A Lentiviral RNAi Library for Human and Mouse Genes Applied to an Arrayed Viral High-Content Screen

Jason Moffat,1,2,4,10 Dorre A. Grueneberg,1,10 Xiaoping Yang,1,10 So Young Kim,1,3,7 Angela M. Kloepfer,1 Gregory Hinkle,1,3 Bruno Piqani,1 Thomas M. Eisenhaure,5 Biao Luo,1 Jennifer K. Grenier,1 Anne E. Carpenter,2,4 Shi Yin Foo,6 Sheila A. Stewart,8 Brent R. Stockwell,9 Nir Hacohen,1,5,7,11 William C. Hahn,1,3,7,11 Eric S. Lander,1,2,4,7,11 David M. Sabatini,1,2,4,11 and David E. Root1,11,*
INTRODUCTION - RNAi

• RNAi widely used now to identify gene function through a loss-of-function screen and also used to find new drugs and therapies

• The sense and the anti-sense strand (dsRNA) together inhibits homologous mRNA expression (Mello and Fire, 1998)
INTRODUCTION- RNAi

• **Synthetic siRNA** (short interfering/silencing RNA) currently used in screening and targeting defined gene families

![Diagram of RNA interference pathway](http://biology.plosjournals.org/perlserv/?request=get-document&doi=10.1371%2Fjournal.pbio.0020133&ct=1)
INTRODUCTION

PROBLEM: many mammalian cell types (ie: stem, primary, tumor cell lines) are resistant to transfection methods needed to introduce synthetic siRNAs into cells

SOLUTION: transduce mammalian cells with viruses carrying expression cassettes that encode short hairpin RNA (shRNA) to generate specific siRNAs within cells
INTRODUCTION - shRNA

shRNA already established method in identifying components of pathways:

• p53 Pathway
• 2 novel tumor suppressor genes
INTRODUCTION - shRNA

INTRODUCTION –

The RNAi Consortium (TRC)

• Goal: to generate genome-scale shRNA libraries in lentiviral vectors
  • Advantages of lentiviral vectors:
    • *Can infect non-dividing cells

**TRC1**- TRC lentiviral shRNA library contains constructs that target more than 22k human and mouse genes (~5 distinct shRNA constructs per gene to avoid off-target effects) → three-years, $18 million initiative

Second phase includes (TRC2) Harvard Medical School (HMS), the Massachusetts Institute of Technology (MIT), Dana-Farber Cancer Institute (DFCI), Massachusetts General Hospital (MGH), the Whitehead Institute for Biomedical Research (WIBR), Bristol-Myers Squibb, the Ontario Institute of Cancer Research, Sigma-Aldrich, and research institute Academia Sinica in Taiwan.
HEK 293T/tTA1-d2EGFP cells were infected with 200 ng of p24 of SIN-W-PGK-nls-LacZ or SIN/H1-siGFP lentiviruses.

METHODS – Library Production

Human U6 promoter drives shRNA expression

*pLKO.1 undergoes very low levels of recombination during cloning and plasmid-purification manipulations

Puromycin-resistance

shRNA sequence

21-bp stem

6-bp loop
METHODS – Library Production

-Yields 94% of designed clones
-Each gene had ~4.7 unique shRNA constructs, with 96% of the genes having at least 4 constructs

About the TRC1:
• 100k vectors
• Targets 12k human and 10k mouse genes
• 45k new constructs generated per month
(information as of March 2006)
METHODS – HT Lentivirus Production

-a high-throughput (HT) method was created to make high-titer lentiviruses

-in 96 well plates, HEK293T cells were transfected in a 3-plasmid lentivirus packaging system

- in a 384 well plate about 3 μl of lentiviral supernatent in each well

→ Yielded sufficient volumes of lentiviral supernatants for ~100 screens from one 96 well plate
METHODS – HT Method Evaluation

Gene suppression sufficiency (Knockdowns in 12 Y of A549):

31% of lentiviruses reduced transcript levels (found by qRT-PCR) by more than 4x
OBJECTIVE

Find genes involved in regulation of mitosis
- used human HT29 colon cancer cells
- Targeted genes include:
  476 protein kinases
  180 phosphates
  372 nonprotein kinases, tumor suppressors and DNA binding and modification enzymes

80% of lentiviruses infected HT29
METHODS – Mitotic-Index Assay

-Automated fluorescence microscopy: to find cells in mitosis that have **histone H3 phosphorylated on serine 10 (pH3)** *(a known marker for mitotic cells)*

-cells were visualized by DNA binding dye to identify nuclei and measure DNA content

-to detect cytoplasmic size and shape phalloidin *(actin stain)* was used

-Mitotic Index (MI): \[
\frac{\text{# cells in pH3}}{\text{total cell #}}
\]
METHODS – Mitotic-Index Assay

A selection of shRNAs in HT29 cells:
some showed increase in mitotic index
→ used to assess the effect of viral dose on cell counts and mitotic index.

Average MI: 5.1
SIGNIFICANT:
MI > 9
MI < 1
Analysis of Known Mitotic Regulators

Checked multiple genes including **CDC2/CDK1** - canonical cyclin-dependent kinase that controls progression through G2/M on A549 cells
- known genes that regulate mitosis had MI>14 or MI<0.3
- transcript levels assessed with qRT-PCR

**Primary MI Screen**

<table>
<thead>
<tr>
<th>E</th>
<th>shCntl MI=5.5</th>
<th>shPLK1-513 MI=30.8</th>
<th>shCDK2-923 MI=34.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclei</td>
<td>phosho-H3</td>
<td>actin</td>
<td>overlay</td>
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</tbody>
</table>

**G2/M Shifts**
- Increased S and G2/M phase

**Normal G1**
Analysis of Known Mitotic Regulators

-shRNAs affected only the desired protein levels (immunoblots):

**BJ-hTERT**

<table>
<thead>
<tr>
<th>sh</th>
<th>Cdk2</th>
<th>Plk1</th>
<th>pH3</th>
<th>α-tubulin</th>
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<td>shCtrl</td>
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</tbody>
</table>

**A549**

<table>
<thead>
<tr>
<th>sh</th>
<th>Cdk2</th>
<th>Plk1</th>
<th>Cdc2</th>
<th>pCdc2 (Tyr15)</th>
<th>Cyclin B</th>
<th>α-tubulin</th>
</tr>
</thead>
</table>

**shPLK1-513**

- Decrease Plk
- No effect on Cdc2 or Cyclin B

**shCDK2-923**

- Decrease Cdk2, which caused the expected drop in Cyclin B

Determining shRNAs induce inform phenotypes in other cell types:

- BJ-hTERT fibroblasts were infected with shPLK1-513 or shCDK2-923 expressing lentiviruses
Finding Novel Mitosis Regulators

**Hit**- at least 2 independent shRNAs target the gene with high/low MI values

(Stringent threshold: MI>14 or MI<0.3)

-screened kinases in *Drosophila S2*, found 80 cell-cycle induced dysfunctions.

tested human 59/80 homologs: 21 alerted mitotic phenotypes

→ 3 found in HT29 screen

→ Others had effect on HT29 cells, but none on fibroblasts → therapy

High MI- 87 genes

Low MI- 15 genes
Finding Novel Mitosis Regulators

Primary analysis knockdowns with shRNAs:

- some sets of genes have similar phenotypes → same pathway?

blue = nuclei, green = pH3, red = actin
Finding Novel Mitosis Regulators

- Selection of 4 novel mitosis regulating genes:
  - **YES1**
  - **TIE1**
  - **ROCK1**

- Strong levels of increased MI and increased pH3

- Unclear correlation between gene knockdown and phenotype

- **MET**
Biological Characterization of Genes

- YES1, TIE1, ROCK1: infection of BJ-TERT fibroblasts induced effective gene suppression.

Arrested in G2/M

Also found induction of apoptosis by cleavage of PARP
Biological Characterization of Genes

Other implications of the genes:

TIE1- receptor tyrosine kinase has roles in angiogenesis and development, thought to function in a complex with TEK receptor tyrosine kinase.

- Can alter MI levels by targeting TEK with specific shRNAs.
- Causes G2/M arrest.

**Found:**

**Products of TIE1 and TIE play a previously unknown role in the control of mitosis in cancer!**
DISCUSSION

• Rapid identification of major regulators of many biological processes (shRNA/RNAi)

• Lentiviral shRNA libraries very important tool in screening in viral form

• Allows for study of a wide range of genes/cell types

• Some problems: need to be done at a larger scale, want to better characterize knock down efficacy

• Very useful in human and mouse genomes

• Found potential therapeutic targets with those that lead to cell death