Genome-scale loss-of-function screening with a lentiviral RNAi library

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The discovery that RNA interference (RNAi) is functional in mammalian cells led us to form The RNAi Consortium (TRC) with the goal of enabling large-scale loss-of-function screens through the development of genome-scale RNAi libraries and methodologies for their use. These resources form the basis of a loss-of-function screening platform created at the Broad Institute. Our human and mouse libraries currently contain >135,000 lentiviral clones targeting 27,000 genes. Initial screening efforts have demonstrated that these libraries and methods are practical and powerful tools for high-throughput lentivirus RNAi screens.

Genome sequencing efforts have transformed the nature of biological inquiry and have led to an increased need for tools that enable functional studies on the genome scale. In model organisms, genome-scale loss-of-function genetic approaches have revealed rich, often unappreciated insights into many biological processes. Properly applied, these approaches can lead to a systems-level understanding of a process as well as the comprehensive identification of the molecular components underlying it. Until recently we have lacked the methods and tools to apply similar genome-scale loss-of-function approaches to mammalian biology. The discovery that RNAi operates in mammalian cells[1] provides an unprecedented opportunity to undertake large-scale loss of function screens in mammalian systems[2–4].

RNAi regulates gene expression through sequence-specific targeting of mRNA, making possible the production of large-scale libraries directed toward entire genomes. Versions of both oligonucleotide- and vector-based libraries now exist, and several groups including our own have reported success in using these tools in genetic screens[5–12]. Despite these encouraging results, it is clear that many challenges remain before such libraries can be considered comprehensive and before the use of such libraries is routine. The ideal RNAi library would permit any skilled scientist to assess specifically and reproducibly the effect of suppressing a gene in any experimental model.

Over the past several years, we have developed a technological platform, based at the Broad Institute, to create genome-scale RNAi libraries and methodologies for their use. Our aim is to produce genome-wide mammalian RNAi libraries that achieve stable and specific gene knockdown in a wide variety of cell types, and that are practical for routine use in a high-throughput screening context. This goal requires ongoing systematic assessment of library performance and the development of reliable and cost-effective procedures for library application. In particular, we have focused on developing an RNAi library that reproducibly suppresses gene expression in a wide range of dividing and nondividing cells, is stable during propagation in a bacterial host, targets each gene at multiple sites within its sequence, and is suitable for both arrayed and pooled screening applications.

The TRC library

Presently, the only high-efficiency delivery method that enables stable long-term gene suppression in a broad range of cell types is virus-mediated integration of an RNAi expression cassette. After integration, the cassette produces a short dsRNA molecule, usually in the form of

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a hairpin structure, short hairpin RNA (shRNA), which is processed into active small interfering RNA (siRNA). Although many types of viruses are suitable for this purpose, lentiviral vectors generate viruses of both high titer and broad tropism, permitting the infection of both dividing and nondividing cells.

shRNA expression cassette and vector backbone. Because arrayed genome-scale loss-of-function screens require the capability to produce tens of thousands of high-titer viral samples, we focused on optimizing our delivery vector to reproducibly generate high-titer lentiviral stocks when pseudotyped with VSV-G envelope protein. The use of several convenient features such as site-specific recombination sequences was tested, but they consistently lowered the viral titer and therefore were not included. We selected a relatively simple library vector design, pLKO.1, to maximize viral titers and resistance to plasmid recombination. The pLKO.1 lentiviral vector is a derivative of pRRLSIN.cPPT.PGK/GFP/WPRE, and is a third-generation self-inactivating lentiviral vector[13–15]. We introduced a human U6 promoter to drive the expression of shRNAs, and the vector contains a PGK promoter driving expression of the puromycin resistance gene to allow selection of transduced cells (**Fig. 1**). Like other third-generation lentiviral vectors, viruses are generated using a three-plasmid packaging system that separates the *gag*, *pol* and *rev*, and the gene encoding the VSV-G coat onto separate vectors to minimize the potential for recombination to create replication competent viruses. Several other modifications in the construction of pLKO.1 include the substitution of *PAC* (puromycin resistance gene) for *EGFP* and *WPRE* (woodchuck post-transcriptional regulatory element) and the addition of a slightly longer cPPT (central polypurine tract) sequence to increase the efficacy of this vector.

The potential for plasmid recombination in bacteria, possibly owing to repetitive sequences in the long terminal repeats (LTRs), is a serious potential drawback of retroviral libraries. As the widespread use of any RNAi library requires considerable amplification and replication, we empirically tested the pLKO.1 vector extensively to ensure that the rate of recombination per clone per growth from bacterial glycerol stocks was below 0.05% (ref. 8).

An alternative RNAi vector design involves placing the shRNA transcript in the context of an endogenous miRNA-precursor sequence[16]. This approach has been incorporated into the most recent version of the Hannon-Elledge library and the newer library exhibited notably improved knockdown compared to the previous version of that library[17]. It will thus be important to determine whether improved knockdown is due to the miRNA context, or whether other shRNA design and vector attributes are more important for enhanced gene suppression.

Selection of shRNA sequences. As of July 12, 2006, the shRNA library of the TRC contained over 135,000 clones targeting 14,500 human and 12,700 mouse genes, and is expanding at the rate of approximately 5,000 clones per month. The genes targeted by the present library encompass most known gene classes including the human and mouse druggable genomes. Each gene is targeted by an average of five distinct shRNA constructs and at least four shRNAs are available for over 96% of targeted genes. The hairpin sequences contain stems of 21 nucleotides that exactly match the target transcript, and are selected using an algorithm designed to increase likelihood of good target gene knockdown, to avoid off-target effects and to disperse the 21-mer sequences across the target transcript. For example, the algorithm strongly favors

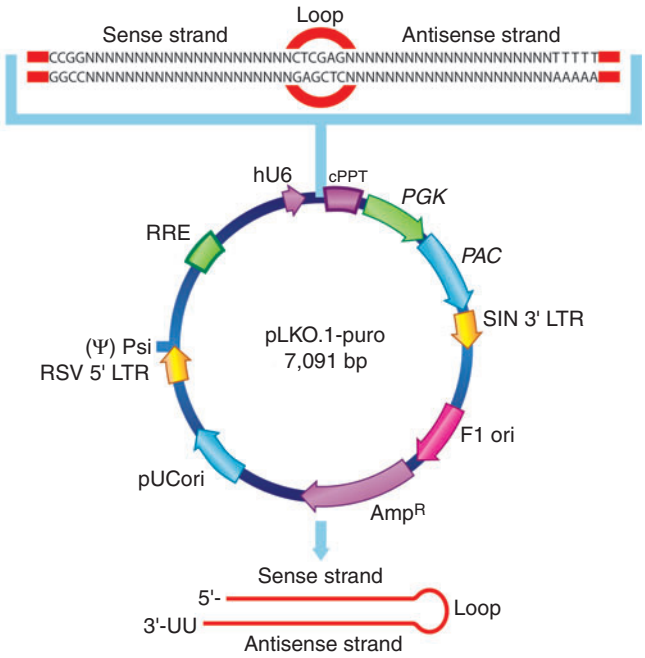

Figure 1 | Vector map for the pLKO.1 lentiviral vector. The self-inactivating lentiviral vector backbone contains elements for efficient viral packaging and shRNA expression. These include Ψ, the lentiviral packaging site; RRE, the Rev-responsive element; cPPT. RSV/ 5′ LTR is a hybrid of the Rous Sarcoma virus promoter and the HIV 5′ LTR. SIN 3′ LTR is the HIV 3′ LTR with a self-inactivating U3 deletion. Expression of the shRNA is driven by the human U6 promoter (hU6). The lentiviral vector also contains the mammalian selection marker puromycin resistance gene (*PAC*) under the control of the *PGK* promoter as a mammalian selection marker. pUCori is the bacterial origin of replication of the plasmid, F1 ori is the single-stranded phage F1 origin of replication, and Amp^R is the ampicillin resistance gene.

21-mer stems that have 30–50% G+C content, with higher G+C content for tighter pairing at the 5′ end and lower G+C content at the 3′ end of the antisense strand[18,19]. The algorithm used to select hairpin sequences is described in more detail online (http://www.broad.mit.edu/genome_bio/trc/rnai.html).

Library construction. TRC library clones are produced in 96-well plates using 60-mer oligonucleotides. Sense and anti-sense hairpin oligonucleotides are annealed, ligated into pLKO.1 at *Age*I and *Eco*RI restriction sites, and transformed into competent bacteria in 96-well plates. The 90 different transformants from each plate are pooled, and 672 colonies are picked for sequence verification. This process yields 94% of the designed shRNA clones. We also use an alternate method of producing library clones through the use of oligonucleotides synthesized on a chip, which are liberated to make an oligonucleotide pool that is then amplified and cloned into pLKO.1. Although this method produces far fewer than 94% of the designed shRNA clones, the reduced oligonucleotide costs result in a lower total cost per clone. We perform several in-line quality control checks including resequencing of the clones selected for the final library and tests for cross-contamination and external contamination of the bacterial stocks. The TRC library is currently distributed by Sigma-Aldrich and Open Biosystems.

Preparation of the viral library

The utility of a viral RNAi library for arrayed screening depends

heavily on the ease with which virus particles can be generated for many thousands of distinct shRNA constructs. Although preparation of transfection-quality plasmid DNA and subsequent packaging of the viral plasmids into viruses is quite straightforward for individual samples, performing this process efficiently in a high-throughput 96-well format is more challenging; furthermore, high-throughput screening demands high and uniform viral titers. We have developed protocols for DNA and viral production for the TRC library. These protocols are frequently updated with improvements and the latest versions can be found online (http://www.broad.mit.edu/genome_bio/trc/protocols.html).

With our current methods of high-throughput viral production in 96-well plates, we obtain viral titers that average approximately 3×10^7 infectious units (I.U.)/ml in A549 cells infected in 96-well plates. In a typical plate, 50% of wells fall within a twofold range of viral titer. With these high average titers, most common cell lines require ≤1 μl of virus per well in a 384-well plate to achieve successful infection rates (for example, ≥25% of cells survive selection in ≥80% of wells; see below). The best viral dose varies depending on the cell line and assay so the dose should be optimized for each screen. Because each 96-well viral preparation yields 300 μl of viral supernatant, a single viral preparation is sufficient to provide virus for several hundred shRNA infections, depending on the transducibility of the target cells.

One consequence of using lentiviral vectors with high titer and broad tropism is the requirement for adequate biosafety procedures for the use of such reagents. The three plasmid–based lentivirus production of self-inactivating viruses nearly eliminates the possibility of recombination to create replication-competent viruses, and the rate of recombination is several orders of magnitude lower than that of comparable amphotropic retroviral systems. Many institutions apply biosafety level 2 practices to work with third-generation lentiviruses. We use biosafety level 2+ precautions for all lentiviral shRNA library work with appropriate containment and decontamination procedures. An example of institutional guidelines for lentiviral work is available online (http://www.ohsu.edu/research/rda/ibc/protocols.shtml).

Library knockdown performance

We have evaluated the performance of a test set of library viruses under screening conditions using untitered virus produced in 96-well plates. Because high-throughput production generates virus stocks of variable titer, this test is expected to underestimate the intrinsic ability of these shRNA constructs to suppress the expression of their target genes. Nonetheless this is a useful test of the library performance under authentic high-throughput screening conditions. We used quantitative reverse-transcriptase PCR (RT-PCR) to systematically test knockdown performance of 256 hairpin sequences targeting 53 genes, and found that 38% of these constructs achieved 70% or better knockdown of the target gene[8] (TRC; unpublished data).

We also tested shRNA-expressing viruses for two genes, *FASTK* and *AKT3*, whose knockdown led to cell death in HT29 cells, using both high and low viral doses. Cells containing a single integrated vector showed nearly the same levels of gene suppression and cell death as did cells harboring multiple integrations, indicating that the level of shRNA expressed from a single integration event can effectively cause gene knockdown[8]. These observations demonstrate that a single integration can be adequate for RNAi-mediated silencing. A pilot study using 4,000 pooled viruses indicated that the frequency with which single integrations achieve useful levels of knockdown is sufficient to support productive pooled screening in our library, as has been the case for other libraries. It would be useful to determine the distribution of knockdown efficiencies of single integrations and its implications for the rate of false negatives in pooled screens.

One of the advantages of arrayed viral screening is that the multiplicity of infection can be increased for greater knockdown. When we titrated shRNAs targeting a *GFP* reporter, we found that increased viral dosage induced more effective gene suppression for some, but not all, shRNAs (TRC; unpublished data).

Applications of the TRC library

We and others have now used TRC library constructs to suppress the expression of many genes in a wide range of human and mouse cells. Additionally, we have confirmed that the ability of this vector to infect primary mammalian cells as well as nondividing cells[8]. In many cases, the availability of multiple shRNAs targeting the same gene has greatly facilitated these studies and diminished the possibility that the observed effects were due to off-target effects of RNAi.

The TRC library is suitable for either pooled or arrayed virus screens but our initial efforts have focused on screening in an arrayed format. Array screens have important advantages that make this capability invaluable. In particular, arrayed screens provide greater sensitivity to discern partial or subtle phenotypes with concomitant reduction in false negatives, and they allow complex, information-rich assays such as those involving cell-cell interactions and high-content imaging.

To test the performance of this library in an arrayed screen, we screened for regulators of mitotic progression in human colon cancer cells (HT29) using a subset of the TRC library targeting 1,000 human genes, primarily kinases and phosphatases[8]. The assay used high-throughput fluorescence microscopy to determine the percentage of cells in each well exhibiting phosphorylation of histone H3 at serine 10, a commonly used marker for mitotic cells. Cells were infected with 5,000 different TRC library viruses in separate wells of 384-well plates. The infections were performed in duplicate; one replicate was treated with puromycin to select for infected cells and the other was left untreated. Only those viruses that conferred at least 25% survival after selection compared to that in the untreated replicate well were considered to represent successful infections. The 80% of high-throughput–generated library viruses that yielded successful infections were used in the subsequent analysis of mitotic index (percent of cells with phospho-H3 staining). Candidate 'hit' genes were those for which two or more distinct shRNAs altered the mitotic index. Additional fluorescent channels were used to visualize DNA and actin using Hoechst and phalloidin staining, respectively. DNA content histograms extracted from the images were used to confirm the distribution of cells in the cell cycle. This screen identified several known regulators of mitosis and ~100 new candidate genes.

Confirming the association between the intended target gene and the observed phenotype is one of the main challenges for RNAi screening. 'Off-target effects', in which genes other than the intended target gene are suppressed, may be present to some extent for any siRNA sequence[20,21]. One can accomplish successful RNAi screens in the background of off-target effects, but putative hit genes nonetheless require substantial follow-up confirmation. We screen 4–5 different shRNA sequences for each gene to increase the confidence of identifying true, specific hit genes in primary screens as distinct hairpin sequences are very unlikely to have similar spectra of off-target effects. In the screen for regulators of mitosis, we required at least two distinct shRNA sequences to cause the mitotic index–altered phenotype[8].

BOX 1 TRC LIBRARY AT A GLANCE

Library vector

Vector description. Library vector: pLKO.1 (see **Fig. 1**; refs. 8,13–15).
Viral vector backbone: self-inactivating lentiviral vector.
Plasmid derivative of pRRLSIN.cPPT.PGK/GFP.WPRE.
Major modifications: (i) substitution of puromycin selectable marker (*PAC*) for *EGFP*, (ii) deletion of WPRE.
Additional features: shRNA expression cassette under the control of hU6 RNA polymerase II promoter.

Library stability. pLKO.1 has not exhibited vector recombination under the conditions used for duplication and regrowth of library bacterial stocks.
We performed 10 sequential rounds of copying and regrowth of 244 clones followed by restriction digests, no recombined clones were found out of the 244 tested.

Infectivity. Lentiviral vectors transduce a broad range of cell types including nondividing cells.
Supplementary data from reference 8 provides a partial list (16 cell lines and 10 primary cell types) that have been successfully infected by pLKO.1.

Silencing. Lentiviral vectors including pRRL have been shown to maintain stable transgene expression for weeks or months. Long-term shRNA-mediated knockdown has been observed in a number of cell lines but is not uniformly achieved for all shRNAs (refs. 22,23, and TRC, unpublished data). For example, THP1, >6 weeks; HT29, >6 weeks; Jurkat, >6 weeks; K562, >6 weeks; human fibroblasts, >3 months; human kidney epithelial cells, >3 months.

Library production

Vector DNA production yield. Transfection-quality DNA is produced in 96-well plates.
The DNA average yield is 3.2 μg/ml of bacterial culture (c.v. = 40%)—as quantified with Hoechst 33258 dye in a 96-well format—with 93% of wells yielding >1.5 μg/ml of culture and 85% of wells yielding >2.0 μg/ml of culture.

Viral titer. Production conditions: The library virus is produced in 96-well format by cotransfection of 100 ng of library plasmid, 100 ng of packaging plasmid (delta8.9), 10 ng of envelope protein plasmid (VSV-G) in HEK-293T. Two virus harvests of 150 μl each are collected at 36 h and 60 h after transfection. Harvests are pooled, aliquoted and stored at −80 °C.
Relative titer is measured in A549 cells in 96-well format by assessing cell viability under puromycin selection following low-multiplicity infections. Viability is measured with resazurin 3 d post-infection (2 d post-selection). A standard virus (~1.0×10^7 infectious particles/ml in A549 cells) is used to create a standard curve.
Typical virus titers of the library range from 10^7 – 10^8 I.U./ml (c.v. for each plate, ~50%).

Library coverage

As of July 12, 2006, the library contains shRNAs directed against:
14,538 human genes (77,301 shRNAs)
12,742 mouse genes (60,944 shRNAs)

Gene coverage distribution. Average: 5 shRNAs per target gene.
0.3% of target genes covered by exactly 1 shRNA.
99.7% of target genes covered by ≥2 shRNAs.
99.0% of target genes covered by ≥3 shRNAs.
96.5% of target genes covered by ≥4 shRNAs.
83.7% of target genes covered by ≥5 shRNAs.
7.1% of target genes covered by ≥6 shRNAs.
Region of transcripts targeted: five shRNAs are designed for each transcript and selected to cover nonoverlapping sequences; 100% of target genes have an shRNA targeting the open reading frame and 93% of target genes also have shRNAs targeting the 3′ untranslated region.
List of target genes: available online (http://www.broad.mit.edu/genome_bio/trc/rnai.html).

shRNA sequences. A description of the design algorithm is available online (http://www.broad.mit.edu/genome_bio/trc/rules.html).
A regularly updated list of shRNA sequence is available online (http://www.broad.mit.edu/genome_bio/trc/rnai.html).

Functional tests

Sequence verification. All library clones are required to have 100% verified sequence match throughout the hairpin region with high read quality. We resequenced 91% of library clones to confirm perfect match[8].

Validation data. Knockdown validation was assessed for shRNAs that generate phenotypes in screens and for overall assessment of library performance. The knockdown efficiency of 283 shRNAs targeting 59 genes was assessed by quantitative RT-PCR of the endogenous transcript (including all shRNAs per target gene; see ref. 8, and TRC, unpublished data).

Knockdown efficiency. Average knockdown efficiency is 47% (statistical representation of library performance; does not include shRNAs selected from phenotype hit lists).
Of the tested shRNAs, 38% (97/256) give more than 70% knockdown.
For 75% of tested target genes (40/53), the library contains at least 1 shRNA that provides >70% knockdown efficiency.
For 43% of tested target genes (23/53), the library contains at least 2 shRNAs that provide >70% knockdown efficiency.

Nonspecific off-target effect. In a test of 8 different shRNAs at three doses each, no induction of the interferon response was observed, as monitored by gene expression of interferon response genes *IFNB1*, *MX1*, *OAS1*, *OAS2*, *IFITM1* and ISGF3G (by quantitative RT-PCR), whereas a transfected poly(I:C) dsRNA control induced expression 10–10^4-fold in all six genes.

Distribution

Glycerol stocks, DNA and virus are available from Sigma-Aldrich.
Glycerol stocks are available from Open Biosystems.

To further test the specificity of the mitotic index–altered phenotypes, we selected five hit genes for additional follow-up experiments in which all of the shRNAs for those genes were tested both for suppression of the target gene and for the mitotic index–altered phenotype. In four out of five cases the phenotype correlated with the level of target-gene suppression. An additional test of target-gene specificity of the active shRNAs was provided by the fluorescence images of DNA and actin distribution. For many of the hit genes, suppression of their expression by RNAi not only altered the mitotic index but also induced a wide diversity of major morphological changes. These changes in cell morphology were very consistent among the effective shRNAs for each hit gene, supporting the specific attribution of these effects to the intended target gene. These changes in cell morphology were very consistent among the hit shRNAs for each hit gene, supporting the attribution of these effects to the intended target gene. Although our observations in this screen reflect favorably on the specificity of gene suppression and the observed phenotype, other assays may exhibit higher susceptibility to off-target effects, so caution is warranted. It may prove feasible to reduce the frequency of off-target effects through improved hairpin-design algorithms, but specificity must still be confirmed through a combination of comparison of complex phenotypes across multiple shRNA sequences, correlation of target gene knockdown with phenotype, and rescue of phenotypes with a nontargeted gene allele.

Outlook

We have created a lentiviral shRNA library for the human and mouse genomes that allows gene silencing in most dividing and nondividing cell types. The major characteristics of the shRNA library developed by the TRC are summarized in **Box 1**. As of July 12, 2006, this library covered 14,500 human and 12,700 mouse genes; full genome coverage of the human and mouse genomes will be completed by spring of 2007. The inclusion of five different shRNA constructs targeting each gene increases the opportunity to achieve strong knockdown, often offers a gradation of knockdown and provides the means to rapidly evaluate the gene specificity of a phenotypic hit. Additional shRNA clones will be added as needed to achieve effective knockdown for each gene.

We will continue to validate target-gene suppression to assess library performance and to facilitate confirmation of target-gene specificity through correlation of knockdown with phenotype. These data, which will be made available to the research community, will also permit us to improve and evaluate hairpin-design parameters and alternative vector designs, such as those containing inducible promoters. As with the present library, we will require that any new vector reproducibly generate high-titer lentiviral particles to make viral screening possible in both arrayed and pooled formats.

Pilot experiments demonstrate that our library is suitable for pooled screens. For example, using unique sequences carried by each vector in the TRC library, we were able to identify negative selection of shRNA targeting a single gene in a pool of ~4,000 shRNA using microarray hybridization of PCR-amplified genomic DNA (TRC; unpublished data). As has been our experience in creating an arrayed screening platform, achieving a reliable, consistent pooled screening system will undoubtedly require the development of a rigorous system to validate the performance of the library under such conditions, and we will continue to optimize both arrayed and pooled strategies.

The use of RNAi for loss-of-function genetics in mammalian systems has already had a dramatic impact on biology. Although it is clear that this technology is still at an early stage and requires continued development to fulfill its potential as a screening tool, we anticipate that further understanding of RNAi biology coupled with rigorous performance evaluation will yield reliable and powerful tools for biological inquiry.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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