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Genome Editing: Past, Present, and Future

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The CRISPR-Cas genome editing tools have been adopted rapidly in the research community, and they are quickly finding applications in the commercial sector as well. Lest we lose track of the broader context, this Perspective presents a brief review of the history of the genome editing platforms and considers a few current technological issues. It then takes a very limited view into the future of this technology and highlights some of the societal issues that require examination and discussion.

INTRODUCTION

This is a marvelous time for genetics, due largely to advances in genetic analysis and genetic manipulation. The impact of innovations in high-throughput DNA sequencing and in genome editing have been felt broadly, from work on model organisms, to evolutionary studies, to improvement of food organisms, to medical applications.

Classically, genetic studies relied on the discovery and analysis of spontaneous mutations. This dependence was true of Mendel, Morgan, Avery, *et al*. In the mid-twentieth century, Muller [1] and Auerbach [2] demonstrated that the rate of mutagenesis could be enhanced with radiation or chemical treatment. Later methods relied on transposon insertions that could be induced in some organisms; but these procedures, like radiation and chemical mutagenesis, produced changes at random sites in the genome. The first targeted genomic changes were produced in yeast and in mice in the 1970s and 1980s [3-6]. This gene targeting depended on the process of homologous recombination, which was remarkably precise but very inefficient, particularly in mouse cells. Recovery of the desired products required powerful selection [7] and thorough characterization. Because of the low frequency and the absence of culturable embryonic stem cells in mammals other than mice, gene targeting was not readily adaptable to other species.

The current genome editing technologies resolved this issue, making directed genetic manipulations possible in essentially all types of cells and organisms [8,9]. In addition, these methods confirmed Nobel laureate Sydney Brenner's notion that, "Progress in science depends on new techniques, new discoveries and new ideas, probably in that order." (http://www.azquotes.com/author/24376-Sydney_Brenner) In this short article, I want to review where the genome editing platforms came from and speculate about where we are headed through their use. I will leave description of the technologies themselves to other contributors.

GENOME EDITING PLATFORMS

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The secret to high-efficiency genome editing is the ability to make a targeted DNA double-strand break (DSB†) in the chromosomal sequence of interest. Realization that such a break would stimulate gene targeting and local mutagenesis did not arise *de novo*, but came from research on DNA damage and repair. Recombination between homologous sequences is stimulated in meiosis by intentional DSBs [10], and DSBs generated by ionizing radiation lead to sister chromatid crossovers [11]. Model experiments with highly specific nucleases showed stimulation of homologous repair in yeast and mammalian cells and pointed the way for programmable genome editing [12-15]. Broken ends are also rejoined by a process called nonhomologous end joining (NHEJ) [16]. The ends are often joined precisely, restoring the original sequence; but occasionally errors are made, leading to local small insertions and deletions (indels). When these mutations occur in a gene, they will frequently inactivate it.

We are currently endowed with three powerful classes of nucleases that can be programmed to make DSBs at essentially any desired target: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-Cas [8]. Although the latter platform now dominates in research laboratories around the world, the other two are still in use for research and in various agricultural and medical arenas. All of these platforms arose from investigations into natural biological processes and not from intentions to find genome editing reagents.

ZFNs are hybrids between a DNA cleavage domain from a bacterial protein and sets of zinc fingers that were originally identified in sequence-specific eukaryotic transcription factors. TALENs employ the same bacterial cleavage domain, but link it to DNA recognition modules from transcription factors produced by plant pathogenic bacteria. CRISPR-Cas is a prokaryotic system of acquired immunity to invading DNA or RNA.

Let us take a closer look at components of each of the platforms. ZFNs: The first eukaryotic sequence-specific transcription factor to be characterized was found to have zinc-binding repeats in its DNA-binding domain [17]. Related sequences from other transcription factors were shown to be peptide modules that made stereotyped contacts with base pair triplets [18]. Changing a few residues in a single zinc finger altered its DNA-recognition specificity, and fingers could be devised to recognize many different DNA triplets [19]. TALENs: Some plant pathogenic bacteria secrete into host cells proteins that bind to and regulate the activity of host genes to promote the infection. There is a simple and robust one-to-one code of recognition between modules in the protein and base pairs in the DNA target [20,21]. ZFNs and TALENs: Some bacterial restriction enzymes cut DNA a few base pairs away from their recognition sites. This is because they have physically separable binding and cleavage domains [22]. The cleavage domain has no inherent sequence specificity, and it can be linked to novel DNA-binding domains, which alters where it cuts [23,24]. One such domain was linked to zinc finger arrays and TALE arrays to generete ZFNs and TALENs.

CRISPR-Cas: This story begins with the discovery of a cluster of odd, short repeats in a bacterial genome [25]. Between those clustered regularly interspaced short palindromic repeats (CRISPR) are short sequences that were eventually shown to match viral genomes [26-28]. Some CRISPR-associated (Cas) proteins encoded adjacent to the repeat clusters mediate capture of these viral sequences, while others mediate cleavage and inactivation of invading viral genomes, guided by short RNAs (crRNAs) transcribed from the CRISPR arrays [29,30]. The final piece of the puzzle was the identification of the small trans-acting RNA (tracrRNA) that participates in both processing of the crRNAs and cleavage of the invading DNA in *Streptococcus pyogenes* [31]. Putting together the crRNA with tracrRNA and the one protein needed for cleavage in this system (Cas9) led to the editing reagent that is now most widely used [32].

In summary, powerful tools come from unexpected sources.

GENOME EDITING ISSUES

Remarkably, all that the genome editing nucleases do is to make a break in chromosomal DNA. The key, of course, is that the break is targeted and thus very specific. Everything that happens after the break, however, depends on cellular DNA repair machinery. The two broad pathways of DSB repair are homology-dependent repair (HDR) in which a donor sequence matching the target is copied, and NHEJ in which putting ends back together can lead to mutations at the break site [8,9]. Most somatic cells in higher eukaryotes generate mutations via NHEJ more frequently than they copy sequences from a user-supplied donor. This bias is acceptable if all you want to do is to knock out a protein coding sequence, but not so good if you want to introduce sequences of your own choice. Limited success has been achieved in modulating the ratio between homologous and non-homologous products [33,34], but no general solution is yet at hand, and the ratio in some cell types is very biased toward NHEJ. Several recent reports suggest that small-molecule inhibitors of key NHEJ activities may be effective [35-37], but more research is needed to produce simple and reliable reagents. Another way to influence the efficiency of homology-dependent events is through design of the donor DNA [38], linkage of the donor sequence to the guide RNA [39], and consideration of specific mecha-

nisms to mediate sequence insertions [40,41].

All of the nuclease platforms can be very effective, but none of them has perfect specificity. Recent modifications of both Cas9 protein and guide RNA have enhanced their discrimination against secondary targets [42]. How much one cares about off-target cleavage and mutagenesis depends on the application. In many model organisms, there are ways to validate the effects of an introduced sequence change, including making independent mutations in the same gene, crossing into a clean background, and complementing with a wild type gene. In cases of organisms that can be rapidly expanded, like crop plants, founder genomes can be fully sequenced, and founder phenotypes can be analyzed thoroughly. Even in some medical applications, off-target mutations may be tolerable, as long as they do not lead to a novel clinical condition.

LOOKING AHEAD

Research advances. It is safe to say that genome editing will continue to be a widely-used tool in research and in commercial and medical applications. One question that arises is whether CRISPR-Cas is the last word in programmable nucleases, or perhaps there is something even better on the horizon. With limited vision into the future, it is difficult to imagine a protein-based system that is fundamentally simpler than recognition by base pairing and cleavage by a single protein. Perhaps the protein could be smaller and be endowed with additional beneficial properties, but that constitutes variations on the same theme rather than something completely novel. Maybe a fully chemically-based reagent could be developed, based on small synthetic compounds that combine DNA recognition with DNA cleavage. Research toward this end has been going on for decades – from triplex-forming oligonucleotides [43], to peptide nucleic acids [44], to polyimines [45] – without producing a platform with adequate cleavage efficiency and recognition range. It seems likely that if novel methods emerge, they will come, like the current ones, from research into natural processes, not from an intent to improve on CRISPR.

A variation on the theme of DSB-induced genome editing is the introduction recently of CRISPR-mediated base editing [46-49]. This platform makes use of Cas9 nickase, that cuts only one strand of the target DNA, linked to a base-modifying activity. Conversion of C to U within a few base pairs of the RNA-guided binding site leads to specific coding changes in that very narrow area. Future uses of this approach include fusions to alternative activities and modeling and correction of human disease alleles.

Medical applications. A few somatic therapies that involve genome editing have been approved for Phase I clinical trials. The earliest trials used ZFNs to knock out the CCR5 co-receptor gene in T cells of HIV-positive patients [50], thereby making the T cells resistant to the virus. The results were encouraging, and an extension to earlier hematopoietic precursors is planned. TALENs have been used to enhance the efficacy of therapeutic CAR T cells [51], and at least two trials using CRISPR-Cas9 for this purpose have been approved [52,53]. These examples rely on editing of cells in the laboratory – in some cases cells derived from the person being treated – and transfer to the patient. Such *ex vivo* treatments allow facile delivery of the editing reagents and preliminary characterization of the edited cells. As stem cell therapies are developed, genome editing is a natural adjunct. Particularly when stem cells are derived in culture from somatic cells of an affected individual, correction of an offending mutation would fall to one of the editing platforms.

In many cases, cell-based therapy is not possible. Clinical trials for treatment of hemophilia and two lysosomal storage diseases, based on *in vivo* delivery of ZFNs with viral vectors, are under way (see clinicaltrials.gov, and search "Sangamo"). These rely on gene editing in the liver, a comparatively accessible organ. Delivery to other *in vivo* sites will require novel vector and non-vector approaches, and possibly the development of well-behaved stem cells for particular tissues. Very active research is directed toward treatments for other genetic diseases, including sickle cell disease and muscular dystrophy. In all cases, whether based on *ex vivo* or *in vivo* treatment, both safety and efficacy must be demonstrated.

Germline editing. Stimulated by recognition of the ease of CRISPR-based editing and the possibility of misuse of the technology, there is considerable current interest in prospects for human germline genome editing. Such applications would involve delivery of the editing reagents to embryos created by *in vitro* fertilization. In the future, it may be feasible to engineer gametogenic precursor cells in prospective parents instead. The advantage to germline correction of disease alleles is that they will forever be gone from the lineage of the treated individual. The risk at present is that the attempt to correct may do more harm than good. Current genome editing technology does not have sufficient efficiency and specificity to be reliably safe. Mutations generated at non-target sites in the genome will also affect the treated person and be transmitted through subsequent generations, and their effects will not always be benign or predictable, nor will they be readily reversible.

Continuing research will make germline editing safer and more effective, and it seems inevitable that it will eventually be used. In the meantime, broad discussion of the ethical issues raised by the prospect should be continued [54]. A thoughtful summary of the practical aspects

of both somatic and germline therapies is provided by Kohn *et al.* [55].

Gene drives. An application of genome editing that has begun to attract attention is the use in a genetic process called gene drive. In brief, a genetic element can spread itself rapidly through a breeding population by copying itself into genomes that previously lacked it. Even if this element causes a moderately deleterious phenotype, it can expand in frequency. Natural gene drives have been identified, but current interest is focused on ones that are mediated by CRISPR-Cas9 [56]. Synthetic gene drives have been developed in mosquitoes that serve as vectors for tropical diseases, including a system that produces sterility in females [57] and one that inactivates genes required for parasite growth [58]. In principle, these approaches could dramatically reduce disease transmission in areas where disease treatment is challenging. The enormous burden of mosquito-borne diseases on human lives and health, particularly in the developing world, provides strong motivation for containing or eliminating the vectors.

The prospect of intentionally, or even unintentionally, releasing organisms carrying gene drives has evoked appropriate concern [59,60]. It is very difficult to predict the consequences for a broad ecosystem of depleting or removing one of its residents. If a particular mosquito population disappears, what will be the impact on organisms that rely on it, perhaps fish, birds, or plants? Other species will soon fill a vacant niche, but will they have the same influence on their surroundings? Will the drive itself become ineffective by mutation or by adaptation of the target organisms? Reversible gene drives are being developed [56], but their efficacy has not been tested. Unfortunately, small-scale laboratory tests will be poor predictors of effects in a natural environment, and we will not know the full impact of gene drives intended for benefit until they have actually been released.

Agriculture. Turning to agriculture, both livestock and crop plants are current targets for genome editing. The organisms produced are literally genetically modified, but they differ from earlier GMOs in important ways [61]. In most cases, no genetic material from another species is introduced, and when it is, it is inserted in a precise genomic location. The changes that are introduced are very often ones that could have occurred naturally, and whole genome sequencing can be done on edited organisms to look for off-target mutations. Because both seeds and semen can be dispersed rapidly into succeeding generations, validated genomes will quickly generate large populations of modified plants or animals.

Among current examples of edited crops are disease resistance in wheat [62], potatoes that don't sweeten on storage [63], and soy plants that produce healthier oil [64]. The prospects for developing other healthier crops are bright. To address economic and animal welfare issues, dairy cows have been generated that lack horns, due to a genetic modification [65]. Cows [66], sheep [66,67], pigs [68], and other food animals that carry more muscle mass (*i.e.*, meat) have also been produced by disruption of a single gene. Genome editing has the advantage over breeding selection that a trait can be introduced in a single generation without disrupting a favorable genetic background. The same beneficial modification can be introduced into different breeds or cultivars that are adapted to different environments, without leading to monoculture. A key question is whether the precise and largely natural genome modifications made by editing will find greater public acceptance than earlier GMOs. As the current resistance is based more on distaste for commercial greed and dominance than on evidence of adverse effects, there is a substantial hurdle to cross.

Beyond food modifications, large animal models of human disease are being produced to facilitate physiological analysis, drug testing and other therapies. It seems likely that genome editing will be applied to companion species, generating new breeds of dogs and cats and correcting genetic susceptibilities in current breeds. Additional work will be needed to uncover the genetic causes for desirable traits, but genetic research in dogs, at least, is making good headway.

Societal issues. Finally, I want to address societal issues that apply to medical and agricultural applications of genome editing. Who will decide what products or treatments are developed, and who will decide who gets them? I call these issues Attribution and Distribution.

In the medical realm, what therapies will be developed based on whom we decide needs to be "fixed"? Devastating diseases, like Huntington's disease and muscular dystrophy, are obvious candidates. What about hereditary deafness or short stature? People with these conditions are often high-functioning, have strong communities, and do not feel themselves to be in need of "correction" [69]. To take an absurd example, is skin color a condition that needs altering? This brings us to purely cosmetic changes that some may find desirable – hair color, eye color, height, athletic ability (assuming we know how to engineer these traits genetically). Should these applications be pursued?[1]

Once methods are developed, who will benefit? Human therapies based on genome editing are currently complex and expensive. Will only the wealthy be able to afford them? Could we distribute a genetic therapy for sickle cell disease to the large populations in Africa and Asia that are most affected?

[1]Two very recent papers highlight the interest in human germline editing and the value of research on human embryos [70,71].

These considerations apply to food organisms as well. Will nutritional improvements be made in specialty crops for the developed world, or in staples that predominate in the developing world? In both plants and animals, will we engineer resistance to diseases that are endemic in wealthy, temperate regions or to ones that limit production in developing regions? Ultimately, who will pay for development and distribution of improved crops and livestock – only the marketplace? Or will generous benefactors emerge?

Things are moving fast in genome editing. Many different applications are being pursued, and the only limit seems to be our imagination. In the midst of this excitement, we need to consider what are the best uses of the technology, what adjustments are needed to make the technology safe and effective, and how its advances will be provided to those who would benefit most. Currently, these decisions are driven by market forces, not humanitarian considerations. Are we comfortable with this, or do we need governmental participation at the national and international levels to change the situation? Count me as an advocate for the latter.

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