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PAM Nucleotide Deviation with CRISPR-Cas9 and sgRNA

Abstract

CRISPR-Cas9 is a system that it is not only revolutionary in expeditious sequencing of deoxyribonucleic acids (DNA) but also serves to induce genomic alterations within the sequence as well. The methodology that allows CRISPR to behave in such a manner is its ability to function in conjunction with ribonucleic acids (RNA). A facet of this RNA is its ability to scale from micro to macro organisms and by doing so, represents an observable deviation in its sequence. These sequential changes in RNA are observable in the form of protospacer adjacent motif (PAM) nucleotides which directly associate with the single guiding RNA (sgRNA) systems that direct DNA to the Cas9 enzyme. These PAM nucleotides are segments on RNA that are changed when DNA is altered and can be monitored. Cas9 is the protein component of CRISPR that is responsible for genome editing. However, the problem is that there hasn't been conclusive evidence that demonstrates sgRNA will scale in complex genomes such as mice or humans. My objective is to observe how the PAM nucleotides behave in a multicellular Mammalia organism such as Mice to determine if RNA is directing DNA to the Cas9 enzyme and inducing genetic alterations. To observe these PAM nucleotides, the Mice genome will be subject to the introduction of Cas9 systems to be observed with PCR, gel electrophoresis, and Illumina sequencing. These are techniques that will yield data that signify if CRISPR is effective in altering mice genomes. With significant observable deviations in the PAM nucleotide sequences, the conclusion will be that CRISPR-Cas9 is able to induce alterations in complex genomes such as Mice and eventually humans.

Introduction & Background

Although the human genome has been sequenced for some time now, only recently has there been significant advancements in its alteration (Louwen et al. 2017). The methodology that is paving the way for genomic alterations in organisms such as *Gallus gallus Doesticus* (chickens), *Escherichia coli* (bacteria), and others, is CRISPR-Cas9. CRISPR is a sequence of DNA that was initially identified in bacterial organisms and was determined to be the sole reason that the bacteria are able to fight off diseases and viral infections so efficiently (Louwen et al. 2014). After further experimentation, the CRISPR sequence was extracted from the bacteria in the form of Cas9 proteins to be implemented in a variety of organisms (Louwen et al. 2017).

One organism that the Cas9 proteins were implemented in was chicken embryos (Zhang et al. 2017). This implementation not only served to establish the basis of the effects of CRISPR, but definitively identified how the system works in a model organism. The embryonic stem cells were

subject to an external-to-organism (in-vitro) cleavage that demonstrated that CRISPR was able to perform as a genetic alteration tool in conjunction to RNA to alter the genome of the *Gallus* organism (Zhang et al. 2017). RNA's role was crucial in guiding DNA to the Cas9-introduced CRISPR system within the organism and the discovery was supported by Donovan et al., who utilized the same Cas9 proteins to uncover gene functions (Zhang et al. 2017 & Donovan et al. 2017). With this new role of RNA discovered, CRISPR was determined a unique, efficient, and precise tool to introduce alterations to the sequence of DNA.

With such a simple yet efficient system, the anticipation quickly became the alteration of the human genome. This concept of introducing the human genome to foreign alteration systems such as CRISPR was and still is a controversial topic. To further add to this controversy, there was new and upcoming applications of CRISPR that were previously considered impossible such as inducing double strand breaks within DNA, identifying error-prone DNA, mediating homologous recombination, and all were conclusive with their respective model organisms (Sommer et al. 2014, Pulido-Quetglas 2017, & Hui-Song et al. 2016). Cumulatively, they led to CRISPR's recognition as a system that has diverse applications on the genotype of an organism. With such an effective system, we will conduct an experiment with the genome in mice to outline a system that may be applied to humans. The goal of this experiment is to further solidify the role of CRISPR within an analogous phylum to humans with a nearly identical genome (Louwen et al. 2017).

Statement of Need

To discuss the necessity of the experimentation of CRISPR with the mice phyla, one must consider the biological hierarchy of systems involving human experimentation. With the experimentation of CRISPR upon mice, any outlying observations that were not previously accounted for in the chicken embryos will be identified and addressed. An example of this is the uncertainty of Cas9 in the RNA mechanism leading to DNA being is the CRISPR enzyme. The problem of this mechanism of designation is that it relies on the ability of tracrRNA, pre-crRNA, and RNaseIII to bind to the Protospacer adjacent motif (PAM) nucleotides to bind to DNA via specific target site selection. These types of RNA merely target DNA to prepare for alteration. This target site selection has not only never been proven in mice, but has never been so critical as in the system of CRISPR. This experiment will seek to identify specific changes (if any) in the PAM nucleotides after Cas9 introduction via PCR, gel electrophoresis, and Illumina Sequencing of an experimental genome of mice and will be compared to a control group to recognize deviations.

Strategies & Goals

The importance of this experiment will be to determine the success of Crispr-Cas9 in the A61DL/9 Mice genome and will be conducted by a lab principal investigator along with 2 research oriented students from UC Merced and 1 graduate student.
We hypothesize that the introduction of Crispr-Cas9 into Mice *via Transcription activator-like effector nucleases(TALEN) will be conclusive in inducing alterations in the genome of* mice *and this will be observed by significant deviations within the PAM nucleotides.*

To address this hypothesis, the specific aims of the experiment are:
Specific Aim 1: Establish a control group of Mice to which no genetic alterations are applied and conduct PCR upon their genomes to establish a control group genome.
In this experiment, 100 A61DL/9-allele Mice (Jackson Laboratories, Sacramento, CA) will be divided such that 75 will be the experimental group and the 25 will be the control group. The control group will be kept unaltered and their genomes replicated via PCR & gel electrophoresis (ThermoFisher) and placed in open reading frame(ORF) libraries (Addgene, Cambridge, MA). The purpose of the ORF libraries is to establish the initial Mice genome to be compared at any stage of the experiment. The ORF libraries will be subject to incubation followed by recrystallization (Thermofisher, Pleasanton, CA) and extraction (Thermofisher) to remove impurities from solution. The remaining solution is added to a 3 µL Spinocerebellar ataxia type 1 (Sca1) (Addgene) and restriction endonuclease 1 (EcoR1) (Addgene) solution for conservation. Sca1 & EcoR1 serve as restriction enzymes to segment foreign genomes, but may be modified to use on host genomes. Conservational procedure includes placement in 0°C ice bath for duration of experiment (6 months).
The ORF libraries will not only serve to hold the genome until further analysis, but allows alteration and duplication provided that the integrity of the genome remains intact to allow displacement back into the original Mice organism of replication (Donovan et al. 2017).
Specific Aim 2: Identify initial Crispr-Cas9 capabilities within the genome of Mice via transportation of Crispr-Cas9 DNA into Mice via transcription activator-like effector nucleases.
Transcription sequences will be extracted from *Xanthomonas* bacteria (ATCC, Manassas, Virginia). The bacteria require level 1 containment which UC Merced is able to provide. To extract TALEN from *Xanthomonas,* 3.5µL Sca1 (Addgene) and EcoR1 (Addgene) enzymes must be injected via pico-liter microinjector (Harvard Apparatus, Holliston, Massachusetts) into the bacterial colony. The pico-liter microinjector can transfer 10^{-12} L solution with greater precision than with a micropipette. To achieve this, the bacterial colony should be collected without touching the bacterial plate itself via scoopula (provided by UC Merced) and placed in a test tube. The test tube contents are introduced to 1.5µL of Sca1 restriction enzyme (Addgene) and 2µL EcoR1 enzyme

(Addgene) via 10µL micropipette (Harvard Apparatus). The solution is centrifuged 45 minutes at 3300 RPM to eliminate solid residue of bacteria to collect the TALEN gene-transferring liquid. After the TALEN concentrate is obtained, 1 µL Cas9/gRNA (Addgene), will be injected into A61DL/9 Mice via 20µL micro syringe (Harvard Apparatus). The Mice is then subject to 42°C heat shock on ice for 85 seconds. Following the heat shock, 5-10µL of Luria Broth (Addgene) is added to the Mice followed by .1% ampicillin (Addgene) for 14-20 h. The result will be positive clones of recombinant plasmid DNA that will be extracted via Plasmid Minispin Kit (Dinguo Biotech. Shanghai, China). The Plasmid Minispin kit extracts plasmid DNA and is therefore utilized to obtain 1-5mL of liquid culture high-copy plasmid DNA samples for further experimentation.

Specific Aim 3: Examine the precise genome alterations of CRISPR-Cas9 (or lack thereof) upon Mice via PCR assay analysis upon two groups of Mice, the experimental and control.

After injecting the mice plasmids with the TALEN integrated Crispr-Cas9 and applying the Plasmid Minispin Kit, the sequence is segmented for identification. To identify the altered genome of experimental mice, one may apply the Tiling pool screen analysis approach in conjunction with PCR amplification. The Tiling pool screen analysis is a technique used to generate sgRNAs that CRISPR requires. This method requires Phytoplasma-Specific PCR Primers (ThermoFisher), such as those utilized for MAP2K1 at site K59 (Donovan et al. 2017). The Phytoplasma-Specific PCR primers will serve to segment the genome of the altered plasmid mice organism. After segmenting the genome, the specific sequences targeted for observance are the BRAF nucleotides. The BRAF nucleotides may establish themselves as the basis for the genetic alteration to be measured (Zhang et al. 2017). To identify these BRAF nucleotides, the methodology of in-vitro cleavage assay of sgRNA activity will be applied (Song et al. 2017). To perform in vitro cleavage, the; following solution will need to be prepared; Nuclease-free deionized water, 10X Cas9 Nuclease Reaction Buffer (Addgene), 1 µL Cas9 Nuclease (Addgene), 300nM sgRNA (Addgene) and 30nM substrate DNA (Addgene) to create a total solution volume of 30 µL. The solution will be exposed to microcentrifugation for 4250 RPM for 40 minutes and incubated at 47 °C for 2 h. The decanted solution will be subject to PCR amplification and gel electrophoresis (ThermoFisher) to visualize genotypical alterations.

Specific Aim 4: Compare the Crispr-Cas9 effects in the experimental Mice to a pre-established control group of which consisted of no genome alterations and create a table of deviations in PAM nucleotides.

To compare the effects of Crispr-Cas9 upon the Mice genome, the initial unaltered genome initially stored in the ORF library is compared to the altered genome. The same methodology of segmenting the genome via PCR primers to isolate and identify BRAF nucleotides is applied to the initial ORF library-contained genome of the control group of Mice. For further analysis of the

initial unaltered and altered genome, the technique of Illumina sequencing (Illumina, San Diego, CA) is applied to the PCR amplified samples via SPRI purification primer (Addgene) and MiSeq desktop sequencer (Illumina). SPRI primer purifies and allows easier access to the genome to which Illumina and MiSeq can sequence the genome such that it uncovers all genomic qualities that a researcher may inquire about. At this point of the experiment, the sequence of the mice is exposed for identification from the Illumina sequencing as well as gel electrophoresis. Included below is a table of the resulting sequence which may provide a concise manner of presentation of the results.

Table 1: Analysis of BRAF Nucleotides & PAM Deviations

Trial	BRAF Nucleotides	PAM Deviations
gRNA- Mice Example	AACTGCAGTAAGACC	TGG

Specific Aim 5: Determine if CRISPR-Cas9 is sufficient in inducing significant genetic changes in Mice.

Upon the completion and sequencing of the altered and unaltered Mice genome, a standard must define to which extent the alterations in DNA are phenotypical. With the information from the ORF libraries of the PCR amplified and gel electrophoresis purified genome, the BRAF nucleotides will phenotypically represent any significant changes in A61DL/9 Mice genome. To observe these changes, a timeline will be applied after alteration such that the students, under PI supervision, will periodically note any physical alterations within the Mice. The specific period will be every 6 h for a total time of 128 h. The results may be extrapolated to observe significant alterations.

Timeline

Month-Procedure	1	2	3	4	5	6
PCR/Gel Electrophoresis Control Group into ORF Libraries	x					
Extract TALEN from *Xanthomonas* and inject Cas9/gRNA into Mice (experimental group)		x	x			
Apply Plasmid Minispin Kit and Tiling Pool Screen to segment genome				x		
PCR/Electrophoresis and Illumina Sequence Experimental genome				x	x	
Analyze Results and Observe PAM Deviations					x	x

Ethics

Suitable living conditions will be provided for the Mice and will be sacrificed following the conclusion of the experiment by following of the IACUC and IRB guidelines. The lab principal investigator will assign the UC Merced student research participant with the task of assuring Mice living conditions are up to par with the standards of the IRB, IACUC, and the OCC. The mice living conditions will be upkept by the graduate student and at the end of the experiment, the mice will be euthanized.

Budget

Item	Manufacturer	Expense
A61DL/9 Mice *Organism*	Jackson Laboratory, Sacramento, CA	13.25x100=$1325
Polymerase Chain Reaction Inducer	ThermoFisher, Pleasanton, CA	$647
Xanthomonas bacteria Organism	ATCC, Manassas, VA	$5.5/per kg
Cas/gRNA 100mL Vial	AddGene, Cambridge, MA	$99
Plasmid Minispin Kit	Dinguo Biotech Company, Shanghai, China	$365
PCR Primer 100mL	ThermoFisher, Pleasanton CA	$69.00
Illumina Sequencing Machine	Illumina, San Diego, CA	$21,599
SPRI Purification Primer & Sca1/EcoR1	AddGene, Cambridge, MA	$46
ORF Libraries	AddGene, Cambridge, MA	$50
Picolitre Microinjector	Harvard Apparatus, Holliston, MA	$75
Micro Pipette	Harvard Apparatus, Holliston, MA	$50
Micro Syringe	Harvard Apparatus, Holliston, Massachusetts	$75
In-Vitro Cleavage Assay Components	AddGene, Cambridge, MA	$200

Necessary Person(s)	Time	Salary
Principal Investigator	Duration of Experiment (6Mo)	$24,000
1 Graduate Student	Duration of Experiment (6Mo)	$10,000
	Total Expense	**57,280**

Significance/Impact in the Field

Upon the conclusion of the experiment, the Cas9 system will be determined for its application in the genome of A61DL/9 Mice and the hypothesis of conclusively inducing alterations within the genome will be answered. Additionally, uncertainties such as the sgRNA scalability from microorganisms like bacteria to the larger organisms such as Mice will be addressed by determining the PAM nucleotide deviations after introducing Cas9 via TALEN transportation (Sommer et al. 2017,

Donovan et al. 2017 & Louwen et al. 2014). Whether the PAM nucleotides significantly deviate from the control group of Mice will be an indication of the effects (or lack thereof) of Cas9 upon Mice. The goal for the experiment is to satisfy the biological necessity of conducting genome alterations in analogous genome organisms such as Mice prior to the conduction of human trials. Human trials will serve to address the ongoing battle with disease as well as contribute to the research of the human genome.

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