Genetic Suppressor Elements that Halt the Proliferation of Breast Carcinoma Cells.

Thomas Primiano

University of Illinois College of Medicine Chicago, Illinois, USA

The completion of the draft sequence of the human genome has provided us with a partial list of known and putative human genes, the total number of which is estimated between 30,000 and 45,000 (1, 2). These genes provide many potential targets for drugs, some of which may be useful in stopping the growth of cancers. The development of gene-targeting anticancer drugs could be greatly facilitated by the ability to narrow down the list of human genes to those that are necessary for the growth of tumor cells. To find such genes, we have used a general strategy, which is based on the principle that genes required for cell proliferation should give rise to genetic suppressor elements (GSEs) that inhibit cell growth.

GSEs are short biologically active cDNA fragments that interfere with the function of their cognate gene (3, 4). GSEs may encode antisense RNA molecules that inhibit gene expression or peptides corresponding to functional protein domains, which act as dominant negative inhibitors. The general strategy for the isolation of biologically active GSEs involves the preparation of an expression library containing randomly fragmented DNA of the target gene or genes. This library is then introduced into recipient cells, followed by selection for the desired phenotype and the recovery of biologically active GSEs from the selected cells. By using a single cDNA as the starting material for GSE selection, one can generate specific inhibitors of the target gene and map functional domains in the target protein. By using a mixture of multiple genes or the entire transcriptome as the starting material, GSE selection allows one to identify genes responsible for a specific cellular function, since such genes will give rise to GSEs inhibiting this function. GSEs derived from genes involved in cell proliferation are expected to inhibit cell growth. To select for growth-inhibitory GSEs, we have used bromodeoxyuridine (BrdU) suicide as the selection strategy. To carry out such selection from a library that would represent the entire transcriptome of breast carcinoma cells, we have now generated a large library of normalized (reduced-redundance) fragments of total cellular cDNA in a retroviral vector that allows for high-efficiency transduction and isopropyl-β-thio-galactoside (IPTG)-regulated gene expression in mammalian cells (5). Using this library, we have succeeded in identifying GSEs that inhibit the
growth of human breast carcinoma cells that are fragments of 57 genes. Nearly two-thirds of these genes are known oncogenes and other positive regulators of cell growth, such as growth factor receptors, cell cycle proteins, protein kinases involved in growth-regulatory signaling, factors in DNA, RNA and protein synthesis. The remainder of the genes discovered had not been previously implicated in cell proliferation, including 5 novel genes called Growth in Breast Cancer (GBC). The genes identified by GSEs include targets for new drugs undergoing clinical testing, and other genes identified in this study may serve as potential targets for future anticancer drugs.

References
Genetic Suppressor Elements that Halt Proliferation of Breast Carcinoma Cells

Thomas Primiano, Ph.D.

Department of Molecular Genetics
University of Illinois College of Medicine
Chicago, Illinois

Identifying new target genes for cancer treatment

• Approximately 10,000 genes expressed in a tumor cell: which of these are essential for tumor cell proliferation?

• Approach: Identify genes, inhibition of which will produce growth arrest in a tumor cell.

• Such genes should give rise to Genetic Suppressor Elements that inhibit tumor cell growth.
Genetic Suppressor Elements (GSE):

Short gene fragments inducing a biological effect, usually opposite to the effect of the whole gene

GSEs encode:
Peptides (dominant negative mutants)
Antisense RNAs (inhibit gene expression)

Gudkov and Roninson (1997)

Antisense RNA inhibitors

\[\text{mRNA} \quad \text{antisense}\]
Protein domains as dominant inhibitors

Functional domains overexpressed

Parent protein cannot form the active complex

Active complex

• Goal: starting from a cDNA fragment library of all genes expressed in breast carcinoma cells, select GSEs that inhibit breast carcinoma cell growth.

• The selected GSEs should be derived from genes that are essential for breast cancer cell growth, i.e. potential drug targets
Normalized cDNA fragment library:

- 100-400 bp random fragments from normalized cDNA of MCF-7 breast carcinoma (ER-positive, p53 wild-type)
- Library size: about 50 million clones, 87% recombinant
- Vector: LNXCO3 (IPTG-inducible, retroviral)
MDA-MB-231-3'SS31: recipient cell line for growth-inhibitory GSE selection

- Starting cell line: MDA-MB-231 breast carcinoma (ER-negative, p53 mutant)
- Transduced with ecotropic retroviral receptor (up to 80% infection rate with ecotropic virus)
- Transfected with LacI repressor modified for nuclear localization (10-fold IPTG inducibility)

Library-transduced cells show increase in IPTG-dependent survival of BrdU suicide with consequent steps of selection

Library-transduced cells  -IPTG  +IPTG
G418-selected
After one round BrdU
After two rounds BrdU
Identification of genes enriched by selection for growth-inhibitory GSEs

- Recover genomic DNA from cells that survived two rounds of selection
- Rescue (by PCR) and reclone retroviral inserts into LNXCO3
- High-throughput sequencing of about 3,000 rescued clones
- About 1,500 cDNA sequences identified and assigned to individual genes
- 69 genes are represented by 3 or more clones or by 2 or more different sequences
Summary Table of Isolated GSEs

<table>
<thead>
<tr>
<th>Function</th>
<th>Clones</th>
<th>Sequences</th>
<th>Genes</th>
<th>Confirmed</th>
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<tr>
<td>Transcription Factors</td>
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<tr>
<td>Cell Cycle</td>
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<td>Signal Transduction</td>
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<td>23</td>
<td>8</td>
<td>3</td>
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<tr>
<td>Growth Factors/receptors</td>
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<td>25</td>
<td>8</td>
<td>4</td>
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<td>Protein Processing</td>
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<td>Protein Synthesis</td>
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<tr>
<td>Others</td>
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<td>Intracellular Transport</td>
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<td><strong>Total</strong></td>
<td>1138</td>
<td>179</td>
<td>69</td>
<td>45</td>
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</table>

Selected genes include:

- 38 genes known to be involved in cell proliferation, including 12 oncogenes: selection works as expected
- 29 genes that were not known to be involved in cell proliferation: new potential drug targets
Functional Testing of Individual GSEs

1. Test GSE-transduced cell populations for IPTG-dependent increase in the survival of BrdU suicide.

2. Isolate GSE-containing cell lines that are growth-inhibited by IPTG.

3. Specific assays for the inhibition of GSE-cognate genes.
Cell Adhesion Molecule L1

Promotes neurite migration and outgrowth
Highly expressed in neuroblastoma and melanoma
Embryonic knockout leads to MASA
Activates MAPKs
Associated with paxillin and FAK
Binds RGD Integrins and ankyrins

Time Course for Cell Growth

- IPTG+
- IPTG-

Cell Number

0 50000 100000 150000 200000 250000 300000 350000

Time (Days) 0 1 2 3 4 5 6 7

CAM
GSE from L1CAM induces formation of dendritic projections

no induction

4 days IPTG

Membrane associated integrin ITGB5

• Activate receptor-associated tyrosine kinases
• Activates cell matrix assembly genes
• Acts as positive regulator of cell growth
• Upregulated in cancers (endothelial)
• Knockout is not embryonic lethal

ITGβ5

1

INB

CYS

CT

3401

799
GSE from ITB5 induces formation of spindle shaped cells

no induction 4 days IPTG

Immunofluorescence analysis of L1CAM and INTGB

Cellular Clones
L1CAM
ITB5

Fluorescence
GBC3 is on chromosome 3q29

- An EST database search for the GSE sequence pulled out a single 5' EST which overlapped and formed a cluster with four 3' ESTs, which together form a 693 bp sequence.
- The ESTs were localized to chromosomal band 3q29 by performing a human genomic BLAST search.
- Analysis of the sequence revealed a potential ORF starting at base 49 encoding a 106-aa protein.

GBC3 mRNA from Genomic sequence, Hs.186804

GSE 7-106
STSG63361

PCR Cloned Sequence 4-503 (497bp)

(693 bps)

putative ORF, 106 AA

5' AA443027 Pooled human melanocyte, fetal heart, and pregnant uterus

3' AA677855 Soares_fetal_liver_spleen

3' A1742356 pooled

3' A1051003 parathyroid tumor

3' AW044522 pooled

GBC11 is on chromosome 14q24.2

An EST database search for the GSE sequence pulled out three 5' EST which formed a cluster with three 3' ESTs, which together form a 1300 bp sequence.

The ESTs were localized to chromosomal band 14q24 by performing a human genomic BLAST search.

GBC11 GSE

500 1000 1500 2000 2500

AL602248 Homo sapiens 5' EST

W84777 20 week fetal spleen/liver 3' EST

W84824 20 week fetal spleen liver 5' EST

AI368822 3' EST Soares total 8-9 fetus 3' EST

AI310112 3' EST pooled kidney tumors (clear cell type)
RT-PCR analysis GBC3 and GBC11.

<table>
<thead>
<tr>
<th>GBC3</th>
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<th>GBC11</th>
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<tr>
<td>-RT</td>
<td>+RT</td>
<td>ladder</td>
<td>water</td>
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370 bp

161 bp
Conclusions

- GSEs are used to identify genetic targets mediating a biological process
- GSEs can be coupled with high throughput screening systems to rapidly discover new drug entities.