From expression microarray to the Development and design of novel cancer treatment strategy

Cho-Lea Tso, Ph.D.
Assistant Professor
Department of Medicine
Division of Hematology-Oncology
UCLA School of Medicine
tso@mednet.ucla.edu
Expression microarray

- Better classification
- Improve diagnosis
- Predict clinical outcome/risk
- Decision making for treatment

Identification of novel therapeutic target gene(s)?

Functional siRNA screening assay:
A loss of function screening of essential genes using a functional model
**siRNAs**

- Long dsRNA
  - Dicer
  - 21-23 nt short siRNA
  - RISC
  - siRNA unwinding
  - Activated RISC
  - Association with target mRNA
  - Target mRNA cleavage

**miRNAs**

- short hairpin RNA (shRNA)
- miRNA gene
- pre-miRNA
- Nucleus
- Cytoplasm
- Dicer
- miRNA duplex
- One strand incorporated into RISC

**RNA interference (RNAi) pathway: sequence-specific silence of gene expression**

- **Dicer**
  - dsRNA endoribonuclease
  - against dsRNA and pre-miRNAs
  - cleaves a pre-miRNA stem-loop or dsRNA to a 20- to 23-base-pair dsRNA fragment with a 2 nt 3’ overhang at each end
  - initiates the formation of RISC

- **RISC**
  - RNA-induced silencing complex
  - uses the siRNA as a template for recognizing complementary mRNA
  - activates RNase and cleaves the RNA
  - siRNA unwinding

- **Argonaute**
  - catalytic components of RISC
  - bind siRNA fragments
  - endonuclease activity against mRNA complementary to siRNA fragment

*siRNAs arising by cleavage of long ds precursors or by chemical synthesis*
RNA interference (RNAi) pathway: from nature to the laboratory

Developmental Regulatory genes

Lab

Lentivirus or plasmid expressed shRNA

or

synthetic siRNA

24-72 h

-transducing
-lipotransfectants

destruction of homologous mRNA
or blocking its translation
SiRNA-gene silence
Microarrays of lentiviruses for gene function screens in immortalized and primary cells

Purpose: Screening of gene function in mammalian cells

Strategy: Lentivirus-infected cell microassay

Nature methods, 3:117, 2006
Whitehead Institute & Massachusetts Institute of Technology
Technical strategies for gene function screens

- High throughput/genome-scale loss/gain of function study
  - cell microarray platform
  - high-density spotting of thousands of gene transducing materials on one slide

- Study of gene function
  - Loss of function – hairpin RNA mediated sequence-specific silencing
  - Gain of function – overexpressed cDNA

- High-efficiency gene transduction
  - Lentiviruses encoded with shRNA or cDNA
  - VSV G-pseudotyped (envelope) infects an almost universal set of cells
  - Infect non-dividing cells
  - Stable transducing cells

- Functional screens
  - Cells land on the printed 'features' become infected with lentivirus
  - Phenotypic changes post particular gene knockdown or gene overexpressed
Infect cell clusters with Lenti-GFP cDNA and Lenti-Lamin shRNA

Lv-Lamin shRNA-gene knockdown
Lv-GFPcDNA- gene overexpression

Viral specific, do not cross react
Quantitative: dose response

Universal infectious particles

not cell type-specific transformed cells

dividing and non-dividing cells adherent and non-adherent cells normal cells
Compatible with a wide variety of promoters and viral backbone

Phosphoglycerate kinase promoter/GFP (LKO.1 plasmid)

Ubiquitin C promoter/RFP (LentiLox 3.7 plasmid)

Ubiquitin C promoter/Thy-1.1 (LKO.2 plasmid)

Preserve characteristics of VSVG LV

Arrays can be stored

Supress cell growth with mTOR shRNA

mTOR target gene
Complex cellular phenotypic changes

mTOR shRNA
phospho-S6-Cy3
- fewer cells
- smaller cells
- cell shape

Lamin A/C shRNA
Lamin-Cy3

Software
CellProfiler: image analysis software for identifying and quantifying cell phenotypes

Genome Biology 2006, 7:R100
Discovering loss-of-function phenotypes

- RNAi in mammalian cells allows rapid specific gene knockdown
- Using the fully annotated human genome to design siRNA libraries to knock down every human gene
Lentiviral short hairpin RNA (shRNA) library production for high throughput loss of function screen and biological discovery


(22,000 genes)
- Pool-based dropout screen barcode microarrays
- Unbiased whole genome screens
- Stable suppression of Target Protein
- Impact phenotype with
  - Cell proliferation
  - Viability/survival
- Identify common and cancer-specific growth regulatory pathways
- Drug targets
Drop-out shRNA screen (negative selection) for genes required for cancer cell proliferation

- 8203 distinct shRNAs targeting 2924 genes
- kinase, phosphatase, ubiquitination, cancer-related genes
- DLD-1: colon cancer
- HCT116: colon cancer
- HCC1954: breast cancer
- HMEC: immortalized mammary epithelial Cells

Protein required for cell proliferation!!

shRNAs with unique barcodes

Dropout shRNA screen (negative selection) for genes required for cancer cell proliferation
Cancer cell-lethal screen

Pool-based dropout screen barcode microarrays
Screen for Cancer-lethal Genes

shRNAs were ranked based on mean normalized log2 Cy3/Cy5 ratios

Common and Specific Lethal genes

Antiproliferative shRNAs and Genes

Clustering of the four cell lines with the antiproliferative shRNAs identified in the screen
Common shRNAs (blue) and genes (red) identified in the screen

<table>
<thead>
<tr>
<th>shRNAs</th>
<th>DLD-1</th>
<th>HCT116</th>
<th>HCC1954</th>
<th>HMEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLD-1</td>
<td></td>
<td>44</td>
<td>33</td>
<td>88</td>
</tr>
<tr>
<td>HCT116</td>
<td>61</td>
<td></td>
<td>53</td>
<td>95</td>
</tr>
<tr>
<td>HCC1954</td>
<td>36</td>
<td>57</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>HMEC</td>
<td>68</td>
<td>104</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>

19 common genes among all 4 cell lines

23 common shRNAs among all 4 cell lines
Genes commonly required for proliferation or survival of normal and cancer cells

Representative candidate shRNAs that reduce viability of all four cell lines.

Core cellular modules required in all four cell lines

Validation of selected shRNAs reduce viability of all four cell lines (growth)
Genes selectively required for proliferation or survival of cancer cells

A

Normalized log2 ratio

B

Cell viability (%)

C

PRPS2 shRNA

D

PRPS2 shRNA

E

MDM2 shRNA

F

Nutlin-3 (μM)

G

HCC1954

H

BUB1 shRNA

BUB1 siRNA
Functional genetic approach

- Identify antiproliferative shRNAs specific to particular cell lines
- Different cancer cells have distinct growth and survival requirements
- Cancer-selective therapeutics
- Generating cancer lethality signatures for different cancer types
- Identifying cancer type–specific lethal genes representing potential drug targets.
Clinical problem: GBM is a highly migratory cancer cells that prevent curative surgical resection

Hypothesize: Blocking the internal cues from promoting the GBM stem cell migration will suppress the infiltrating nature of GBM tumor they initiated

Objective: Identify genes and pathways that confer the migration nature of GBM stem cells

Approaches:
1. Molecular profiling of GBM stem cells
2. Designing siRNA library against self-expression genes
3. Loss of function screen using siRNA library and in vitro functional model
4. Validation of gene function in animal by injecting of GBM stem cells stably express shRNAs against migratory machinery

In vitro model

In vivo model
Clinical problem: Temozolomide (TMZ) became standard-of-care therapy for GBM patients, but majority of patients will always develop tumor recurrence.

Hypothesize: TMZ-resistant GBM stem cells are responsible for the post-treatment tumor returns.

Objective: Identify genes and pathways that confer the GBM stem cell resistance to TMZ treatment.

Approaches:
1. Isolation of TMZ-resistant GBM stem cell clones by propagating GBM stem cells in the presence of high-dose TMZ.
2. Molecular profiling of TMZ resistant GBM stem cell clones compared to Non-selected clones.
3. Designing siRNA library against self-expression genes associated with TMZ resistance.
4. siRNA-based loss of function screen in the presence of TMZ.
5. In vivo treatment sensitivity
   - Resistant clones vs. clones express shRNA against TMZ resistant genes.
   - Survival study of TMZ treatment (high Vs. Low dose).
The worst phenotype for Temozolomide therapy

- alkylate/methylate DNA at O-6 positions of guanine residues
- standard care therapy for GBM
- clinical achievable: <5μM in CSF and 50μM in plasma
- O-6-methylguanine-DNA methyltransferase (MGMT), a DNA-repair protein that protects cells from cytotoxic and mutagenic effects of TMZ
Treatment and selection of TMZ resistant GBM stem cell clones
High dose TMZ induced cell cycle arrest in GBM stem cell cultures
Induction of significant cell apoptosis in GBM stem cell cultures by 500 μM TMZ
Expression microarray and siRNA based loss of function screens

Identifying genes and pathways that promote CD133+ GBM stem cells
- self-renewal
- migration
- survival/quiescence
- resistance to TMZ treatment

Developing novel therapeutic strategies to diminish the tumor recurrence
- small molecules
- gene therapy
- cancer stem cell vaccine
- siRNA therapy
Seek for molecular targets of cancer stem cells to develop effective cancer therapy