

Pooled RNAi screens in xenograft mouse models



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Abstract

We present results of clonal analysis studies aimed at characterizing the heterogeneity of in vivo cancer cell growth in xenograft models. Using a high-throughput sequencing method, we followed the fate of thousands of individually bar-coded cancer cells grown in vitro, or in vivo upon sub-cutaneous implantation in immunocompromised mice. By precisely and reproducibly quantifying clonal cancer cell growth in vivo, we found that xenograft growth is marked by a phenomenon of clonal dominance, in which a small subset of cancer cell sub-clones account for the bulk of the resulting tumor mass. This phenomenon has important implications for the field of in vivo shRNA screening. Subsequently, we developed a new proprietary clonal RNAi screening platform specifically designed to circumscribe the clonal dominance effect in xenograft model viability screens. We used this technology to perform xenograft "drop-out" screens in a panel of tumorigenic cell lines, including human breast, ovarian and colon carcinomas. Preliminary data will be presented. These results demonstrate that complex pooled shRNA libraries provide a highly efficient, flexible, and unique tool for in vivo screens aimed the discovery of potential cancer therapy targets.

shRNA Expr. Library Construction & Screening

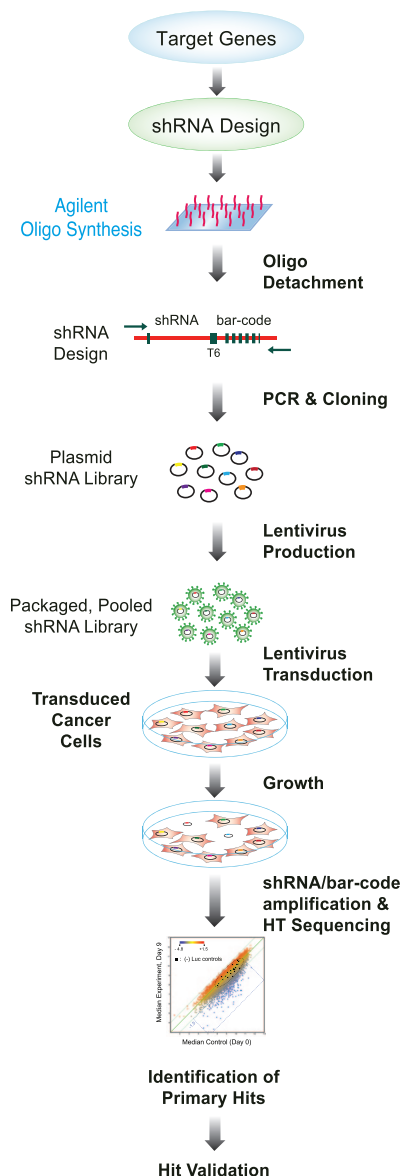


Figure 1. A library of 27,000 shRNA expression constructs targeting 5,000 human genes (DECIPHER 27K shRNA Library) was constructed by cloning pooled oligonucleotide inserts (Agilent) into a lentiviral vector under the control of the U6 promoter. Each shRNA contains a unique 22-base sequence (i.e., barcode) downstream of the hairpin termination site.

The complex plasmid library was packaged into VSV-g pseudoviral particles by co-transfecting into HEK 293 cells with plasmids expressing the lentiviral packaging proteins.

In a standard RNAi screening "drop-out" screen to identify genes essential for cell viability, 5 million or more cells are transduced with the pooled library, then culture for 6-10 doublings. After which, the relative number of each barcode in the remaining population of cells is determined by high throughput sequencing of the barcodes present in their genomic DNA.

Pooled shRNA Library with Clonal Tracking Barcodes

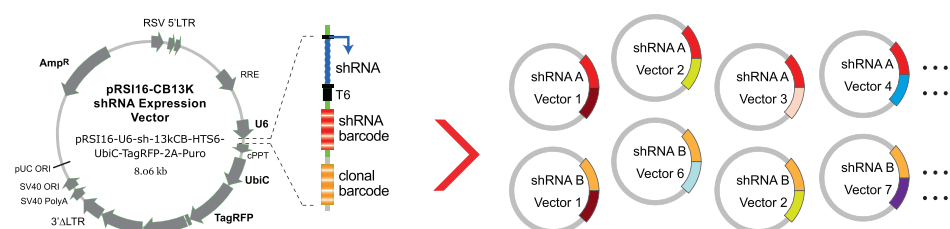


Figure 2. To enable clonal tracking using the pooled shRNA library, an additional vector barcode is introduced just downstream of the shRNA-associated barcode. This was done by first creating a library of barcoded vectors using 13,000 barcodes. 27,000 defined shRNA inserts (as shown in Fig 1) were then cloned into this vector library.

Clonal Growth Profile of Tumor Xenografts

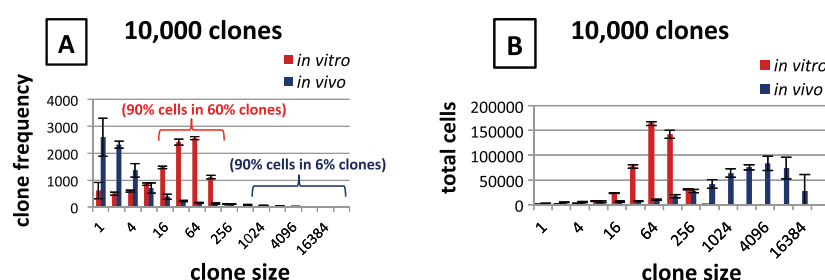


Figure 3. HTC 116 cells were transduced with a library of lentiviral constructs containing 27,000 barcodes (no shRNA). Immediately after transduction, 10,000 barcoded cell samples were injected into NSG mice, and additional 10,000 cell samples were cultured in vitro. After two weeks, genomic DNA was isolated from each of the populations and the barcodes were sequenced. **Figure A** shows the relative abundance of individual clones (i.e., unique barcodes) as a function of clone size, and **Figure B** shows the aggregate number of cells as a function of clone size. In vitro, the majority of cells in vitro doubled 5-6 times. In vivo, about 90% didn't grow at all or doubled just a few times, and 90% of the total tumor came from just 6% of the initial cells.

Clone size distribution of typical Toxic and non-Toxic shRNAs

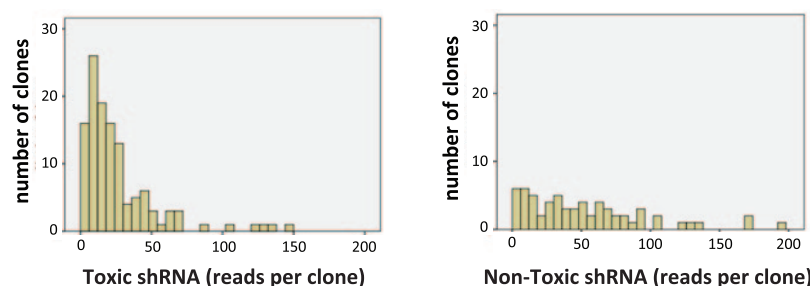


Figure 4. Observed clone size distribution for one toxic and one non-toxic control shRNA from an in vitro RNAi screen using clonal barcoded pooled shRNA library. Differences in the distribution of growth between the ca. 75 cells with the toxic shRNA (larger fraction of clones with poor growth) vs. those with the non-toxic shRNA (clones with a wider distribution of growth) are evident.

In Vivo (Xenograft) Clone Size Distribution of Toxic vs. Non-Toxic shRNAs

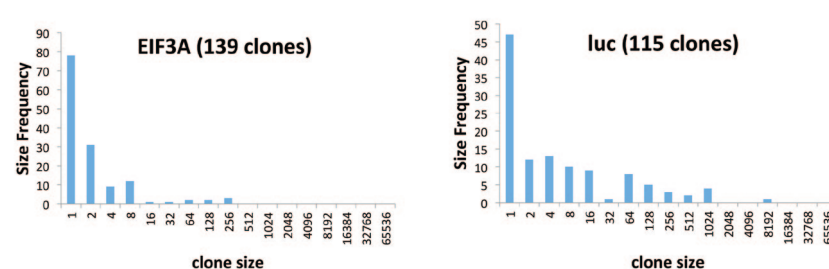


Figure 5. Observed clone size distribution for one toxic and one non-toxic control shRNA from an in vivo RNAi screen using clonal barcoded pooled shRNA library. As shown in Fig 3, a larger fraction of the clones did not grow in vivo. However, as above with the in vitro shRNA screen (Fig 4), differences in the distribution of growth between the ca. 100 cells with the toxic shRNA (EIF3a) vs. those with the non-toxic shRNA (luc) are evident.

Cell Growth Results from an In Vivo RNAi Screen

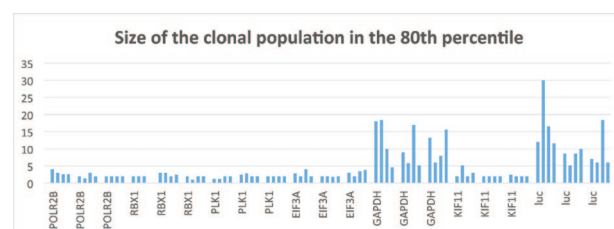


Figure 6. The above graph shows the number of cells in the clonal population found at the 80th percentile in size for each of the shRNA indicated. Shown are results from 4 replicates of 3 different shRNA targeting each gene from an in vivo RNAi screen. Since a majority of the clones do not grow in the tumor, inspection of the size of the clonal populations above the median percentile better indicate how a specific shRNA influences affects the growth of cells in which it is expressed.

Conclusion

By incorporating a second vector barcode in addition an shRNA-specific barcode, we can produce a complex pooled shRNA library that allows us to assess the fate of the hundreds of independent clonal populations produced by each cell transduced at the start of an RNAi screening. Thus, the shRNA-induced phenotype can be assessed independently for each clone derived from each transduced cell for every shRNA in the pool.

Clonal assessment is a departure from the traditional method of measuring the overall effect on the total population of cells with a given shRNA. In essence, the standard approach averages the effect of the shRNA across all clones in the population which results in an inherent bias toward the largest clones. With clonal barcodes, it is possible to assess how each cell that received a particular shRNA responds. This level of resolution enables better discernment of the shRNA's versus the environment's effect on growth.

Finally, in viability screens with clonal tracking, no "zero point" is necessary since all the clones start as a single cell. As a consequence, shRNA library complexity does not need to be maintained. It is the distribution of clone sizes that is used to assess shRNA toxicity, not total number of clones or cells.