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Literature Review

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Evaluating CRISPR-Cas Nuclease Suitability for Human Applications

CRISPR (clustered, regularly interspaced, short palindromic repeat) is a novel genome-editing technique utilizing a class of novel RNA-guided proteins. Previous genome-editing tools have relied on the protein's structure solely in order to identify the location at which to induce mutation, which limited their use as they required significant redesign to target different locations. CRISPR proteins bind to RNA and search the DNA for analogous sections, which then become the modification sites. With continued refinement, CRISPR-based methods could offer an easier alternative to the older, fully protein-based genome editing methods, but require further improvements in specificity compared to traditional methods to see widespread medical use.

One academic article finds that CRISPR nucleases may be active on imperfectly-matched RNA-DNA interfaces, manifesting in inaccurate alterations. (Fu et al. 822) In a paper published in Nature Biotechnology in June 2013, they sought to measure the loss of added fluorescence signal from human cells in order to "[assess] the effects of systematically mismatching various positions within multiple gRNA–target DNA interfaces." (Fu et al. 823) Their results showed that mutation at off-target locations was very high, averaging 40% of the mutation rate obtained at the proper location. (Fu et al. 825) Additionally, the tolerance to mutations proved variable to the point of skewing the results due to the site-specific nature of the surrounding DNA. (Fu et al. 824) Overall, the authors thought that the CRISPR nucleases were highly active off-site, which could "confound their use in research and therapeutic applications." (Fu et al. 822) This further lends to a scientific consensus opinion of inaccurate results from CRISPR-Cas systems owing to lack of refinement.

Later that year, researchers sought to lower the rate of off-site CRISPR activity, culminating in a November 2013 paper in which S. W. Cho et al. managed to "[reduce] below the detection limits of deep sequencing by choosing unique target sequences in the genome and modifying both guide RNA and Cas9." (Cho et al. 132) Off-site mutations that were two base pairs off of the active site were able to avoid off-target sites that were just two base pairs off, despite seeing higher rates of mutation at off-target sites that were a single base pair off. (Cho et al. 138) They concluded that their technique of picking unique target sites without any homologous sequences in the genome was a good method of avoiding or minimizing off-target effects from CRISPR nucleases. (Cho et al. 139) This comes with a caveat, given as follows: "We cannot rule out the possibility that the 11 RGENs we created in this study induce off-target mutations at sites not examined here, which could be revealed by deep sequencing at other less-homologous candidate sites or by whole genome sequencing." (Cho et al. 139)

Whole genome sequencing with CRISPR would see progress the following year. One set of researchers attempted to use CRISPR-Cas9 to perform knockout testing on the genome-scale. Genome-scale CRISPR-Cas9 knockout (GeCKO) screening was Utilizing CRISPR in this way allowed Shalem et al. to supplant the previously widespread RNA interference method, whose "utility is limited by the inherent incompleteness of protein depletion by RNAi and confounding off-target effects (6, 7)."(84) The screening was also performed via contemporary methods as a control. Consistency measured with two methods: P values for the top 100 hits via the RNAi Gene Enrichment Ranking algorithm (RIGER), and a more detailed comparative analysis for the top 10 genes: "First, we plotted the P values for the top 100 hits using either RIGER (Fig. 4A) or redundant siRNA activity (RSA) (fig. S9) scoring. Lower P values for the GeCKO versus shRNA screen indicate better scoring consistency among sgRNAs. Second, for the top 10 RIGER hit

genes, 78 ± 27% of sgRNAs targeting each gene ranked among the top 5% of enriched sgRNAs, whereas 20 ± 12% of shRNAs targeting each gene ranked among the top 5% of enriched shRNAs (Fig. 4B).” (Shalem et al. 86) The CRISPR screening suffered from lower consistency in both measures. It’s important to note that the GeCKO screening differed mechanistically from RNAi, as explained by Shalem et al.: “GeCKO screening provides a mechanistically distinct method from RNAi for systematic perturbation of gene function. Whereas RNAi reduces protein expression by targeting RNA, GeCKO introduces loss-of-function mutations into genomic DNA.” (86) A mechanistic difference can mean the introduction of a wide variety of confounding variables, depending on how well the mechanism is understood, which would need to be further analyzed and tested to account for their interference. However, Shalem et al. state in the article that CRISPR has potential applications across many genomic elements that are not possible through RNAi such as promoters, enhancers, introns, and intergenic regions (86), and as such, while their CRISPR procedure yielded less consistency than the RNAi procedure, Shalem et al. explain than any success at all shows that CRISPR is worthy of further research to uncover its full potential: “In the GeCKO screens presented here, the efficiency of complete knockout, the consistency of distinct sgRNAs, and the high validation rate for top screen hits demonstrate the potential of Cas9:sgRNA-based technology to transform functional genomics.” (87) Thus, the work of Shalem et al. demonstrates a basic proof-of-concept demonstration of genome-scale use of CRISPR.

Refinement of CRISPR-Cas methods continued to see progress in 2016. In an article by Kleinstiver et al., a novel CRISPR-Cas component was designed, “SpCas9-HF1, a high-fidelity variant harbouring alterations designed to reduce non-specific DNA contacts.” (490) This variant was tested first for accuracy at on-function sites, via a control set up performing the same

modifications on the same target sites, but using SpCas9, a more conventional Cas component. “Overall, SpCas9-HF1 possesses comparable activities (greater than 70% of wild-type SpCas9 activities) for 86% (32/37) of the sgRNAs we tested.” (Kleinstiver et al. 491) Then, testing was done to consider the off-target effects of the SpCas9-HF1, as to compare them to the standard SpCas9. The researchers found that while SpCas9 acted on 2-25 off-target sites on seven of the eight samples (the last of which being a control sample chosen for its low likelihood of SpCas9 errors), SpCas9-HF1 produced only one such error of eight, and matched the control sample activity. “The SpCas9-HF1 variant characterized in this report reduces all or nearly all genome-wide off-target effects to undetectable levels as judged by GUIDE-seq and targeted next-generation sequencing, with the most robust and consistent effects observed with sgRNAs designed against standard, non-repetitive target sequences.” (Kleinstiver et al. 493) The article expresses that these results indicate a potential refinement in CRISPR-Cas specificity, as part of a suite of orthologous tools to be chosen from depending on which was least likely to generate errors for the specific alterations to be made: “Overall, our results demonstrate that the approach of mutating non-specific DNA contacts is highly effective at increasing SpCas9 specificity and suggest it might be extended to other naturally occurring and engineered Cas9 orthologues [38, 39, 40, 41, 42], as well as other CRISPR-associated nucleases [43, 44].” (Kleinstiver et al. 494) Unfortunately, the orthologues appear not to have been explored in further papers whatsoever, meaning that the proposed SpCas9-HFx suite remains open to further experimentation.

Most recently, a group of researchers have used CRISPR-Cas to research macroautophagy in humans. Macroautophagy is an important process in the cell involved in many processes, as O’Prey et al. explains its potential in many different fields of research: “Macroautophagy (hereafter referred to as autophagy) is one of the main processes involved in

the degradation and turnover of cytoplasmic molecules and organelles within cells (Rosenfeldt & Ryan, 2011)… Autophagy regulates a variety of cellular processes such as cell survival, metabolism, motility, and death, and has been shown to play vital roles in both normal physiology and diseased settings (Choi et al., 2013 ; Ravikumar et al., 2010). [Autophagy is involved in] many human conditions including aging, inflammation, cancer, and neurodegenerative disorders (Jiang and Mizushima, 2014 ; Rubinsztein et al., 2011) [.]" (O'Prey et al. 80) A new method to study autophagy with CRISPR was tested, with CRISPR targeting the autophagy-inducing ATG genes. The authors claim a qualitative improvement, with a figure depicting markers for ATG5-12 showing a marked decrease from the control sample. (O'Prey et al. 88) The authors continue in the discussion to theorize ways to further increase specificity, a primary concern for their research. They discuss possible modifications to achieve greater specificity: "One of the main concerns of the CRISPR/Cas9 system, which is also an issue common with many techniques, is the possibility of off-target activity… Modified versions of Cas9, which have been reported to exhibit higher specificity, are also currently available. These include the Cas9 D10A mutant (or Cas9 Nickase) with a mutation at the HNH nuclease domain of Cas9 (Ran et al., 2013), the eSpCas9 mutants with alanine substitutions within the HNH/RuvC groove (Slaymaker et al., 2016), and the Cas9-HF1 (Cas9-High-fidelity version 1) with four base substitutions (N497, R661, Q695, Q926) shown to reduce nonspecific DNA contact (Kleinstiver et al., 2016)." (O'Prey et al. 102) Interesting to note is the suggestion to utilize the Cas9-HF1 designed by Kleinstiver et al.- this promotes a more confident scientific consensus in the HF family researched by Kleinstiver et al.

For CRISPR-Cas to see practical human application, it is necessary for further improvements to specificity. (O'Prey, et al. 80) It is clear to see from the research that CRISPR

started with very low specificity, as per Fu et al., but has seen consistent continual improvement over the recent years since. The general direction of research in this field appears to be toward further refinement and eventual medicinal use in humans, with no papers found posing fundamental problems to the use of CRISPR-Cas in humans. The next step seems to be in the direction of further research into the high-fidelity suite of Cas9 variants as per Kleinstiver, so as to be able to choose the most appropriate variant for most specificity for the change desired, as site-specific differences can lead to varying specificity. (Fu et al. 824)

Works Cited

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