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

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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-redundancy) 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
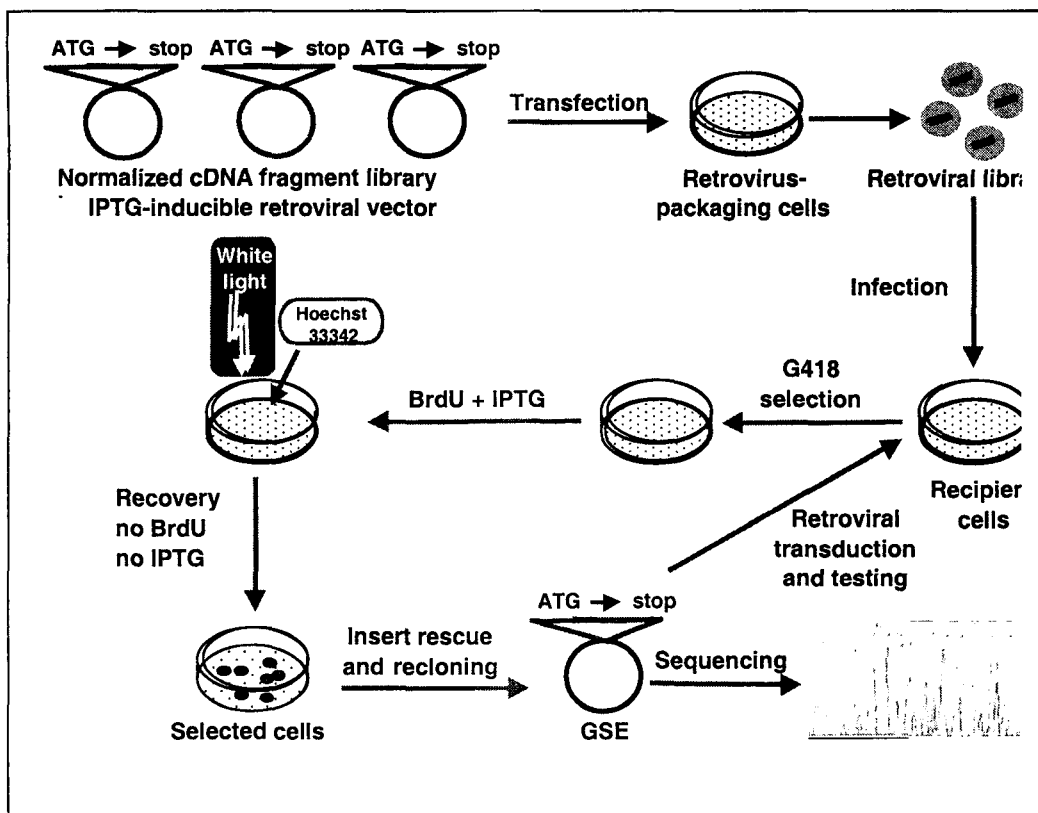
growth of human breast carcinoma cells that are fragments of 57 genes. Nearly two-thirds of these genes are known oncogenes and other positive regulators of cell growth, such as growth factor receptors, cell cycle proteins, protein kinases involved in growth-regulatory signaling, factors in DNA, RNA 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

1. J. C. Venter, et al., *Science* **291**, 1304-51. (2001).
2. E. S. Lander, et al., *Nature* **409**, 860-921. (2001).
3. I. B. Roninson, et al., *Cancer Res.* **55**, 4023-4028 (1995).
4. A. Gudkov, I. B. Roninson, R. Brown, *Science* **285**, 299a (1999).
5. B. D. Chang, I. B. Roninson, *Gene* **183**, 137-142 (1996).

Genetic Suppressor Elements that Halt Proliferation of Breast Carcinoma Cells

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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
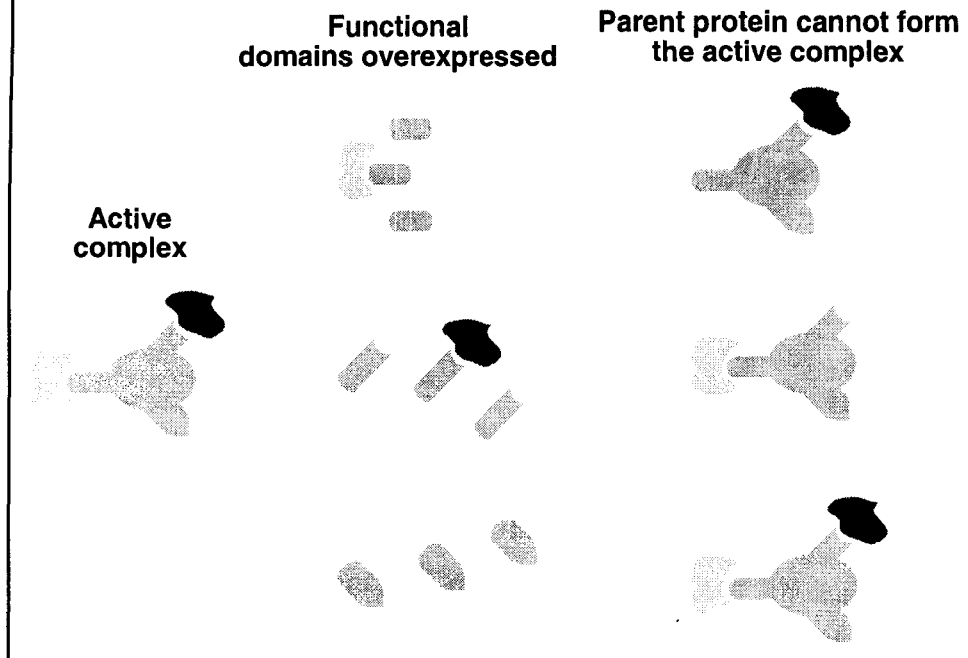to the effect of the whole gene**

**GSEs encode:
Peptides (dominant negative mutants)
Antisense RNAs (inhibit gene expression)**
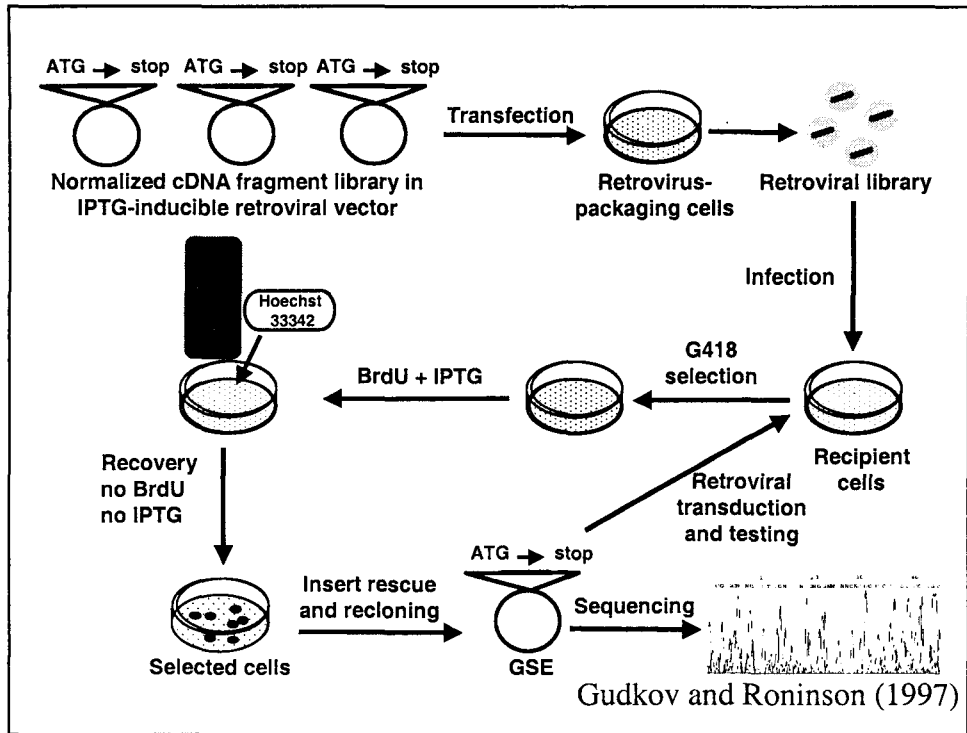
Gudkov and Roninson (1997)

Antisense RNA inhibitors

mRNA
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**

Figure 1 (top panel)

ATG → stop ATG → stop ATG → stop

Normalized cDNA fragment library in IPTG-inducible retroviral vector

Transfection → Retrovirus-packaging cells → Retroviral library

Infection

Hoechst 33342

BrdU + IPTG

G418 selection

Recipient cells

Retroviral transduction and testing

Recovery
no BrdU
no IPTG

Selected cells

Insert rescue and recloning

ATG → stop

GSE

Sequencing

Gudkov and Roninson (1997)

Figure 2 (bottom panel)

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)

cDNA fragment

neo

CMV

lac operators

Chang and Roninson (1996)
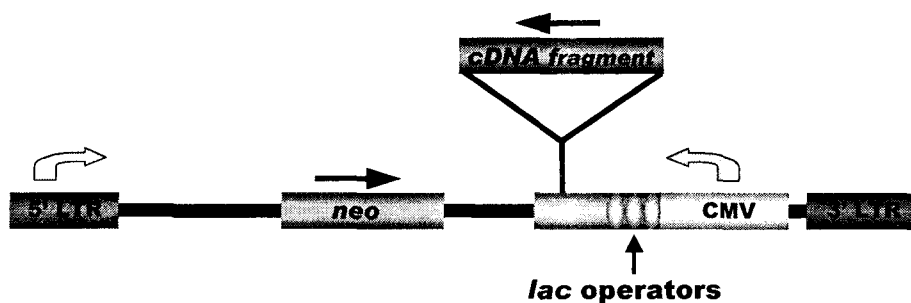
MDA-MB-231-3'SS31: recipient cell line for growth-inhibitory GSE selection

•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)

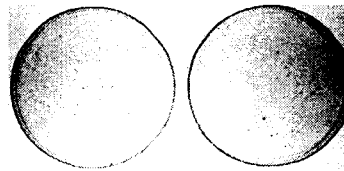•Transfected with LacI repressor modified for nuclear localization (10-fold IPTG inducibility)

Library-transduced cells show increase in IPTG-dependent survival of BrdU suicide with consequent steps of selection
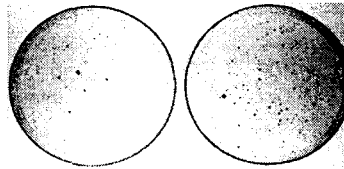
Library-transduced cells   -IPTG   +IPTG
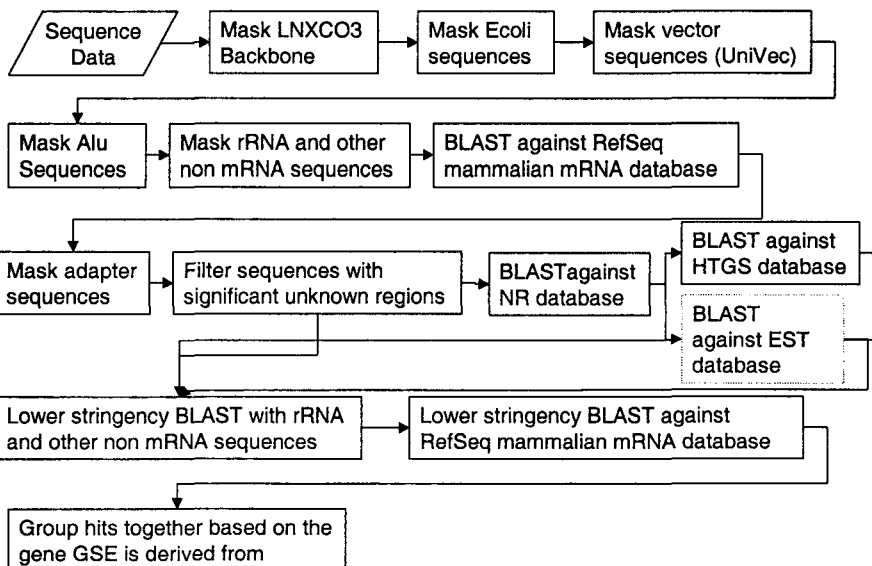
G418-selected
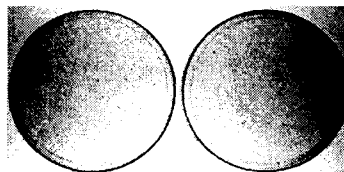
After one round BrdU

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 of DNA Sequence Analysis

Sequence Data → Mask LNXCO3 Backbone → Mask Ecoli sequences → Mask vector sequences (UniVec)

Mask Alu Sequences → Mask rRNA and other non mRNA sequences → BLAST against RefSeq mammalian mRNA database

Mask adapter sequences → Filter sequences with significant unknown regions → BLAST against NR database → BLAST against HTGS database / BLAST against EST database

Lower stringency BLAST with rRNA and other non mRNA sequences → Lower stringency BLAST against RefSeq mammalian mRNA database

Group hits together based on the gene GSE is derived from

Summary Table of Isolated GSEs

Function	Clones	Sequences	Genes	Confirmed
Transcription Factors	592	35	11	9
Cell Adhesion	101	32	5	4
Unknown	89	5	6	6
Cell Cycle	82	22	8	5
Signal Transduction	73	23	8	3
Growth Factors/receptors	52	25	8	4
Protein Processing	52	6	4	3
Protein Synthesis	33	10	5	3
Others	29	11	6	4
Intracellular Transport	21	7	5	1
RNA Maturation	14	3	3	3
Total	1138	179	69	45

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
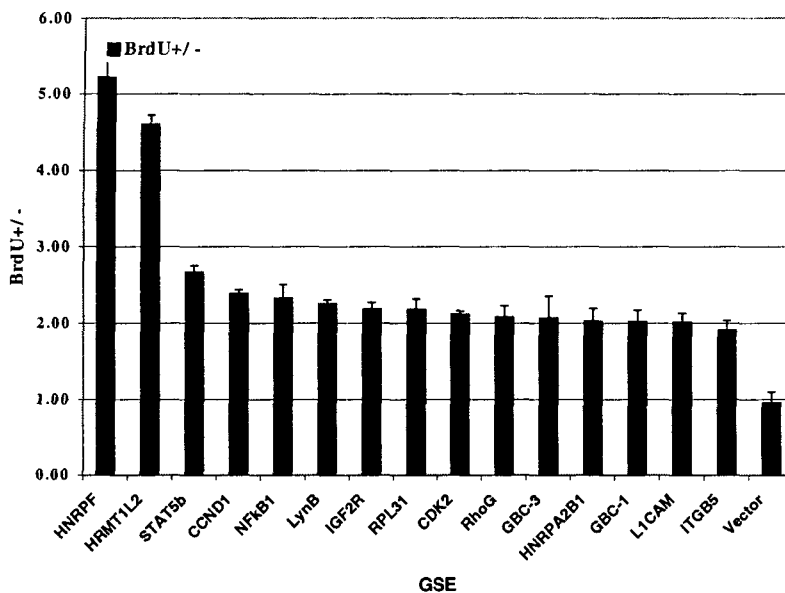
Functional Testing of Individual GSEs

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.

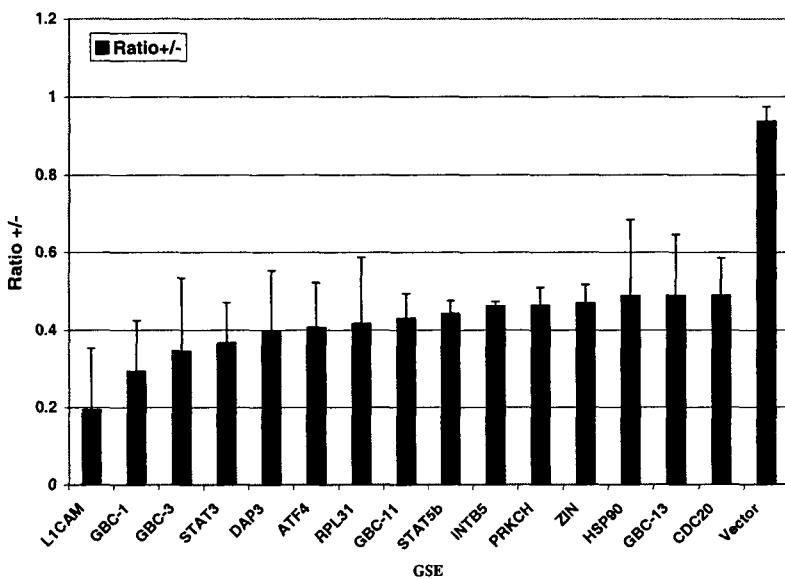3. Specific assays for the inhibition of GSE-cognate genes.

Testing individual clones in the second round of BrdU selection

-IPTG   +IPTG   -IPTG   +IPTG   -IPTG   +IPTG

LNXCO3   Stat5b   GBC-1

p21   Stat3a   L1CAM
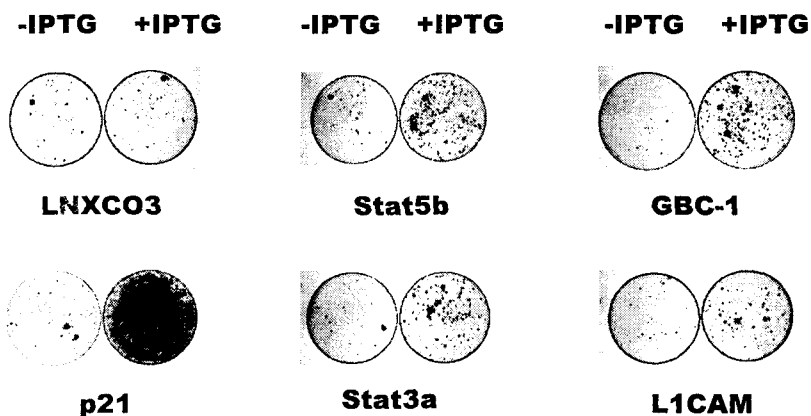
Testing individual clones by BrdU assay

BrdU+/−

GSE: HNRPF, HRMT1L2, STAT5b, CCND1, NFkB1, LynB, IGF2R, RPL31, CDK2, RhoG, GBC-3, HNRPA2B1, GBC-1, L1CAM, ITGB5, Vector

Testing individual clones by growth inhibition assay

Ratio+/−

GSE: L1CAM, GBC-1, GBC-3, STAT3, DAP3, ATF4, RPL31, GBC-11, STAT5b, INTB5, PRKCH, ZIN, HSP90, GBC-13, CDC20, Vector

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

1   *4525*

1   IgGL   FNL   ICD 1258
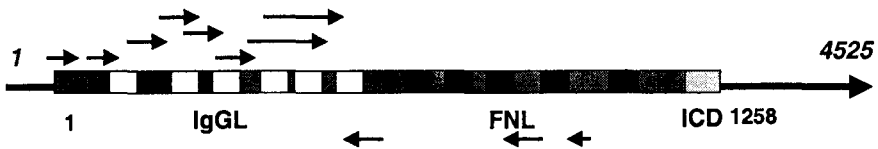
Time Course for Cell Growth

IPTG+
IPTG-

Cell Number

350000
300000
250000
200000
150000
100000
50000
0

0   1   2   3   4   5   6   7

Time (Days)

CAM
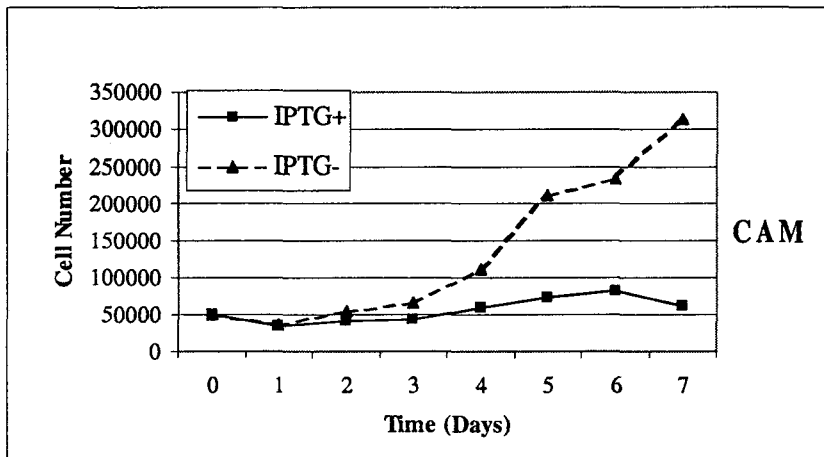
GSE from L1CAM induces formation of dendritic projections
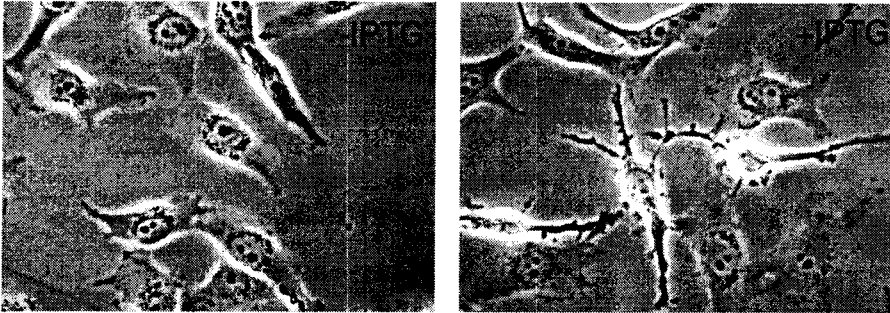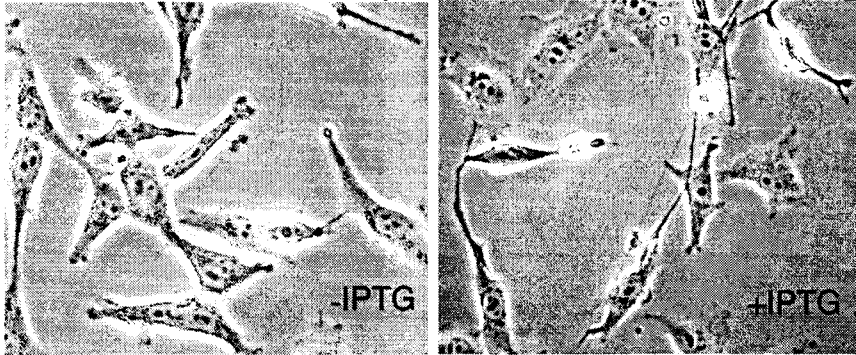

no induction   4 days IPTG

Membrane associated integrin ITGB5

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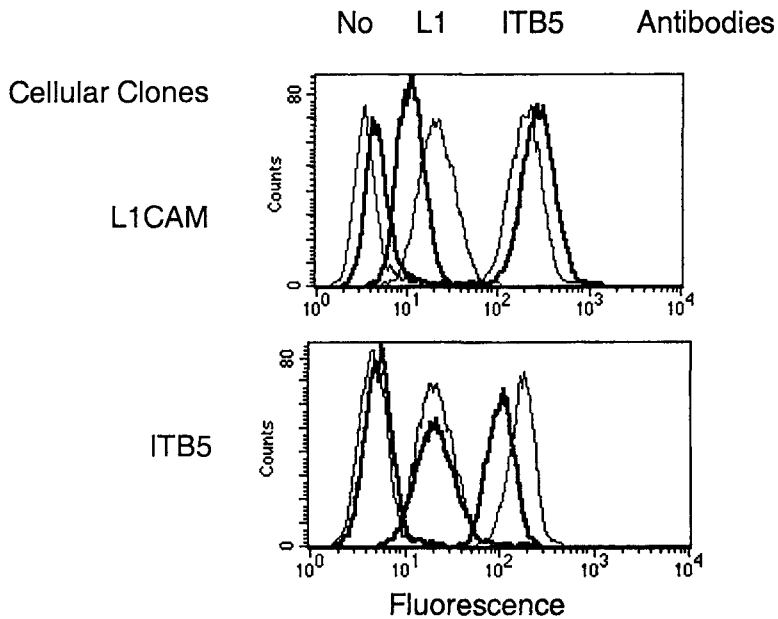- Upregulated in cancers (endothelial)
- Knockout is not embryonic lethal

ITGβ5

1 ... 3401

1 ... INB ... CYS ... CT ... 799
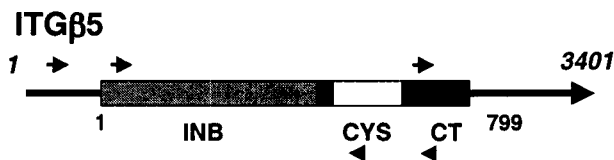
GSE from ITB5 induces formation of spindle shaped cells

-IPTG

+IPTG

no induction

4 days IPTG

Immunofluorescence analysis of L1CAM and INTGB

No   L1   ITB5   Antibodies

Cellular Clones

L1CAM

ITB5
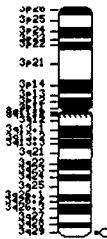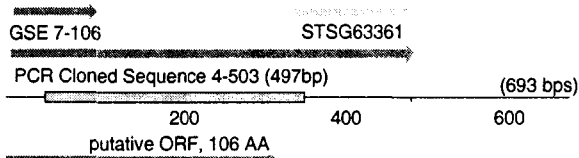
Fluorescence

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

Chromosome 3

GBC3 mRNA from Genomic sequence, Hs.186804

GSE 7-106   STSG63361

PCR Cloned Sequence 4-503 (497bp)

(693 bps)

200   400   600
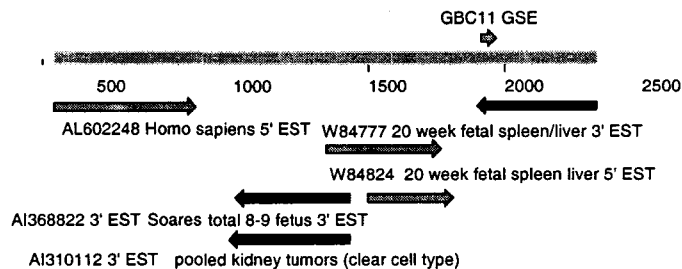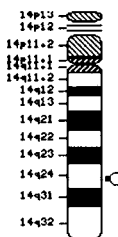
putative ORF, 106 AA

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

3' AA677855 Soares_fetal_liver_spleen

3' AI742356 pooled

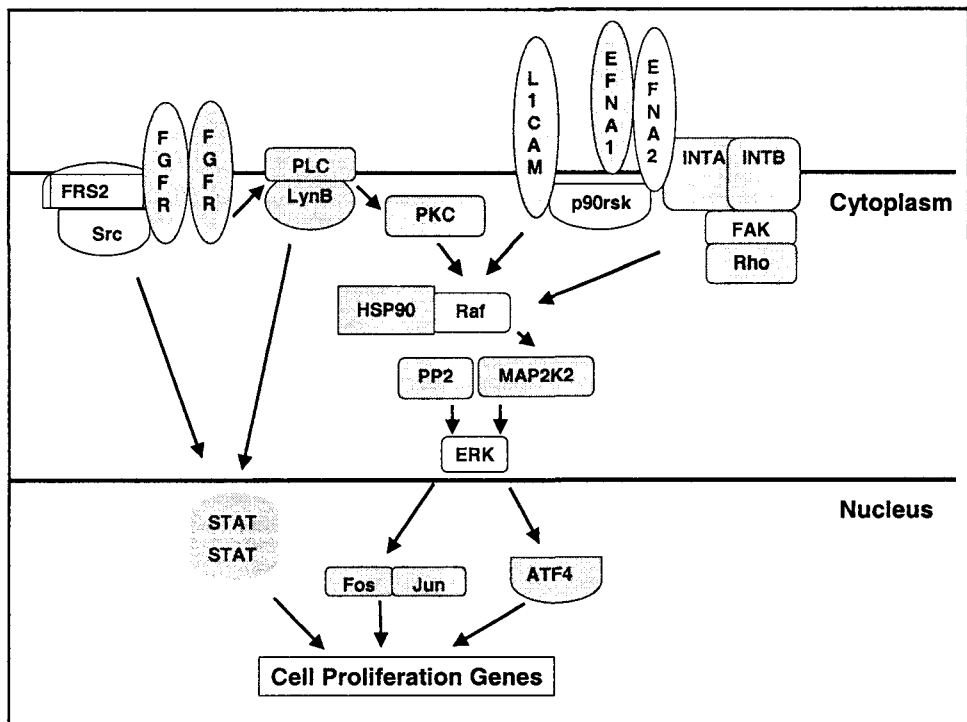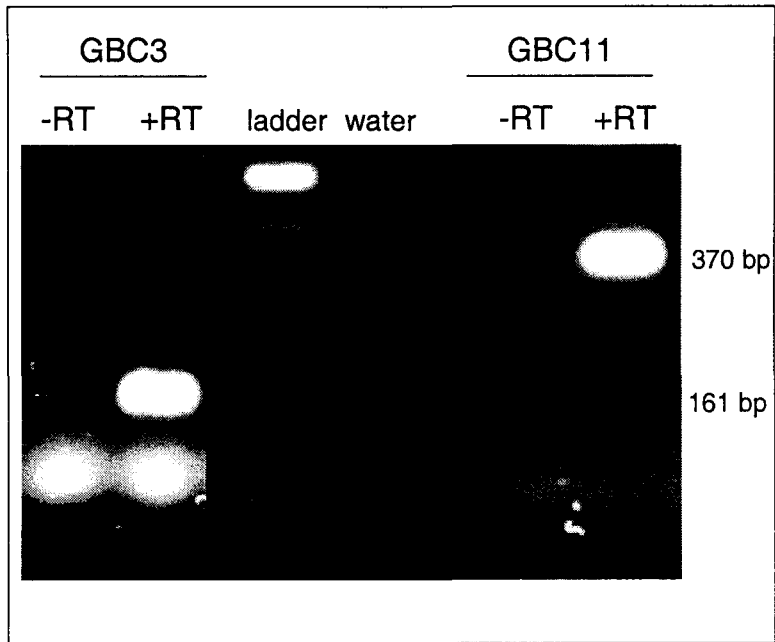3' AI051003 parathyroid tumor

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 three3' 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.

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.