Cell Systems

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Voices

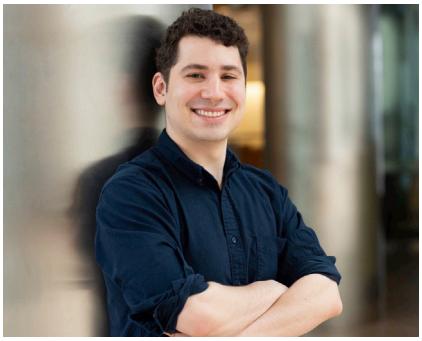
What are the current bottlenecks in developing and applying CRISPR technologies?

Elizabeth H. Kellogg
Cornell University

Diversity is the bottleneck

I think of CRISPR as being a subset of "adaptive" bacterial systems that promote host survival. These adaptive systems are macromolecular systems that enhance host survival in a myriad of ways: by destroying foreign DNA, by mediating horizontal gene transfer, or even by altering host metabolism, among many other diverse functions. In this sense, CRISPR represents just the tip of the iceberg; there are likely many unexplored and uncharacterized adaptive systems that would be practically useful for genomic manipulation. Therefore, identification and characterization of functionally useful adaptive systems is a major bottleneck in the development of new technologies. Bacterial genomes are fast-evolving. Furthermore, sampling of bacterial genomes is sparse compared to the vast diversity of bacterial species. All of this makes computational identification of novel adaptive systems challenging. High-throughput screens are useful if a suitable assay can be identified. Alternatively, common mechanistic principles can be obtained using biochemical and structural characterization, which could guide identification of adaptive systems.

In order to circumvent the need to "fish" for systems with desirable properties, protein engineering techniques can be used to fill in the gaps between what is needed for technology development and what is available naturally. Directed evolution approaches have already proven to be incredibly fruitful in this area and further highlight the potential of re-engineering to greatly expand the functional diversity of known adaptive systems.

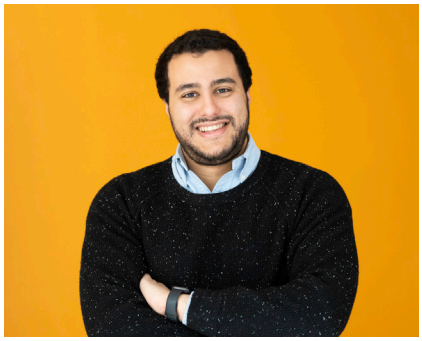


Jonathan Gootenberg
Massachusetts Institute of Technology

Overcoming delivery challenges

The impact of CRISPR-based tools on genome editing in the past decade cannot be overstated, but as these approaches mature and move into the clinic, we are reminded of a timeless adage: location, location, location. Genome editing therapies must always reach cells and tissues of therapeutic relevance at levels that enable effective treatment. Narrow tropism of both viral and non-viral vectors has restricted most *in vivo* CRISPR therapies to the liver or eye, and packaging constraints of viral technologies have precluded the delivery of larger genome editing tools, such as those for programmable genome integration (see "Next-generation genome insertion technologies"). Improvements in delivery will unlock new therapeutic modalities, including *in vivo* engineering of cell therapies, drastically increasing their accessibility.

This demand has spurred a wave of promising new delivery technologies. Delivery of CRISPR components as mRNA and guide RNA encapsulated in lipid nanoparticles (LNPs) has entered the clinic, and further development of LNPs will expand targeting capabilities outside of the liver. Adeno-associated virus vector engineering has enabled tropism to tissues including brain and muscle. Newer platforms, including viral-like particles and helper-dependent adenoviral vectors, promise increased payload sizes and reduced immunogenicity. In addition, technologies limiting expression to certain cell types, such as the RADARS systems, can improve the safety of new therapies. These new delivery technologies will extend the promise of genome editing therapeutics to numerous new diseases.



Omar Abudayyeh
Massachusetts Institute of Technology

Next-generation genome insertion technologies

Ten years after the first mammalian cell CRISPR experiments, we are witnessing the immense promise of the technology in humans. In the past year alone, a number of CRISPR human trials had successful readouts, such as 42 thalassemia patients no longer needing regular blood transfusions and transthyretin amyloidosis patients being successfully treated for more than a year with a single dose. Despite this great start for the era of human gene editing, there are over 7,000 genetic diseases requiring treatments, with only a sliver of them having approved therapies. How do we accelerate development of cures for all these diseases?

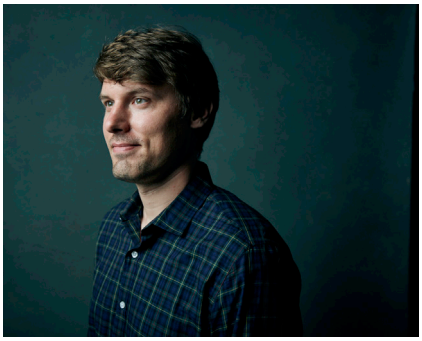
One answer lies in next-generation gene editing technologies. Newer technologies like base editing and prime editing enable precise editing of many genetic variants, addressing more diseases. However, many diseases, such as cystic fibrosis, have hundreds, if not thousands, of genetic variants, each requiring development of a unique CRISPR therapy. Faster progress could be made with tools that allow for programmable genome insertion (PGI), such as PASTE and Twin-PE. PGI tools, which insert complete genes into native locations, can treat all variants of a disease with a single therapy and offer opportunities for *in vivo* cell engineering. As these tools become more complex, involving additional enzymes and multiple guide RNAs, challenges with efficiency and delivery surface (see "Overcoming delivery challenges"), but with time, these challenges will be addressed, and a mature genome editing toolbox will offer revolutionary treatments for scores of patients in the next decade to come.

Alan S.L. Wong
The University of Hong Kong

Engineering CRISPR enzymes at scale

The facile programmability of CRISPR enzymes makes them important tools for genome editing. CRISPR nucleases could cure specific kinds of genetic diseases in which disruption of genes can yield therapeutic effects. Base editing and prime editing technologies are two additions to the genome editing toolbox that advance CRISPR technologies by going from "search-and-cut" to "search-and-replace" of DNA sequences. They expand the type of edits that can be cleanly installed and could achieve a greater efficiency than the nuclease-dependent approach via homology-directed repair. To unleash their full potential, it is imperative to further develop a broader range of variants of nucleases, base editors, and prime editors with higher editing efficiency, different targeting scope, and minimal off-target editing. For many kinds of applications with a vast amount of target sequences, each of them likely requires a distinct class of editor or its variant to make a particular type of edit.

The options presented by currently available genome editors are still limited. Efforts to design and characterize genome editors one by one are unlikely to quickly yield a wide variant repertoire sufficient for different applications. To scale up the efforts to engineer genome editors, enabling technologies such as advanced protein structure prediction, deep learning, and massively parallel combinatorial mutagenesis are vitally important. These technologies would also help us understand the complex rules for designing genome editors tailored for each specific context and for real-world applications that require high precision and efficiency.



James E. Dahlman
Georgia Institute of Technology and Emory University

Simple delivery to new tissues

One key bottleneck to applying CRISPR technologies in the clinic is delivering them to the right cell type *in vivo*. To date, clinically relevant *in vivo* CRISPR delivery has been achieved in hepatocytes, and to a lesser extent, the eye. In one promising example, lipid nanoparticle (LNP)-mediated delivery of Cas9 mRNA and sgRNA to hepatocytes, led by Intellia Therapeutics, resulted in robust, durable gene silencing in patients. More recently, Verve Therapeutics, using base editing technology licensed from Beam Therapeutics, initiated a liver-targeting clinical trial.

These data underscore the potential clinical impact of next-generation LNPs (or other drug delivery systems) that target new tissues with genetically defined diseases such as lung, heart, or bone marrow. Subsequent efforts to improve non-liver delivery progress must keep something in mind, though: while chemically complex delivery systems have always worked in small animals, clinical drug delivery has, to date, required chemically simple delivery systems. If scientists and engineers remember to keep it simple, I am optimistic that CRISPR drugs will be used to treat many diseases across diverse tissues.

Audrone Lapinaite
Arizona State University

Years of CRISPR and beyond

Since the discovery of the DNA double helix, researchers have been working toward programmable site-specific editing of genomic DNA. The approaches based on targeted DNA cleavage (ZFNs, TALENs) generated considerable excitement; however, complex protein design and subpar efficiency limited their use.

10 years ago, two seminal papers showed that the bacterial immune system (CRISPR)-derived Cas9 can be programmed to cut DNA at specific sites by simply changing the sequence of its guide RNA. This revealed the potential of CRISPR as a programmable, genomic DNA editing tool. It quickly became the preferred genome editing approach, revolutionizing basic research due to its superb efficiency and ease of reprogramming. However, CRISPR is not perfect: it performs off-target editing that yields unintended genomic changes. This prompted the characterization of novel bacterial immune systems (expanding the genome engineering toolbox) and the design of precision genome editing tools that can perform more precise DNA modifications (e.g., DNA base editors). The decade culminated in multiple clinical trials using CRISPR *ex vivo* to treat monogenic diseases like sickle cell.

Future efforts will focus on further enhancing the specificity and safety of genome editors and developing tissue- and site-specific delivery approaches, making precision genome editing in the human body possible. Beyond biomedicine, CRISPR will further revolutionize other fields (agriculture, environmental sciences) and provide innovative solutions to today's pressing issues such as climate change and food shortage.

Cameron Myhrvold
Princeton University

Effector characterization

The CRISPR field has advanced rapidly over the past decade. New effector proteins and new orthologs of existing effectors continue to be discovered at a breakneck pace. However, comprehensive biochemical characterization of CRISPR effector activity takes much longer than bioinformatic discovery of new effectors. This is in part because effector activity is context-specific (depending on the biochemical conditions or organism it is being used in) and in part because many assays for effector activity are low-throughput. For example, off-target cleavage by SpCas9 is different *in vitro* compared to in cell lines or in animal models. Even within a particular model system, the delivery modality and expression levels of the effector and guide RNA also affect activity and can be difficult to control for.

We also don't fully understand how primary sequence and secondary structure influence effector activity. To be clear, significant progress has been made toward understanding how Cas9 cleaves dsDNA and the extent to which it is able to cleave at off-target sites in the genome. Some progress in understanding the rules that govern Cas12 and Cas13 activity has been made, but many unanswered questions remain. A better understanding of the kinetics and the principles that govern CRISPR effector

proteins will allow us to unlock the next generation of CRISPR-based technologies. I am excited to see what the next decade has to bring to the field.

Chang C. Liu
University of California, Irvine

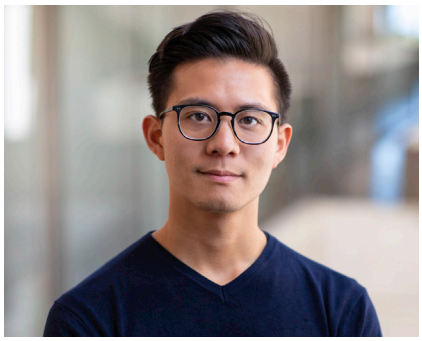
Molecular engineering goals

The realized and potential impact of CRISPR is truly exceptional. Thus, there is little doubt that bottlenecks in developing and applying the ecosystem of CRISPR technologies will be overcome by the motivated CRISPR community. Nonetheless, there are a few challenges in CRISPR biomolecular engineering worthy of special attention.

First is efficiency. The overall efficiency of CRISPR-based genome editing per unit of time still has plenty of room for improvement. Moreover, variation in editing efficiency across cell types, cell states, and target sites needs to be better understood in order to guide engineering approaches that minimize the variation or exploit it in predictable ways.

Second is off-target activity, especially over long timescales. Understanding and overcoming off-target or spurious editing activity over long times is important for advanced applications where extended timescales are involved, such as in gene therapy or the use of CRISPR systems for continuous barcoding and molecular recording.

Third is guide RNA induction. There is great potential for the study and control of cell biology through the multiplexable induction of CRISPR guide RNAs by important biological signals. However, the versatility and efficiency of inducible guide RNA expression systems is currently limited. Advances in this arena are also needed.



Patrick D. Hsu
Arc Institute and University of California, Berkeley

Higher-complexity operations

"Find and replace," "Drag and drop," "Gene surgery"… Despite immense progress in the last decade, we have popularized a lexicon around the concept of genome editing in human cells that conjures a level of technical ease and reliability that exceeds our actual experimental capabilities. Current gene editing approaches with CRISPR-Cas nucleases rely on complex cellular DNA repair pathways with highly variable outcomes that compete against each other. As a result, large-template editing experiments are limited by low efficiency and undesired mutations, while more precise methods can only change one to tens of nucleotides. Further, in computer terms, we try to "edit" three billion base pairs of biological code by changing the 0s and 1s of our hardware with an assembly-level programming language. To unlock the true potential of genome programming, bioengineers need to develop and deliver platform technologies that can control larger sections of the genome at a higher level of abstraction with more complex operations than knock out or add one gene.

We are particularly excited about two areas. The first is the development of biologically inspired genome editing tools sourced from naturally occurring mechanisms, such as diverse phage integrases that have evolved to insert tens to hundreds of kilobases of DNA into specific target sequences. The second is the advancement of multi-component synthetic biology concepts such as genetic sensors and actuators, largely developed in bacterial or yeast systems, into robust genetic circuits in human cells. Together, these capabilities will enable new frontiers for synthetic human biology.

Prashant Mali
University of California, San Diego

The CRISPR pill

If one gets a fever, they are prescribed a pill. If the fever intensifies, the dose is increased. If this instead causes side effects, they are drawn down by progressively lowering the dose. And if the fever relapses, the whole cycle is started again. These attributes of targetability, tunability, reversibility, redosability, and ultimately ready accessibility of pills, while normally taken for granted, are the very attributes that make small-molecule drugs the most potent effectors of human medicine. These are thus also key attributes that CRISPR medicines must aspire towards.

In this regard, the greatest roadblock to applicability of CRISPRs is not CRISPRs, but instead their efficient and safe delivery. To most effectively leverage the unprecedented degree of genetic precision and multiplexability that CRISPRs enable, their effective deployment as medicines must entail *the CRISPR pill* to be (1) *tissue-targeted*, so that gene-edits and epi-edits are engineered only in the desired cell types; (2) *tunable*, so gene editors are adequately expressed to maintain the balance of specificity and activity, and epi-editors are adequately expressed to maintain the balance of specificity and regulation of their target genes to levels within the desired Goldilocks zone; (3) *redosable*, i.e., the editors and their delivery vehicles must evade the innate and adaptive immune defenses to enable readministration as desired, and which in turn ensures treatments don't need to be one-time and hence high-dose; (4) *reversible*, so one can draw down adverse events, as well as enable tackling of complex diseases where permanent edits may not be desirable; and (5) *accessible*, such that accompanying manufacturing is affordable and scalable from the *n = many* down to the *n = 1*.

Lei Stanley Qi
Stanford University

Ultra-large-scale chromosome engineering

CRISPR has made it very easy to edit and engineer the genome with high precision. This precision enables targeted alterations to small pieces of DNA or even a single base pair. However, this precision is currently limited to small regions in the genome, and we don't yet have a method to engineer the genome on a larger scale (>megabases or involving many genes). Because the human genome consists of 23 pairs of chromosomes (>3 billion bp) with delicate 3-dimensional structures, this inability is a bottleneck that restricts many applications.

Take genetic diseases as an example. Current CRISPR technologies are most suitable for studying monogenic diseases, but many common diseases are caused by groups of genes. While each gene may have a small pathogenic effect, collectively they exhibit a devastating disease phenotype. Studying or treating such diseases will require technologies to manipulate larger pieces of chromosomes. It may require deleting or knocking in megabase-sized DNA fragments consisting of dozens of genes and regulatory elements. In other cases, it may require targeting many genes or regulatory elements (such as introns or enhancers) simultaneously by activating some and silencing others.

Now is the right time to broaden the concept of genome engineering. Chromosome engineering (genome engineering on the ultra-large scale) goes beyond a single gene to target many genes. This will require more than editing. Chromosome engineering should instead embrace new technologies for precisely manipulating large chromosomal segments and for engineering the epigenome. I find ultra-large-scale chromosome and epigenome engineering is an unprecedented opportunity for broad applications in basic research and clinical studies.