Optimization of a genomic editing system using CRISPR/Cas9-induced site-specific gene integration

Jillian McCool
STAR Fellow at Lawrence Livermore National Lab
California State University, Chico
What is the CRISPR-Cas System?

- Bacterial defense and adaptive immunity against foreign genetic material
- Clustered Regularly Interspaced Short Palindromic Repeats
- CRISPR-Associates Proteins
- Many CRISPR pathway types
  - Type 1 - Cleave/Degrade DNA
  - Type 2 - Cleave DNA
    - Acquisition
    - Expression
    - Interference
  - Type 3 - Cleave DNA or RNA
- CRISPRs are transcribed into short RNAs, and guide Cas-protein to cleave genomic material
Traditional creation of transgenic lines

- **Techniques**
  - microinjecting the transgenic construct into a fertilized egg
  - Retrovirus or bacterial vector
  - transfecotransfect a transgenic construct into mouse embryonic stem (ES) cells and then mouse blastocysts

- **Limitations**
  - Variations in the mouse responses
  - Injections and zygote harvesting is a skill
  - Consistency
  - Generational systems
  - Poor transgene incorporation
  - Transgene size

- **ZF (zinc finger) and TALEN (transcription activator-like effector nuclease)**
  - Repetitive nature this timely construct development
  - Expensive and difficult to compare to or alter multiple genes,
**ROSAβ26**

- ROSAβ26 - used as a target for reporter genes through genomic insertions
- “Safe harbor” locus which allows for
  - transgene integration
  - single copy insertion
  - cis-regulatory elements
- Not prone to gene-silencing effects or decreases in cell viability.
- Completely sequenced so easy to target
- Non-fluorescent so screening involves a different process
pmKate2-N

- pmKate2-N - expression vector encoding for far-red fluorescent protein
  - mKate2 is a monomeric protein
  - Highly stable
  - Fluoresces between 588 and 633 nm

Transiently transfected HeLa cells
http://evrogen.com
Optimized CRISPR Protocol:

1. Design sgRNAs targeting the ROSA26 and mKate2

2. Construction of sgRNA-scaffold-target via PCR

3. *In vitro* Transcription

4. RNA Recovery via EtOH precipitation

5. *In vitro* digestion with Cas9 nuclease
Gel Validations of Transcription and Cas9 Digest

*Bands between 250 and 350 indicate successful transcription of the gRNAs

*Uncut mKate2 plasmid and ROSA locus compared with the the Cas9 digested mKate and ROSA
Optimized CRISPR Protocol Continued:

6. Transfection of gRNA, Cas9, and mKate2 plasmid into mouse osteoblast (MC3T3) cells and incubate.

7. Flow Cytometry for validation of integration of mKate2 into ROSA26 through fluorescence.

*Schematic of the 24-well plate set up for transfections into MC3T3 cells.

- Green=Plasmid in solution
- Purple= No plasmid in solution
- ROSA and mKate refer to sgRNA type added in transfection of MC3T3 cells.
CRISPR/Cas9 Results: Fluorescent Imaging

*Cells imaged 48 hours post-transfection under brightfield (top rows) and fluorescence (bottom rows)*
What is Flow Cytometry?

- Laser-based, biophysical technology
- Cell counting, sorting and biomarker detection
- Cells are suspended in fluid and passed by electronic detection apparatus.
- Allows for analysis of physical and chemical characteristics of cells.
CRISPR/Cas9 Results: Flow Cytometry

* Flow Cytometry conducted 96 hours post-transfection
CRISPR/Cas9 Results: Flow Cytometry

* Flow Cytometry conducted 96 hours post-transfection
Conclusion

• <2.5% positive red cells in all control samples

• mKate plasmid without CRISPR activity produced 13.8% positive red cells

• 17% of cells were positive in the presence of the ROSA locus and UC mKate plasmid

• An increase in fluorescence by 10%-30% was found in cells transfected with ROSA sg-RNA and mKate sg-RNA compared to those cells with only a single sg-RNA

• Cells transfected with a sg-RNA had a 5%-10% increase compared to the fluorescence levels of the mKate plasmid with no sg-RNAs.

• The most efficient integration was found in samples containing sg-RNA targeting both the genomic locus and plasmid, specifically the R1 and M1 combination
  • suggests linearized mKate fluorescent plasmid improves integration
Future Work....

- T7 endonuclease screening
  - Cleavage of NHEJ from CRISPR/Cas9 Activity

- Check for off target effects
  - Southern Blot/Sequencing

- Expression vs. integration

- Guide efficiency: ROSA vs. mKate
  - Which works better and why?

- New transgene incorporation into the ROSA locus

- Additional cell lines and altered sgRNA loci for knocking out genes

- Final Goal: *In vivo* experimentation to create new transgenic lines through embryonic injections
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References


