Evaluating the Efficiency of Transgene Knock-In by CRISPR/Cas9- Mediated Nonhomologous End Joining in Zebrafish

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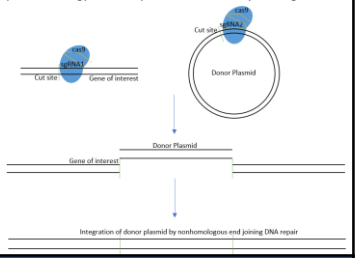
Introduction

CRISPR/Cas9 genome engineering has received a significant amount of attention in recent years for its ability to cut DNA at a specific site. This technology has the potential for genetic research, biotechnology, as well as in medicine for treatment of many genetic diseases such as cancer. However, current methodologies for DNA integration typically have low efficiencies and high levels of off-target effects. The development of methodologies which allow for efficient CRISPR-mediated knock-in transgenesis with low off targeting effects is essential in optimizing this technology.

Zebrafish, are a powerful model for studying CRISPR-based technologies, since the embryonic development of the organism can be closely monitored, in a relatively short span of time. To visualize a phenotypic change as a result of CRISPR mediated transgenesis, the gene SLC45A2 was chosen for the "knock-out" site. Mutation of this locus will cause albinism. Insertion of green fluorescent protein under a strong ubiquitous promoter into this site, would indicate a successful knock in by Non-homologous end joining(NHEJ). The goals of this research are to knock in a large reporter gene by NHEJ and to develop methodology which improves the efficiency of integration and reduces off targeting effects

Figure 1: CRISPR-Mediated Nonhomologous End Joining Zebrafish embryos can be injected at a one cell stage with cas9 mRNA, donor plasmid, and co-injection of sgRNAs which will simultaneously cut the genome and plasmid, leading to integration of the donor by NHEJ.



Materials & Methods

Cas9 mRNA and sgRNA *In Vitro* Transcription

The zebrafish codon optimized cas9 plasmid DNA template was linearized using *Xba*I restriction endonuclease enzyme. mRNA was transcribed *in vitro* using the mMessage mMachine T3 Transcription kit. sgRNA DNA templates designed to contain a T7 promoter sequence, a 20 bp RNA-DNA binding sequence, and a 15 bp tail designed to anneal to a universal primer; as described in Vejnar et al. The 117 bp sgRNA DNA template was generated by fill-in PCR. sgRNAs were then generated by the MEGAscript T7 In vitro Transcription Kit.

Cloning SLC45A2 Cut Sequence into ubi:EGFP plasmid

The SLC45A2 locus sgRNA-DNA recognition sequence was designed with flanking overhangs complementary to the plasmid sticky ends generated by *Sac*I and *Bcu*I restriction digestion. Ligation of the insert was confirmed by Sanger sequencing at the Yale Keck Sequencing Facility in New Haven CT.

Breeding and Injection of Embryos

Zebrafish were bred and embryos were collected and injected in accordance with IACUC standards for laboratory animal use. Each embryo was injected at a one cell stage with approx. 1nL of solution containing 100 ng/uL of cas9 mRNA, 20 ng/uL of sgRNA, and 10 ng/uL of donor plasmid DNA.

Analysis of Mutant Phenotypes

Albino phenotype was determined by standard light microscopy and were screened for between 24 and 48 hours post fertilization. GFP expression in embryos was analyzed by ZOE fluorescent imaging. GFP was screened for at 6, 24, 48, and 72 hours post fertilization.

Results

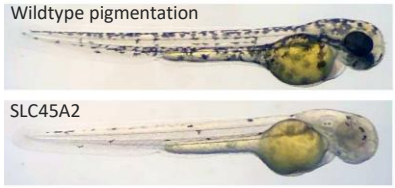


Figure 2: Disruption of SLC45A2 Activity, 48 hours post fertilization. Embryos were injected at a 1 cell stage with cas9 mRNA, and sgRNA targeting the SLC45A2 locus of the zebrafish genome. Disruption of this gene leads to albinism, predominantly in the developing eyes.

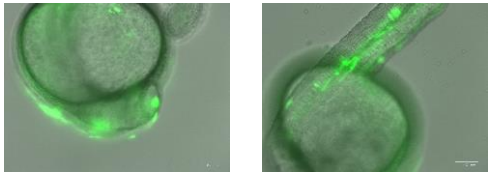


Figure 3: 24 hours post fertilization. Embryos were injected at a 1 cell stage with cas9 mRNA, sgRNA1(genome), sgRNA2 (plasmid) and ubi:EGFP plasmid DNA. At the 24 hour stage, there is visible integration of GFP in the developing eyes, as well as the trunk region, which includes the notochord, neuronal, and muscle cell types.

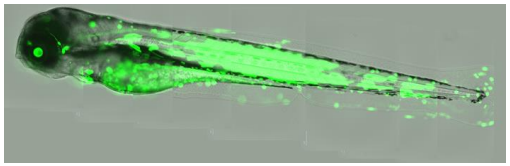


Figure 4: 72 hours post fertilization. Embryos show continued development of GFP expression pattern. Fluorescence is visible in the developing heart and circulating blood cells, as well as in the germ cells, indicating germ line transmission may be possible.

Conclusions

- CRISPR Cas9 mediated NHEJ can be utilized to integrated large reporter genes with high efficiency.
- Some researchers have reported success with integration of donor plasmids up to 5 kb (Kesavan et. al, Sawatsubashi et al). This research has demonstrated that integration is possible with much larger fragments, upwards of 9kb.

Future Work

- Experiments have been designed to analyze the extent of off targeting effects.
- PCR amplification of SLC45A2 cut site will be utilized to confirm integration of GFP.
- Further research is planned to investigate methodologies that may increase the efficiency of CRISPR-Cas9 mediate NHEJ, such as integration by a single sgRNA rather than coinjection of two sgRNAs.

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