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14. ABSTRACT The application of RNAi has transformed the way we approach mammalian cell genetics. Over the past year we have made significant progress in several areas that have enhanced the use of shRNAs as a tool for genetic screens. Our second-generation shRNA retroviral library now covers 32,202 genes (86,128 clones) for human and 30,629 genes (76,896 clones) for mouse genomes. These shRNA can be delivered into cells, both in vitro and in vivo, using our optimized viral expression vectors. More importantly, with these tools we have successfully demonstrated the feasibility of performing genetic screens in mouse and human cells through our pilot efforts. Using the 'synthetic-lethal' genetic approach, we can now perform drug-induced synthetic-lethal screens by using cancer drugs that are currently in clinical trials. This is important as cancer cells can often become resistant to the toxic effects of chemotherapeutics. Bortezomib (Velcade) is the first targeted therapeutic to the proteasome approved by the FDA for treatment against multiple myeloma and is currently in phase II clinical trials for breast and lung cancers. Our goal is to identify genes that mediate resistance against Velcade that could serve as potential drug targets. RNAi technology is a powerful tool that could potentially be used to study and treat other human diseases through its application to mammalian cell genetics.					
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Introduction:

RNA interference (RNAi) is a conserved biological process in response to double-stranded RNA (dsRNA)¹. DsRNAs are processed into short interfering RNAs (siRNAs), about 22 nucleotides in length, by the RNase enzyme Dicer. The siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary messenger RNAs. The most well characterized source of endogenous triggers for the RNAi machinery are the microRNA genes^{2,3}. Numerous studies have demonstrated that, in animals, miRNAs are transcribed to generate long primary polyadenylylated RNAs (pri-miRNAs)^{4,5}. Through mechanisms not yet fully understood, the pri-microRNA is recognized and cleaved at a specific site by the nuclear Microprocessor complex⁶⁻¹⁰ to produce a ~70-90 nucleotide microRNA precursor (pre-miRNA) which is exported to the cytoplasm^{11,12}. Only then is the pre-miRNA recognized by Dicer and cleaved to produce a mature microRNA. This probably involves recognition of the 2 nucleotide 3' overhang created by Drosha to focus Dicer cleavage at a single site ~22 nucleotides from the end of the hairpin¹³.

This process can be programmed experimentally in order to repress the expression of any chosen gene. We have constructed shRNA libraries (shRNA-mir) that uses our advanced understanding of miRNA biogenesis. ShRNA-mirs are modeled after endogenous miRNAs, specifically contained in the backbone of the primary miR-30 microRNA¹⁴. We have produced and sequence-verified more than 200,000 shRNAs covering almost all of the predicted genes in the mouse and human genomes¹⁵.

Our shRNA library can function in both individual cell based assays and pooled screens. We have linked a unique 60-nucleotide DNA barcode to each shRNA vector to allow us to follow the fate of shRNAs in populations of virally transduced cells. If, for example, a particular shRNA provided resistance to a growth inhibitor stimulus, then the representation of its associated barcode should be increased after treatment. If a given shRNA sensitized a population to a specific stress, then the relative abundance of its barcode should diminish after the stress. This is measured by hybridizing genomic PCR products containing the barcodes to custom microarrays that contain the complement of these sequences. One can assess cellular response to different treatments by comparing barcode representations of cell populations expressing known shRNA. The development of this highly efficient RNAi library together with the ability to screen pools of genes, provide us with the unique opportunity to investigate the entire genome.

Velcade, the only proteasome targeted therapeutic approved by the FDA, is currently in Phase II clinical trials in breast cancer, though its molecular mechanism is highly disputed. I am examining the genes responsible for granting resistance and susceptibility to Velcade using our complex short hairpin RNAi library that results in the silencing of specific target genes. This technology will illustrate resistance to chemotherapy as a gain of barcode representation and increased susceptibility to chemotherapy as loss of barcode representation in a population of cells.

Body:

Screening methods

Recent breakthroughs in Velcade's mechanism point to possible controls for our screen. Velcade induces Noxa, a specific BH3 only protein, in abundance in multiple cancer types. Velcade differentially upregulates Noxa in cancer cells in comparison to normal cell types and while Noxa is known to be a downstream effector of p53, suprisingly, Velcade's induction occurs regardless of p53 status. Knockdown of Noxa through RNAi decreases response to Velcade in mantle cell lymphoma, melanoma and multiple myeloma. The Soengas lab showed that Velcade induces Noxa robustly in the MDA-MB231 breast cancer cell line, which I am using for my experiments. It has been postulated that different environmental triggers can activate cell death through distinct BH3 only proteins in different cell types, although the orchestrated activity of more than one BH3 only protein is possible. Recently, it has been published that the Noxa/MCL1 axis functions as a rheostat under conditions of glucose deprivation or in pathological circumstances when cells compete for nutrients.

I have confirmed Noxa induction by Velcade in MDAMB231 and H292 lung cancer cells and through Western blot analysis using Noxa antibodies. In addition, I have found 3 different hairpins from our library that each silence Noxa to different degrees. The degree of hairpin knockdown roughly correlates to the amount of Velcade resistance as measured by an MTT assay. I have completed a screen in MDAMB231 cells using a focused collection of library hairpins (7400 hps). The screen was conducted in triplicate utilizing 3x15cm plates per biological replicate. MDA MB231 cells were retrovirally infected with our shRNA library focusing on human kinases, phosphatases, and a set of 1000 genes that been linked to cancer progression, the Harvard Cancer 1000. Kinases and phosphatases are attractive drug targets as their dysfunction can result in a variety of diseases including cancer. In recent years, several

protein kinase inhibitors have performed successfully in clinical studies. They now account for 20–30% of the drug discovery programs of many companies.

After infection, cells with hairpin were selected for using a puromycin resistance marker. These hairpin infected cells were separated into three groups. Two groups received specific drug treatment and the third group was treated with DMSO as a control. Hairpin infected cells were treated at an ineffectual dose 5nM (IC20) where 80% of the cells survive or at a high dose 10nM (IC50). The low dose exposes genes that will increase the tumor's sensitivity to the drug by causing an increase in cell death. Genes that synergize with a suboptimal drug treatment will allow us to make the drug more potent at a lower dose. In addition, since the chemotherapy could potentially be used at lower levels, some of its toxic side effects could be relieved. The high dose of 10nM will expose genes that are resistant to Velcade treatment. Time points were taken where genomic DNA was extracted from a portion of the cells every time the cells reach confluency which was approximately every four to five days. A total of two timepoints (not including T0) were taken after puromycin selection.

Microarray Analysis

Changes in cell growth are monitored through our cutting-edge DNA barcoding technology. Each hairpin is linked to a unique 60 nucleotide sequence, which serves as a barcode, and allows us to virtually count the number of cells that contain a specific hairpin in a cell population. Small changes in barcode copy number can be monitored through the use of microarray technology. Our hybridization quality has improved considerably due to previous experiments that resulted in changing our probe size, labeling methods, probe amounts and hybridization conditions. We now amplify a 350mer segment of genomic DNA including the barcode and hairpin allowing us to use both the barcode and the sense hairpin as probes. In addition, by collaborating with bioinformaticist, Joel Parker, we have been able to identify major sources of variability for our hybridizations including DNA preparation, PCR and a “day effect” caused by day of hybridization. Joel's analysis has helped us refine our microarray hybridizations.

In addition, we are also developing a method for analyzing our results based on a method called SAFE from the Wright lab at UNC. SAFE allows to increase our ability to identify target genes that have been enriched or depleted. Hairpins that show a modest downward shift in p values might not be noticeable when examining a large group of target genes. A single hairpin may not be statistically significant, unless it is taken together with independent estimates (different hairpins) of the same target. To account for this, first a local statistic (simple T test) is taken and then the p values are applied to a larger global statistic (SAFE method) with the goal

of detecting a shift in the local statistic within a gene target to more extreme values. The significance of the global statistics is assessed by repeatedly permuting the values and recomputing the statistic.

My next step is to hybridize the screen using our Agilent eight-plex microarray platform. After hybridization, Loess Normalization will be performed on all the chips to account for intensity dependent differences and principal component analysis will be used to account for variability due to day of hybridization. I will then use the SAFE method and the SAM program to look for genes of statistical significance. Priority will be given to genes that are consistently enriched/depleted over all time points and over both doses. Kinases with known drug inhibitors are given priority as these results could be expanded on in chemotherapy combination trials in the clinic. I am also focusing on candidate genes that illuminate known pathways as this may indicate a gene network that is involved in increasing vulnerability to chemotherapy. In addition, since our library has multiple hairpins per gene, several hairpins that score for the same gene are a good indication of a true positive. I will then validate these targets by on an individual hp basis and characterize biological function of genes that modify sensitivity. Our mouse mammary fat pad model presents the best combination of convenience and biological relevance for testing our validated *in vitro* interactions for their effects *in vivo*.

Key Research Accomplishments:

- A RNAi screen in MDAMB231 breast cancer cells identifying genes that modify sensitivity to the proteasome inhibitor, Velcade.
- Optimization of microarray conditions using Agilent eight-plex platform and development of different microarray analysis methods.

Reportable Outcomes:

Presentations:

Minisymposium Talk:

Siolas, D., Chang K, Silva J, Rollins F, Powers S, Parker J, Hannon GJ. (2007) High throughput RNA interference barcode screens as a tool for discovering gene function. *American Association for Cancer Research Conference Minisymposium Presentation*, Los Angeles, California, USA

Awards:

Siolas, D. (2007) AACR-WICR Brigid G. Leventhal Scholar Award in Cancer Research Award. American Association for Cancer Research, Philadelphia, PA, USA.

Conclusions:

Using our shRNA pool strategy, I performed a genome-wide RNAi in vitro screen in MDAMB231 breast cancer cells seeking genes that modify sensitivity to the proteasome inhibitor, Velcade. This screen was conducted at two different dosages allowing us to detect genes that will enhance sensitivity or increase resistance to Velcade. My next step is to hybridize my tissue culture screen to a barcode microarray. I have been developing our microarray platform and analysis methods to allow us to detect viable candidates. These candidates will then be validated *in vitro* and *in vivo*.

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