



MURRAY STATE
UNIVERSITY

Murray State's Digital Commons

Honors College Theses

Honors College

Spring 5-13-2017

Risk Assessment in CRISPR Transgenic Organisms

Jeffrey B. Warner

Follow this and additional works at: <http://digitalcommons.murraystate.edu/honorsthesis>



Part of the [Molecular Genetics Commons](#)

Recommended Citation

Warner, Jeffrey B., "Risk Assessment in CRISPR Transgenic Organisms" (2017). *Honors College Theses*. 11.
<http://digitalcommons.murraystate.edu/honorsthesis/11>

This Dissertation/Thesis is brought to you for free and open access by the Honors College at Murray State's Digital Commons. It has been accepted for inclusion in Honors College Theses by an authorized administrator of Murray State's Digital Commons. For more information, please contact msu.digitalcommons@murraystate.edu.

Risk Assessment in CRISPR Transgenic Organisms

Submitted in partial fulfillment
of the requirements
for the Murray State University Honors Diploma

Jeffrey Warner
May, 2017

Table of Contents

<i>Abstract</i>	<i>ii</i>
<i>Introduction</i>	<i>1</i>
<i>What is CRIPR/Cas?</i>	<i>1</i>
<i>How is CRISPR/Cas Being Used?</i>	<i>1</i>
<i>Human Genome Editing – Ethical Issues facing CRISPR Technology</i>	<i>4</i>
<i>Off-Target Cleavage Prediction and Prevention</i>	<i>6</i>
<i>Screening for Off-Target Cleavage and CRISPR-Induced Mutations</i>	<i>8</i>
<i>Risk Assessment Strategy in CRISPR Transgenic Organisms</i>	<i>12</i>
<i>Article Selection</i>	<i>13</i>
<i>Results and Discussion</i>	<i>13</i>
<i>Conclusions</i>	<i>17</i>
<i>References</i>	<i>18</i>

Abstract

CRISPR/Cas, a gene editing tool modified from an antiviral response in bacteria, has been demonstrated to faithfully and precisely introduce double strand breaks (DSB's) in DNA at sites designated by guide RNA's (gRNA's). The system is applicable to many model organisms and shows promise in the study of gene function, creation of model organisms, and, importantly, treatment of heritable disease. However, the specificity of the Cas endonuclease is such that DSB's are often introduced at sites similar to the target sequence, called off-target sites. Minimization of off-target DNA cleavage is important to prevent unknown and unwanted mutation, which can raise significant safety concerns. Thus, it is important for researchers using the tool to predict, prevent, and screen for these off-target DSB's. Luckily, many methods have been developed to meet these needs. In the present analysis, the risk assessment efforts of several representative studies using various model organisms are investigated and summarized. Most often, researchers focus on *predictive* risk assessment strategies, leaving *preventative* and *screening* measures rarely or never used. CRISPR/Cas is a very potent gene editing tool whose greatest potential will likely be achieved in human germline cell editing. However, implementation in human embryos will likely only occur on a large scale after the tool is more fully understood. The use of all applicable risk assessment strategies could help further our understanding of CRISPR/Cas, lessening the time before it can be applied to human embryos in the way of heritable disease treatment.

Introduction

What is CRISPR/Cas?

The development of efficient means of controlling gene expression has long been a goal of molecular genetics research, with the most recent and prominent discovery coming from a bacterial adaptive immunity mechanism against phages. The clustered regularly interspersed short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas) system can easily be combined with single guide RNAs (sgRNAs) to induce double-strand DNA breaks (DSB's), single strand nicks, and DNA binding with high fidelity and precision across almost any species. The sgRNA binds to the protospacer region of genomic DNA, downstream of a conserved protospacer-adjacent motif (PAM) sequence, and when a custom sgRNA is introduced, this binding can occur at a specific location as desired by the researcher. Once DSB's are introduced, the damage can be repaired with either non-homologous end joining (NHEJ) or homology directed repair (HDR), allowing for precision genome editing.

How is CRISPR/Cas Being Used?

While the primary application of CRISPR/Cas is genome editing, research groups have been modifying the Cas protein in clever ways to achieve other genetic effects, described here:

Transcriptional Inhibition/Activation

Quite simply, the functional Cas9 protein can be replaced by a "dead" Cas protein (dCas) which still uses gRNA to locate a certain DNA sequence, but has no endonuclease ability. In this way, researchers can provide a physical barrier preventing

RNA Polymerase and other enzymes to associate with a particular area of DNA. This mechanism, called CRISPRi, provides a way to knock out genes of interest, allowing study of their biological function without changing the DNA sequence (Qi et. al, 2013). Similarly, a mechanism was developed by which CRISPR/Cas could be used to activate genes of interest. This system, called CRISPRa, involves the attachment of a transcriptional activator to endonuclease-deficient Cas9, allowing modulation of gene expression 1000-fold (Gilbert et. al, 2014).

Epigenetic Modification

CRISPR/Cas have been shown useful in the addition of epigenetic markers (acetyl groups, methyl groups, etc.) to specific DNA loci by use of gRNA at a level of precision previously unattainable. Similar to CRISPRi or CRISPRa, epigenetic writer enzymes can be coupled to a dCas protein and carried to a specific location in the DNA sequence. In this way, the epigenetic influence of certain markers on a gene of interest can be studied, adding to our bank of knowledge of epigenetic regulatory systems (Hilton et. al, 2015).

Studying Enhancers

The study of non-coding DNA regions (like enhancers and non-coding RNA's) is important considering that non-coding genes account for greater than 98% of the human genome. Cas9's endonuclease activity has been applied to such regulatory sequences of DNA to create knockouts, letting researchers examine phenotypic changes in the absence of the natural upregulation of a gene. It is, however, more

difficult to target specific non-coding RNA's, because they're typically very short, and may not contain a PAM sequence. For this reason, researchers are searching for other Cas-like endonucleases with more relaxed specificities. For example, a protein family called Cpf1 has been identified by a team at MIT that might prove useful for targeting short RNA's due to its ability to recognize a thymine-rich PAM sequence instead of the typical 5'-NGG-'3 sequence (Zetsche et. al, 2015).

Inducible Genome Editing

In some research applications of gene editing, it is desirable to toggle the expression of a gene of interest. A research laboratory at Duke University has modified the CRISPR/Cas system to do just this. The results of their work showed that coupling an inactivated Cas9 protein (dCas9) to both a transcriptional activator and a light-inducible protein complex (in addition to the usual complementary gRNA) can allow target genes to be toggled in this manner. Simply exposing the cells to a particular frequency of light activates the light-inducible proteins, triggering the upregulation of the gene at that locus. This system, called the light-activated CRISPR/Cas effector (LACE) system, gives researchers the power to transiently control gene regulation, a tool very helpful in understanding cell fate (Polstein et. al, 2015). A similar inducible CRISPR (iCRISPR) system was developed by a research team in New York that utilizes doxycycline to control the spatial activation of genes in mice. The research is useful in loss-of-function studies and creating transgenic mice that mimic human colon cancer patients (Dow et. al, 2014).

While these and multiple other uses for CRISPR/Cas exist, this study will focus primarily on the gene editing function of the tool.

Human Genome Editing – Ethical Issues facing CRISPR Technology

One of the long term goals for use of the CRISPR system is human germline gene editing, wherein modification of the genome in the early embryonic stages can prevent rare heritable diseases like Huntington's disease, an effect that would carry through subsequent generations. However, major ethical concerns are raised in opposition to editing human embryos that would otherwise grow into healthy babies. To circumvent such an ethical dilemma, one group at Sun Yat-sen University in Guangzhou, China, has used CRISPR to edit the genomes of tripronuclear (3PN) zygotes. 3PN zygotes are those that are fertilized by two sperm instead of one, preventing them from growing into healthy babies – these zygotes are often rejected by fertility labs for use in *in vitro* fertilization. The study was successful in cleaving and rewriting the endogenous β -globin gene (HBB) with synthetic gRNA in only 4 of 86 injected eggs, resulting in “mosaic” embryos (a mix of modified and unmodified cells) and multiple off-target mutations (Liang et. al, 2015). The results of this study highlight the need for further study into modification of human embryos, but show promise in treating heritable diseases like beta-thalassemia (caused by mutations in the HBB gene). In fact, the HBB gene has previously been the target of unsuccessful gene-editing attempts, further showing the promise of CRISPR as a useful tool.

Because of the discrepancy in ethical values between the United States and China, some believe modification of human embryos will spark a “space race-esque”

face-off between the two superpowers. At this point in time it is clear that China has been green-lighting more research in this field than the United States has – in fact, China has already used CRISPR/Cas9 to treat lung cancer with modified immune cells and edit human embryos five times (Adee, 2016). One of those attempts was recently performed on normally fertilized two pronuclear (2PN) human embryos that would have otherwise developed into healthy fetuses, not those that were double fertilized (3PN) and typically do not survive, like previous studies. This group's study showed successful corrections of mutations in the HBB and G6PD (codes for an enzyme central to favism, a red blood cell disorder) genes in *some* of the single-cell embryos. While the efficiency rate is still quite low and the study only attempted editing in six embryos, it did point to the conclusion that use of CRISPR is more efficient in 2PN embryos compared to 3PN embryos (Tang et. al, 2017). Mosaicism, a phenomenon wherein fetuses may still show a diseased phenotype despite *most* cells being modified correctly, was still a problem in this study. In fact, mosaicism continues to be a formidable road block preventing CRISPR/Cas-edited embryos from being implanted in women, because of the difficulty of correctly and identically editing *all* cells in an embryo.

The controversial nature of such studies prevents this type of embryonic editing from occurring in the United States, where a block on federal funding for research on human embryos is in effect (Adee, 2016). The current societal consensus is that further understanding of CRISPR technology should precede its use in human embryos, leaving the main targets of CRISPR research in the US to be livestock animals and common model organisms like mice and zebrafish (Adee, 2016). The ethics surrounding gene editing in other well known organisms, like trees for example, can serve as a

simpler analogy. For example, a group of researchers polled young educated people on their attitudes toward the growing and cultivation of transgenic trees, and found that they mostly approve of the use of transgenic trees, but showed a lack of understanding for potential benefits and risks of the process (Kazana et. al, 2016). This finding parallels the general gap in knowledge about gene editing techniques that may be preventing most nations from using technologies like CRISPR on human embryos.

Off-Target Cleavage Prediction and Prevention

Because of the CRISPR/Cas9 tool's ability to, with high fidelity, create DSB's in genomic DNA, it is inherently important to control the site of that break. DSB's at an unintended off-target site could result in unwanted mutation or simply failure to achieve the desired genetic result. The best strategy in dealing with off-target DSB's is to predict off-target cleavage sites *before* applying the CRISPR/Cas technology. Mostly, this is done *in silico* through bioinformatics tools being developed by several research groups, including nucleotide BLAST searching (Hopes, 2016), and algorithmic programs like Cas-OF Finder, E-CRISP, and Breaking-Cas (Martin et. al, 2016). Furthermore, programs like EuPaGDT and CasOT are available, which use genome libraries to search for PAM sequences and complementary DNA sequences within the specified length of the guide RNA. The commonly used Cas protein Cas9, derived from *S. pyrogenes*, has an optimal PAM sequence of 5'-NGG-3', but can also cleave at sites with a 5'-NAG'-3' or 5'-NGA-3' sequence (Martin et. al, 2016), highlighting the importance of screening for these suboptimal PAM-like motifs *prior* to gene editing.

While predicting off-target sites before introduction of the endonuclease is ideal, other methods can be employed to control the presence of off-site double-strand breaking. The most direct approach is to simply design the sgRNA such that each base pair is written with perfect precision. This method can be difficult in so far as genomic libraries do not yet exist for every model organism with the level of detail needed to construct perfect sgRNA's or locate proper target DNA sequences, but work is being done on this front. In fact, several online tools have been developed to help locate target DNA sites in well understood organisms (Belhaj et. al, 2013). To make matters more complicated, it is debated whether a total match in the seed region of the sgRNA (the 7-12 base pairs extending from the 3' end) is more appropriate to achieve target site recognition, or if a longer, better matching sgRNA should be employed. It has been shown that shorter sgRNA's have a lower activity but a higher specificity, and that longer sgRNA's have a higher activity but lower specificity. Thus, depending on how well a researcher can "write" his or her sgRNA, the activity of off-site cleavage can be controlled (Pattanayak et. al, 2013). In less well understood model organisms, it may be more appropriate to implement a longer sgRNA, because a number of mismatches in the 5' end of the sgRNA can be tolerated (Hsu et. al, 2013). However, if a detailed genomic library exists, a shorter, more specific sgRNA could be used to decrease off-site cleavage activity and lower unwanted mutation risk.

Additionally, studies have shown that shortening sgRNA's from 20 base pairs to 17 base pairs (Fu et. al, 2014) or adding two guanine residues to the 5' end (Seung Woo, et. al, 2014) can reduce off-site cleavage by RNA-guided Endonuclease (RGEN) proteins, below what is detectable by deep sequencing methods. Outside of modifying

sgRNA length, off-site activity can be controlled by limiting RNA:Cas9 complex expression (Pattanayak et. al, 2013). Higher concentrations of RNA:Cas9 (a potent, efficient endonuclease complex) can very easily result in multiple unwanted cleavage sites in or around the PAM.

Screening for Off-Target Cleavage and CRISPR-Induced Mutations

The tendency of the CRISPR/Cas system to produce unwanted mutations by mechanism of off-target cleavage introduces the potential for the creation of non-viable embryos, mosaic embryos, or other base pair mutations with unknown effects. Such mutations can be dealt with in one of two broad ways: 1) screen for off-target DSB's (as previously discussed), or 2) screen for mutant cells. Both result in "finding" problem cells for the researchers to eliminate.

Screening for off-target DSB's is important because all CRISPR/Cas9 systems (the most commonly used CRISPR/Cas system) produce them, and the resulting breaks can produce insertions or deletions (indels) that can be particularly risky in therapeutic use of CRISPR/Cas9 (Martin et. al, 2016). While it is ideal to predict and prevent off-target cleavage *in silico*, it is sometimes too costly, laborious, or not enough is known about a particular model organism's genome to take this approach. Furthermore, it is rare for even a good algorithm to correctly predict all potential unwanted activity because of the complex nature of Cas9's specificity. Likewise, even if prediction of off-target sites is doable, researchers must still analyze their genetic products for mutations at those sites. PCR analysis of predicted off-target sites, analysis of heteroduplex formation (double-stranded DNA fragments resulting from homologous recombination),

and high-throughput sequencing of PCR products are all standard methods for searching for off-target activity (Martin et. al, 2016). The laboratory work of Martin et. al has identified several more strategies that can be combined with standard methods to provide a more complete picture of all endonuclease activity:

1 – Cross-Linking Chromatin Immunoprecipitation of Endonuclease-Mutant Cas9 (ChIP-dCas9)

This approach employs cross-linking immunoprecipitation (ChIP), a method that reveals protein-nucleic acid interactions, to show association between an inactive Cas9 (dCas9) protein and DNA. Following immunoprecipitation, genome sequencing shows the presence of these DNA-gRNA-dCas9 interactions in transfected cells at both on-site and off-site locations. Some researchers have shown that this method does not always reveal 100% of interaction sites, and theorize that the use of a mutant Cas9 protein may alter its DNA specificity (Teytelman et. al, 2013).

2 – Integrative-Deficient Lentiviral Vectors (IDLV) Capture

IDLV's have been used in the detection of DSB's since before CRISPR/Cas9, in other nucleases like zinc finger nucleases. It is a promising strategy because of IDLV's ability to efficiently enter cells, including human primary cells (which are typically difficult). The lentiviral vectors associate with the DNA breaks and can then be identified by high-throughput DNA sequencing. The technique, although

commonly used with other nucleases, has shown promise in detecting CRISPR-based DSB's (Wang et. al, 2015).

3 – *GUIDE-seq Methods*

This method, called genome-wide unbiased identification of DSB'S evaluated by sequencing (GUIDE-seq), involves the use of modified double-stranded oligonucleotides (dsODN's) to associate with DSB's (similar to IDLV's). The dsODN's act as tags used to amplify DNA elements that have breaks in them. The results, coupled with a reference genome, can show the researcher where Cas9 endonuclease activity has occurred, even in off-target sites that appear at a 0.1% frequency (Tsai et. al, 2015). This method is successful because of dsODN's ability to associate with DSB's at a high level, but limited in that it requires a high plasmid transfection rate, which is not achievable in all cell types.

4 – *LAM-HTGTS*

Linear amplification mediated PCR-based high-throughput genome-wide translocation sequencing (LAM-HTGTS) is an unbiased DSB detection method that uses the identification of chromosomal translocation that sometimes occurs when two ends of a DSB fuse. The junctions of these fusion sites are cloned using LAM-PCR, attached to adaptor sequences, amplified, and then stored. A growing library of these junctions allows a bioinformatic approach that allows identification of off-target sites by predicting where translocations will occur. This

method's limitations lie in the scarcity of translocation events – not every DSB “nick” will fuse with another, leaving some off-target sites to go undetected (Frock et. al, 2015).

5 – Whole Genome Sequencing (WGS)

Because molecular geneticists now have the ability to sequence the entire genome of a cell, off-target DSB analysis can be performed by simply sequencing the genome of the target cell before and after editing by CRISPR/Cas, and looking for mutation outside the intended locus. This method is useful in single cells, clones, and F1 genome-edited animals, but lacks the specificity of other methods like GUIDE-seq (Smith et. al, 2014).

In the context of microorganisms or cell cultures where controlling the production of mutants is difficult, it becomes important to screen for mutant clones. Therefore, the discovery of new mechanisms for mutation screening has become the focus for several researchers. For example, a high-throughput screening strategy that allows for analysis of up to 96 clones at a time has been shown to effectively identify mutations in the homeobox gene *Evx1* in mouse embryonic stem cells through next-generation sequencing (Bell et. al, 2014). This type of strategy is faster, less laborious, and more cost effective than previous methods, and shows promise in the generation of transgenic organisms as well as checking the accuracy of CRISPR/Cas homology directed repair.

An interesting strategy for the control of transgenic organisms is the use of CRISPR to target CRISPR/Cas-made mutations. This strategy, called DNA interference (DNAi) uses the type I-E CRISPR system derived from *Escherichia coli* to target modified DNA elements, and can be toggled on and off by addition of a sugar specified by the researcher. The system acts as a “kill switch” capable of shutting off CRISPR/Cas-induced genetic effects at any desired time, and is seen to have many applications in biotechnology (Caliando et. al, 2015).

Risk Assessment Strategy in CRISPR Transgenic Organisms

Risk assessment studies are often carried out in agricultural contexts to prevent transgenic crops from harming non-target organisms (Raybould et. al, 2010). However, the risk assessment approach is quite easily transferrable to other practices as well. Accurate, powerful gene editing tools like CRISPR/Cas serve as excellent candidates for risk assessment efforts, due its large societal and scientific implications. It is imperative that researchers use CRISPR/Cas with the highest degree of precision possible, in order to minimize unwanted off-target genetic mutation, the creation of mosaic embryos, or the creation of hazardous cell lines. A large body of research, as partially described in this introduction, aims to describe safe practices for the use of CRISPR/Cas through bioinformatics and molecular tools that can screen for mutations. It is the goal of this study to describe the risk assessment efforts being taken by current scientists in the field using CRISPR/Cas for their research and identify areas of improvement in the best interest of the proliferation of the tool.

Article Selection

Recently published experimental studies using CRISPR/Cas for gene editing from various journals and various fields were chosen for risk assessment analysis. Because risk assessment efforts depend on how well understood the genome of a particular model organism is, several model organisms are represented. Researchers' strategies were broadly considered as being predictive, preventative, or screening.

Results and Discussion

The risk assessment strategies taken by the researchers involved in the selected studies were documented, and are described in the following paragraphs. Applicable strategies are those described previously, including efforts to predict off target activity through bioinformatics, modify gRNA's to modulate off-target effects, modify Cas9 expression, and screen for mutants or mosaics after gene editing.

Use of CRISPR/Cas9 editing in mammals is particularly relevant to the ongoing ethical debate over the use of gene editing technologies, as we approach its spread to human embryos. Linlin Zhang et. al used CRISPR/Cas9 to generate a transgenic mouse line to use in disease treatment research. During the course of their study, they discovered that by injecting only sgRNA into single-cell-stage mouse embryos, rather than sgRNA and Cas9 mRNA (standard procedure), mosaicism could be reduced significantly. Injecting early-stage in this manner means that subsequent cell divisions will hand down CRISPR/Cas9 components to daughter cells, increasing the efficiency of the process, and at the same time creating embryos whose cells all share the same expression of genetically modified loci (Zhang et. al, 2016). The study's use of

CRISPR/Cas9 was low risk in that three distinct steps were taken to predict and mitigate potential damage: off-target analysis, analysis of mosaicism, and analysis of off-target effects. An open tool called SeqMap was used to predict 16 potential off-target sites, all of which were subjected to PCR amplification and T7EN1 assay. The PCR products were cloned and analyzed for off-target effects. Analysis of mosaicism showed a lower allele complexity in sgRNA injection only mice compared to control mice, resulting in less mosaic embryos, allowing for a shorter breeding schedule (mosaicism retards mouse breeding). Yani Zhang et. al conducted a similar study, using CRISPR/Cas9 to create transgenic cell lines in the domestic chicken *Gallus gallus* to study the effects of the gene *Stra8* on embryonic stem cell differentiation (Zhang et. al, 2017). In contrast, in this study, the only mention of risk assessment strategy is one sentence describing the use of whole-genome searching to find potential off-target cleavage sites. The exact bioinformatic program is not listed, nor are any efforts to screen for DSB's at those potential off-target sites (say, by PCR amplification and T7EN1 assay, for example) described. It is concerning that transgenic chicken cell lines are being created that likely have unknown mutations at whichever sequences might mimic the loci targeted by the study's three constructed gRNA's. Certainly, if *Gallus gallus* embryos were to be transfected with CRISPR/Cas9 plasmids containing these gRNA's, more precautionary steps should be taken to prevent off target DSB's and mosaicism.

The creation of genetically modified food crops and plants has long been the interest of geneticists and agricultural scientists (Ghanian et. al, 2016). Introduction of CRISPR/Cas to this field has proven very useful, spawning many efforts to use it to create transgenic plants with a higher level of precision in as many species as possible.

For example, Chandrasekaran et. al use CRISPR/Cas9 to develop virus resistance in the cucumber *Cucumis sativus* by disrupting the eIF4E gene (Chandrasekaran et. al, 2016). As should be expected, the researchers used bioinformatics software (CRISPR-P) to determine potential off-site DSB's, and followed that prediction with PCR amplification and sequencing at those sites. Luckily, no off-target activity was found, eliminating the need for screening for mutant cells. In fact, Peterson et. al describe how off-target effects are typically lower when using CRISPR/Cas9 in non-plant systems, which may help explain some apparent gaps in risk assessment effort in plant system studies (Peterson et. al, 2016). Consistent with this notion, Qi et. al introduced a tRNA-based multiplex editing system (wherein multiple gRNA's are encoded by one plasmid, previously demonstrated in rice) to maize, and provide no off-target analysis despite introducing mutations in multiple genes (Qi et. al, 2016).

Hopes et. al applied CRISPR/Cas to the prokaryotic diatom *Thalassiosira pseudonana* to edit the urease gene as a proof of concept study. Controlling DSB's becomes important with a model organism that reproduces so quickly, and the researchers meet this need through bioinformatic prediction of off-target activity. A nucleotide BLAST search was employed, and resulting homologous sequences were searched for nearby PAM sequences at the 3' end. In addition, the researchers also used the CasOT program to identify potential off target sites, effectively doubling their efforts to predict off-target DSB's (Hopes et. al, 2016). The risk assessment analyses of all studies were categorized into predictive, preventative, and screening categories, and are summarized below (**Table 1**).

Table 1 summarizes risk assessment efforts undertaken by researchers in several representative studies using CRISPR/Cas for gene editing.

Title	Authors	Year	Journal	Model Organism	Risk Assessment Strategy		
					Predictive	Preventative	Screening
Generation of an Oocyte-Specific Cas9 Transgenic Mouse for Genome Editing	Zhang et. al	2016	Public Library of Science	Mouse	X	X	X
CRISPR/Cas9 Mediated Chicken Stra8 Gene Knockout and Inhibition of Male Germ Cell Differentiation	Zhang et. al	2017	Public Library of Science	Domestic Chicken	X		X
Development of Broad Virus Resistance in Non-Transgenic Cucumber Using CRISPR/Cas9 Technology	Chandrasekaran et. al	2016	Molecular Plant Pathology	Cucumber	X		X
Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression	Qi et. al	2016	Cell	Maize	X		X
Editing of the Urease Gene by CRISPR-Cas in the Diatom <i>Thalassiosira pseudonana</i>	Hopes et. al	2016	Plant Methods	Diatom	X		X

Overall, as expected, researchers using CRISPR/Cas for gene editing followed guidelines for safe use of the technology, with the *Gallus gallus* study by Zhang et. al being an important exception. However, most efforts being taken by those studies are focusing on *prediction* and *screening*, and not *prevention* of off-target activity. The techniques described by Fu et. al and Seung et. al to truncate or add guanine residues to gRNA, or to limit the expression of the Cas9 nuclease as described by Pattanayak et. al are techniques that should be put to good use. The absence of these strategies in experimental studies is likely attributable to the fact that they are relatively new, and because of this, in the near future they should be seen more and more. Because of CRISPR/Cas' ability to very faithfully induce DSB's in the DNA of many model organisms, it is very important to control the exact sites of those breaks. The techniques summarized in this paper could be crucial in achieving this, and their use is highly recommended in CRISPR/Cas gene editing projects. Using CRISPR/Cas in the most efficient, most precise way will help advance our understanding and application of the

tool, leading to its use in disease treatment in humans more quickly, which is arguably its most relevant and exciting use.

Conclusions

CRISPR/Cas, a gene editing tool derived from an antiviral response in bacteria and modified for use in eukaryotes, has proven to be a powerful and precise gene editing tool to add to the molecular biologist's tool chest. However, the specificity of the Cas nuclease is such that off-target double strand breaks are often introduced, creating a need for strategies to prevent, predict, and screen for these unwanted mutations. Bioinformatic software, gRNA modification, and post-edit mutation screening have all shown to be useful tactics. The goal of this study was to analyze these risk assessment efforts in several representative studies, highlighting the need for improvement where necessary. For the most part, risk assessment efforts were adequate for safe use of the tool, with several important exceptions. However, researchers using CRISPR/Cas tend to focus on prediction of and screening for off-target cleavage, and often fail to take efforts to prevent those double-strand breaks.

References

- Adee, Sally. "Will the US and China Face off over CRISPR?." *New Scientist*, vol. 232, no. 31031, 26 Nov. 2016, p. 21. EBSCOhost, waterfield.murraystate.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=119687559&login.asp&site=ehost-live&scope=site.
- Belhaj, Khaoula, Angela Chaparro-Garcia, Sophien Kamoun, and Vladimir Nekrasov. "Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system." *Plant Methods* 9.1 (2013): n. pag. Web.
- Bell, Charles C., et al. "A High-Throughput Screening Strategy for Detecting CRISPR-Cas9 Induced Mutations Using Next-Generation Sequencing." *BMC Genomics*, vol. 15, no. 1, Dec. 2014, pp. 1-15. EBSCOhost, doi:10.1186/1471-2164-15-1002.
- Caliando, Brian J., and Christopher A. Voigt. "Targeted DNA degradation using a CRISPR device stably carried in the host genome." *Nature Communications* 6 (2015): 6989. Web.
- Chandrasekaran, Jeyabharathy, et al. "Development of Broad Virus Resistance in Non-Transgenic Cucumber Using CRISPR/Cas9 Technology." *Molecular Plant Pathology*, vol. 17, no. 7, Sept. 2016, pp. 1140-1153. EBSCOhost, doi:10.1111/mpp.12375.
- Dow, Lukas E., Jonathan Fisher, Kevin P. O'rourke, Ashlesha Muley, Edward R. Kasthuber, Geulah Livshits, Darjus F. Tschaharganeh, Nicholas D. Socci, and Scott W. Lowe. "Inducible in vivo genome editing with CRISPR-Cas9." *Nature Biotechnology* 33.4 (2015): 390-94. Web.
- Frock, R.L.; Hu, J.; Meyers, R.M.; Ho, Y.J.; Kii, E.; Alt, F.W. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat. Biotechnol.* 2015, 33, 179–186.
- Fu, Yanfang, et al. "Improving CRISPR-Cas Nuclease Specificity Using Truncated Guide Rnas." *Nature Biotechnology*, vol. 32, no. 3, Mar. 2014, pp. 279-284. EBSCOhost, doi:10.1038/nbt.2808.
- Ghanian, Mansour, et al. "Attitudes of Agricultural Experts toward Genetically Modified Crops: A Case Study in Southwest Iran." *Science & Engineering Ethics*, vol. 22, no. 2, Apr. 2016, pp. 509-524. EBSCOhost, doi:10.1007/s11948-015-9653-1.
- Gilbert, Luke A., Max A. Horlbeck, Britt Adamson, Jacqueline E. Villalta, Yuwen Chen, Evan H. Whitehead, Carla Guimaraes, Barbara Panning, Hidde L. Ploegh, Michael C. Bassik, Lei S. Qi, Martin Kampmann, and Jonathan S. Weissman.

- "Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation." *Cell* 159.3 (2014): 647-61. Web.
- Hilton, Isaac B., Anthony M. D'ippolito, Christopher M. Vockley, Pratiksha I. Thakore, Gregory E. Crawford, Timothy E. Reddy, and Charles A. Gersbach. "Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers." *Nature Biotechnology* 33.5 (2015): 510-17. Web.
- Hopes, Amanda, et al. "Editing of the Urease Gene by CRISPR-Cas in the Diatom *Thalassiosira Pseudonana*." *Plant Methods*, vol. 12, 24 Nov. 2016, pp. 1-12. EBSCOhost, doi:10.1186/s13007-016-0148-0.
- Hsu, Patrick D., David A. Scott, Joshua A. Weinstein, F. Ann Ran, Silvana Konermann, Vineeta Agarwala, Yinqing Li, Eli J. Fine, Xuebing Wu, Ophir Shalem, Thomas J. Cradick, Luciano A. Marraffini, Gang Bao, and Feng Zhang. "DNA targeting specificity of RNA-guided Cas9 nucleases." *Nature Biotechnology* 31.9 (2013): 827-32. Web.
- Kazana, Vassiliki, et al. "Public Attitudes Towards the Use of Transgenic Forest Trees: A Crosscountry Pilot Survey." *Iforest - Biogeosciences & Forestry*, vol. 9, no. 2, Apr. 2016, pp. e1-e10. EBSCOhost, doi:10.3832/ifor1441-008.
- Liang, P., Xu, Y., Zhang, X. et al. *Protein Cell* (2015) 6: 363. doi:10.1007/s13238-015-0153-5
- Martin, Francisco, et al. "Biased and Unbiased Methods for the Detection of Off-Target Cleavage by CRISPR/Cas9: An Overview." *International Journal of Molecular Sciences*, vol. 17, no. 9, Sept. 2016, pp. 1-9. EBSCOhost, doi:10.3390/ijms17091507.
- Pattanayak, Vikram, et al. "High-Throughput Profiling of Off-Target DNA Cleavage Reveals RNA-Programmed Cas9 Nuclease Specificity." *Nature Biotechnology*, vol. 31, no. 9, Sept. 2013, pp. 839-843. EBSCOhost, doi:10.1038/nbt.2673.
- Peterson, Brenda A., et al. "Genome-Wide Assessment of Efficiency and Specificity in CRISPR/Cas9 Mediated Multiple Site Targeting in *Arabidopsis*." *Plos ONE*, vol. 11, no. 9, 13 Sept. 2016, pp. 1-11. EBSCOhost, doi:10.1371/journal.pone.0162169.
- Polstein, Lauren R., and Charles A. Gersbach. "A light-inducible CRISPR-Cas9 system for control of endogenous gene activation." *Nature Chemical Biology* 11.3 (2015): 198-200. Web.
- Qi, Lei S., Matthew H. Larson, Luke A. Gilbert, Jennifer A. Doudna, Jonathan S. Weissman, Adam P. Arkin, and Wendell A. Lim. "Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression." *Cell* 152.5 (2013): 1173-183. Web.

- Raybould, A., et al. "Ecological Risk Assessments For Transgenic Crops With Combined Insect-Resistance Traits: The Example Of Bt11 × MIR604 Maize." *Journal Of Applied Entomology* 136.1/2 (2012): 27-37. *Academic Search Complete*. Web. 6 Feb. 2017.
- Seung Woo, Cho, et al. "Analysis of Off-Target Effects of CRISPR/Cas-Derived RNA-Guided Endonucleases and Nickases." *Genome Research*, vol. 24, no. 1, Jan. 2014, p. 2. EBSCOhost, doi:10.1101/gr.162339.113.
- Smith, C.; Gore, A.; Yan, W.; Abalde-Atristain, L.; Li, Z.; He, C.; Wang, Y.; Brodsky, R.A.; Zhang, K.; Cheng, L.; et al. Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. *Cell Stem Cell* **2014**, 15, 12–13.
- Tang, L., Zeng, Y., Du, H. et al. *Mol Genet Genomics* (2017). doi:10.1007/s00438-017-1299-z
- Teytelman, L., D. M. Thurtle, J. Rine, and A. Van Oudenaarden. "Highly expressed loci are vulnerable to misleading ChIP localization of multiple unrelated proteins." *Proceedings of the National Academy of Sciences* 110.46 (2013): 18602-8607. Web.
- Tsai, S.Q.; Zheng, Z.; Nguyen, N.T.; Liebers, M.; Topkar, V.V.; Thapar, V.; Wyvekens, N.; Khayter, C.; Iafrate, A.J.; Le, L.P.; et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* **2015**, 33, 187–197.
- Wang, X.; Wang, Y.; Wu, X.; Wang, J.; Qiu, Z.; Chang, T.; Huang, H.; Lin, R.J.; Yee, J.K. Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENS using integrase-defective lentiviral vectors. *Nat. Biotechnol.* **2015**, 33, 175–178.
- Zetsche, Bernd, Jonathan S. Gootenberg, Omar O. Abudayyeh, Ian M. Slaymaker, Kira S. Makarova, Patrick Essletzbichler, Sara E. Volz, Julia Joung, John Van Der Oost, Aviv Regev, Eugene V. Koonin, and Feng Zhang. "Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System." *Cell* 163.3 (2015): 759-71. Web.
- Zhang, Linlin, et al. "Generation of an Oocyte-Specific Cas9 Transgenic Mouse for Genome Editing." *Plos ONE*, vol. 11, no. 4, 27 Apr. 2016, pp. 1-10. EBSCOhost, doi:10.1371/journal.pone.0154364.
- Zhang, Yani, et al. "CRISPR/Cas9 Mediated Chicken Stra8 Gene Knockout and Inhibition of Male Germ Cell Differentiation." *Plos ONE*, vol. 12, no. 2, 24 Feb. 2017, pp. 1-12. EBSCOhost, doi:10.1371/journal.pone.0172207.