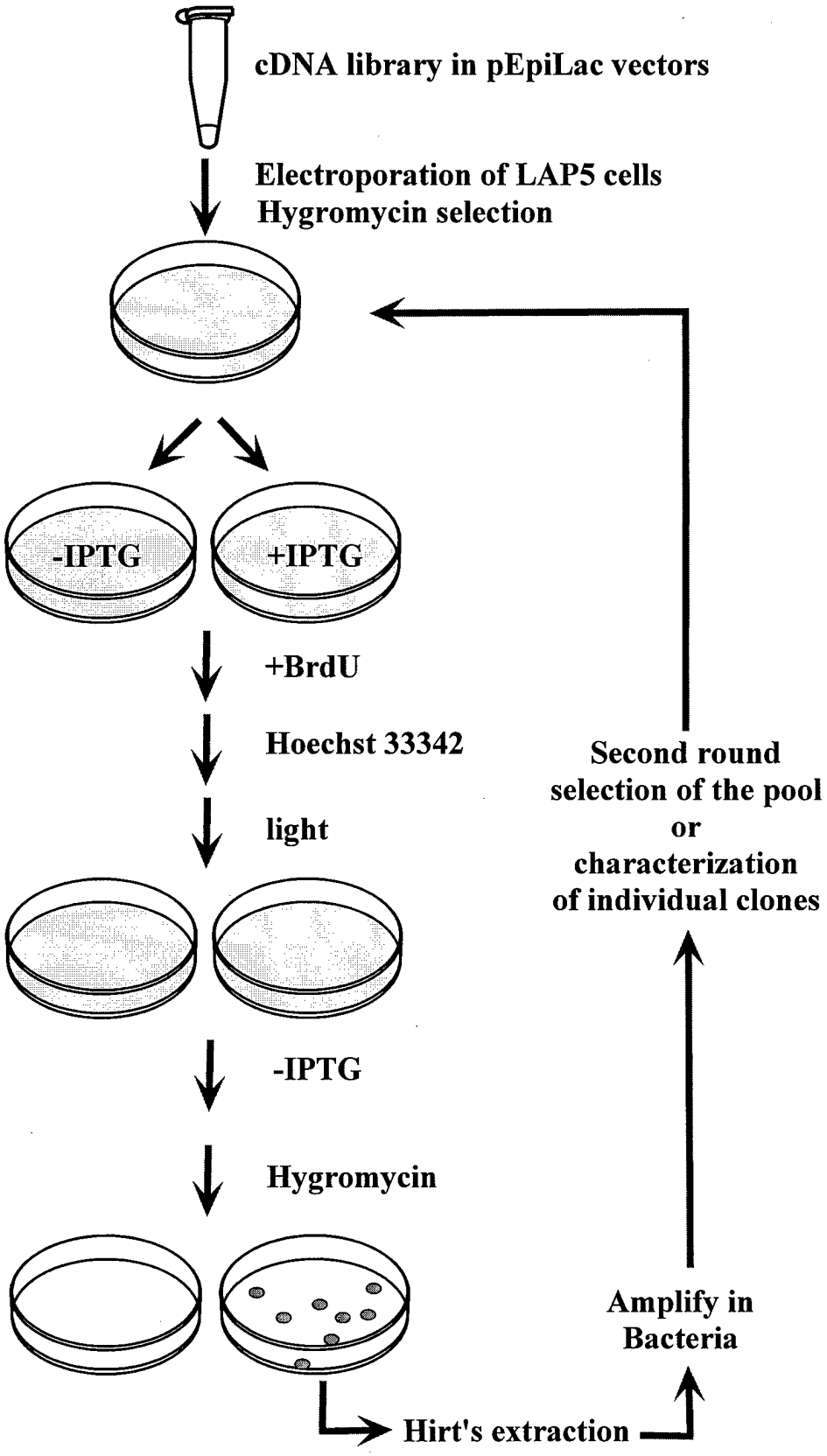
Using this novel system of selection for growth inhibitors (TGIF) in MCF7 cells, we have isolated and sequenced 12 growth inhibitory sequences. Many of these sequences represent fragments of known genes, while others represent previously uncharacterized genes. It is noteworthy that the TGIFs isolated thus far are fragments of the full length cDNAs, suggesting that their encoded gene products are acting as dominant negative variants of the normally expressed full-length protein products. It is possible that these dominant negative variants comprise the more potent growth inhibitors when compared to normally expressed growth inhibitors, and therefore they are more likely to be isolated in a selection procedure designed to identify growth inhibitors. Inasmuch as we have already made sure that our cDNA library is comprised of mostly full-length cDNAs, the SETGAP procedure itself most likely exerts the selective forces in determining whether full-length cDNAs or partial cDNAs might be preferentially selected. It is also important to note that thus far only a small fraction of the complete library has been put through the SETGAP selection (6000 hygromycin resistant clones). Further selections to examine a larger fraction of the library will likely yield new results. A new round of SETGAP selection is now on-going.

Publications resulting from this work:

1. Pestov, D.G., Grzeszkiewwicz, T.M., Lau, L.F. (1998) Isolation of growth suppressors from a cDNA expression library. Oncogene 17: 3187-3197.

2. Li, Y. and Lau, L.F. (1999) An IPTG-inducible episomal expression system for exogenous genes in primate cells. Submitted and under revision.

Figure 1. The improved SETGAP procedure. A cDNA library constructed in the pEpiLac1 episomal vector is transfected into LAP5 cells, and selected against hygromycin B (75 μg/ml) for three weeks. Hygromycin B-resistant cells are plated in mediums containing none or 1 mM IPTG. Forty to forty-eight hours later, the mediums as well as IPTG are refreshed and BrdU is added to the final concentration of 15 μM. After 40-48 h incubation, Hoechst dye 33342 is added into the medium to 2.0 μg/ml. Three hours later, expose cells to light for 30 min. Afterwards, cells are washed twice with PBS and incubated in fresh medium for 3 days followed by two week incubation in mediums containing hygromycin. Extra-chromosomal DNAs can be extracted using Hirt's method and amplified in bacterial cells. These DNAs can be applied to the next round of selection as a pool, or they can be further analyzed as individual sequences.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+IPTG
+BrdU
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Bacteria
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selection of the pool
or
characterization
of individual clones

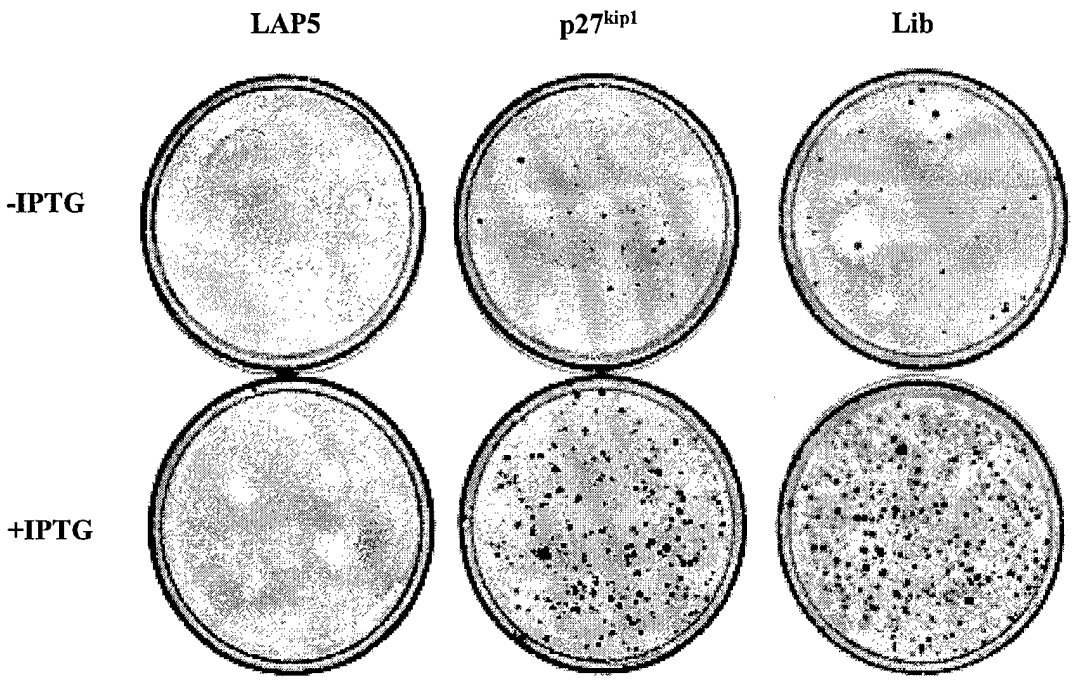

Figure 2. Selection of potential growth inhibitory sequences in SETGAP. A cDNA library representing normal human breast mammary gland cells was constructed in the pEpiLac1 vector and applied to SETGAP procedure (Fig. 1) in MCF7/LAP5 cells. After two rounds of selection, cells surviving the selection were rescued by the removal of IPTG, grown to form colonies and were stained with 1% crystal violet. The growth inhibitory effect was shown as increased colony numbers after IPTG stimulation. Lib, cells transfected with library DNA; $p27^{KIP1}$, cells transfected with mouse $p27^{KIP1}$ CDK inhibitor gene; LAP5, non-transfected MCF7/LAP5 cells.

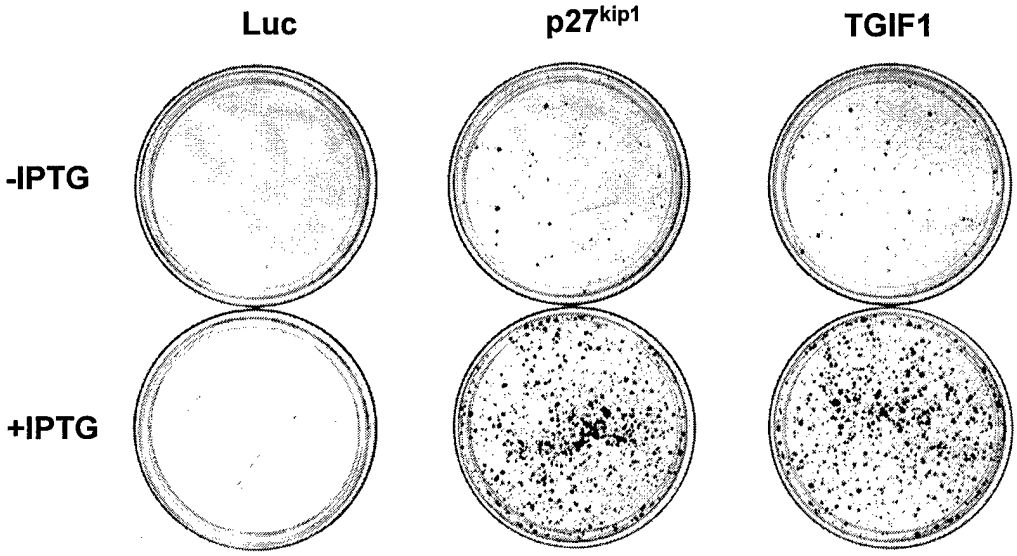

Figure 3. Over-expression of *TGIF1* inhibited LAP5 cell growth in SETGAP. LAP5 cells were transfected with either pEpiLac1-TGIF1 (20 μg), pEpiLac1-p27 (20 μg), or pEpiLac1-Luc (20 μg) using electroporation technique. Cells survived hygromycin B selection were applied to SETGAP. Over-expression of both *TGIF1* and $p27^{kip1}$ gene, but not luciferase gene, inhibited LAP5 cells growth.

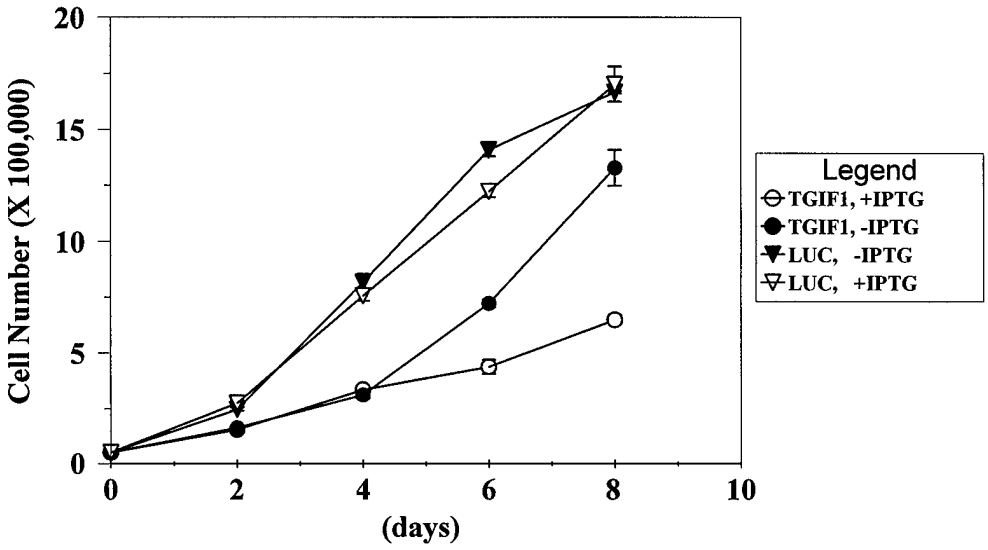

Figure 4. Expression of *TGIF1* inhibited LAP5 cell proliferation. LAP5 cells were cotransfected with pLac3TGIF1 (20 μg) or pLac3Luc (20 μg) and pHyg (2 μg) using electroporation technique. A pool of hygromycin B-resistant cells was obtained for each gene. Duplicated cultures (1 X 10^5 cells per 60 mm dish) were either not treated or treated with 1 mM of IPTG for 2-8 days. At indicated time points, the cell number in each dish was counted using a Coulter Counter. The averages from two independent experiments were shown with standard deviation. Expression of TGIF1, but not luciferase gene, inhibited LAP5 cell growth.

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LAGKRPKAPH GEEAHLPPVS DLTVEFDKLN LQNIGRSVSK TPDESTKTKD QILTSRINAV   540

ERDLLEPSPA DQLGNGHRRT ESEMSARIAK MSLSPSSPRH EDQLEVTREP ARRLFLFGEE   600

PSKLDQDVLA ALECADVDPH QFPAVHRWKS AVLCYSPSDR QSWPSPAVKG RFKSQLPDLS   660

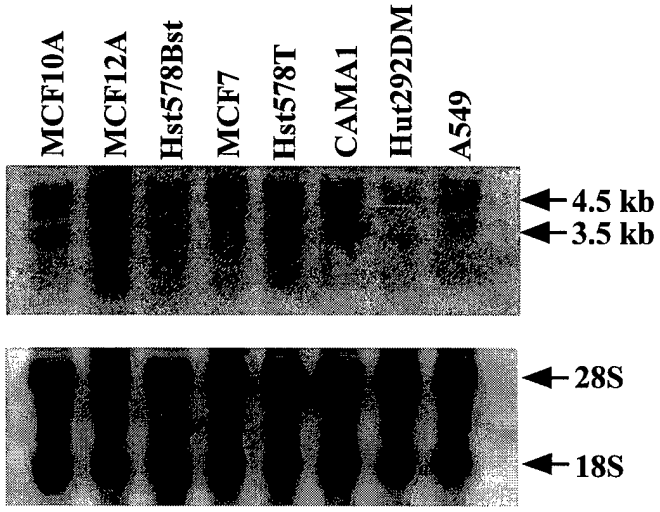
GPHSYSPGRN SVAGSNPAKP GLGSPGRYSP VHGSQLRRMA RLAELAAL*

Note: = marks the start coden in TGIF1
      E450 is K in TGIF1;
      H490 is R in TGIF1;
      G661 is A in TGIF1
```

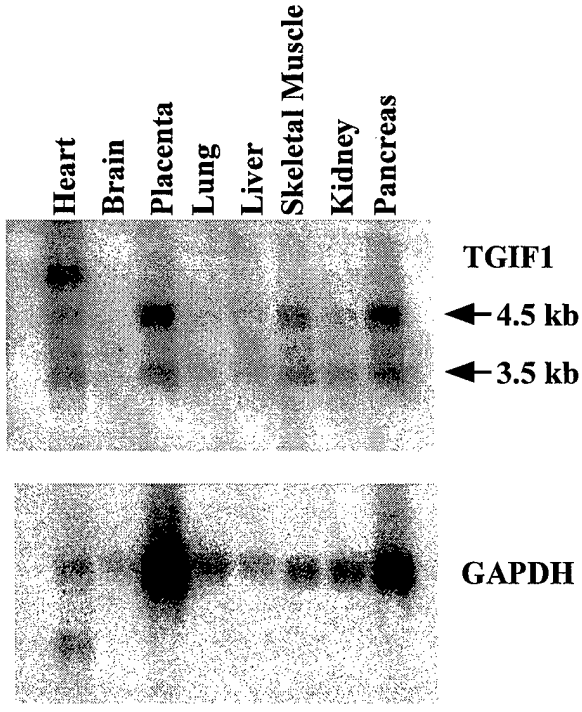
Figure 5. Deduced protein sequences encoded by TGIF1 and its corresponding full length cDNA. The deduced protein sequence encoded by the full-length cDNA corresponding to TGIF1 is shown. The start codon for TGIF1 fragment is marked by (=) at position 416. When compared to the KIAA0692 sequence (Ishikawa et al., 1998), the TGIF sequence showed three nucleotide substitutions that resulted in different encoded amino acids. The amino acid alterations have been underlined.

Figure 6. *TGIF1* expression. TGIF1 expression is determined by Northern blot analysis using ^{32}P-labeled cDNA probes in 8 human cell lines (A) and in 8 human tissue samples (B). A. Ten micrograms of total RNAs from logarithmatically growing normal human mammary gland cells (MCF10A, MCF12A, and Hst578Bst), human breast cancer cells (MCF7, Hst578T, CAMA1, and Hut292DM) or the human lung cancer A549 cells were electrophoresed and transferred to membrane for Northern blot analysis. The nylon membrane was stained with methylene blue to show the equal loading in each lane (lower panel). B. The Multiple Tissue Northern Blot (Clontech) was used to examine *TGIF1* expression in different human tissues. The loading in each lane was shown by the expression of human GAPDH, lower panel.

A


MCF10A
MCF12A
Hst578Bst
MCF7
Hst578T
CAMA1
Hut292DM
A549
4.5 kb
3.5 kb
28S
18S

B


Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
TGIF1
4.5 kb
3.5 kb
GAPDH

clone number	Description
TGIF1	1.4 kb fragment of uncharacterized sequence KIAA0692 (Ishikawa K. et al. 1998. Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res. 5(3):169-176.)
TGIF2	1.2 kb sequence. About 100 bp was sequenced at 5', which is about 97% identical to 695-876 interval of the human ras-related krev-1 gene (1579 kb long).
TGIF3	0.6 kb sequence, partial DNA sequencing showed similarity to the ribosomal protein L11 gene
TGIF4	0.7 kb sequence, partial DNA sequencing showed similarity to the human eIF4D and eIF5A
TGIF5	1 kb sequence, partial DNA sequencing showed similarity to HGR74 cDNA
TGIF6	1.7 kb sequence, partial DNA sequencing showed similarity to the Ca++ and phospholipid-binding protein synaptotagmin 4.
TGIF7	0.9 kb unidentified sequence
TGIF8	0.7 kb fragment of a novel gene which gave a transcript of about 2 kb as determined in Northern blot analysis.
TGIF9	0.7 kb sequence similar to human chaperonin 10 cDNA
TGIF10	250 bp fragment similar to ZNF195 Kruppel zinc finger protein.
TGIF11	0.7 kb sequence, partial DNA sequencing showed similarity to human metallothionein-II cDNA
TGIF12	0.7 kb sequence, partial DNA sequencing showed similarity to the abundant gene for human ribosomal protein L21.

Table 1. Characterization of TGIF sequences isolated through SETGAP. The 12 TGIF sequences isolated through SETGAP selection in LAP5 cells were isolated and sequenced. The sizes of the TGIF sequences are shown, as are their sequences characterization.

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