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

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ABSTRACT

The CRISPR-Cas system is an adaptive immune system found in bacteria which helps protect against the invasion of other microorganisms. This system induces double stranded breaks at precise genomic loci *(1)* in which repairs are initiated and insertions of a target are completed in the process. This mechanism can be used in eukaryotic cells in combination with sgRNAs *(1)* as a tool for genome editing. By using this CRISPR-Cas system, in addition to the "safe harbor locus," ROSAβ26, the incorporation of a target gene into a site that is not susceptible to gene silencing effects can be achieved through few simple steps. PCR amplification of the target genes , ROSA26 and mKate2, with a sgRNA scaffold and T7 promoter followed by *in vitro* transcription aim to produce an RNA product. This sgRNA product can be run through a digestion with Cas9 to validate cleavage of the genomic ROSA DNA template or mKate plasmid. Osteoblast mouse cells are transfected and labeled through partial uptake by the CRISPR mechanism, by cutting in the ROSA loci and repairing with pieces of the fluorescent mKate2 plasmid. These cells were measured via flow cytometry to give a percentage of red cells. This data shows the scaffolding construct created is targeted by the Cas9 endonuclease and through homologous repair the cells will incorporate the mKate2 target gene *in vitro* in MC3T3 mouse cells.

BACKGROUND

The CRISPR-Cas system is a defense and regulatory mechanism which bacteria have evolutionarily acquired to cope with a number of the diverse stressors within their environment. Host bacteria use this to protect against other microorganisms by incorporating short sequences from the invader's genome into its own *(5)*. These sequences are distinguished by clustered regularly interspaced short palindromic repeats (CRISPRs). These CRISPRs are transcribed into short RNAs, and lead a multifunctional Cas-protein to distinguish and cleave other extraneous genomic material as an adaptive immunological response to outside antagonists *(3)*. This research uses the CRISPR-Cas9 mechanism to develop an effective and reproducible technique to generate targeted alterations to the genome of mouse cells.

The ROSAβ26 (ROSA) is a locus in mice that is widely used for constitutive, ubiquitous gene expression, as well as used as a target for various reporter genes through genomic insertions *(2)*. This site is considered to be a "safe harbor" locus, which allows for several advantages when producing a targeted transgenic model. Specifically, this ROSA site allows for transgene integration, single copy insertion, cis-regulatory elements, and versatility with transgenic modeling without alteration of the phenotype *(4)*. The ROSA site is located within the first intron of the gene and can be targeted by homologous recombination. Using this site enables ubiquitous expression of insertions and is not prone to gene-silencing effects, as other sites are, or a decrease in cell viability. By combining the CRISPR/Cas9 machinery with this ROSA site, the possibility for easy and consistent gene editing is a possibility. By using the Cas9 cutting mechanism and the CRISPR recognition site that is constructed through sg-RNAs and *in vitro* transfection, an insertion of a target gene can be made within the ROSA locus with no disruption of other valuable cell processes.

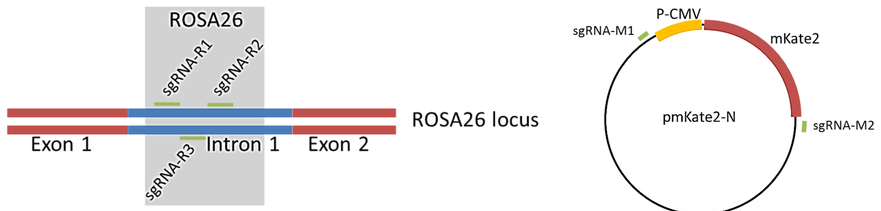
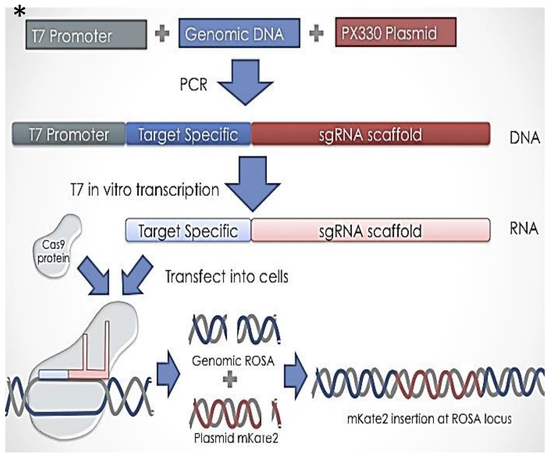


Figure 1: Schematic of the ROSA26 locus (left) and the mKate2 plasmid (right) as well as primer locations on those sites.

METHODOLOGY



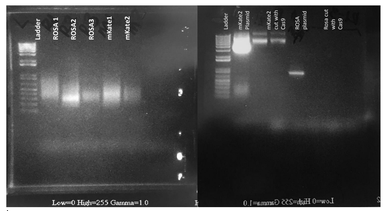
▲**Figure 2:** Flowchart of the sgRNA-scaffold-target construction and CRISPR/Cas9 mechanism for genomic insertion.

Protocol:

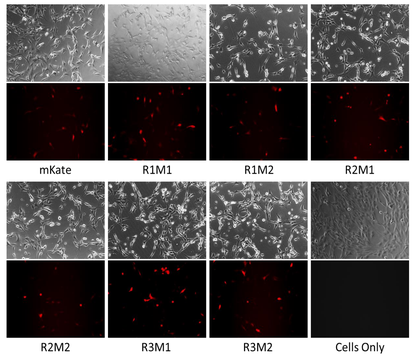
1. Design 3 sgRNA targeting the ROSA26 locus in the mouse genome and 2 sgRNA targeting mKate2 (Figure 1)
2. Construction of sgRNA-scaffold-target* via PCR and TAQ Polymerase (55ºC)
3. *In vitro* Transcription using Ambion mMessage mMachine T7 Ultra Kit *(3)*
4. RNA Recovery via LiCl precipitation
5. *In vitro* digestion with Cas9 nuclease to validate sgRNA site-specific DNA recognition and cleavage of DNA Template.
6. Transfection of gRNA, Cas9, and mKate2 plasmid into mouse osteoblast (MC3T3) cells.
7. Flow Cytometry for validation of integration of mKate2 into ROSA26 through fluorescence.

NOTE*: *Validation of step 2: sgRNA construct and step 3: T7 transcription via gel electrophoresis (Figure 3)

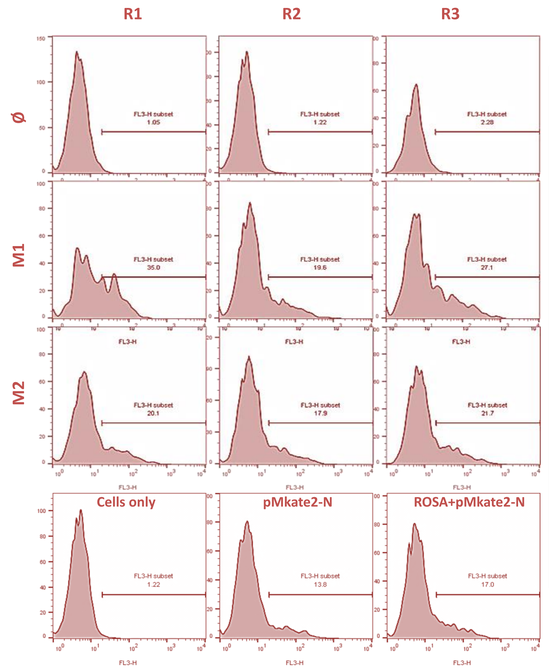
RESULTS



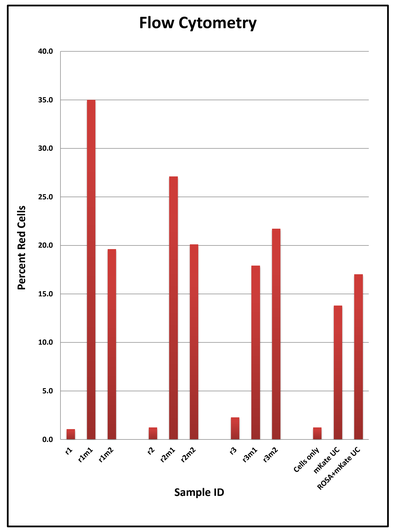
▲**Figure 3:** Gel Electrophoresis UV imaging for validation of T7 Transcription (left) and Cas9 endonuclease activity on the ROSA26 loci and mKate2 plasmid (right)



▲**Figure 4:** Brightfield microscopy (top) and red fluorescent microscopy (bottom) of MC3T3 mouse pre-osteoblast cells. Cells imaged 48 hours post-transfection.



▲**Figure 5:** Flow cytometry results showing the distribution of mKate2 integration. The x-axis denotes fluorescent intensity and y-axis denotes quantity of cells. Populations within the gate represent successful genomic integration. This data suggests increased integration of mKate2 from the CRISPR/Cas9 system inducing double stranded breaks in both the genomic and plasmid DNA.



▲**Figure 6:** Analysis of flow cytometry showing the percent of red cells in each sample as compared to the whole cell count recorded. Where samples with both ROSA and mKate are being compared to the positive controls containing the mKate plasmid and negative control containing only cells with no plasmid.

CONCLUSION

The CRISPR/Cas9 system is an innovative and reliable way to create novel transgenic cellular systems which can be used *in vivo* and *in vitro*. These experiments optimized a CRISPR/Cas9 editing mechanism that is time and cost effective. This process limits the pleiotropic effects due to position effects, multiple insertions, as well as time restrictions faced when performing gene integration.

By constructing the scaffolding for the *in vitro* transcriptions, and using the T7 Transcription Kit, the amount of time it would normally take for cells to run through the process naturally is restricted. This enhanced system allots for a short time requirement for an otherwise lengthy and complex mechanism. By using this approach and pairing it with what is known about the intron based "safe harbor" ROSAβ26, cells were transfected with a gene of interest, or target sequence. This target sequence is screened for through flow cytometry and by looking for an expression of a fluorescent protein from a mKate plasmid. Once screened, data showed <2.5% positive cells in all control samples, mKate plasmid without CRISPR activity produced 13.8% positive red cells, and a slightly greater, 17%, of positive cells when CRISPR at solely the ROSA locus was active. Further analysis showed an increase in fluorescence by 10%-30% of those cells transfected with a ROSA sg-RNA and an mKate sg-RNA, compared to those cells with a single sg-RNA, as well as a slight increase of 5%-10% above the fluorescence levels of the far-red fluorescent mKate plasmid with no sg-RNAs. The most efficient integration of the reporter gene was found in samples containing sgRNA targeting both the genomic locus and plasmid, specifically R1 and M1 combination. This data in turn suggests that linearized mKate fluorescent plasmid improves integration into the genome.

Future analysis will include a T7 endonuclease screening system, which will act as a secondary validation that the CRISPR/Cas9 system created does in fact work. Additionally future research will focus on application of this method for new transgene incorporation into the ROSA locus, additional cell lines, and altered sgRNA loci for knocking out genes. Once this system has reached a satisfactory and completely optimized level of gene integration it can be used *in vivo* to assist in the alteration of embryonic stem cells and help create new transgenic lines with ease.

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