

Application Forum

Identification of Genes Specific for Viability of Prostate Cancer Cells using a Pooled Lentiviral shRNA Expression Library

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Introduction

As an alternative to arrayed RNAi screens that require significant resources and infrastructure, we pursued a screening approach for functional genes using standard cell culture techniques with a complex pooled RNAi expression library. We applied this RNAi screening approach to identify genes specifically essential to cells from prostate cancer adenocarcinoma (PCA), one of the most frequently diagnosed malignancies. The pathologies of PCA cells that express androgen-receptor (AR+) and those that do not (AR-) differ so both PCA sub-types were included in the screening.

Methods

Construction of shRNA Libraries

A short hairpin RNA (shRNA) expression library targeting 1,200 human genes known to be expressed in prostate cells was made by synthesizing 7,000 oligonucleotides on the surface of a custom-designed microarray (Agilent Technologies, Santa Clara, CA). Each oligonucleotide was designed to express an shRNA molecule, a 25-nt sense sequence (based on the target gene), and an 18-nt unique sequence identifier ("bar-code"). The bar-codes, specific for each shRNA sequence, were used to uniquely identify the hairpin sequences from high throughput (HT) sequencing data. Synthesized oligonucleotides were amplified, cloned into the Cellecta pRSI-H1Tet-CMV-TetRep-2A-Bleo lentiviral vector, and packaged into VSV-g pseudotyped viral particles. The quality of the library was assessed by HT sequencing (Figure 1).

Library Transduction

The viral packaged 7K shRNA Prostate library was transduced at an MOI of 0.35 into 2 million cells of each of five PCA cells lines (LNCaP, C4-2, CWR22R, DU145, and PC3) and 3 non-prostate cancer lines (HeLa, HEK293, and Hep3B) in 6 independent replicates. Each transduction produced approximately 700,000 transduced cells/replicate (i.e., an average of 100 transduced cells for each shRNA). 24 hours after transduction, 3 replicates of each cell line

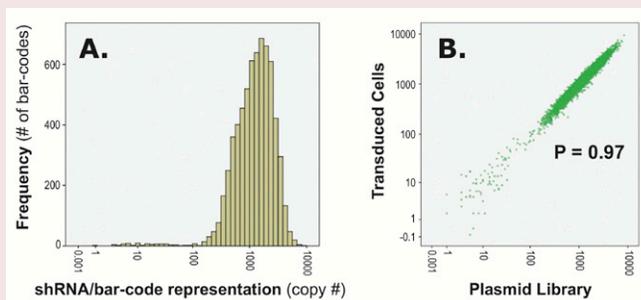


Figure 1. Sequence distribution of shRNA library in plasmids and transduced cells. Analysis of 20 million HT sequencing reads of shRNA-specific bar-codes from the Prostate Library plasmid DNA demonstrated more than 95% of the cloned sequences appeared within a 100-fold distribution range (panel A). The shRNA distribution in cells harvested 24 hours after transduction of the packaged library is almost equivalent to the original plasmid library (panel B).

were harvested as untreated negative-control samples and the remaining replicates were selected with bleomycin and treated with doxycycline (0.1 $\mu\text{g/ml}$) to induce shRNA expression. After an additional 7 population doublings, induced cells were harvested, genomic DNA was isolated, and the bar-codes uniquely identifying each shRNA sequence were PCR-amplified and sequenced.

Determination of shRNA counts in Screened Samples

After the pool of bar-codes was amplified with vector-specific and HTS2/Gex1 primers from total genomic DNA isolated from each sample of cells, it was subjected to a 20 million read sequencing run on the GAllx analyzer (Illumina, San Diego, CA). Deconvolution and normalization produced the number of reads for each bar-code and the determination of the fractional representation of each shRNA sequence in the sample. Comparison of the levels of each shRNA with the corresponding levels in the plasmid library and non-treated controls enabled identification of the shRNAs significantly depleted in the samples, thereby indicating those that negatively affect the growth and proliferation of the cells presumably because they target essential genes.

Assessment of Individual shRNAs on Cell Viability

For each selected gene, we synthesized and cloned two shRNAs into the same tetracycline-inducible expression vector used to construct the library. Each shRNA expression

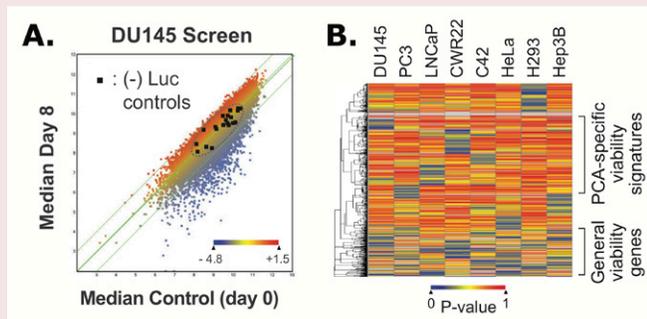


Figure 2. Screening results of library expressing 7,000 shRNA targeting 1,200 prostate-specific genes. Panel A plots the number of reads (out of 20 million) of each shRNA-specific bar-code present in DU145 cells after 8 days of growth (average counts over triplicate samples) compared with the corresponding number of bar-codes read 24h after transduction of these cells. The shRNA hairpins that negatively affect cell growth decrease in the population over time. Thus, the number of bar-codes specific to these shRNA is lower at day 8 than they are just after transduction, and they appear significantly below the diagonal. Panel B is a “heat map” that depicts significance of depletion levels for each shRNA in each of 8 cell lines screened. Red or orange indicates no significant change in the shRNAs targeting the gene over time, whereas blue indicates genes with the most significantly depleted shRNAs.

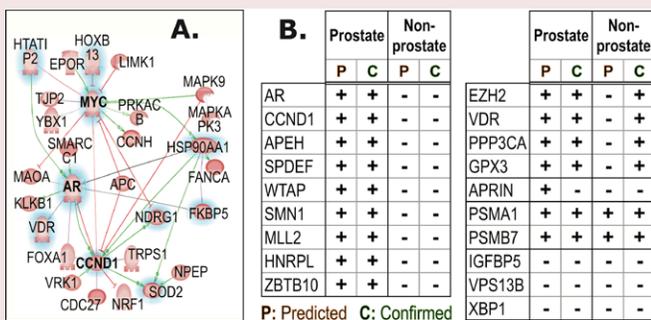


Figure 3. Pathway analysis and confirmation of hits. Panel A shows a network model based on screen results. Predicted PCA-specific viability network model based on genes targeted by shRNAs significantly depleted in PCA cells (i.e., genes designated by blue bands in Figure 2B). Genes known to be associated with PCA are highlighted with blue background. Panel B shows the confirmation of screening results with individual shRNA expression constructs targeting genes significantly depleted in the screen. Two shRNAs targeting each selected gene were synthesized, cloned, and transduced into all cell lines. The table compares the anticipated effect of the shRNAs on the cells based on the screening data (predicted) with the effect of the shRNAs.

construct was transduced into each of the 8 cell lines. After 48 hours, cultures were split and both treated with bleomycin to select for transduced cells, but only one treated with doxycycline to induce expression of the shRNA. Cells were grown for an additional 7 days and then counted to evaluate shRNA cytotoxicity.

Results and Discussion

Cytotoxic shRNA sequences in the induced populations were identified by their depletion in the doxycycline-treated samples vs. control “no-dox” samples and plasmid library. Statistical analysis of the screening results revealed about 184 genes with significantly depleted shRNA in at least 3 PCA lines but not the control, non-prostate cells (Figure

2). Interaction analysis of the 184 genes that putatively appeared non-essential for most cells but essential for viability in prostate cancer cells revealed several defined clusters with the largest in the androgen receptor pathway (Figure 3A).

To confirm the lethality profiles of selected shRNA with single shRNA expression constructs, we selected 14 genes whose lethality appeared specific to PCA cells, as well as 2 genes whose shRNAs appeared depleted in all cell lines and 3 that did not appear essential in any cells. We tested two shRNA for each of these genes for their effects on viability in all cell lines. Of the 14 targets that appeared specifically essential to PCA cells in the screen, shRNA targeting 9 of these (64%) were lethal only for PCA cells; individual shRNA to 4 of the targets (29%) were also somewhat lethal to non-PCA cells, and shRNA to one target (7%) was not significantly lethal to any of the cells (Figure 3B). The results confirmed the general specificity of the screening.

Due to the relatively small number of genes targeted with this library, only a few lethality differences between AR+ and AR- prostate cancers were observed. Of particular interest was the differential lethality of CCND1, which appeared selectively essential to the AR+ subset of PCA lines, regardless of RB gene status. In an attempt to identify more detailed genetic differences in AR+ vs. AR- prostate cells, we are adapting this study to a genome-wide screen using a series of pooled shRNA expression libraries, where each library targets 5,000 non overlapping genes (the DECIPHER Libraries; www.decipherproject.net).

The results demonstrate the utility of RNAi screening using complex pooled shRNA expression libraries to obtain, in a single screen, comprehensive and informative data with large numbers of target genes. This type of approach provides a better overall understanding of the lethality profile and the genetic controls regulating proliferation in a single screen than does discrete gene-by-gene RNAi analyses that often identify small sets of disparate genes. However, the effectiveness of this type of screen relies heavily on the use of a well-characterized library with good representation and optimized hairpin design, and a screening procedure that maintains shRNA representation throughout the screen.

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