December 10, 2018

Division of Dockets Management
(HFA-305)
Food and Drug Administration
5630 Fishers Lane, Rm. 1061
Rockville, MD 20852

Re: **Docket No. FDA-2008-D-0205: Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)**

Dear Dr. Sir/Madam,

The Innovative Genomics Institute (IGI) is pleased to submit the attached comments in response to the issuance by the Food and Drug Administration ("FDA") of a request for comments in its Federal Register Notice entitled "Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)" (hereinafter, the "Draft Guidance"). We are grateful to the FDA for preparing a Draft Guidance that addresses products created by Gene Editing (GE) technologies, and appreciate the opportunity to contribute our expertise to the regulation of these therapies.

The Innovative Genomics Institute (IGI) is a non-profit, academic research organization formed through a partnership between the University of California, Berkeley and the University of California, San Francisco, two of the world's leading scientific research institutions. After co-inventing CRISPR-based systems for rewriting DNA, Jennifer Doudna founded the IGI to bring together researchers in diverse disciplines with a powerful combined expertise in order to apply this technology to address some of humanity's greatest problems. In addition to our efforts in the life sciences, the IGI is committed to advancing scholarship on the ethical, legal, and social impacts of this transformational technology.

We have approached these comments on the FDA's Draft Guidance in a similar interdisciplinary vein. What follows is the result of a multi-disciplinary discussion among bench researchers, ethicists, legal professionals, and industry scientists. However, because IGI's expertise is primarily in CRISPR-based genome editing technology, we limit our discussion to CRISPR-based therapies.

We appreciate your consideration and are happy to discuss further if desired.

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Brief Summary / Outline of Comments

The Draft Guidance refers primarily to conventional Gene Therapy drug substances (DS) and Drug Products (DP) with only occasional references to gene editing technologies or ex vivo genetically modified cells. We ask the FDA to provide more specific and tailored guidance and recommendations for drug substances and products created by GE technologies, particularly those therapeutics that are created ex vivo using editing reagents that are not intended to persist (for example, purified Cas9 protein in complex with its guide RNA, termed "RNP"). By identifying CMC requirements tuned to the particular risks presented by the drug substances and products created by this GE approach, we believe that the FDA can better assure a demonstration of product safety, identity, quality, purity, and strength (including potency) without imposing undue or unnecessary burdens on sponsors of therapeutics that provide important patient and public health benefits.

We focus our comments on the Draft Guidance on a particular subset of GE therapies - ex vivo RNP edited cells. We chose this subclass for two reasons. First, we expect that many of the early GE therapies will rely on this ex vivo approach. Second, we think that this ex vivo approach highlights the limitations inherent in regulating GE therapies based on conventional GT technology.

Summary of Key Suggestions: For the case of GE undertaken ex vivo using RNP introduced through non-viral delivery:

- the FDA should state in the Guidance that the Drug Substance (which would have to be manufactured under cGMP) is the edited cells and the Drug Product is those cells re-formulated in injection buffer for delivery to the study subject or patient.

- The guide RNA and Cas9 protein do not persist and are not intended to be part of the final drug product. They should be considered critical reagents that the FDA should accept upon a rigorous Certificate of Analysis (CoA) for high quality research grade. The FDA should state that they
    - do not have to be produced cGMP, and
    - do not require Batch Record
    - do not have to be clinical grade.

cGMP, Batch Record and clinical grade requirements will unnecessarily increase sponsor costs and delay clinical testing without materially increasing safety to the study subjects or patients since these reagents do not persist and are not intended to be part of the drug substance.

- The Guidance should specifically address approaches that can be used to validate critical reagents in ex vivo RNP GE. For example, use of mass spectrometry (MS) for synthetic, non-viral, animal free, pure reagents should be sufficient to demonstrate identity and purity.

- An important additional characteristic of CRISPR-based gene editing technologies is the modularity of the reagents. We suggest that the FDA consider including guidance that can help sponsors best maximize this feature of emerging gene editing therapies. For example, discussion of where sponsors can leverage existing data or cross reference previous INDs when using the same manufacturing processes and many of the same constant modules of the reagents, such as Cas9 would be helpful.

- The FDA should also consider the feasibility and utility of preparing a separate guidance document to address the CMC information necessary to be provided for INDs for therapeutics created by GE.

Background

A majority of the concerns from conventional GT stem from the presence of a transgene and the non-consistent, semi-random nature of the modification to the genome, particularly for those GT that use integrating viruses. In contrast, GE therapies often don't derive their therapeutic effect from a transgene, and have highly precise, targeted changes to the genome. In addition to risks of insertional mutagenesis, conventional GT requires the presence of a transgene to lend a therapeutic effect. Thus, even for those gene therapies that use non-

integrating viruses, the reliance on a transgene means that GT presents risks of genotoxicity stemming from over or under expression of the transgene, particularly when the transgene is a growth factor. In contrast, rather than randomly augmenting cells with a transgene, one of the virtues of gene editing is its ability to directly target an endogenous gene. Even in those cases where the therapeutic effect is derived from gene augmentation, GE allows the augmenting gene to be precisely integrated at an endogenous location, providing consistent and predictable expression levels, and thereby reducing the risk of genotoxicity. Additionally, in contrast to products from conventional GT, even in those cases in which off-target modifications occur with GE, the effects are consistent, non-random, deterministic and can be robustly de-risked

Thus, products created by GE therapies do not exhibit the same risk characteristics as GT products. The FDA has understandably built their framework for risk evaluation on over 20 years of GT experience. GE therapies, however, improve on many of the risks of GT and present a distinct risk profile as illustrated in Tables 1 and 2 in our Appendix.

In addition to the risks lent from the genome editing agents themselves, factors particular to a proposed therapy may modify those risks or contribute additional risks. These factors include route of administration in the patient (systemic, targeted, or ex vivo), persistence of editing reagents (whether the reagents are delivered as RNA, RNP, or DNA), and whether viral vectors are used for delivery. In Figure 1 of our Appendix, we illustrate how these factors combine to create a spectrum of risk for Cas9-based GE therapies. Example a) in Figure 1 represents a GE approach that we expect will create products that present a lower overall risk to the research subject and patient: GE executed ex vivo using RNP introduced through non-viral delivery. We expect many early applications of CRISPR-based genome editing will take this approach. In the following sections, we present our comments on the Draft Guidance by focusing on this type of GE therapy.

Discussion and General Comments

The Draft Guidance provides definitions of Drug Substance (DS), Drug Product (DP), and Reagents.

- A DS is "...defined as an active ingredient that is intended to furnish biological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the human body" (lines 213-216).
- A DP is defined "...as the finished dosage form that contains the DS, generally, but not necessarily in association with one or more other ingredients (e.g., excipients)" (line 217-219). The FDA acknowledges that it can be difficult to determine how different therapies fit these definitions and suggests that sponsors justify how they decide to apply them.

For the example case of ex vivo editing using RNP delivered non-virally (Figure 1a), we suggest that the FDA explicitly confirm in the final Guidance that the DS is the edited cells and the DP is those cells re-formulated in injection buffer for delivery to the patient. The rationale for making this assignment is partly based in the FDA's definition of a reagent. Starting at line 519, the FDA states that "reagents (or ancillary materials) are those materials used for manufacturing (e.g., cell growth, differentiation, selection, purification, or other critical manufacturing steps) that are not intended to be part of the final product". When editing using RNP, the editing agents, Cas9 in complex with guide RNA, do not persist and are not intended to be introduced into patients. Proteases and nucleases act to degrade proteins and RNA in the cell, and thus without the presence of transgenes encoding the editing agents, the Cas9 and guide RNA are degraded and lost to dilution. This has been shown in technology development experiments by multiple labs[reviewed in 1 and 2]. Degradation and dilution of the RNP has many advantages for mitigating risks in gene editing, including reducing the probability of off-target edits as well as temporally confining the immunogenicity risk posed by Cas9. The intent of using RNP is to ensure that Cas9 and guide RNA are not part of the final product that gets administered to patients. Thus, by definition, Cas9 and the guide RNA should be considered "reagents" rather than drug substances.

This definition is also upheld by the biology of the intended drug. In this example of ex vivo RNP editing, the "active ingredient that is intended to furnish" the therapeutic effect to the research subjects and patients is the genetically corrected cell, without the editing agents. Thus, the drug product is that cell reformulated for

infusion into patients. This is in contrast to in vivo GE therapies where the substance introduced into the patient having a direct effect is the editing agents themselves, packaged in a delivery vehicle.

Clarifying this definition also has implications for where sponsors need to focus their efforts on developing cGMP manufacturing or banking. Both of these processes are critical for safety to patients, but also impose substantial issues of scale and cost of drugs.

As the Draft Guidance states, "each DS should be manufactured under appropriate Good Manufacturing Practice (GMP) conditions". We agree that it is critical that the drug substance, which will be part of the drug product, be manufactured under the highest safety standards. As discussed above, in the case of editing using RNP delivered ex vivo, the drug substance is the edited cells, thus the process of editing in culture should be conducted under cGMP.

The guidance is less clear as to the required grade or standard of manufacturing regarding reagents and how this will apply to Cas9 protein and guide RNA. We ask that the FDA clarify its thinking for these reagents and suggest the following as a possible approach.

Reagents such as Cas9 proteins and guide RNAs are non-viral, pure agents. Furthermore, as compared to other reagents such as sera, Cas9 and guide RNAs are not complex mixtures with high variability from batch to batch. Nor are they animal-derived. The guide RNA is often synthesized chemically, avoiding any exposure to animal tissues or microbial agents, limiting the possibility of adventitious biological agents or contaminants.

Rather than imposing cGMP requirements on these reagents, which do not persist and are not intended to be part of the final product, we suggest that the FDA consider accepting these reagents upon a sufficiently rigorous Certificate of Analysis (CoA) for high quality research grade. As purified agents, both the guide RNA and Cas9 protein are readily amenable to testing for the four required characteristics of the critical reagents as identified by the FDA: Identity, Purity, Potency, and Safety. Where manufacturers or sponsors can use a technique such as mass spectrometry to test identity and purity of the critical reagents, additional compliance requirements for manufacturing during early stages of a clinical trials will not likely lead to a material increase in safety to the research subject. Furthermore, when gene editing reagents are able to be applied early in the manufacturing process for the drug substance, the risk of contaminating or adventitious agents is lowered owing to the opportunity for in process testing and dilution of the material through cell expansion.

The FDA acknowledges this balance of risk vs burden during its discussion starting at line 1067: "Process validation studies are generally or typically not required for early stage manufacturing, and thus, most original IND submissions will not include process performance qualification. We recommend that you use early stage manufacturing experience to evaluate the need for process improvements and to support process validation studies in the future." Our suggestion that for early stage clinical trials the FDA consider allowing research grade editing reagents to be accepted upon a rigorous CoA echoes this provision of the Draft Guidance.

An important additional characteristic of CRISPR-based gene editing technologies is the modularity of the reagents. Targeting a new gene for a new indication requires only a change in the sequence of the guide RNA; the Cas protein is constant and unchanged, as is the general structure and function of the guide. This modularity is one of the drivers of the potential for this technology to treat human disease, and provides a real opportunity to create not just safe and effective therapies, but accessible therapies by potentially streamlining the requirements for an IND. It is not clear in the guidance how this modularity might be best leveraged using the current CMC requirements for reagents or drug substances. We suggest that the FDA consider including guidance that can help sponsors best maximize this feature of emerging gene editing therapies. For example, discussion of where sponsors can leverage existing data or cross reference previous INDs when using the same manufacturing processes and many of the same constant reagents, such as Cas9 would be helpful.

Specific Comments

Line Reference	Current Content	Discussion or Suggested Changes
Lines 52-54	The Draft Guidance states, "Some examples of gene therapy products include nucleic acids, genetically modified microorganisms (e.g., viruses, bacteria, fungi), engineered site-specific nucleases used for human genome editing, and ex vivo genetically modified human cells."	The text suggests that all site-specific nucleases used for human genome editing could be considered DP. It should be revised to clarify that this treatment only applies to such nucleases used in vivo and not those used ex vivo.
Line 443ff	"You should describe whether the DS will be formulated into the DP for direct administration or whether it will be formulated for ex vivo genetic modification of cells, as outlined in section IV.B."	This sentence assumes the therapy is classic viral gene therapy, and therefore the DS would be delivered to the cells in vivo or ex vivo. The Guidance should provide that for RNP ex vivo GE, the DS is the edited cells that have been expanded.
Line 443ff	This discussion implies that the vectors should be produced cGMP.	The FDA should clarify that the gRNA and Cas proteins would not fall under this requirement (see below the definition of Reagent)
Lines 506ff	The section requires sponsors to "provide documentation that the materials used for manufacturing meet standards appropriate for their intended use (e.g., test results, certificates of analysis (COAs), package inserts). . . We recommend that you use FDA-approved or cleared or other clinical-grade materials, when they are available."	The FDA should specify that reagents such as Cas proteins that are synthetic, non-viral, animal free, pure agents, and used ex vivo, should require only a COA and not a Batch Record and that these reagents do not have to be clinical grade. Batch Record requirements will unnecessarily increase sponsor costs and delay clinical testing because these reagents do not persist and are not intended to be part of the drug substance. Additionally, their salient characteristics can be sufficiently identified through alternative means as described below.
Lines 512ff	The Guidance makes the generalized statement that "If the material is not FDA-approved or cleared (or in the absence of recognized standards), additional information on the manufacturing and/or testing may be needed to evaluate the safety and quality of the material. The extent of testing will depend on the specific material and the manner in which it is used in the manufacturing process."	The Guidance should specifically address approaches that can be used to validate critical reagents in ex vivo RNP GE. For example, use of mass spectrometry (MS) for synthetic, non-viral, animal free, pure reagents should be sufficient to demonstrate identity and purity.

Line 519ff (Definition of Reagent)	This section states that "For purpose of this guidance, reagents (or ancillary materials) are those materials used for manufacturing (e.g., cell growth, differentiation, selection, purification, or other critical manufacturing steps) that are not intended to be part of the final product."	The FDA should explicitly include in this definition the gRNA and Cas proteins used in ex vivo GE. In addition, it would be beneficial for the Guidance to include a section that specifically addresses key production, handling and documentation requirements for these reagents. We address some of the matters that we recommend including in such a separate section in the comments that follow. The Guidance should include a specific statement that gRNA and Cas proteins used in ex vivo GE that meet the requirements of the definition of reagents do not have to be cGMP. These reagents are a critical starting material, which should be well characterized and appropriately controlled but not to the same extent as a Drug Substance.
Lines 724ff	"A banking system improves control and consistency in the manufacturing of many biologics. Banking assures an adequate supply of equivalent, well-characterized material for production over the expected lifetime of production. For these reasons, banked materials are a common starting point for many routine production applications."	Where the guidance discusses requirements for plasmids, we infer that the FDA is referring to plasmids used to make viral vectors, the sequences of which will become a persistent part of the drug product. For applications of GE that use RNP ex vivo, plasmids are used to make the reagents but do not become part of the drug substance. Confirmation by the sponsor of the identity and purity of the plasmids used to express the Cas9, coupled with quality controls on the purified protein that include identity and purity tests such as mass spectrometry, are capable of identifying contaminating proteins produced from contaminating plasmid sequences. We ask that the FDA specify in the guidance that plasmids used to make reagents do not have to be made and obtained from banking sources that comply with the banking requirements of Section V.A.2.c.vii-x.

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Appendix

Table 1. Gene Editing Offers Improvement on Many Risks Presented by Conventional Gene Therapy

	Classic Gene Therapy		**Gene Editing**	
	Risk/Safety Issue	**Description**	**Risk/Safety Improvement**	**Continued Risk/Safety Concern**
1	Non-consistent modification of the genome	For integrating vectors ● Each batch of transduced cells will have a different distribution of insertion sites, leading to variability of the intended genetic modification ● In vivo modification will also contain a distribution of insertion sites and will vary both within the individual and between individuals ● Unpredictable integration sites	Consistent, testable, targeted modifications ● Alleviates the concerns about (semi) random nature of modification in classic gene therapy ● Provides opportunity to gather data about the locus being targeted, as well as the off-target changes, since they are also not random ● Means that different batches of cellular products of editing will be modified in the same target locations, and thus don't require repeated characterization of genetic modification as those that have varying integration sites.	
2	Permanent nature of genetic modification	If using integrating vectors ● The genetic modification is permanent and transmittable along the cell lineage. ● If the modification has associated adverse effects,		● If the on-target edit has unforeseen adverse effects, those will be permanent and transmittable along the cell lineage.

		those effects have the potential to be permanent.		• If an off-target edit is induced, that will also be permanent and transmittable along the cell lineage.
5	Immune response	Immune response to viral components • Serious systemic immune responses have been observed in patients treated with viral vectors. • AAV reduced immune response but still present • Ex vivo applications have reduced this risk • There may also be non-systemic cellular responses to foreign DNA (non-integrated DNA)	• If not using viral delivery in vivo, then avoid risks of viral immune response. • Immune response to CRISPR components may be manageable (see Table 2)	• Potential for immune response to CRISPR components (see table 2). • If delivering GE reagents using viral delivery vectors, then the same immunogenicity risks apply as do the cellular responses to any introduce foreign DNA - thus not an increase in risk from these sources.
6	Insertional mutagenesis	For integrating vectors • insertional mutagenesis leading to misregulation or disruption of a nearby gene • Disruption of oncogenes or proto-oncogenes can lead to cancer	If not using integrating viral delivery of CRISPR components: • Don't run the risk of insertional mutagenesis from viral components or gene product expression cassettes.	
7	Genotoxicity	Genotoxicity from incorrect level of expression of a transgene • Expression of added gene is from a non-endogenous location, so expression levels may not match healthy levels and may vary depending on integration site or regulatory elements used in the expression cassette.	• Reduced risk of genotoxicity from aberrant or incorrect expression levels of the correctional gene since many GE therapies correct at the endogenous locus rather than adding a transgene. For those that do use a transgene in a non-endogenous location, the integration location is specific,	

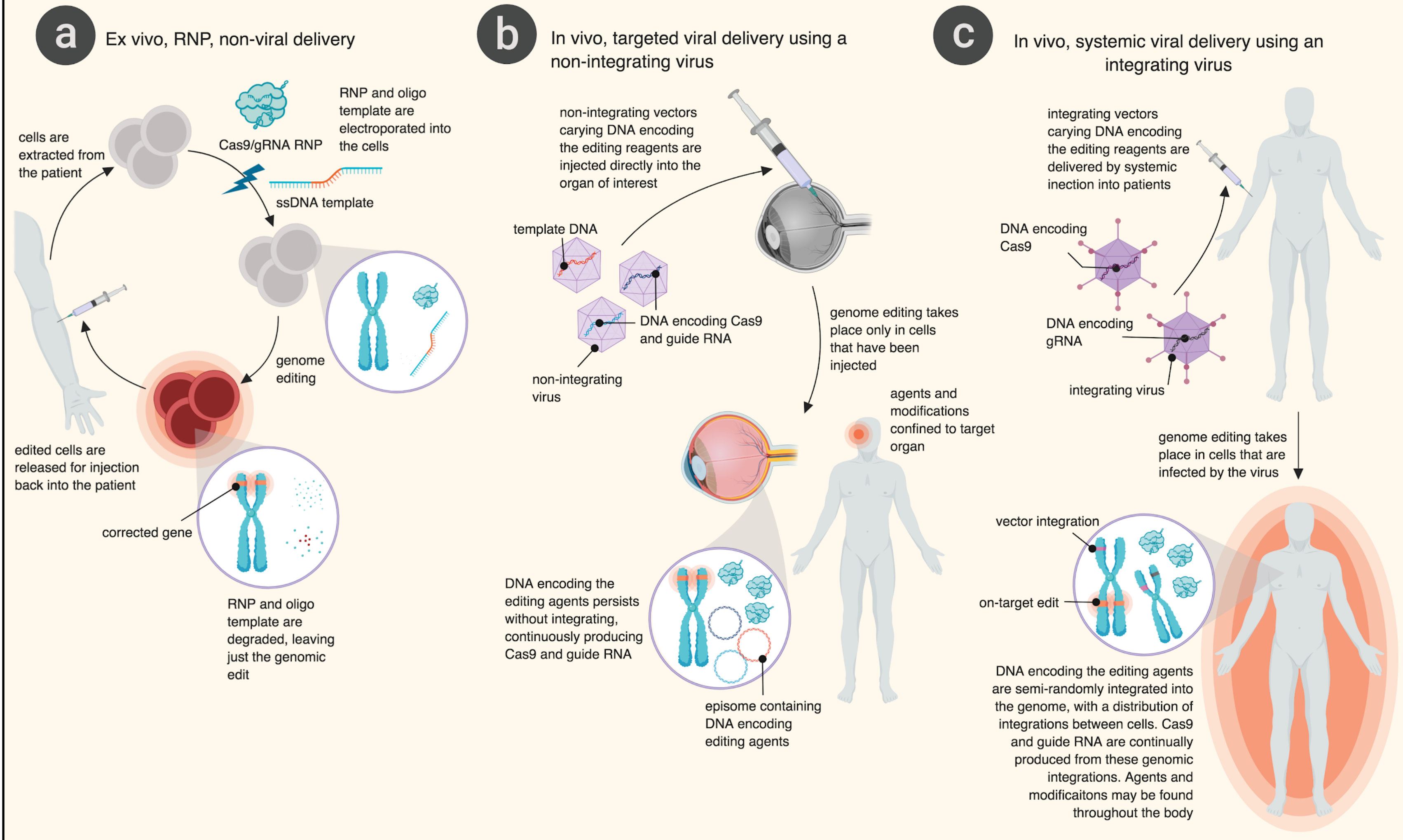
		• Whether the transgene integrates into the genome or not, it is intended to persist and will have a distribution of copy numbers in the patient's cells. Non-consistent copy number may contribute to genotoxicity concerns.	precise, and consistent, and thus expression is well known and pre-determined.	

Table 2. Mechanisms and Solutions for Identified Potential Risks Associated with Gene Editing

	Risk	Mechanism	Solutions
1	Off-target effects	● Indels resulting from off-target cutting. ● Chromosome rearrangements resulting from off-target cutting at more than one site. ● Potential for translocating sections of endogenous DNA or parts of the template DNA due to microhomology. ● All these events have the potential to happen in regions that may adversely affect the expression or structure of a gene that is critical for the patient or that could lead to malignancy.	● Off-target cutting or modification can be mitigated by any one or combination of the following[reviewed in 1, 2, 3, 4]: ○ reducing the exposure of the genome to the editing reagents ■ Delivering the reagents as a pre-assembled ribonucleoprotein (RNP) provides the most transient exposure. These molecules do not persist and are not transmittable along cell lineages. ■ Delivering mRNA encoding Cas along with the guide RNA. These molecules do not persist and are not transmittable along cell lineages. ■ AAV delivery of expression vectors into dividing cells allows the transgene to be diluted out while the modification is transmitted to daughter cells. ■ Using anti-CRISPR molecules delivered shortly after exposure to CRISPR reagents[5]. ● This is a very new area of research ■ Using inducible or conditional promoters when delivering editing reagents as transgenes[6, 7]. Or enabling self inactivation[8]. ○ by increasing the specificity of the enzyme ■ Many groups have engineered the protein and guide RNAs to

			increase the specificity of the enzyme significantly[9, 10, 11, 12] ■ New variants of Cas proteins may provide improved specificity profiles
2	Immune response to CRISPR components	● Cas9 protein[reviewed in 13] ○ Immune response to dosing with Cas9 protein observed in mice[14, 15] ○ Anti-Cas9 antibodies and immune cells observed in untreated humans, suggesting possibility of an immune reaction to a Cas9 therapy[16]. ■ Current evidence of immune recognition of the Cas protein is for the most common form of CRISPR technology - SpyCas9 and saCas9. These Cas9 proteins are taken from species of bacteria that infect humans. This is the likely explanation for why there may be prior antibodies found in humans that haven't been dosed with a Cas9 treatment. ○ Concern that treating a subject once may preclude treating the same subject again. ● Donor DNA ○ has the potential to elicit a foreign DNA response in the cell, but this is true of any therapy that introduces DNA into the cell[17] ● In vitro transcribed RNAs (guide RNAs or mRNA for translating Cas9) ○ Identified as eliciting viral cellular response leading to cell death[18, 19].	● Cas9 Protein ○ Limit exposure to editing reagents (see above) ■ If the treatment uses RNP ex vivo, then immune response to the CRISPR effector protein is not concern because the cells that get re-infused into the patient can be determined to not have the CRISPR editing reagents anymore ○ Take immune suppressors during administration of the editing reagents ■ Same as is done in classic gene therapy since immunogenicity of the viral capsid is still a problem. ○ Use orthogonal CRISPR effector proteins that are not derived from a species that is known to infect humans. This may mitigate the pre-dosing immune presence[20]. ○ Concerns about immunogenicity precluding subjects and patients from receiving Cas9-based therapies in the future are not materially different from any other therapy that induces an immune response, including conventional gene therapy using viruses. ○ A recent publication shows that engineering modification on Cas9 protein can immunosilence the reagent while preserving its function and specificity[21]. ● In vitro transcribed RNAs

			○ this issue has been resolved by using synthetic RNAs or de-phosphorylating IVT RNAs[19, 22].
3	Selection for cells with dysregulated DNA damage response	• p53 is a DNA damage response regulator. The hypothesis that is presented by a couple of recent papers is that for cells to efficiently repair Cas9 induced DSBs, p53 will have to not be working correctly[23, 24]. Thus, successfully edited cells may be cells that have a mutant p53. Mutations in p53 are often part of creating a cancerous cell.	• Importantly, this risk has not been tested. This is a hypothesis based on two studies that did not look at the selection for mutant p53 in their experiments, nor the propensity to produce tumors in animal models. • The cell types used were not clinically relevant. Clinical data from Zinc Finger trials doesn't support this, nor do animal models that have been developed using gene editing. Additionally, Daniel Bauer's group has looked at primary patient cells and doesn't see this selection for defective p53[25]. • This issue is not resolved and should be further studied. Importantly, however, this issue is still hypothetical and the agency should not regulate hypothetical risk.



Increasing Risk
a
Ex vivo, RNP, non-viral delivery
cells are extracted from the patient
Cas9/gRNA RNP
RNP and oligo template are electroporated into the cells
ssDNA template
genome editing
edited cells are released for injection back into the patient
corrected gene
RNP and oligo template are degraded, leaving just the genomic edit
b
In vivo, targeted viral delivery using a non-integrating virus
non-integrating vectors carrying DNA encoding the editing reagents are injected directly into the organ of interest
template DNA
DNA encoding Cas9 and guide RNA
non-integrating virus
genome editing takes place only in cells that have been injected
agents and modifications confined to target organ
DNA encoding the editing agents persists without integrating, continuously producing Cas9 and guide RNA
episome containing DNA encoding editing agents
c
In vivo, systemic viral delivery using an integrating virus
integrating vectors carying DNA encoding the editing reagents are delivered by systemic inection into patients
DNA encoding Cas9
DNA encoding gRNA
integrating virus
genome editing takes place in cells that are infected by the virus
vector integration
on-target edit
DNA encoding the editing agents are semi-randomly integrated into the genome, with a distribution of integrations between cells. Cas9 and guide RNA are continually produced from these genomic integrations. Agents and modificaitons may be found throughout the body

Figure 1. Genome editing-based therapies present a spectrum of risk profiles that are dependent on a variety of factors

We show here three examples that represent increasing risk profiles. **a)** Cells are edited ex vivo using purified, pre-assembled RNP (Cas9 and guide RNA) and single stranded template DNA. Delivering Cas9 and guide RNA as purified RNP has the advantage of limiting off-target events by controlling the concentration of editing reagents and constraining the amount of time the genome is exposed to the reagents before they degrade or are lost to dilution. Off-target-tissue risks are limited by editing ex vivo, as are risks of immunogenicity since the exposure to Cas9 happens outside the body and the editing reagents do not persist and are therefore not part of the final drug product re-infused into the patient. Any risks due to changes in the genome are restricted to only those cells edited ex vivo. Additionally, because the body is never exposed to the editing agents, the risk of immunogenicity precluding patients from future CRISPR-based treatments is alleviated. **b)** Cells are edited in the body but confined to the target tissue by physically targeting the organ of interest. When directly administering to an immunoprivileged organ such as the eye[26], immunogenicity risks presented by persistent Cas9 may be reduced. Using non-integrating viruses to deliver the DNA encoding the editing reagents allows sponsors to reduce the risk of insertional mutagenesis. However, the vectors encoding the editing agents may still persist for an extended time[27, 28]. This may lead to a continued risk of off-target effects or immune response for as long as the vectors are still expressed and present in the cells. Additionally, it may be hard to control the concentration of editing reagents due to varying copy numbers in each cells[29]. **c)** Cells are edited by systemic administration of integrating viral vectors encoding the editing agents. The intended tissue type is targeted via a reliance on tropism, leaving a greater risk of non-target tissue modification as compared to examples a) and b). Using integrating vectors presents a risk of insertional mutagenesis. Furthermore, because the vectors are integrated into the host genome, the ability to express the editing reagents may persist and be transmitted to daughter cells, leading to continued surveillance of the genome by Cas9 (or other effector protein), thus increasing the possibility of off-target modifications. Because infection happens on a Poisson distribution[29], the number and location of integration sites per cells varies from patient to patient, and as in b) the concentration of editing reagents may also vary from cell to cell. Risks of immunogenicity are increased with systemic administration and where the Cas9 protein is expressed in cells, possibly even for as long as the lifetime of the patient. It should be noted, however, that despite these varying levels of risk, there are still applications using viral vectors that have reported low or no off-target mutations[30]. (Figure created using BioRender.)

Appendix References

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