CRISPR/Cas9 system can be used to target essential genes

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Abstract

The CRISPR/Cas (clustered regularly interspaced short palindromic repeat and CRISPR-associated nucleases) system has revolutionized the way we generate animal models. While it is very efficient, fast, and less expensive than standard techniques; it is not a well characterized system. One limitation of this system was thought be the difficulty to generate animal models by targeting genes that may be essential. If both alleles of an essential gene are disrupted, the embryos would die. We wanted to test if CRISPR/Cas9 system can be use to target a potential essential genes, Diexf. We used two sgRNAs to target exon1 of Diexf, one of which yielded live animals with deleted allele. Even though both alleles were targeted in most animals, one or both alleles were always in-frame resulting in embryo survival. We observed that all alleles were transmitted via germline to the next generation. After germline transmission of the Diexf allele with frame-shift deletion, we observed that Diexf null animals are not viable. A major concern of the CRISPR/Cas9 system is the modifications (insertion or deletions) at off-target sites, however, no off-target effect was observed. In summary, we learned that Diexf is essential for the viability of the embryos and that CRISPR/Cas9 can be used to target essential genes .

Introduction

The CRISPR/Cas9 system uses a guide RNA (sgRNA) and an endonuclease (Cas9) to introduce a double stranded break (DSB) at a specific site in the DNA(1). The sgRNA has a 20 bases long unique sequence and guides the Cas9 to a region in DNA with the complementary sequence followed by a PAM (protospacer adjacent motif) sequence. Cas9 endonuclease cleaves the DNA at that site, initiating a NHEJ (non homologous end joining) or HR (homologous recombination) mediated repair.

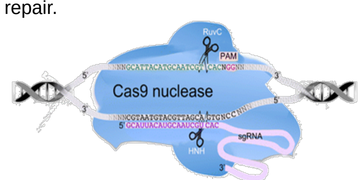

Figure 1. CRISPR–Cas9-mediated genome editing.

CRISPR/Cas9 system has been used to generate animal models(2) by directly injecting sgRNAs and Cas9 mRNA, targeting specific genes, into embryos. It has enabled the rapid generation of animals with multiple modified alleles in a single step (2,3,4). However, we wondered if CRISPR/Cas9 is a suitable system to knockout essential genes given that biallelic disruption results in embryonic lethality. Therefore, we wanted to knockout a potential essential gene, Diexf, using CRISPR/Cas9 system. Diexf is a pan-endodermal-enriched factor that is essential for the growth of digestive organs in zebrafish(4). Diexf null mutant zebrafish dies between 8 and 11 dpf (days post-fertilization), suggesting that is an essential gene.

In zebrafish, loss of Diexf resulted in hypo-plastic phenotype in digestive organs because of cell proliferation arrest. Given that Diexf is amplified in many cancers, we were interested to know the role of Diexf in cell proliferation. Therefore, we decided to use CRISPR/Cas9 system to target the Diexf gene in mouse embryos.

Hypothesis

CRISPR/Cas9 system can be used to target essential genes.

Methods and Results

Identification of CRISPR/Cas9 target and off-target sites

We screened and identified two target sites within exon 1 of Diexf using CRISPR Designing Tools at genome-engineering site (http://crispr.mit.edu/).

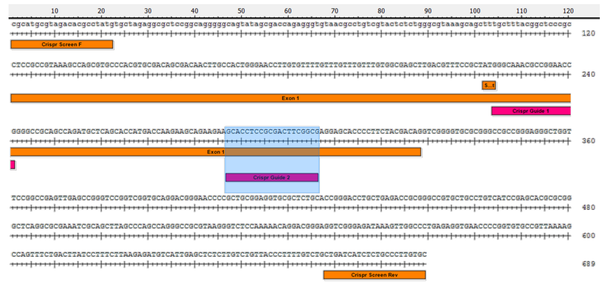

Figure 1. Diexf screen for CRISPR/Cas sgRNA guide sequence. Two ~20nt seed sequence followed by PAM were identified.

We reviewed for potential off- target sites for each sgRNA using the CRISPR Designing Tools. Later, we searched for insertion/deletions in this site using deep sequencing.

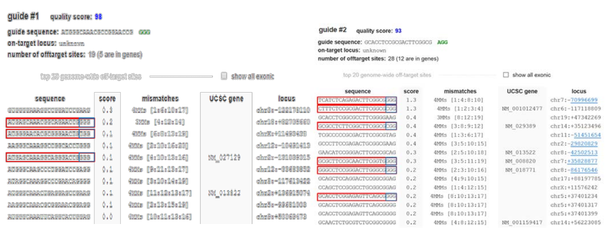

Figure 1. Off target site was evaluated for sgRNA1 and sgRNA2. Three potential off-target site were identified for sgRNA1 and 6 for sgRNA2

sgRNA Design and Synthesis

We hybrized 120 base long oligos using the sgRNA template in Figure 2. sgRNA was synthetized in-vitro using the MEGAshortscript kit.

sgRNA1 template:
GAAATTAATACGACTCACTATAGATGGGCAAACGCCGGAACCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT

Figure 3. The sgRNA template consisted of a T7 promoter (yellow), 20 base unique targeting sequence (yellow) and the remaining sequence.

Targeting embryos and Screening

The Cas9 mRNA, sgRNA1 and sgRNA 2 were co-injected into the pro-nucleus of the zygote, and transplanted to pseudo-pregnant mice. Live pups were screened for alteration in Diexf alleles by PCR and sequencing.

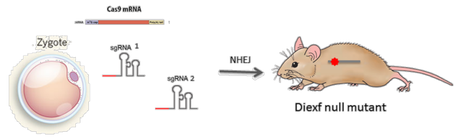

Figure 2. Diexf targeting by CRISPR/Cas9 system.

Screening Results

We screened 16 animals and found that 14 animals had targeting in one or both alleles of Diexf. 2 animals (highlighted in red) which had a frame shift alteration in one allele where crossed with wild type animals for germline transmission of the mutant allele.

A

B

Num. Mouse	Allele 1	Allele2	Effect in the protein level
1 Mouse	3 Base deletion	26 Base deletion	23 aa- frame shift; 62 aa- Stop
1 Mouse	9 Base deletion	1 base insertion + 12 base insertion (Rev. comp. of downstream sequence):	RDFGEEH… → RDLS-STOP
1 mouse	26 base deletion	WT allele	
7 mice	WT allele	9 base deletion	RDFGEEH → REEH (Deletion of aa 26-28)
1 mouse	Homozygous 9 base deletion		
1 mouse	9 base deletion	5 deletion + 7 base insertion (Rev. comp. of downstream sequence)	
1 mouse	9 base deletion	7 base deletion + 6 base deletion and insertion of 1 base	In-frame
1 mouse	9 base deletion	26 base deletion	

Figure 4. (A) Alterations in Diexf allele. (B) Alterations in individual mice.

Loss of Diexf resulted in embryonic lethality

Diexf mutant mice were treated as founders and crossed with WT animals for 2 generations. The Diexf mutant allele was transmitted via germline to the next generations. The Diexf$^{-/+}$ males and females were mated to check if the Diexf$^{-/-}$ animals are viable. We found that Diexf$^{-/-}$ mice are embryonic lethal.

Diexf $^{+/-}$ x Diexf $^{+/-}$

	WT	-/+	-/-
Diexf$^{\Delta 26/+}$	10	21	0
Diexf$^{13ins/+}$	5	11	0
Total	15	32	0

P = 0.0004 based on chi-square test.

Figure 5. Diexf $^{+/-}$ crosses results.

Conclusions

- We were able to successfully target Diexf gene in mouse embryos using CRISPR/Cas9 technology.
- Homozygous loss of Diexf resulted in embryonic lethality in mice suggesting that it is an essential gene.
- No off-target effect were observed indicating high specificity of CRISPR/Cas9 system.
- CRISPR/Cas9 system can be used to target essential genes.

Continued Collaboration

- We are crossing Diexf $^{\Delta 26/+}$ x Diexf $^{\Delta 26/+}$ to harvest embryos at different time points to see at what development state they are dying.
- We plan to identify how the Diexf null embryos are dying and Diexf functions and/or interactions.

References

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