

# Lentiviral-transduction of RAW264.7 cells for stable shRNA-mediated RNAi

Tamara Roach<sup>†</sup>, Bob Rebres<sup>†</sup> and Iain Fraser<sup>‡</sup>  
<sup>†</sup>Macrophage Biology Lab., San Francisco and <sup>‡</sup>Molecular Biology Lab., Pasadena

## BACKGROUND

### RNA Interference (RNAi)

RNAi is a powerful technology for the post-transcriptional manipulation of gene expression that has revolutionized the analysis of gene function in lower organisms. Stable 'gene silencing' or 'knock-down' of target proteins in mammalian cells has been developed in multiple labs by utilizing small hairpin dsRNAs or shRNAs. In mammalian cells, these short dsRNA fragments evade antiviral responses, such as the production of interferon, that are seen with longer dsRNA fragments.

### Advantages of target knock-down using shRNA-mediated RNAi:

- shRNA sequences (carried on plasmids) can be stably expressed
- Transduced cells can be selected by using antibiotics or sorting, resulting in populations of >95% carrying vector sequences
- Lines can be expanded for extensive experimentation
- Lines can be frozen and thawed
- Replicate lines can be used to verify phenotypes
- Multiple knock downs can be achieved in the same line

### A disadvantage of using stable shRNA-mediated RNAi:

Selection of populations can result in outgrowth of 'abnormal' cells. Hence phenotypes require verification by testing replicate lines transduced with shRNA against the same target. We also tested selection conditions to maximize survival of transduced cells.

## AIMS: 1. Optimize lentivirus systems to maximize selection of transduced cells

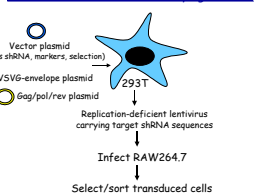
### 1. Lentivirus System for shRNA based RNAi

Optimized selection of transduced RAW264.7 using different lentivirus vectors:

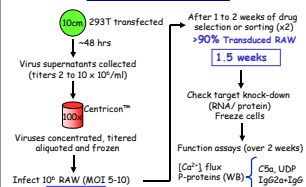
- UG1P = GFP/IRES1/puromycin
- UG12P = GFP/IRES2/puromycin
- UG1H = GFP/IRES1/hygromycin

## Production of shRNA-transduced RAW264.7 lines

### Production of lentiviruses carrying shRNAs



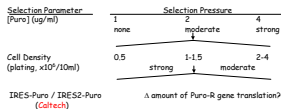
### Production of stable shRNA-transduced RAW264.7 lines takes ~ three weeks



## Results 1: Optimization of antibiotic selection of transduced RAW264.7

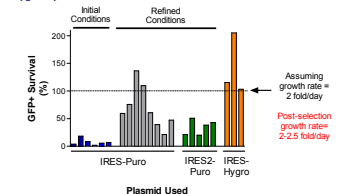
### Puromycin vectors (made with IRES1 or 2) did not confer robust resistance in RAW264.7

- Cells surviving selection <GFP+ fraction
- Must optimize survival of GFP+ to minimize clonality & maximize throughput
- Narrow range of conditions between too little/too much selection pressure
- 5-10% of GFP+ cells survived initial selections
- 20-100% of GFP+ cells survive refined selection conditions (variable)



### Proportions of the transduced RAW264.7 cells surviving antibiotic selections varied with lentiviral vectors.

Hygromycin had the best survival rate for transduced cells



### Fold change in variable which could be tolerated by transduced cells

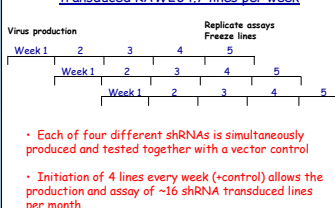
Resistance	Antibiotic [ ]	Cell Density
Puromycin	<0.5	0.5
Hygromycin B	4	8

## AIMS: 2. Obtain an initial dataset using RNAi against FXM target proteins

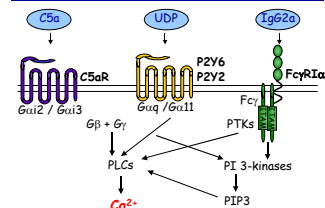
### 2. Interruption of cell signaling using RNAi

- Chose 3 ligands: C5a, UDP and IgG2a
- Focused on signaling pathways proximal to Ca<sup>2+</sup> and PIP3 generation.
- Assayed [Ca<sup>2+</sup>]<sub>i</sub> flux as an output for interruption of signaling
- Used lentiviral transduction of shRNA-based RNAi to achieve target knock-down

### Current production is 4 experimental shRNA-transduced RAW264.7 lines per week



### Initial targets for the testing of shRNA-based RNAi were selected using the AfCS 'parts list'



## Results 2: Phenotypes for shRNA expressing RAW264.7

### shRNA-mediated RNAi: multiple phenotypes for C5a and IgG2a, fewer for UDP

Receptors	No.	Target	Line #	C5a	UDP	IgG2a	Pre-selection		Post-selection	
							Cell Density	Ca <sup>2+</sup> flux	Cell Density	Ca <sup>2+</sup> flux
G-proteins and related	1	Gai2	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	2	Gai2	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	3	Gai2	3	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	4	Gai2	4	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	5	Gai2	5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	6	Gai2	6	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	7	Gai2	7	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	8	Gai2	8	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	9	Gai2	9	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	10	Gai2	10	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	11	Gai2	11	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	12	Gai2	12	0.5	0.5	0.5	0.5	0.5	0.5	0.5
PI 3-kinases	1	PI3K	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	2	PI3K	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	3	PI3K	3	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	4	PI3K	4	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	5	PI3K	5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	6	PI3K	6	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	7	PI3K	7	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	8	PI3K	8	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	9	PI3K	9	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	10	PI3K	10	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	11	PI3K	11	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	12	PI3K	12	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Tyrosine kinases docking proteins	1	PTK	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	2	PTK	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	3	PTK	3	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	4	PTK	4	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	5	PTK	5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	6	PTK	6	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	7	PTK	7	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	8	PTK	8	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	9	PTK	9	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	10	PTK	10	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	11	PTK	11	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	12	PTK	12	0.5	0.5	0.5	0.5	0.5	0.5	0.5

## Conclusion and summary:

### RNAi can be efficiently used in RAW264.7 cells

- Using prescreened shRNA sequences, target knock-down was >80% in almost all transduced lines, >90% in two-thirds, and >99% in one third
- 37 Lines have been studied, covering 31 targets (more in the pipeline, at rate of 4/week)
- Apart from receptor knock-downs, alterations in signaling were observed in:
  - Half of responses to IgG2a
  - One-third of responses to C5a
  - Only one response to UDP (more supported statistically)
- Expected phenotypes are usually (but not always) detected; Unexpected phenotypes were frequent.
- Results could be replicated

## Approaches to validation of RNAi phenotypes:

- Replicate lines with different shRNAs
- Alternate KD strategy (antisense, siRNA)
- Microarrays to look for off-target effects
- Knockdown reversal
- Test (and confirm) a likely hypothesis (e.g., with a 2nd knockdown)