Innovative Genomics Institute

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-2018-D-2173: Long Term Follow-Up After Administration of Human Gene Therapy Products**

Dear Dr. Sir/Madam,

The Innovative Genomics Institute (IGI) is pleased to submit these comments in response to the issuance by the Food and Drug Administration ("FDA") of a request for comments in its Federal Register Notice entitled "Long Term Follow-Up After Administration of Human Gene Therapy Products" (hereinafter, the "Draft Guidance"). We are grateful to the FDA for Draft Guidance that addresses Genome Editing (GE), and appreciate the opportunity to contribute our expertise to the regulation of these therapies.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We have approached these comments on the FDA's Draft Guidance in a similar interdisciplinary vein.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

In analyzing the risk of delayed adverse events, the Draft Guidance refers primarily to therapeutics created by a conventional Gene Therapy (GT) process with only occasional references to gene editing technologies. We ask the FDA to consider altering its long term follow-up (LTFU) requirements for Genome Editing (GE) to better take into account features of the GE product that can provide reduced risk of delayed adverse events. We believe that by identifying LTFU protocol requirements tuned to the particular risks presented by drug products created with different genome editing approaches, the FDA can better protect research subject and patient safety without imposing undue or unnecessary burdens on sponsors of therapeutics that provide important patient and public health benefits.

We discuss two criteria for delayed risk where GE technology presents unique characteristics as compared to conventional GT - mutagenesis and persistence. While both conventional, integrating GT and current GE technologies can lead to unwanted mutations, their mutagenesis risk profiles differ. Conventional, integrating GT presents risks from semi-random insertional mutagenesis that varies from batch to batch, and patient to patient. GE presents risks from non-random, consistent, deterministic off target modifications or undesired indels and translocations at the on-target site that can be robustly de-risked.

In commenting on the guidance, we focus our assessment on the area of GE in which we have leading experts - CRISPR-Cas-based GE therapies.

Summary of Key Suggestions: While we agree that any modification of the genome may present a risk of delayed adverse events, we suggest changes to the Draft Guidance including the following:

- We suggest that the FDA refine the requirements for LTFU protocols based on the distinct characteristics of the different genome modifying therapies as those characteristics affect the risk to research subjects or patients of delayed adverse events.

- We request that the FDA alter the requirement that all products developed using GE technologies automatically require up to 15 years of LTFU. For those GE products that result from applications that (i) do not use integrating vectors, and (ii) use editing reagents that do not persist, the FDA should require up to a 5 year LTFU protocol, consistent with the FDA's requirements for non-integrating viral GT.

- We suggest that the FDA revise those statements in the Draft Guidance that link the innovations of GE to increased risk of delayed adverse events. Novelty of the innovation does not automatically lead to increased risk.

Discussion

We are grateful that the FDA has chosen to formally address Gene Editing (GE) therapies in the Draft Guidance. We would like bring to the FDA's attention a number of ways in which the Draft Guidance's treatment of products created by GE does not reflect distinct features of many GE technologies and the therapeutics created by them. Many of the issues that we identify stem from the observation that the Draft Guidances seem to attempt to fit GE into a risk-management framework that has been built for and tailored to conventional gene therapy (GT) using viral vectors and the introduction of a transgene. Implicit in this choice is the assumption that GE is a new variant of conventional GT technology and can thus be evaluated with the same framework. As we address in greater detail below, we believe that GE is, in contrast, sufficiently distinct in several material ways to warrant more specific and tailored guidance with respect to evaluation of risk of delayed adverse effects.

Genome modification-induced risks from products created by GT and GE differ.
The Draft Guidance notes that GT products are often "designed to achieve therapeutic effect through permanent or long-acting changes in the human body. As a result of long term exposure to an investigational GT product, study subjects may be at increased risk of undesirable and unpredictable outcomes which may present as delayed adverse event(s)." The Draft Guidance hinges its risk assessment on the propensity of a product to permanently modify the genome. The primacy placed on permanent modification underlying the proposed LTFU

protocol obligations is illustrated in Draft Guidance Table 1 (Lines 523-526), along with Figure 1 (Lines 227-237) and the associated criteria to assess potential delayed risks. While we agree that a therapy that seeks to permanently alter the genome may present a risk of delayed adverse events, identifying "Propensity to Modify the Genome" (Line 525ff) as a single, or even primary, risk factor that leads to a potentially burdensome LTFU of up to 15 years incorrectly assumes the same level of risk from products created using GE as from products created by conventional integrating GT. In fact, there are many ways in which GE products have an enhanced safety profile relative to GT products, particularly in the risks associated with the ability to permanently modify the genome.

In Table 1 of our Appendix, we describe the variety of risks presented by conventional GT products and highlight the ways in which GE technologies create products that alleviate those risks or do not present an increased risk. Below we focus on two criteria for assessing the risk of delayed adverse events - questions of mutagenesis and persistence - and compare how these criteria apply to GE versus conventional GT.

Mutation Risks: Distinguishing insertional mutagenesis risks from off-target effects.
While both conventional, integrating GT and current GE can lead to unwanted mutations, the mutagenesis risk profiles presented by these two technologies and the products created by them are importantly very different. Conventional, integrating GT presents risks from semi-random, non-consistent insertional mutagenesis, while GE presents risks as a result of non-random, consistent, deterministic off-target modifications or undesired indels and translocations at the on-target site that can be robustly de-risked.

To demonstrate the significance of this difference, let us consider two examples of products created by ex vivo GT and GE. A typical patient being treated with a GT product for a blood disorder such as beta thalassemia or sickle cell disease using lentivirus administered ex vivo is currently treated with approximately 10 million cells per kilogram of volume[1]. For a 50 kg patient, this equates to 500 million cells transduced with the virus. That is 500 million cells transfused into each subject, some with up to 9 integration events[2], all with the possibility that integration caused misregulation of an oncogene. Moreover, the locations of those integrations are semi-random, divided among cells based on a Poisson distribution[3]. Thus, each batch of transduced cells will have a different distribution of integration sites that cannot be fully sampled or known in advance, making it difficult to design protocols to mitigate against delayed adverse events.

Contrast this to an ex vivo therapy using non-viral, CRISPR-based GE targeting a similar blood disorder. In this case, modification to the genome is programmable by virtue of base pairing interactions. Any off-target modifications (and undesired on-target modifications) will be consistent, non-random and testable as evidenced by extensive data in the literature [reviewed in 4]. Furthermore, the extent of off-target editing is modulated by the concentration of the editing reagents and the length of time they are exposed to the chromatin[5, and reviewed in 6]. When using purified Cas9 with guide RNA (RNP), the researcher can limit exposure to the editing reagents, reducing the risk of unwanted modifications to the genome. Using an approach such as this, genome editing with undetectable levels of off-target modification in clinically relevant cells has been observed[19]. For those applications that have non-zero levels of off-target editing, the locations of those edits and the relative frequencies are testable and consistent, allowing selection of guides that do not exhibit off-target modifications in regions of concern[7, 8].

These examples show why assigning the same mutagenic risk to a targeted mutagenesis technology and a random (by definition) mutagenesis technology is not upheld by the science. For delayed adverse events resulting from gene therapies, the source of the risk is not the concept of modification itself, but rather the genome modifying agent specifically. With conventional GT, the agent is a randomly integrated transgene that persists. With GE, the agent is a programmable nuclease or DNA binder which is non-random, and for many applications of GE, does not persist. While it may not be possible to know with certainty whether a genome edited cell contains adverse mutations, the consistent, deterministic nature of the technology and the advancements in off-target detection make this risk more manageable than conventional, integrating GT.

To further illustrate how the ability to modify the genome is not in itself a useful standard of risk, it is helpful to consider genetic modifications that occur in other contexts. It has been identified that cells undergo a surprisingly high level of background genome modification. Double strand breaks occur frequently as detected in techniques such as GUIDE-seq which map DNA breaks through oligo capture[9]. Every round of cell division introduces about 50 double strand breaks and up to 20 new mutations[10,11,12]. Thus, beyond the naturally occurring modifications that happen in our bodies on a daily basis, simply storing and culturing cells leads to modification

of the genome. There is no evidence to suggest that these background modifications would necessarily be less risky than modifications introduced through genome editing in which off-target modifications are non-random, and can be identified and managed.

Risk Modifying Factors: Persistence.
In Figure 1 of our Appendix, we introduce the concept of a risk spectrum. The propensity for a gene editing therapy to present a risk of delayed adverse effects is dependent not only on the ability to modify the genome, but also on factors including route of administration, delivery method, and persistence of the editing agents. This concept of risk modifying factors is shared by the FDA as illustrated in their list of "Elements that will influence the determination of the duration of LTFU observations..." (lines 607-615). To facilitate the FDA's consideration for how these factors might influence LTFU for GE therapies, we offer an example in part a) of Figure 1 in our Appendix. This example illustrates a combination we expect may present a lower overall risk to the research subject or patient where genome editing is executed ex vivo using RNP (purified Cas protein in complex with guide RNA) rather than viral delivery. 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 rapidly degraded[13], and reviewed in [14]. Degradation and loss of the RNP has many advantages for mitigating risks in GE therapies, including spatially and temporally confining the immunogenicity risk posed by Cas9, as well as reducing the probability of off target modifications. When combined with ex vivo editing, unwanted modifications to the genome are restricted in space to those cells that are exposed to the editing reagents, and restricted in time to the persistence of the RNP. In many cases this leads to low or unmeasurable amounts of off-target mutagenesis. Other delivery methods such as using mRNA also limit the persistence of the editing agents and lower the risk of delayed adverse events.

General Recommendations

While we agree that modification of the genome may present a risk of delayed adverse events, we suggest that the FDA consider the following:

- The mutagenic risk from genome modifying therapies derives primarily from the use of an integrating virus for delivery that induces non-targeted and non-consistent modifications.

- Gene editing using systems such as CRISPR-Cas are materially distinct from conventional gene therapy using viruses that integrate into the genome. These differences are essential to evaluation of risk from products created by these GE systems. Simple reliance on the fact of genetic modification as a trigger that necessitates up to 15 years of long term follow up is not sufficiently nuanced to reflect the materially different risks of delayed adverse events presented by GE and conventional GT products. We suggest that the FDA consider further parsing the requirements for LTFU based on the distinct characteristics of the different genome modifying therapies.

- For those GE products that result from applications that (i) do not use integrating vectors, and (ii) use editing reagents that do not persist, we suggest that the FDA require up to a 5 year LTFU protocol, consistent with the FDA's requirements for non-integrating AAV viral GT. The longer, up to 15 year LTFU protocol requirement, should apply to only those GE or conventional GT applications that use integrating viruses for delivery or that have genome modifying agents that persist in the research subject or patient.

- The FDA seems to assert that because GE is a novel "emerging technology", it presents significantly greater risks of delayed adverse events than classic GT (see table below). Novelty does not necessarily equate to increased risk. The example above highlights how despite a lack of extensive clinical data as compared to GT, many forms of GE may actually present a lower overall risk to the research subject or patient, including risk of delayed adverse events. Thus, we suggest that the FDA consider revising those statements in the Draft Guidance that link the innovations of GE to increased risk merely because of the novelty of the innovation or that suggest that products created by novel GE technologies automatically require an LTFU protocol of up to 15 years.

Table of specific suggested changes to LTFU Draft Guidance

Line in Document	Current Language with Suggested Edit	Comment
144-148	"Herein, we update our recommendations in the Draft Guidance taking into account the clinical experience gained since 2006 in LTFU of investigational GT products (as described in the following section), and the development of novel GT products with emerging technologies such as genome-editing that may be associated with an increased risk of delayed adverse events (as described in section III.D of this document)."	As discussed in these comments, while GE is an "emerging technology", GE products do not present significantly greater risks of delayed adverse events than classic GT products . Novelty does not necessarily equate to increased risk. The discussion above highlights how even in the absence of extensive clinical data as compared to conventional GT, there is strong published pre-clinical support for the proposition that GE technology may actually present a lower overall risk to the research subject, including the risk of delayed adverse events.
180-185	"Novel GT products developed as a result of emerging technologies, such as transposon- based gene insertion and genome editing, also raise concerns for delayed adverse events due to the unique genome modifying activity of such products. Specifically, a vector with a transposon element can insert transgenes into the host chromosome randomly by a direct "cut-and-paste" mechanism, mediated by the transposases (enzyme) activity in the product (Ref. 18)."	The Draft Guidance discusses transposon-based gene therapies and gene editing therapies together. However, while both emerging technologies that significantly different risks of delayed adverse events in research subjects and patients . As the discussed in the Draft Guidance, transposons exert their effect through random integration into the genome. By contrast, genome editing agents exert their effects through non-persisting, targeted modification that should be of substantially lower concern for delayed adverse events, especially if after 5 years of monitoring no such events have been detected in research subjects or patients. Notwithstanding the random integration, under the Draft Guidance, transposon-based therapies may be required to undergo less extensive LTFU than GE.
523, Table 1	"Table 1. Propensity of Commonly Used Gene Therapy Products/Vectors to Modify the Host Genome" Change "LTFU Observations" for Genome editing products from "yes" to "Product Specific"	We suggest that this seems in contrast to the mutagenic risk that the FDA highlights as pertinent to LTFU. Because the risk profile of GE therapies exists on a risk spectrum that includes considerations for the delivery mechanism and presence of a transgene, we suggest that like transposons, and adeno associated viruses, the FDA should consider making LTFU observations for GE products "product specific".
185	A GT product with genome editing components (nucleases) can give rise to non-specific off-target changes in the genome (Ref. 2) and may be associated with unknown and unpredictable risks for developing	Importantly, the off-target changes are not non-specific, but deterministic and assayable. This feature is a direct result of the targeting mechanisms of these agents. For CRISPR-Cas editors, the off-targets are determined by a combination of sequence similarity to the target, as well as

	delayed adverse events in study subjects and patients once approved, the extent of which will vary depending up the targeting mechanisms accompanying these components.	chromatin structure[15,16] and reviewed in [4]. These events are stochastic, but determined by the biology of the reagents. This specificity provides a clear risk management path that includes investigating the presence and identities of any off-targets produced by a sponsor's GE therapy. In addition to our comment regarding the characteristics of off-targets, the FDA should also consider removing the parentheses "(nucleases)". As we highlight in our comments regarding the FDA's definition of genome editing (below), new technologies are being developed that can edit genes without the function of a nuclease. These "base editors" aim to chemically convert DNA bases without inducing a double strand break.
243	**Question 1:** "Does your GT product involve integration of a transgene utilize genome-editing technology?"	As discussed above and in the Appendix to these comments, the primary risk factor for delayed adverse events is transgene integration. Even in the absence of extensive clinical data as compared to GT, research on the mechanism of genetic modification and pre-clinical research support the proposition that the GE technology will create products that present a lower mutagenesis risk to the research subject or patient than do GT products[17,18,8,7].
620-622	• Fifteen years for integrating vectors such as gammaretroviral and lentiviral vectors and transposon elements. • Up to fifteen five years for genome editing products created without using integrating vectors, and where the editing agents are not intended to persist. • Up to fifteen years for genome editing products created using integrating vectors or where the editing agents are intended to persist. • Up to five years for AAV vectors.	See Discussion section above and in our Appendix. The gating reason for requiring an LTFU protocol of up to 15 years for GE is if the GE product uses integrating vectors, in which case the factor determining 15 years is not the GE itself, but rather the integration event or the intention for the editing reagents to persist in the patient or study subject.
1116-1117	**Genome editing:** The processes by which the genome sequence is changed by adding, replacing, or removing DNA base pairs using engineered site-specific nucleases targeted enzymes.	Genome editing is not dependent on a nuclease. "Base editors" are (current) gene editing variants built from CRISPR effectors that aim to chemically convert DNA bases without inducing a double strand break. We expect that as a rapidly evolving field, additional variations of gene editors may further challenge the notion of gene editing requiring a nuclease, and thus we suggest the FDA alter its definition to better reflect the current and future characteristics of the field.

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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.

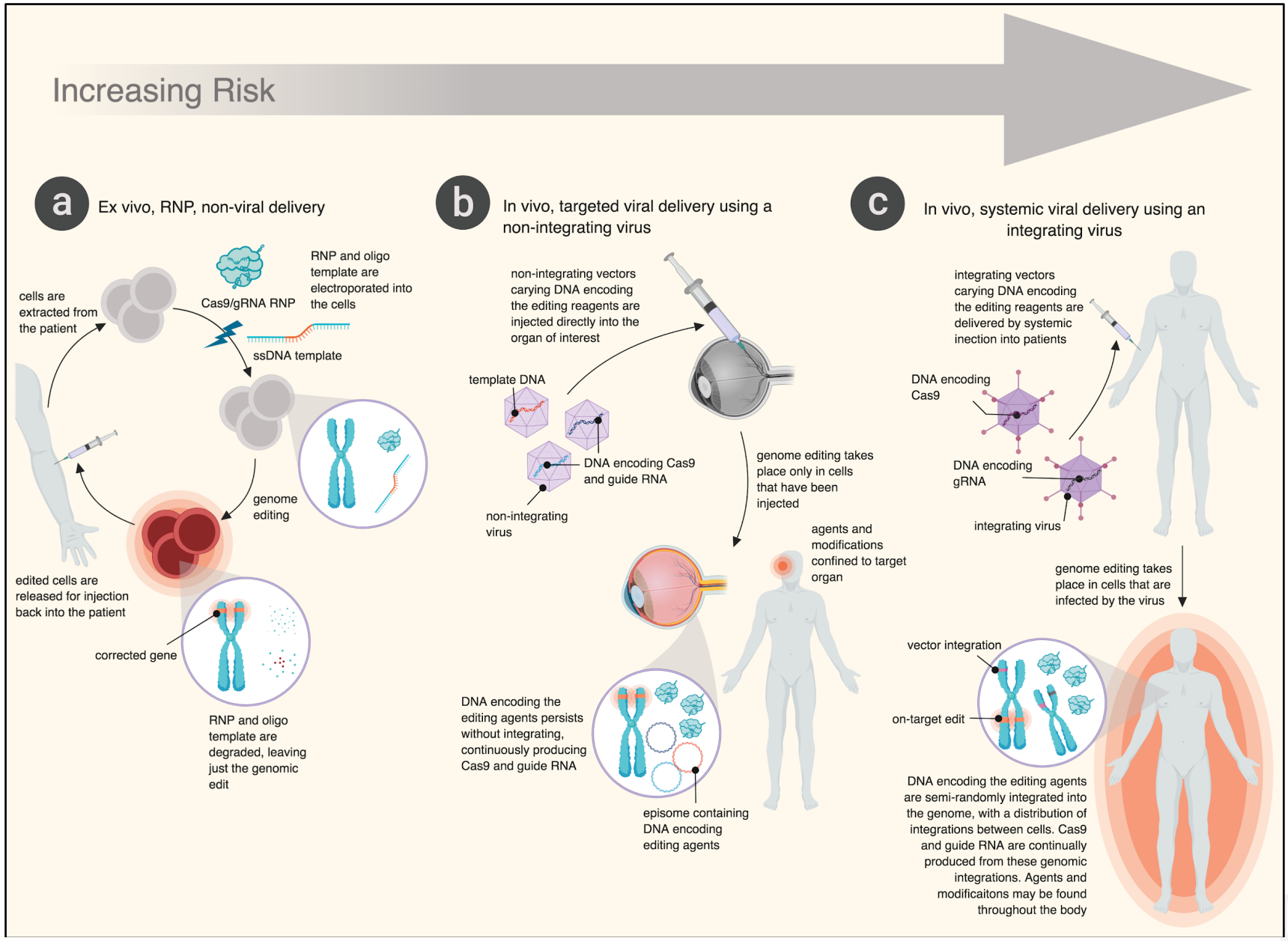
Increasing Risk
a
Ex vivo, RNP, non-viral delivery
cells are extracted from the patient
Cas9/gRNA RNP
RNP and oligo template are electroporated into the cells
ssDNA template
genome editing
edited cells are released for injection back into the patient
corrected gene
RNP and oligo template are degraded, leaving just the genomic edit
b
In vivo, targeted viral delivery using a non-integrating virus
non-integrating vectors carying 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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