Proteome-wide High Throughput Cell Based Assay for Apoptotic Genes

John Kenten, Doug Woods, Pankaj Oberoi, Laura Schaefer, Jonathan Reeves, Hans A. Biebuyck and Jacob N. Wohlstadtter
Abstract

We demonstrate a systematic, cell-based, high throughput approach to the discovery of apoptotic genes. We detected apoptotic genes based on their ability to lower significantly the level of beta-galactosidase expression in a co-transfection assay. Validation of the assay occurred in two cell lines (HEK-293 and U2OS) using BAX as a positive control for apoptosis, followed by a 96-well plate of other, known pro-apoptotic, genes having widely varying function, molecular weight, requirements for translational modification and expression efficiencies. This assay had a high level of performance in the detection of BAX and showed statistical recovery of many other pro-apoptotic genes using our methodology. We selected 18,000 clones encoding 14,000 different proteins (Unigene) from our library of 65,000 full-length cDNAs (24,000 proteins) for co-transfection with beta-galactosidase into U2OS cells. We examined 1,000 clones that gave the lowest levels of beta-galactosidase expression and selected 200 from among these, based on ontology, literature and experiment, for further characterization. Retesting of this set (making new DNA and repeating the transfections several times) allowed us to further cull the list to 28 clones having the greatest effects on death in U2OS cells. We repeated the assay in a cell line much more resistant to cell death, HEK-293, to find BAX-like genes. We found 9 genes that were BAX-like by our criteria, including Caspase 4, a gene already known to be pro-apoptotic. We further assessed the involvement in apoptosis of several of these genes using Caspase 3/7 activation following their transfection into HEK-293 cells. In sum these data demonstrated the validity of our approach to screening and discovery of genes involved in apoptosis. We conclude that our approach to high throughput, cell-based screens through the proteome for pro-apoptotic genes represents a facile route to characterization of this, and by extension, other biological activities.

Beta galactosidase expression is used as a surrogate marker for cell survival

The flow chart outlines the basic steps involved in the assay for the detection of genes that cause loss of cell viability. The gene of interest is co-transfected in with a plasmid encoding a reporter, beta galactosidase in this case, driven by a constitutive promoter. The loss of beta galactosidase activity after the transfection provides a measure of the deleterious effects of the transfected gene. This approach has the inherent benefit that translation of the gene of interest is coupled to that of the reporter gene by co-transfection and thus proves particularly useful where transfection efficiency is low and precludes other methods of the detection of compromised cell viability. The effective amplification of signal that results from this approach is critical to the construction of non-hypothesis driven screens with sufficiently high signal to noise to allow facile and comprehensive surveys of gene libraries.
Proteome-wide High Throughput Cell Based Assay for Apoptotic Genes

3 Signal amplification by co-transfection: how it works

Transfectable cell line 96 well plate
Transfection Beta Galactosidase Plus Gene X
Co-Transfected Cells mixed with Non transfected cells
Non apoptotic co-transfected Gene X

Beta Galactosidase present
Beta Galactosidase reduced Cells Dead

Apoptotic co-transfected Gene X

4 Apoptosis of a cell

Many roads lead to death of a cell. Prominent paths include apoptosis. Apoptotic paths can be directed from the outside in through ligands for cytokine death receptors (e.g. TRAIL, FAS) that activate downstream caspases and trigger general degradation of the cell. Other paths derive from intrinsic signaling ligands (e.g. BAX) that target and permeabilize mitochondrial bodies in the cell causing release of Cytochrome C with subsequent activation of caspases. Stress responses in the endoplasmic reticulum and sentinel processes within the nucleus similarly initiate death throes in cells.
The figure compares the diversity of our cDNA clone library with the mouse and human genome. We group the genes according to their molecular function using public domain annotations provided by the Gene Ontology® Consortium. The data indicate that the contents of our library provide a reasonable representation of the functional diversity of the mammalian genome in both the character and distribution of its members.

Flow chart for automation

1. Add DNA from Master plate
2. Add Fugene
3. Add cells 1.5x10⁴ cells
4. Incubate 24-48 hours at 37°C
5. Remove media add lysis buffer
6. Add assay reagent incubate
7. Measure Beta Galactosidase activity

Our protocol for the automation of transfections from DNA preps. We investigated a number of different conditions for the addition of DNA to cells and settled on the outlined approach as the best match to our automation while maintaining good signals and low backgrounds. The sequence of additions allowed the use of a near optimal work flow with good reagent stability and high reproducibility (see below). The protocol was general for many types of cells, demonstrated here with two adherent, carcinoma derived cell lines, HEK-293 and U2OS. Runs of up to 30 plates per batch were typical of our screen without a loss of performance of the assay. We ran quality control plates comprising a number of well characterized genes to assess assay performance and the condition of the cells every tenth plate of the screen.
Proteome-wide High Throughput Cell Based Assay for Apoptotic Genes

Response to co-transfections in U2OS vs HEK-293

An illustration of the marked difference in response between two different cell lines, U2OS and HEK-293. Other cell lines showed still different responsiveness probably related to the unique characteristics of each as regards cell cycle and apoptotic machinery. The vertical and horizontal lines on the graph demarcate the point three standard deviations from the negative controls for each cell line and highlight the differences between them with respect to noise and known pro-apoptotic effects. We selected U2OS cells for our primary screen as they appeared particularly well suited to the study, are known to have a substantially intact apoptotic signaling pathway (i.e. are wild type p53), and gave us sensitive signals in the assay.

*HEK-293 is a primary human embryonal kidney transformed by sheared human adenovirus type 5 DNA. U2OS is a cell line derived from a moderately differentiated sarcoma of the tibia of a 15 year old girl.

Assessment of intraday variation in the assay

The reproducibility of our quality control plate was high over 5 plates and ten hours with a number of different genes having different transfection efficiencies and effects on cell viability. These plates were evenly interspersed throughout our screening plates that contained the rest of the library (see below). The controls are genes we expected to have no viability effect on the U2OS cell line used here. We use these controls throughout our work to provide a measure of the baseline activity of beta galactosidase and its variability in the cell line. Each point represents a unique gene from a unique clone. The average of the first three plates is the X axis and the average of the last two plates, run several hours later, is the Y axis. We used this type of averaging throughout the screen to improve its statistics and to catch aberrations. The line is a fit to data and has a slope of 0.9. Overall we used these data to validate the stability and robustness of the assay in pre-screen development.
Proteome-wide High Throughput Cell Based Assay for Apoptotic Genes

Assessment of interday variation in the assay

A study with our control QC plate illustrated the low variability of the assay between the averages of five plates run on consecutive days. The line is a fit to the data with a slope of 0.4. We concluded that while the absolute signal level fluctuated from day to day with different batches of cells, inter and intra gene correlations remained high augmenting our confidence in the approach. Axes and data are like those in the previous figure.

Data from the screen of 18,000 clones

A sample of plates from of our collection of 18,000 clones is typical of the range and type of data from the screen. We used a first screen to qualify 1,000 clones as candidates for further study. Analysis of this collection revealed many genes expected to affect our signal including genes important to cell cycle, in addition to those already implicated in apoptosis. Quality control plates are shown in blue and were used to calculate percentage activity. The horizontal grey line demarcates the point three standard deviations from the mean of the control genes and provides a visual measure of putative hits. Bioinformatic studies suggested that at least 30% of the known genes might have an a priori influence on beta-galactosidase activity based on their ontology, for example genes that affect protein translation. We used ontology and other literature in combination with our hit picking criteria to select 200 genes to investigate further for apoptotic activity.
We made two different preps of DNA from our selected set of 200 genes and retested them in the beta-galactosidase assay. The graph shows the high correlation between data from each of the two preps. The BAX control gene is one of the blue data points at greater than 4 standard deviations from the controls on the plate, where each point is an average of three runs for a given prep. Eleven clones (green) were further characterized to ascertain the reason of the lowering of the beta-galactosidase signal.

Results from a caspase 3/7 assay on HEK-293 cells transfected with the various genes selected for detailed study of their apoptotic potential demonstrated evidence for apoptosis by this classical pathway.
We demonstrated a new approach to high throughput cell based assays for genes that are involved in apoptosis. The assay exploits the influence of plasma membrane integrity on signal derived from a reporter encapsulated in cells co-transfected with random genes from a library. It had high reproducibility in time, across DNA preps and many different types of genes. Apoptosis may not be the only cause for loss of signal in this approach: transfection efficiency; disruptors of beta-galactosidase and perturbations to normal cell cycle could influence the reporter. We nonetheless recovered a significant number of genes involved in activation of apoptosis using this non-hypothesis driven methodology. Many of these genes were already well known to be pro-apoptotic (e.g. caspase 4). Others were new to this role, underscoring the success of the application to biological discovery. Importantly, we showed that the apoptotic effect of these genes was cell line specific, suggesting that high throughput screens based on the differential effects of genes in cells having diverse lineage can provide new insights into effectors of a pathway. Our approach is readily generalizable to the interrogation of other cellular pathways. The assay system we developed could be used to find genes that rescue a cell from death caused by its co-transfection with an apoptotic gene, for example. We have, by extension, developed high throughput cell based assays for discovery of small molecule inhibitors of apoptosis induced by CASP4, TNFRSF10a and our new apoptotic genes by rescue of lost beta-galactosidase activity after their transfection (data not shown). The basic assay protocol extends to the transfection of genes for many other cell based discovery projects by the use of a cis-acting enhancers linked to a reporter (i.e. NFkB-luciferase) or by the quantitation of cytokines, cAMP and other signaling components. We think that these assays combined with curated libraries of full length cDNAs and high throughput automation provide powerful new algorithms for the discovery of genes involved in cellular pathways.