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14. ABSTRACT By virtue of their accumulated genetic alterations, tumor cells may acquire vulnerabilities that create opportunities for therapeutic intervention. We have devised a massively parallel strategy for screening short hairpin RNA (shRNA) collections for stable loss-of-function phenotypes. We assayed from 6000 to 20,000 shRNAs simultaneously to identify genes important for the proliferation and survival of five cell lines derived from human mammary tissue. Lethal shRNAs common to these cell lines targeted many known cell-cycle regulatory networks. Cell line-specific sensitivities to suppression of protein complexes and biological pathways also emerged, and these could be validated by RNA interference (RNAi) and pharmacologically. These studies establish a practical platform for genome-scale screening of complex phenotypes in mammalian cells and demonstrate that RNAi can be used to expose genotype-specific sensitivities. We are applying these methods to study the drug Bortezomib (Velcade). 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. We are identifying genes that mediate resistance against Velcade that could serve as potential drug targets.					
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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 polyadenylated 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.

Large-scale screens of small interfering RNA (siRNA) and shRNA collections have generally adopted a one-by-one approach, interrogating phenotypes in a well-based format. This requires both considerable infrastructure and a substantial investment for each cell line to be screened. Alternatively, shRNA collections can be screened by assaying enrichment from pools, but this limits the range of phenotypes that can be addressed. Our focus is identifying essential genes or synthetically lethal genetic interactions through shRNAs that are selectively depleted from populations. This type of screen holds promise for the discovery of novel targets for cancer therapy and genetically validated combination therapies. 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. Previously, one such negative screen was reported; however, this tested only ~500 shRNAs in a single pool ¹⁶. We therefore sought methods that allow multiplex analysis of phenotypic outputs on a genomic scale. In order to test whether such a screen can be done, we conducted a pilot screen identifying essential genes that were selectively depleted from populations using shRNAs in breast cancer cells.

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. We are 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:

We therefore sought methods that allow multiplex analysis of phenotypic outputs on a genomic scale. In order to test whether such a screen can be done, we conducted a pilot screen identifying essential genes that were selectively depleted from populations using shRNAs in breast cancer cells.

Pooled libraries drew from our previous collections wherein shRNAs are carried in a backbone derived from miR-30¹⁷. Combining RNA polymerase II promoters with miR-30-based shRNAs permits efficient suppression even with a single-copy integrant^{18,19}. Target cell populations were infected such that each cell contained, on average, a single integrated virus, and each individual shRNA occupied ~1000 cells. Three parallel infections generated biological replicate samples. Because our goal was to identify essential genes, genomic DNA was prepared from each replicate at three time points during a simple outgrowth assay. Each shRNA cassette contains two unique identifiers: the shRNA itself and a random 60-nucleotide barcode. Barcode sequences were determined for the human shRNA library, and custom, multiplex format microarrays were prepared that contained both barcode and half-hairpin (HH) probes ²⁰. Proviral DNA fragments encompassing both shRNAs and barcodes were amplified from genomic DNA pools and hybridized to arrays in competition with a common reference.

We screened complex populations containing 6,000 (6K), 10,000 (10K) or 20,000 (20K) shRNAs. We began with a pooled analysis of 6000 (6K) shRNAs in MCF-10A and MDA-MB-435. The 10K pool was introduced into MDA-MB-231, T-47D and ZR-75-1 breast cancer cell lines. The most complex pool (20K) was introduced into MCF-10A to allow direct comparison with previous screens of smaller complexity. In all cases, cell numbers were scaled to maintain a representation of 1000 cells per shRNA. The quality of each screen was similar, with high correlations between biological replicates. We assessed the consistency of the MCF-10A screens by comparing depleted gene sets for the 20K pools. FDR thresholds were the same for both data sets ($q < 0.1$), but the fold-change criterion was relaxed from 2-fold to 1.5-fold for the 20K screen so that similar numbers of candidates were compared. A set of 172 genes ($P = 1.123 \times 10^{-9}$) overlapped in both data sets, despite some differences in the protocols used to carry out each screen. This suggests that a pool of ~20K shRNAs can be effectively screened.

We established a rigorous data analysis pipeline for analyzing pooled shRNA screens. Correlations between biological replicates were high but diminished at later time points, whereas correlations between the reference channels remained unchanged. Overall, a gene was scored as a candidate if either its barcode or shRNA probe showed greater than 2-fold change with a false discovery rate (FDR) <10%.

Viewing this portrait of shRNA sensitivity in more detail revealed a number of pathways and complexes that were differentially required in MCF-10A. These included epidermal growth factor receptor (EGFR), an effect that could be reproduced pharmacologically using the EGFR inhibitor Tarceva²¹. DNA methyltransferases also scored either above or close to the threshold. In accord with these results, MCF-10A cells showed a more than 50-fold greater sensitivity to 5-aza-deoxycytidine, a methyltransferase suicide substrate²², than the other cell lines. As a final example, numerous proteasome subunits were preferentially depleted from MCF-10A. These cells showed the greatest sensitivity to a proteasome inhibitor, MG-132²³. Interestingly, MDA-MB-435 showed an intermediate level of sensitivity to the drug, and this was reflected precisely in their intermediate level of depletion of proteasomal shRNAs during the screen.

We have validated a highly scalable approach for screening shRNA libraries. Although we used a phenotypic filter reflecting growth and survival, virtually any characteristic that allows separation of phenotypically distinct cells can be applied. We also validated the ability of functional shRNA screening to separate cell lines based on their genetic vulnerabilities in a manner that reflects their already defined characteristics (e.g., immortal versus tumor, basal

versus luminal). Although one could attribute selective dependency to culture conditions in some cases, the overwhelming concordance of the shRNAs that affect proliferation and survival across these lines, many of which are cultured identically, strongly argues against this being a pervasive explanation. In all, this approach enables genome-wide screens for tumor-specific vulnerabilities to be carried out on large numbers of tumor lines. Moreover, it permits rational searches for lesions that synergize with existing therapeutics to produce a path toward genetically informed combination therapies. This pilot screen has allowed us to develop the tools necessary to conduct large-scale negative selection screens using a shRNA library with up to 20,000 hairpins. In addition, we have developed a highly microarray platform with the accompanying statistical methods for analysis. This microarray platform and statistical analysis is currently being applied to our Velcade screen that was conducted in MDA-MB-231 breast cancer cells.

Key Research Accomplishments:

- A RNAi screen identifying genes that are important for the proliferation and survival of five cell lines derived from human mammary tissue.
- These studies establish a practical platform for genome-scale screening of complex phenotypes in mammalian cells and demonstrate that RNAi can be used to expose genotype-specific sensitivities.

Reportable Outcomes:

Publications:

Silva JM, Marran K, Parker JS, Silva J, Golding M, Schlabach MR, Elledge SJ, Hannon GJ, Chang K. Profiling essential genes in human mammary cells by multiplex RNAi screening. *Science*. 2008 Feb 1;319(5863):617-20.

Conclusions:

We have validated a highly scalable approach for screening shRNA libraries in breast cancer cells. We can conduct screens with up to 20,000 hps and identify depleted genes from a complex population. Our pilot screen identified genes that are important for the proliferation and survival of five cell lines derived from human mammary tissue. We will use these microarray and statistical tools for to study genes that modify sensitivity to the proteasome inhibitor, Velcade. This screen was conducted in MDAMB231 breast cancer cells at two different dosages allowing us to detect genes that will enhance sensitivity or increase resistance to Velcade. We have developed 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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