Molecular Therapy
Review

Variability in Genome Editing Outcomes: Challenges for Research Reproducibility and Clinical Safety

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Genome editing tools have already revolutionized biomedical research and are also expected to have an important impact in the clinic. However, their extensive use in research has revealed much unpredictability, both off and on target, in the outcome of their application. We discuss the challenges associated with this unpredictability, both for research and in the clinic. For the former, an extensive validation of the model is essential. For the latter, potential unpredicted activity does not preclude the use of these tools but requires that molecular evidence to underpin the relevant risk:benefit evaluation is available. Safe and successful clinical application will also depend on the mode of delivery and the cellular context.

New therapies that employ genome editing tools (GETs) are being developed for a broad range of diseases, including cancers, β-thalassemia, sickle-cell disease, and Duchenne muscular dystrophy (reviewed and discussed in Porteus,[1] Pickar-Oliver and Gersbach,[2] Mullard,[3] Ledford,[4,5] and Hamilton and Doudna[6]). Meanwhile, although GETs are efficient at inducing specific double-strand breaks (DSBs), the outcome of DNA repair remains unpredictable. Validation of edited models, checking both targeted allele and potential off-target (OT) changes, is therefore essential to ensure research reproducibility. With more than 30 clinical trials underway or concluded,[1] the safety and efficacy of GETs as therapeutic agents are being explored. The safety challenges associated with using these tools (see Box 1 for key messages) and potential solutions are discussed in this review.

Off-Target Effects: The Tip of the Iceberg?

Closely associated with the efficacy to target specific genomic sequences is the risk of generating unwanted mutations. Until recently, OT cuts have been the main focus of discussions: initial use of GETs on human cultured cells raised concerns about the specificity that could be achieved, as frequent OT cutting events were recorded.[7–10] Subsequent studies of CRISPR/Cas9 unwanted effects suggested that in mice, OT events were rare.[11–13] The same conclusion was reached in various other models: tomato,[14] rice,[15,16] *Arabidopsis*,[17] zebrafish,[18] and human cell culture models[19–24] and with the use of transcription activator-like effector nucleases (TALENs).[20,21] The conclusion of these studies was challenged by Schaefer and colleagues[25] who reported frequent and unexpected types of genetic variations in a published article that was subsequently withdrawn when its methodology came under scrutiny. It is noteworthy that with the exception of this retracted paper, high-frequency OT effects have most commonly been reported in cell culture with sustained Cas9/guide expression.[7,8,10,24,26] The level of expression achieved, genetic impact of maintaining cells in culture, transfection method, cell type, guide sequence, constitutive nuclease expression, or a combination of any of these factors may explain the differences between high and low frequency of OT events. More recent studies[11,27–31] reinforce the conclusion that the frequency of OT effects when GETs are used *in vivo* is lower than the natural occurrence of genetic drift.[32]

Less highlighted, but more important, is the variety of repair events that occurs subsequent to DSBs on target. Evidence of CRISPR/Cas9-triggered genomic rearrangements was published early in their use,[33] and related potential safety issues for patients in therapeutic settings were largely unnoticed. Further work demonstrated that DSBs generated by one or more single-guide ribonucleic acids (sgRNAs) also led to a high frequency of various and sometimes complex (and often unwanted) events in a range of cellular contexts (early embryos, embryonic stem cells, primary cultures, and established cell lines):[33–41] these included deletions larger than the genomic segments defined by the sgRNAs, duplications (see examples in Birling et al.[34] and Lee et al.[40]), inversions (examples in Birling et al.[34] and Lee et al.[40]), insertions of unrelated DNA sequences,[41] translocations (examples in Jiang et al.[35]), and combinations of these rearrangements.[33–35,37,39] But overall, the most common unexpected repair event that was described was a deletion of a small number of kilobases

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Box 1 Currently Recognized Risks of Using Nucleases for GET: Key Messages

- GETs fit the definition of "targeted mutagens" as agents that increase the frequency or extent of a mutation at a given locus.
- With an experimental design that favors GET transient expression, the frequency of OT mutation is likely to be lower than the natural occurrence of genetic drift for any given generation.
- GET-associated safety risks are also on-target mutations that are the consequences of unwanted DSB repair mechanisms.
- Each GET is different, with its own efficiency and target specificity compared to other GETs.
- Experimental models produced with GETs require extensive molecular validation to ensure research reproducibility.
- The use of GETs as therapeutic agents is associated with three main risk factors: those directly associated with DNA cutting and the resulting repair events, those associated with the delivery means, and those attributable to the biological context in which GETs are employed (that is, which organ systems or cell types are involved).
- GETs need to be assessed in the cellular context(s) for which they are intended in therapy, as the mechanism by which targeted cells repair specific DSBs may differ among cell types, cell-cycle phase, metabolic status, and level of differentiation.

DNA, deoxyribonucleic acid; DSB, double-stranded break; GET, genome-editing tools; OT, off target.

around the site of CRISPR/Cas9 nuclease or nickase activity.[36,42] Unexpected sequence rearrangements may potentially induce confounding phenotypes; for example, an initially unrecognized bystander tandem duplication associated with an enhancer deletion allele in *Ilr2a* causes immune dysregulation through increased cellularity and relative representation of memory T cells.[43]

With the addition of donor templates, the complexity of repair outcomes increases, as the donor sequence also may integrate partially or incorrectly.[44–46] Recently, deep scanning of large numbers of events in cell culture with long-read sequencing illustrated the diversity of repair obtained, with a knock-in attempt resulting in a complex population of indels and partial, rearranged, or concatemerized on-target donor insertion, in addition to the expected integration event.[47] Furthermore, OT donor integrations (partial or integral) are not uncommon with both double- and single-stranded DNA templates alike.[44,46]

The precise DNA repair mechanisms that yield this vast variety of repair events are largely unknown. DSBs are highly toxic events[48] and elicit cellular responses (for example, p53-mediated DNA damage response and cell-cycle arrest[49,50]). One of several possible complex repair mechanisms is induced,[51,52] which most likely determines the resulting repair event. Examples of such potential consequences may include unintentional mutations in the cancer driver genes *VHL* or *KRAS*, as a result of GET activity yielding neoplastic events.[53] More intense research is needed to understand, and perhaps in the future control, which repair pathway is activated following DNA cutting by GETs. In this context, as molecules that increase the frequency or extent of mutations, GETs fit the definition of "targeted mutagens." Therefore, it is unrealistic to expect that GETs alone will guarantee the desired outcomes of their use. In conclusion, whether they are used alone or in combination with donor templates, although GETs are astonishingly efficient at generating sought-after sequence changes, which DNA repair mechanisms are triggered is poorly understood and even less well controlled. On-target variability has been understudied. The gravity of the risks associated with it will depend on the biological function of modified loci. In addition, this variability also means that the desired sequence may not be obtained, preventing the generation of the mutation of interest or the achievement of the therapeutic goal. In the biomedical research setting, these findings highlight challenges for ensuring research reproducibility (see the following paragraph). In the clinic, the emphasis turns to evaluating the benefit-to-risk ratio of gene-editing therapy, including consideration of the full range of possible outcomes.

Reproducibility Studies in Basic and Preclinical Research Inform Clinical Strategies

The variability of GET-induced mutations described in the previous paragraph emphasizes the importance of experimental design in considering the possibility of unexpected outcomes of DSB repair, differentiating it from the effect of genetic drift,[27–31] and excluding unwanted events during the validation of genome-edited models, whether in cell culture or in animals. In particular, it is recognized that insufficient molecular validation of *in vivo* models plays an important role in the misinterpretation of scientific results.[54] To be explicit, detailed characterization of the models generated by GETs is required to ensure research validity.

With the apparent ease of generating mutated founder animals, the first generation produced by the action of GETs in embryos was initially heralded as a desirable model for phenotyping.[55] However, it is now clear that the founder generation may only be used for specific phenotypic screens.[56] Association of a biological trait to a specific genetic variation requires the mutation to be established and validated in generations subsequent to the founder stage to exclude any unpredicted on-target events. Additionally, repeated crossing of the GET-generated mutant with wild-type animals will segregate most OT mutations, thus lowering the likelihood that phenotypes could be caused by OT rather than on-target sequence changes.

Importantly, the understanding and control of the variability of the outcome of GET use in laboratory animals inform the clinical field of potential undesired effects and will provide solutions to deal with

these risks. However, full assessment of GET activity and its impact will await clinical trial results.

What Is Already Known in Humans?

Early therapeutic attempts to use GETs in humans employed zinc finger nucleases (ZFNs), targeting the *CCR5* gene for the treatment of HIV-infected patients.[57] The same strategy has now been tested in both autologous T cells and hematopoietic stem cells (HSCs) without genotoxic side effects. The first direct DNA modification using ZFNs involved adeno-associated virus (AAV)-mediated *in vivo* delivery for the treatment of Hunter metabolic disease.[58] After 4 months, in the two men who received the highest dose, glycosaminoglycan sugar levels dropped substantially, but iduronate 2-sulfatase—the enzyme that breaks these sugars and is not functional in Hunter syndrome—remained undetected in blood, and furthermore, no signatures of genome-edited tissue were recovered.[59] These results are therefore difficult to interpret in terms of the safety and efficacy of this therapy and are not conclusive.

TALENs were also employed to engineer universal chimeric antigen receptor T cells by disruption of T cell receptor (*TCR*) and *CD52* genes, leading to remission of acute lymphocytic leukemia in two, 1-year-old infants.[60,61] The cells were used in a time-limited manner to obviate safety concerns, and no genotoxic side effects were detected. Over 20 subjects have since been treated using similar cells with no TALEN-related toxicity described.[62] Of note, in these multiplex-edited cells, the risk of translocations between edited chromosomes was recognized and quantified.[63]

The first studies employing *ex vivo* CRISPR-modified cells have been initiated in China with the *ex vivo* targeting of the programmed cell death protein 1 gene (*PDCD1*) in T cells of patients with metastatic non-small-cell lung cancer,[64] followed by infusion of the cells back into patients. However, clinical data have not been published. A number of additional studies modifying TCR, human leukocyte antigen (HLA) class I, and *PDCD1* using CRISPR/Cas9 editing have been reviewed by regulatory agencies. The first results of a phase I human clinical trial with patients suffering from refractory cancer were published very recently.[65] The authors showed persistence of the engraftment of cells with edits in three loci: *TRAC*, *TRBC*, and *PDCD1*, after 9 months, showing the feasibility of such therapy for cancer immunotherapy. The trial identified no safety issues, although it also illustrated genetic heterogeneity in the grafted cells, including chromosomal translocations.

Other studies had addressed the possibility of initiating HIV resistance through the modification of *CCR5*.[66] Although the therapeutic goal was not met, no issues of immune reaction or other adverse effects were reported.[66,67]

Injection of CRISPR/Cas9-modified $CD34^+$ HSCs was shown to ameliorate the pathology of both sickle-cell disease and β-thalassemia.[68] A first *in vivo* CRISPR/Cas9 trial, administered via subretinal injection, was launched in March 2019 to treat an inherited form of blindness caused by a mutation in the *CEP290* gene (ClinicalTrials.gov: NCT03872479[3,5]). These trials herald promise for the use of GETs for tissue engineering or somatic therapies.

In November 2018, the announcement of the alteration of the genome of twin girls using CRISPR/Cas9 in human early-stage embryos shocked the scientific community.[69] This first gametic trial raised major practical and ethical concerns around both the absence of full scientific evaluation and licensing and the safety and efficacy of *CCR5* gene inactivation to prevent HIV infection but chiefly, about the ethically contentious use of GET for human germline modification and the potential for a drift toward eugenism.

Today, over 30 GET trials are registered on ClinicalTrials.gov, but not all will reach patient recruitment.[1] However, few data on short-term toxicity[57,60] and no data on long-term toxicity are yet available. Early *in vivo* or *ex vivo* GET trials are either to assess safety in healthy individuals or are mostly fast-tracked studies of therapy for terminally ill patients.

Threshold of Benefit:Risk Analysis

As described above, GETs, along with other therapeutic tools, ranging from radiotherapy[70] and chemotherapy[70] to integrating viral vectors, inherently carry risks of genotoxicity. Both radiotherapy and chemotherapy cause random genomic alterations, from single nucleotide changes to large-scale chromosomal rearrangements. The application of gamma-retroviral vectors for the modification of HSCs in children with inherited severe combined immunodeficiency (SCIDX1) resulted in serious adverse effects from insertional mutagenesis.[71] Similar complications were later reported in chronic granulomatous disease[72] and Wiskott-Aldrich syndrome.[73] The issue seems to have been resolved by switching to self-inactivating lentiviral[74] and gamma-retrovirus[75] with deleted long-terminal repeat U3 enhancer sequences. Interestingly, variations remain between disease settings, with no transformation events reported for the enzyme deficiency adenosine deaminase SCID.[76] Other conditions, in which carefully regulated gene expression is likely to be required to avoid transformation, have not, to date, been tackled by "gene-addition" strategies but could be well suited to GETs. In addition, the nature of the target cells being modified is critical, with reported genotoxicity limited to studies modifying stem cells and no similar occurrences in other *ex vivo*-modified T cells, keratinocytes, or fibroblasts.[77] Alternative nonviral systems, such as the sleeping beauty transposon gene-delivery platform, have also reached clinical-phase testing.[63] Constitutive expression of transposase was known to be highly mutagenic, and yet, transient expression during *ex vivo* modification of cells has, to date, been safe.

GETs only affect specific targets and potentially, a small number of DNA sequences with a high degree of homology. Furthermore, GETs represent a heterogeneous group and differ in efficiency and target specificity.[78] Therefore, each GET carries specific risks, in terms of both the frequency of the target and highly related sequences, and the specific risks attached to the targets' loci.[79]

For clinical use, the mode of delivery of GETs influences the risks and depends, among other factors, on whether the genetic defects have a somatic or developmental etiology. Depending on this, therapy will involve tissue engineering and somatic delivery or may require germline intervention, each of which brings fundamentally different challenges. Efficiency and safety with each given delivery mode for use in the clinic must be validated together for any genome editing-based therapeutic intervention (for reviews on delivery, see Shim et al.[80] and Lino et al.[81]). As with any other therapeutic agent, the delivery mode itself of GETs brings inherent safety risks: viruses can be associated with a high risk of gene disruption[82] or can cause problems of toxicity and immunogenicity.[83] Likewise, transfer of naked DNA to cells is known to activate an immune response.[84]

Delivery of any new DNA into a cell has the potential to generate unexpected genomic sequence insertions.

In conclusion, evaluation of the risk by nuclease type (that is, ZFN, TALEN, or CRISPR/Cas9) is not possible. The initial perception of these molecules was that of an entirely specific and predictable family of tools. Potential secondary effects have since been revealed, and earlier over-optimistic expectations have been shattered, leading to debate over the risks associated with the use of GETs.

In light of the benefits expected from the use of GETs, some cancer risk may become acceptable for lethal diseases, such as Duchenne muscular dystrophy, for which the use of CRISPR/Cas9 may show promise,[85] and numerous preclinical studies are ongoing.[86] Already, clinical trials to treat refractory cancer are also underway,[65] but for less immediately critical conditions, such as HIV infection,[87] the threshold of acceptability will be different. Overall, relevant risks for potential secondary cancer development or for other adverse effects remain to be evaluated.

Clinical Risks Derive from the Unpredictability of GETs and Are Modulated by Delivery Mode and Target Cells

With regard to safety in therapy, the genome-editing community initially focused on the potential OT activity of GETs.[7,9] However, these events are proving to be relatively rare in comparison with local rearrangements following on-target activity,[34–36,39,46] which are newly recognized and are likely to be studied further. Many questions arise, such as the size of the rearrangements, frequency of each type of event (for example, larger than designed deletions, inversions, duplications, and combinations of these), and specific consequences attached to the modification of each targeted locus (for example, potential activation of an oncogene or other pathogenic gene in the vicinity). The question of specificity can also be applied at several levels: do GETs solely cut where expected, and what are the functional consequences of the repairs in each genomic region affected? The answer to these questions requires sensitive means of detecting mutagenic events that are themselves still the objects of improvement.[88] These studies are essential to ensure the reproducibility of all research involving GET use, but in the clinic, unlike in biomedical research, only cases where those changes are both functional and pathogenic will represent a safety concern.

The unwanted consequences of using GETs also need to be assessed in the relevant systems. Studies in human cells will inform on safety in relation to the interaction of GETs with the human genome[7,19–22] and on expected repairs of the genetic interval containing the targeted locus.[36] The choice of *in vitro* model is paramount, as cellular repair systems are lineage specific.[89] Animal models may not represent human repair systems but will inform on safety at the level of the whole organism and are also useful for studying the consequences of DNA repair activity. Humanized mouse strains in which the mouse allele is replaced by the full human sequence ally the advantages of working with the relevant target locus in the context of a whole organism.[90] Evidently, different models have different biases; cell culture itself is known to introduce changes that affect genome integrity.[91] Furthermore, the mode of delivery in cell culture is different to that *in vivo*, as it often involves sustained expression of GETs. Conversely, animal models may lack the specific (human) genetic sequences (and potential OT sites) for which the GETs are intended. Full evaluation must include the consideration of safety issues associated with GETs and their mode of delivery. Cell culture and animal models are therefore both complementary and essential but cannot replace safety trials in patients.

Initial attempts to understand unwanted effects of GETs on the genome included whole-genome sequencing and capture of GET-related events in cell culture models.[7–10,24] These approaches, although informative, did not identify large-scale changes, such as chromosomal rearrangements and larger deletions. As the research field matures, methods for the detection and characterization of these initially unexpected repair events are being developed and applied in a more systematic fashion, uncovering a hitherto underestimated variability in outcomes. Table 1 summarizes the most relevant techniques employed to analyze the genomic consequences of GET use. In particular, methods for counting the copy number of specific alleles and long-read sequencing have changed our perception of GET activity.[34,36,46,92] Such methods will be instrumental tools for the validation of research models, assessment of GET genotoxicity, and definition of DNA mutation signatures associated with risks. Next-generation sequencing (NGS) detects a large panel of single nucleotide polymorphism mutations and small indels in the entire genome. However, unless a very large depth of coverage is achieved, short-read NGS does not reliably detect structural variation mutations, and some genomic regions (for example, repetitive or structurally challenging sequences) are poorly amplified or aligned. By contrast, linear amplification-mediated, high-throughput, genome-wide sequencing detects DSBs with high sensitivity by employing a fixed "bait" DSB to capture them prior to cloning and short-read NGS sequencing. This method allows for a nucleotide-level definition of genomic rearrangements without the requirement of predefining potential OT sites. Long-read NGS methodologies are also beginning to be employed to analyze the outcome of genome editing through targeted sequencing for in-depth analysis of specific loci or for whole-genome sequencing in replacement of or combination with short-read NGS.[93]

Table 1. Examples of Techniques for Analyzing Genomic Consequences of GET Activity

Technique	Advantages	Disadvantages	References
Droplet digital PCR	detection of deletion or duplication on any selected sequence	only a few selected regions can be checked; for example, potential oncogene or other gene pathogenic in the GET target vicinity	34,46
Short-read, whole-genome NGS	detection of SNP and small indel mutations in the entire genome, no required OT prediction	large rearrangements not seen	11
Long-read, whole-genome NGS	more accurate contig generation	error rate for some instruments	93
Linear amplification-mediated, high-throughput, genome-wide translocation sequencing	detection of OT nuclease activity, higher sensitivity, no required OT prediction	large number of events must be analyzed for complete inventory of OT effect	94
Targeted long-read sequencing (nanopore or PacBio)	precise detection of rearrangement in the vicinity of the target region, high sensitivity	rearrangements greater than a few kilobases may not be detected	36,46,47
Chromosomal microarrays	detection of structural variant	not all structural variations will be detected; no inversion can be detected by this method	95
FISH	detection of chromosomal rearrangements	large rearrangements only are detected	38
Next-generation mapping (i.e., Bionano Genomics and Genomic Vision) and molecular painting (Fiber FISH)	detection of structural variant	access to technology and cost for next-generation mapping; as Fiber FISH is based on using probes, it cannot detect variation in the whole genome	92

In all instances, appropriate controls are required to differentiate genome-editing activity from naturally occurring sequence changes. A full characterization of the genome-editing impact requires the implementation of complementary methods. FISH, fluorescence *in situ* hybridization; GET, genome-editing tool; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

For all whole-genome methodologies, the number of mutation events detected can be high, making it difficult to distinguish between causative and nonessential mutations. Cytogenetic methods[38] and next-generation mapping sensitively identify large genomic variations that can be missed by PCR-based techniques, NGS, or chromosomal microarrays.[92] Again, the ability to distinguish between pathogenic structural variants and benign mutations remains challenging.

As new diagnostic tools are developed, new features of GET activity are revealed, and no single approach currently encompasses the detection of all possible mutagenic events. Today in the clinic, the DNA mutation signature obtained through the combination of these diagnostic tools generally precludes a definition of the risk of toxicity. The prediction of what these data mean remains highly speculative, but if toxicity arises, then the data will be essential to define adequate and unacceptable DNA mutation signatures.

Additionally, the impact of genomic alterations will differ according to the targeted cell type. For example, therapies that propose to disrupt integrated HIV sequences by CRISPR/Cas9[96] are likely to generate multiple genomic rearrangements: as multiple copies of HIV can be integrated into a single-cell genome,[97] DNA repair of multiple DSBs could trigger large rearrangements or translocations. Transformation risk is, however, low for T cells compared to other cell types. By contrast, for cystic fibrosis, after GET-mediated correction of the patient's induced pluripotent stem cells (iPSCs), no significant OT events were detected,[98] but the cancer transformation risk of iPSCs is much higher.

In summary, the clinical risk will be defined by a complex combination of factors: the induced genomic variations, the delivery mode of GETs, and the cellular context (Figure 1).

How Can We Control Safety Risks?

Three therapeutic pathways have been proposed for GETs in the clinic: *ex vivo* tissue engineering followed by grafting,[80] somatic delivery of GETs,[80] and early embryo genome modification that will be transmitted through the germline.[99] In all three cases, GET design, in particular, the choice of sgRNAs for CRISPR/Cas9-based approaches, is the first issue to consider. Many studies indicate that GET OT activity depends on sgRNA sequence;[7,21] designs should be optimized to minimize the OT activity, in particular, with the relevant mode of delivery. On-target mutations following cell genomic repairs will be equally if not more important to assess when designing a GET, as they can involve a large variety of changes, including larger-than-expected deletions and chromosomal rearrangements. These issues need to be assessed in the cellular context(s) for which they are intended in therapy, as the way in which targeted cells repair specific DSBs may differ among cell types, cell-cycle phase, metabolic status, and levels of cell differentiation.[89] Interestingly, some studies propose that not only is the mutational outcome dependent on both the target sequence and cell line but also that the outcome is also partly predictable.[100] Careful validation of the design of GETs to limit the range of mutations generated at each target is essential to a reduction in risks associated with their use. However, even with the knowledge of the patient's genome sequence and sophisticated prediction software, an exhaustive OT risk profile may not entirely be defined in preclinical studies or in clinical trials and may require investigation of reactivity of the GETs in the cells of each patient.

Other considerations are more specific to the given mode of GET delivery. If edited cells can be selected *in vitro* prior to grafting or reimplantation, then infrequent mutations may represent less of a barrier to GET use. In such cases, if the therapeutic design allows for the

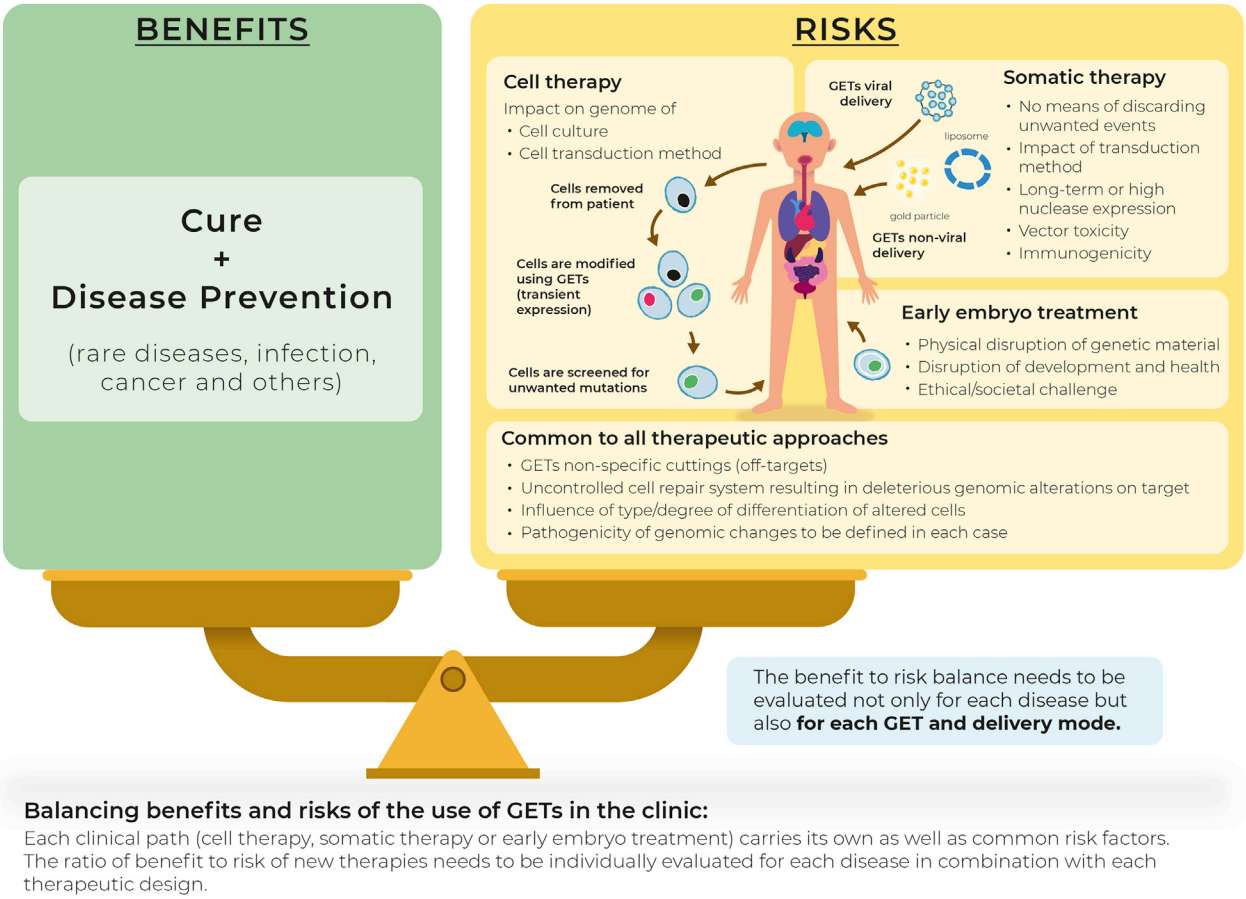

Figure 1. Balancing Benefits and Risks of Use of GETs in the Clinic

Each clinical path (cell therapy, somatic therapy, or early embryo treatment) carries its own, as well as common, risk factors. The ratio of benefit to risk of new therapies needs to be individually evaluated for each disease in combination with each therapeutic design.

selection of clones or pools of cells that only contain an acceptable outcome of genome editing, any unwanted events could then be discarded. Recent studies have reported that GETs induce DNA damage response. Thus, they may carry a risk for cancer, due to selection against cells with a functional *P53* but also with *VHL* and *KRAS* mutations.[49,50,101] Quality control of engineered tissue should also address other features related to the use of *in vitro* methods. For example, karyotype and epigenetic changes are common occurrences that are linked to tissue culturing and to methods of transfection[91] and may represent as significant a carcinogenic risk as the use of GETs themselves.

Somatic delivery may present different challenges.[102] Following safety issues with viral sequence insertions,[71] current trials are mainly focused on nonintegrative approaches.[80] However, even if integration-deficient viruses are used, insertion of viral sequences may occur in time.[103] Critically, adenovirus or AAV-mediated delivery may lead to long-term expression of a targeted mutagenic agent[104] and therefore, carries an increased risk of OT events. Strategies may therefore favor transient expression using ribonucleoprotein (RNP) or mRNA that will reduce the time of exposure to a Cas9 nuclease,[105] supported by a viral vector[106] or other modes of delivery, such as liposomes or association to gold particles.[107] Furthermore, the expression itself of nucleases may carry the risk of immune reaction, as shown by the identification of immunogenicity against *Staphylococcus aureus* and *Streptococcus pyogenes* (*Sp*) Cas9 proteins in humans.[108,109] Such issues may be circumvented by the use of alternative nucleases, transient Cas9 expression, or the combination of GET with transient immunosuppressant treatment. The frequency of such pre-existing adaptive immunity is not fully understood and may reflect a balance between the activities of *Sp*Cas9-reactive regulatory T cells and *Sp*Cas9-reactive effector T cells.[108]

Genome editing of the early human embryo or gamete is not the subject of current registered trials, but methods applicable to such interventions are being developed.[110] Early attempts to modify human embryos, including unlicensed trials, have encountered the same challenges as those that are experienced when using animal models,

such as mosaicism or unpredictability of the repair outcome.[99] Existing methods employed in prenatal diagnostics, such as preimplantation diagnosis after *in vitro* fertilization, may offer lower risk by selecting offspring free of genetic diseases. However, these techniques are not applicable to all families (for example, in cases when no unaffected embryo is available). Such issues continue to be subjects of ethical debate on germline modification in humans.[111]

Potentially applicable to all three routes of delivery is the ongoing development of new GETs. A wide variety of strategies have been tried, including the engineering of nucleases with higher specificity,[112,113] the coupling of Cas9 fusions with specific protein domains to enhance the frequency of DSB repair by homology-directed repair (see Devkota[114] for review), and the abolition of all nuclease activity with the coupling of another effector, such as modules that edit DNA base identity.[115] The latter seems particularly attractive, as many safety issues stem from our inability to direct the mode of DSB repair that follows GET activity. However, current versions of these base-editing tools do not yet allow the precise prediction of the outcome of their use, and whereas point mutations other than C → T and A → G are not feasible to date,[115] there may be immediate applications aimed at creating targeted gene knockouts, and new generations of base editors are being developed.[116] Nickase variants of GETs may also represent possible safety improvements,[117] but the molecular mechanisms that are involved when nickases are used for genome editing remain poorly understood, and all safety caveats associated with the use of nucleases may also apply.[42] A most recent addition to the GET set is a system with two nickases coupled to a reverse transcription activity to be used in conjunction with hybrid guides that also contain a repair template sequence for genome modification by prime editing.[118] Appraisal of the applicability and reliability of such advanced strategies will await extensive trials in both the research and preclinical settings. Looking further ahead, methods to direct DSB repair cell mechanisms transiently to yield the desired outcome would represent an attractive solution that so far has eluded the field.[119]

Conclusions

In spite of their initial promise, GETs have not yet yielded a flawless capacity to modify genomic sequences specifically and predictably, either in research or in the clinic. Although their use is highly efficient, the outcomes can be unpredictable. In the research environment, it is important that the experimental design takes into account the possibility of unwanted DNA repair outcomes, excludes undesired events by validation of edited models, and employs appropriate controls to ensure research reproducibility. In the clinic, there are likely to be ongoing improvements to genome-editing efficiency, delivery systems, and techniques to map on- and off-target effects. The first clinical applications are already underway in areas of unmet need, such as hematological malignancy, using *ex vivo*-modified somatic and differentiated T cells. Applications using *ex vivo* CRISPR/Cas9-edited HSCs for hemoglobinopathies are imminent, and patients will be subjected to close long-term monitoring, as are patients who receive *in vivo* therapy for certain life-threatening metabolic conditions. Careful monitoring and in-depth consideration, including deep molecular interrogation of any adverse effects, will be the key to successful exploitation of these tools.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing of the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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