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The conversion of a normal cell into a cancer cell proceeds through a series of genetic and epigenetic alterations. We have proposed to use well established genetic methodologies to identify novel anti-cancer targets via their specific, genetic interactions with common cancer mutations. In short, we will identify genetic alterations that are neutral in normal cells, but that are lethal when combined with cancer mutations. This "synthetic lethality" approach may identify potential therapeutic targets that are highly specific to the cancer cell.

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Introduction

My laboratory has long been dedicated to the application of genetic principles to both the understanding of cancer biology and the search for novel anticancer targets. Since the 1970s, the war on cancer has been based on the notion that studying the disease will lead to the discovery of vulnerabilities, which can be exploited in the clinic. While many underlying genetic determinants of cancer have been identified, this knowledge has failed to translate into new therapeutic strategies, with only a handful of exceptions. One hypothesis is that this failure has largely been due to the genetically intractable nature of cultured mammalian cells. In part due to work from my laboratory, dsRNA-induced gene silencing, or RNA interference (RNAi), in mammalian systems has emerged as a tool that is likely to re-invigorate the field of somatic cell genetics and in the process revolutionize the study of human disease. During the past year, we have reached a point at which any gene in the human genome can conceivably be targeted using small dsRNA gene silencing triggers – *small interfering RNAs (siRNAs) or expressed short hairpin RNAs (shRNAs).* I have recently received an Innovator award from this same program that will fund the construction of shRNA-expression cassettes on a large, and possibly whole-genome scale. We will be able to use these tools within the present programs to test the notion that synthetic lethality is a plausible approach to the discovery of novel anticancer targets. I note that the goals of this grant and that of the Innovator award are distinct. The innovator award is to develop high-throughput procedures to create a resource for the entire cancer community. This program will apply that resource to a very specific problem in cancer biology.

Body

A Genome-wide shRNA library

Over the two years and with the support of an Innovator award, we have constructed a library of RNAi-inducing constructs that covers ~20,000 cancer relevant genes with from 1 to 6 shRNAs each (total to date of ~50,000 sequence-verified constructs). Additionally, last year, we constructed libraries consisting of ~20,000 mouse shRNAs targeting ~15,000 mouse genes. These libraries are the tools that are necessary to move forward with large-scale screens for synthetic lethality in mammalian cells. The library itself is being constructed with support from a number of sources (consider that the overall cost will be ~$9,000,000). Since it is a key reagent for our studies, it is worthwhile to describe the resource here.

The first-generation library described in last year's report covered 10,000 selected genes. This year our efforts have expanded in our second generation library to include all predicted genes in the human and mouse genomes. These were constructed using optimized strategies both for shRNA expression and for bioinformatics predictions of effective shRNAs.

shRNA optimization

While our first-generation constructs were quite effective as suppressors of gene expression, last year saw the discovery of an additional step in the processing of small endogenous triggers of the RNAi machinery. MicroRNAs produced from their natural genes are first transcribed by RNA polymerase II as long primary transcripts that are processed by a recently described complex (the Microprocessor) into pre-miRNAs. It was the pre-miRNA structure that all first generation shRNA expression systems tried to mimic. However, we have found that if we design our vectors to express a modified pri-miRNA structure based upon miR30, we can achieve approximately 10-fold higher expression of a small RNA in vivo than was achieved with the first generation vector systems (Figure 1). We have therefore used this design in the construction of our second generation library.

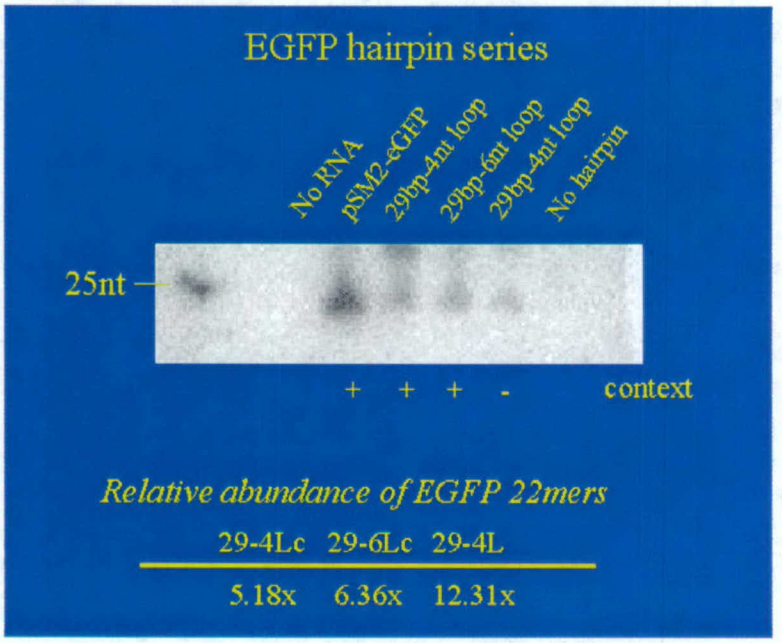

Figure 1. pSM2, the second-generation shRNA vector, is compared to first generation vectors for small RNA production. Specifically, 29bp-4nt loop lane shows the small RNA production from the first generation vector.

shRNA library construction

Using piezo-inject oligonucleotide synthesis, we have constructed second generation shRNA libraries that are now available to the academic community through Open Biosystems. As of this date, more than 500,000 vectors have

been distributed from this source to the academic and commercial research communities.

Loss of function genetics

The original goal of this grant was to use genetic technologies to enable loss-of-function approaches toward finding new anti-cancer targets in mammalian cells. The discovery that RNAi could be used as such a tool has been a tremendous boon. The availability of a large-scale RNAi-based library now makes our original goals possible.

We are taking two approaches toward identifying synthetic lethal interactions. The first is to use very standard approaches, using either morphological markers of cell death or fluorescent reports of caspase activity in vitro to identify lethal interactions. The second is to use highly-parallel approaches based upon the use of barcode arrays to identify such interactions.

One-by-one approaches

We have validated the second generation library in a screen for factors that modulate the activity of the p53 tumor suppressor pathway. To begin, we used a selected set of ~500 shRNAs that mainly target protein kinases. These were transfected into recipient cells individually in combination with reporter constructs that respond to p53 activation and with a construct that permits normalization for transfection efficiency. From this pilot study several kinases whose suppression leads to p53 activation were identified. Notably, when multiple shRNAs to the same gene were present in the collection, all the shRNAs to that gene gave the same phenotype. There were also instances in which we found both kinases and regulatory subunits of the same complex through our screen (figure 2). We are now studying how these kinases connect to the p53 pathway. Additionally, kinase inhibitors that activate p53 may present therapeutic opportunities with fewer side-effects than conventional p53 activators (e.g. DNA damaging agents). Even with the relatively limited screen that we have performed so far, several of the kinases that we have found are being evaluated as drug targets by Oncogene Sciences.

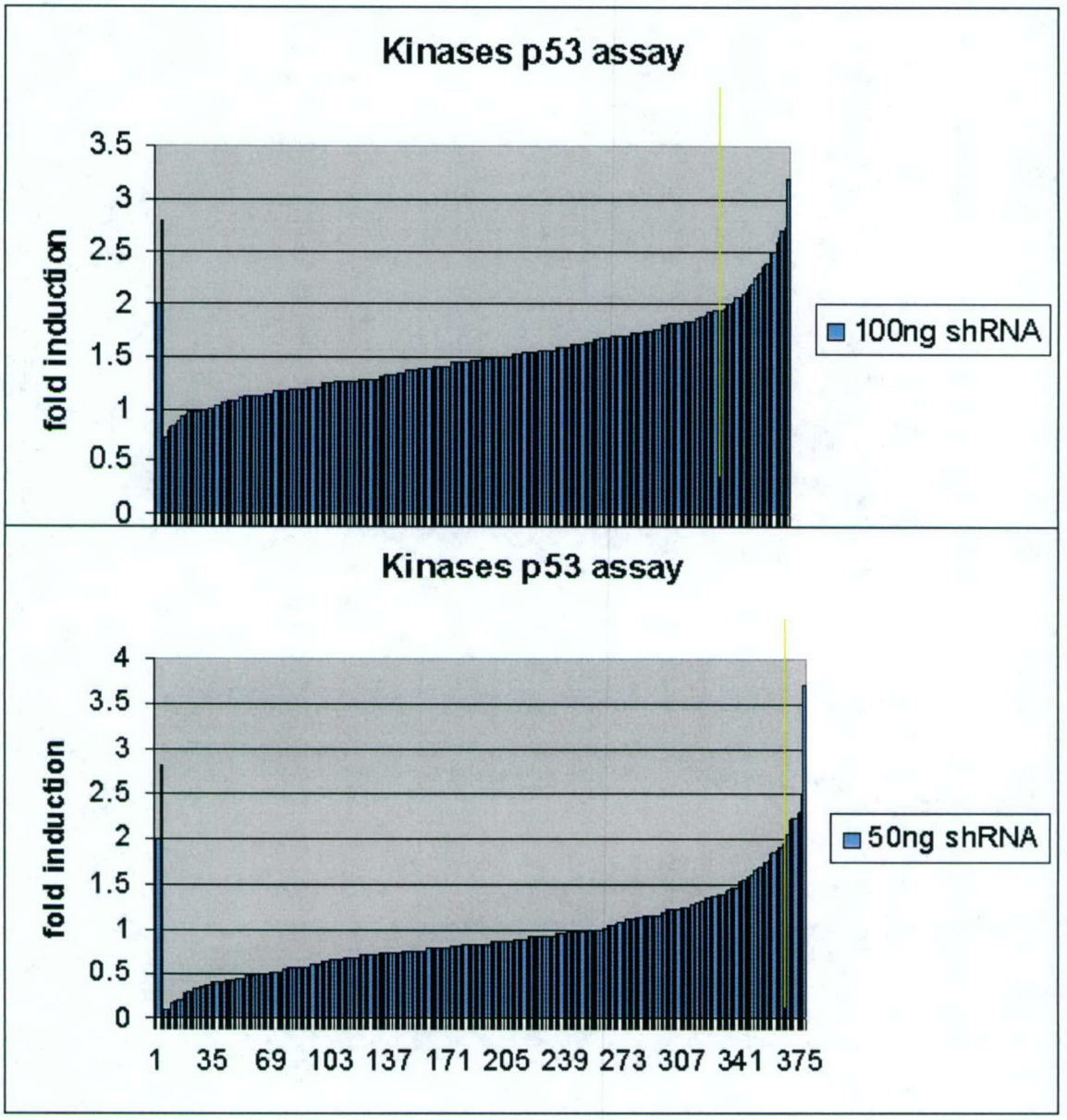

Figure 2. A screen of a partial human kinase collection for inducers of p53. Positive signals to the left of each graph are shRNAs to mdm2 as controls.

Highly parallel approaches

Since individual vectors contain unique barcodes that have been associated with each shRNA by sequence analysis, we can use a population-based approach to identify genes that interact with specific pathways. In short, a population of cells is infected with the mixed library under conditions in which each construct is represented by multiple individual infected cells (~1000 cells/construct). This population is subjected to a stress (genetic, environmental or chemical) while a control population remains untreated. The barcodes

representing individual vectors are then recovered from the populations by PCR. The frequency change in each clone within the population can then be measured by competitive hybridization of the barcode material to DNA microarrays. For those shRNAs that confer resistance to a treatment, we expect enrichment. Conversely, for those barcodes that sensitize a cell to a particular treatment, we expect a reduction in frequency.

The overall approach is depicted schematically in Figure 3.

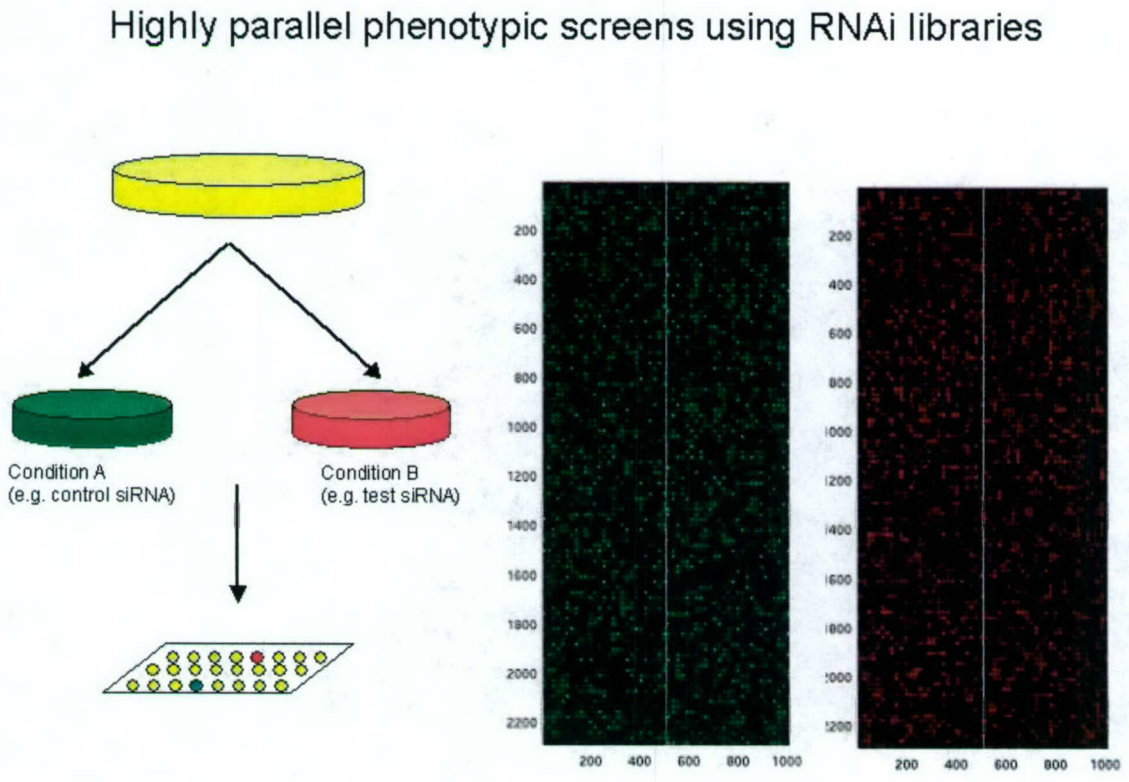

Figure 4 Highly parallel approaches toward synthetic lethal phenotypes.

Last year, we showed that one could assay barcode populations with a complexity of ~20,000 in mixed pools at time 0. However, as we analyzed later time points we saw tremendous amounts of noise. This made the barcode strategy unworkable as it stood. Over the past year, we have examined numerous conditions for stabilizing barcoded populations and have found that using pools of restricted complexity (presently 1000 hairpins per pool) that we can get reproducible results from parallel populations cultured for as much as 10 days. We are now using collections of cells engineered with 1400 kinase shRNAs to test for synthetic interactions between these kinase shRNAs and

targeted therapeutics. Should these assays work, we can proceed to using siRNAs in place of drugs to search for bona fide synthetic lethal interactions.

Key Research Accomplishments

- Preparation of 80,000 shRNA expression vectors from the library
- Validation of the library in a screen for p53 activators
- Identification of 15 shRNAs that reproducibly activate p53 in multiple cell lines.
- Design of viable strategies for using barcodes to screen for phenotypes

Reportable outcomes

None

Conclusions

We are now proceeding to identify targets for combination therapy through searching for shRNA drug interactions in cultured cells. Should this succeed, we can undertake more complicated searches for synthetic lethal interactions.