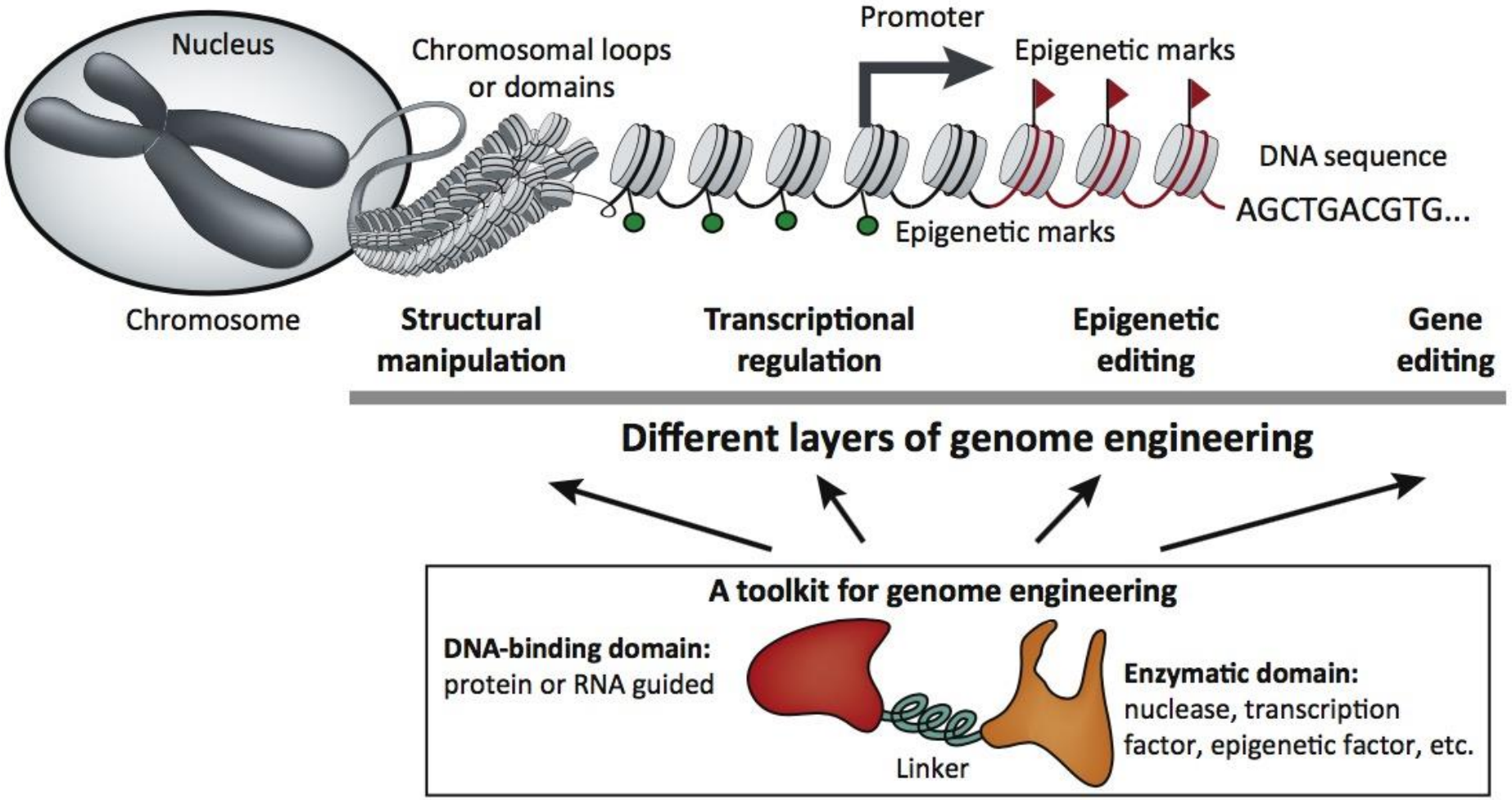
Genome Engineering using Programmable Nucleases



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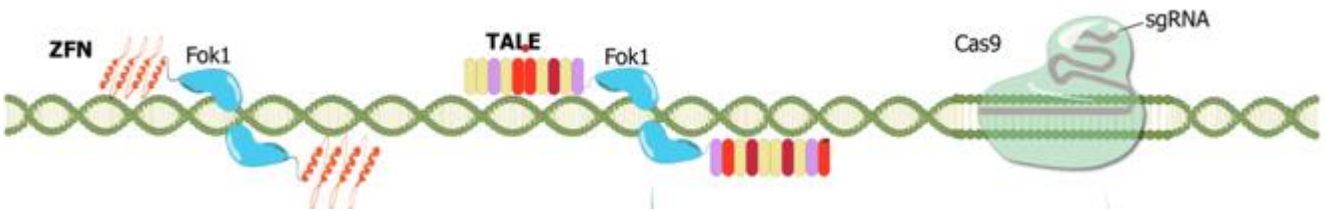
Next Generation Gene Therapy



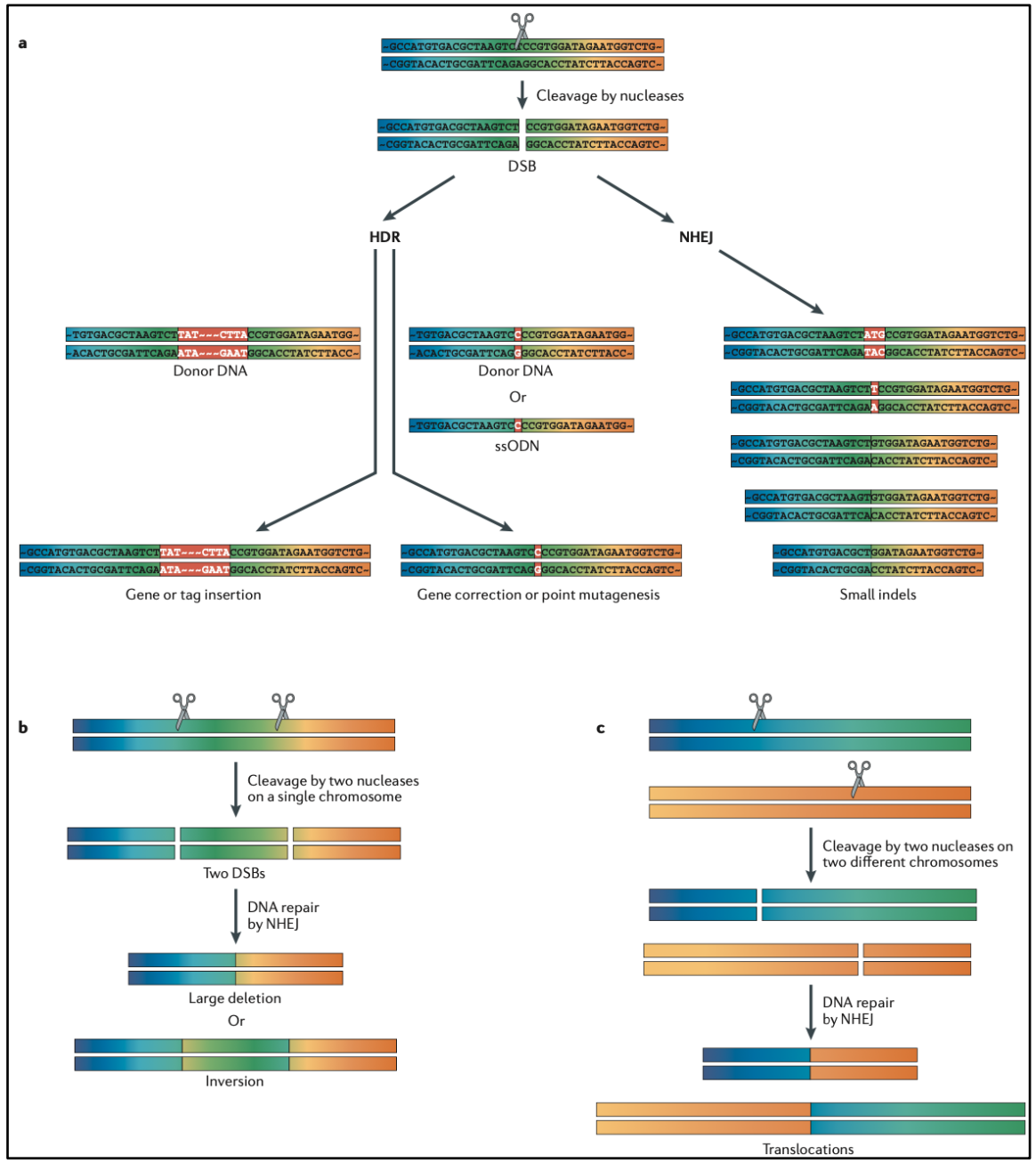
Jennifer Doudna and Emmanuelle Charpentier
2020 Nobel Prize Laureates in Chemistry

Genome editing (also called gene editing) is of great interest in the prevention and treatment of human diseases. Targeted genome editing is a continually evolving technology employing programmable nucleases to specifically change, insert, or remove a genomic sequence of interest. A number of genome editing technologies have emerged in recent years, including zinc-finger nucleases (***ZFNs***), transcription activator–like effector nucleases (***TALENs***) and the RNA-guided ***CRISPR-Cas*** nuclease system (***RGENs***), which create double-strand breaks at specific target sites in the genome, and repair DNA either by homologous recombination (HR) in the presence of donor DNA or via the error-prone non-homologous end-joining mechanism.

The first two technologies (ZFNs and TALENs) use a strategy of tethering endonuclease catalytic domains to modular DNA-binding proteins for inducing targeted DNA double-stranded breaks (DSBs) at specific genomic loci. By contrast, Cas9 is a nuclease ***guided by small RNAs*** through Watson-Crick base pairing with target DNA, representing a system that is markedly easier to design, highly specific, efficient and well-suited for high- throughput and multiplexed gene editing for a variety of cell types and organisms. Gene targeting via homologous recombination is not practical in higher eukaryotic cells due to low efficiency, hampering their routine use. On the other hand, **programmable nucleases** generating site-specific DSBs were found to increase **homologous recombination efficiency by at least 100 fold** and/or activated the error-prone NHEJ mechanism.

Ethical concerns arise when genome editing, using technologies such as CRISPR-Cas9, is used to alter human genomes. Most of the changes introduced with **genome editing** are **limited to somatic cells**, which are cells other than egg and sperm cells (germline cells). These changes are isolated to only certain tissues and are not passed from one generation to the next. However, changes made to genes in **egg or sperm cells** or to the genes of an **embryo** could be **passed to future generations**. **Germline cell** and **embryo genome editing** bring up a number of ethical challenges, including whether it would be permissible to use this technology to enhance normal human traits (such as height or intelligence). Based on concerns about **ethics** and **safety**, **germline cell** and **embryo genome editing** are **currently illegal** in the United States and many other countries.

Citation: Eksi YE, Sanlioglu AD, Akkaya B, Ozturk BE, Sanlioglu S. Genome engineering and disease modeling *via* programmable nucleases for insulin gene therapy; promises of CRISPR/Cas9 technology. *World J Stem Cells* 2021; 13(6): 485-502



Outcome of genome editing using programmable nucleases

a | Nuclease induced double-strand breaks (DSBs) can lead to sequence insertion, nucleotide correction or change (red box) through **homology-directed repair** (HDR) in the presence of a donor DNA or a single-strand oligodeoxynucleotide (ssODN), both of which contain homology arms. DSBs can also be repaired through error-prone **non-homologous end-joining** (NHEJ), which does not require donor DNA or ssODN and consequently often leads to small insertions and deletions (indels). Typical indel sequences and the number of inserted (+3 and +1) or deleted (–2, –4 and –10) bases are shown.

b | When two DSBs are generated in cis on a single chromosome by programmable nucleases, the flanking region can be deleted or inverted.

c | When two DSBs are generated on two different chromosomes, chromosomal translocations can be induced.

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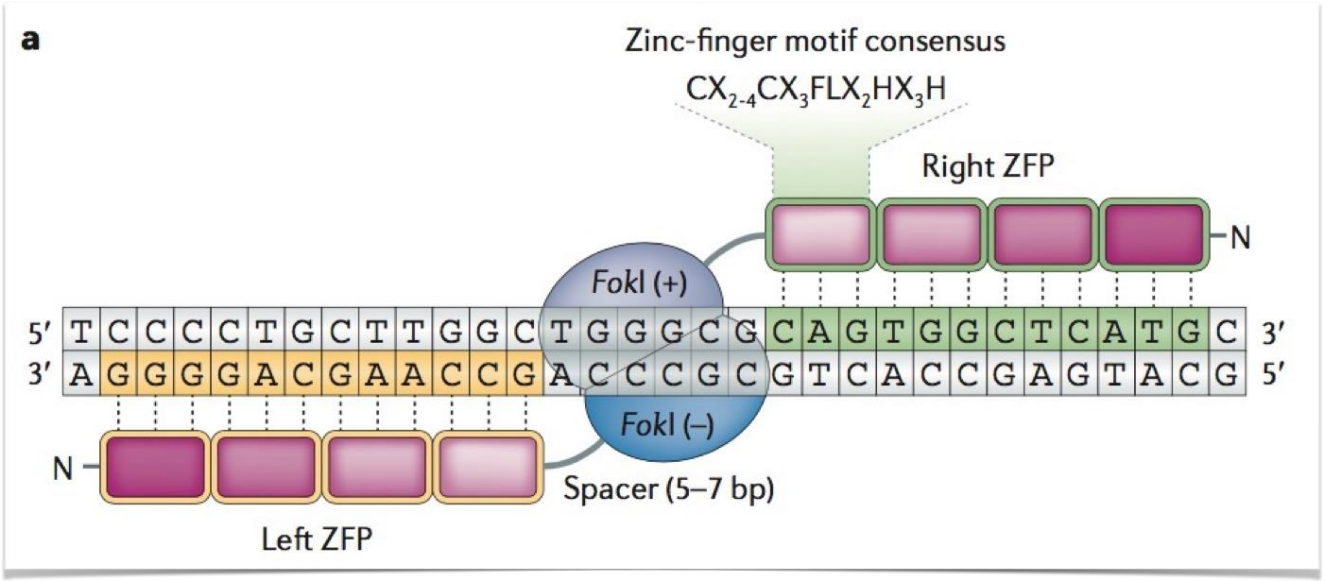
Zinc-finger nucleases

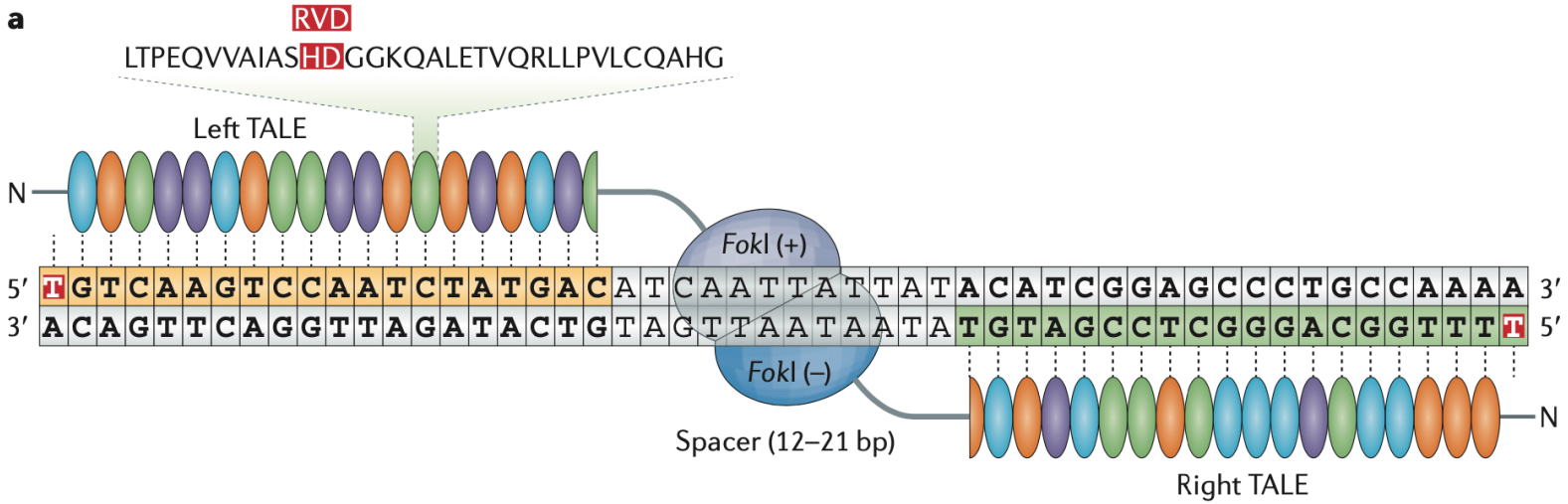


Structure of ZFNs. A ZFN has a modular structure that is composed of two domains: a DNA-binding zinc-finger protein (***ZFP***) domain and the nuclease domain derived from the ***FokI*** restriction enzyme. The FokI nuclease domain must dimerize to cleave DNA. Thus, two ZFN monomers are required to form an active nuclease; each monomer must bind to adjacent half-sites that are separated by spacers of 5–7 bp. This requirement for dimerization doubles the length of recognition sites, which substantially increases the specificity of ZFNs. However, **the wild-type FokI domain can still form homodimers to cleave DNA when one monomer binds to DNA**, which often leads to **unwanted off-target effects**.

The sequence specificity of ZFNs is determined by ZFPs, which consist of tandem arrays of C2H2 zinc fingers the most common DNA-binding motif in higher eukaryotes. **Each zinc-finger recognizes a 3-bp DNA sequence**, and 3–6 zinc-fingers are used to generate a single ZFN subunit that binds to DNA sequences of 9–18 bp. Importantly, the DNA-binding specificities of zinc-fingers can be altered by **mutagenesis**, which is a key feature of constructing a programmable nuclease.

There is **no straightforward and easy way to construct zinc finger domains** to bind a comprehensive stretch of nucleotides with high affinity. Furthermore, commercial ZFN modules are **quite expensive**, there are certain difficulties to perform replacement of large fragments (which is pivotal for inducible knockouts), and the technique necessitates screening in order to identify targeted events in animal models.

Transcription activator-like effector nucleases

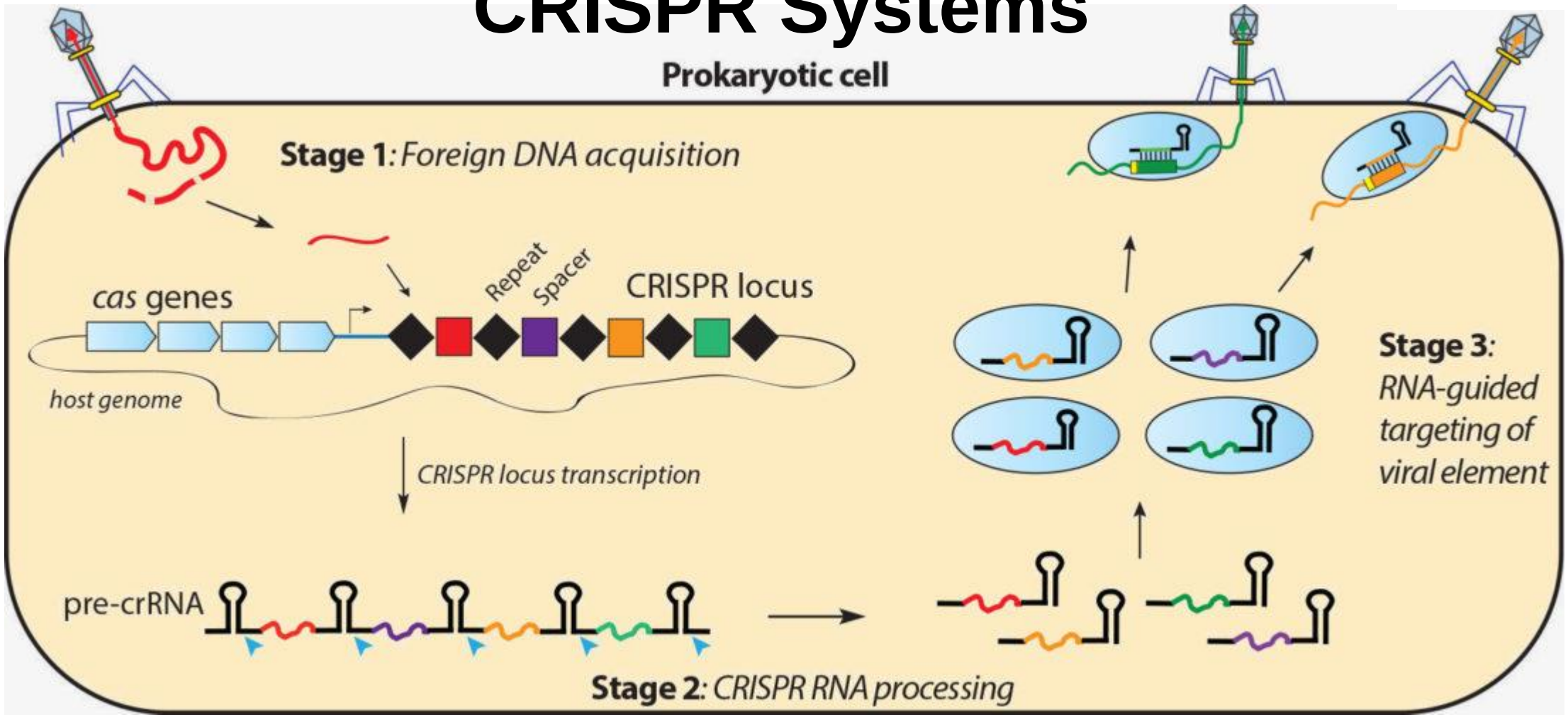


The general structural organization of TALENs is similar to that of ZFNs. Like ZFNs, TALENs contain the ***FokI*** nuclease domain at their carboxyl termini. However, they use a different class of DNA- binding domains known as transcription activator-like effectors (***TALEs***), which are derived from the plant pathogenic Xanthomonas spp. bacterium. TALEs are composed of tandem arrays of 33–35 amino acid repeats, each of which recognizes a single base-pair in the major groove. The nucleotide specificity of each repeat domain is determined by the two amino acids at positions 12 and 13, which are called **repeat variable diresidues (RVDs)**. Four different RVD modules — namely Asn-Asn, Asn-Ile, His-Asp and Asn-Gly — are most widely used to recognize guanine, adenine, cytosine and thymine, respectively.

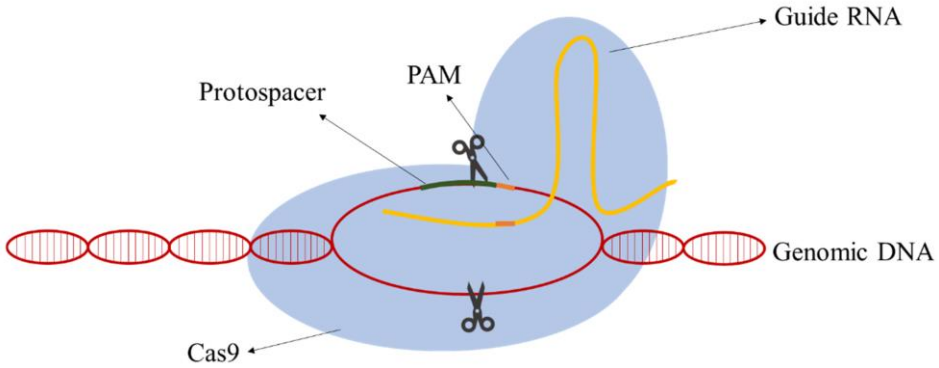
However, **the construction of DNA segments** that encode TALE arrays can be **challenging** and **time-consuming** both because TALENs often consist of up to 20 RVDs and because these **highly homologous sequences** can **recombine** with each other in cells

CRISPR Systems

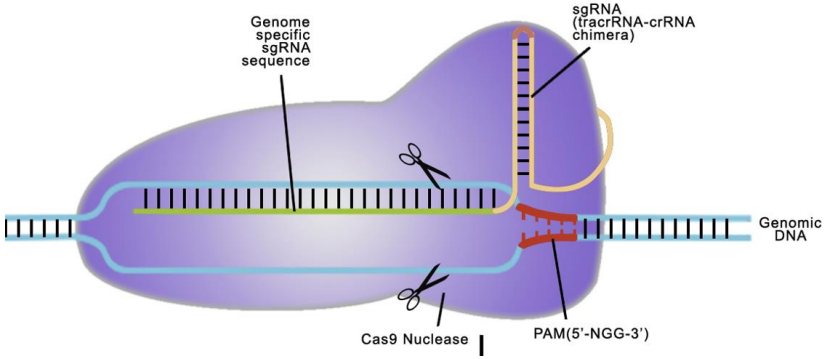
Doudna Lab



Bacteria and archaea possess **adaptive immunity** against foreign genetic elements using CRISPR–Cas systems. Upon infection, new foreign DNA sequences are captured and integrated into the host CRISPR locus as **new spacers**. The CRISPR locus is transcribed and processed to generate mature CRISPR RNAs, each encoding a unique spacer sequence. Each crRNA associates with Cas effector proteins that use **crRNAs as guides** to silence foreign genetic elements that match the crRNA sequence. Type II CRISPR–Cas systems use **an RNA-guided DNA endonuclease**, **Cas9**, to generate double-strand breaks in invasive DNA during an adaptive bacterial immune response. Cas9-mediated cleavage is strictly dependent on the presence of **a protospacer adjacent motif (PAM)** in the target DNA. The ability to program Cas9 for DNA cleavage at specific sites defined by guide RNAs has led to its adoption as a versatile platform for **genome engineering**.



CRISPR-Cas9



A well-known genome editing technology is called ***CRISPR-Cas9***, which is short for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats and **C**RISPR-**AS**sociated protein **9**. The CRISPR-Cas9 system has generated a lot of excitement in the scientific community because it is ***faster, cheaper, more accurate,*** and ***more efficient*** than other genome editing methods.

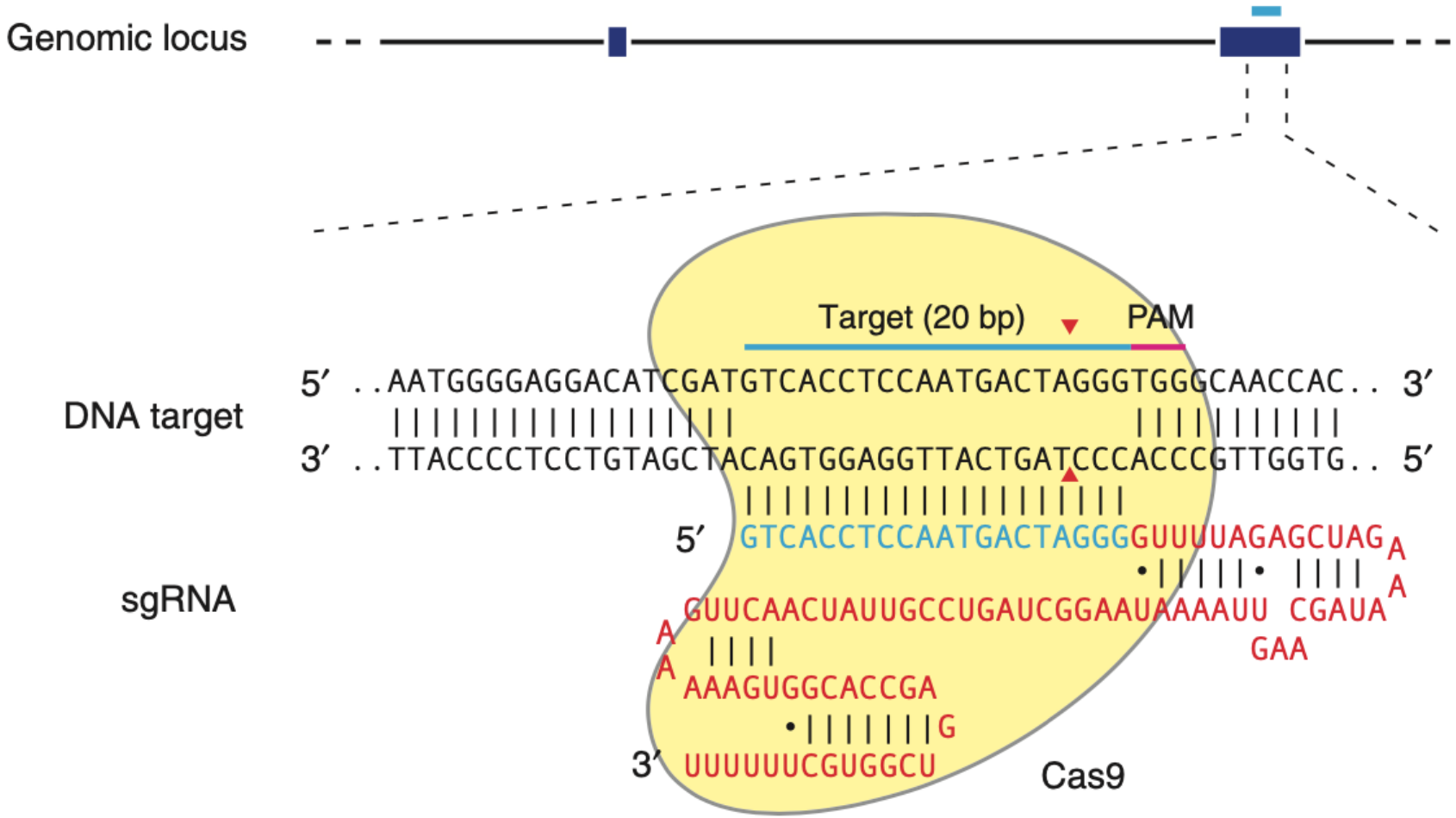
CRISPR-Cas9 was adapted from a naturally occurring genome editing system that bacteria use as an immune defense. When infected with viruses, bacteria capture small pieces of the viruses' DNA and insert them into their own DNA in a particular pattern to create segments known as **CRISPR arrays**. The CRISPR arrays allow the bacteria to "**remember**" the viruses (or closely related ones). If the viruses attack again, the bacteria produce RNA segments from the CRISPR arrays that recognize and attach to specific regions of the viruses' DNA. The bacteria then use Cas9 or a similar enzyme to cut the DNA apart, which disables the virus.

Researchers adapted this immune defense system **to edit DNA**. They create a small piece of RNA with **a short "guide" sequence** that attaches (binds) to a specific target sequence in a cell's DNA, much like the RNA segments bacteria produce from the CRISPR array. This guide RNA also attaches to **the Cas9 enzyme**. When introduced into cells, **the guide RNA recognizes the intended DNA sequence, and the Cas9 enzyme cuts the DNA at the targeted location**, mirroring the process in bacteria. Although Cas9 is the enzyme that is used most often, other enzymes (for example Cpf1) can also be used. Once the DNA is cut, researchers use **the cell's own DNA repair machinery** to add or delete pieces of genetic material, or to make changes to the DNA by replacing an existing segment with a customized DNA sequence.

In the field of genome engineering, the term "**CRISPR**" or "***CRISPR-Cas9***" is often used loosely to refer to the various CRISPR-Cas9 and -CPF1 (**Cas12**), (and other) systems that can be programmed to target specific stretches of genetic code and to edit DNA at precise locations, as well as for other purposes, such as for new diagnostic tools. With these systems, researchers ***can permanently modify genes*** in living cells and organisms and, in the future, may make it possible to ***correct mutations*** at precise locations in the human genome in order ***to treat genetic causes of disease***. Other systems are now available, such as CRISPR-Cas13's, that target RNA provide alternate avenues for use, and with unique characteristics that have been leveraged for sensitive diagnostic tools, such as SHERLOCK.

Cas13 is an outlier in the CRISPR world because **it targets RNA**, **not DNA**. Once it is activated by a ssRNA sequence bearing complementarity to its crRNA spacer, it unleashes **a nonspecific RNase activity** and **destroys all nearby RNA regardless of their sequence**. This property has been harnessed in vitro for **precision diagnostics**. These systems can also be used for efficient, multiplexable, and specific **RNA knockdown** or **RNA sequence editing** in mammalian cells. This makes Cas13 a potentially significant therapeutic for **influencing gene expression without altering genome sequence**.

F Ann Ran[1–5,8], Patrick D Hsu[1–5,8], Jason Wright[1], Vineeta Agarwala[1,6,7], David A Scott[1–4] & Feng Zhang[1–4]



Schematic of the RNA-guided Cas9 nuclease. The Cas9 nuclease from S. pyogenes (in yellow) is targeted to genomic DNA (shown for example is the human EMX1 locus) by an sgRNA consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence pairs with the DNA target (blue bar on top strand), directly upstream of a requisite 5′-NGG adjacent motif (PAM; pink). Cas9 mediates a DSB ~3 bp upstream of the PAM (red triangle).

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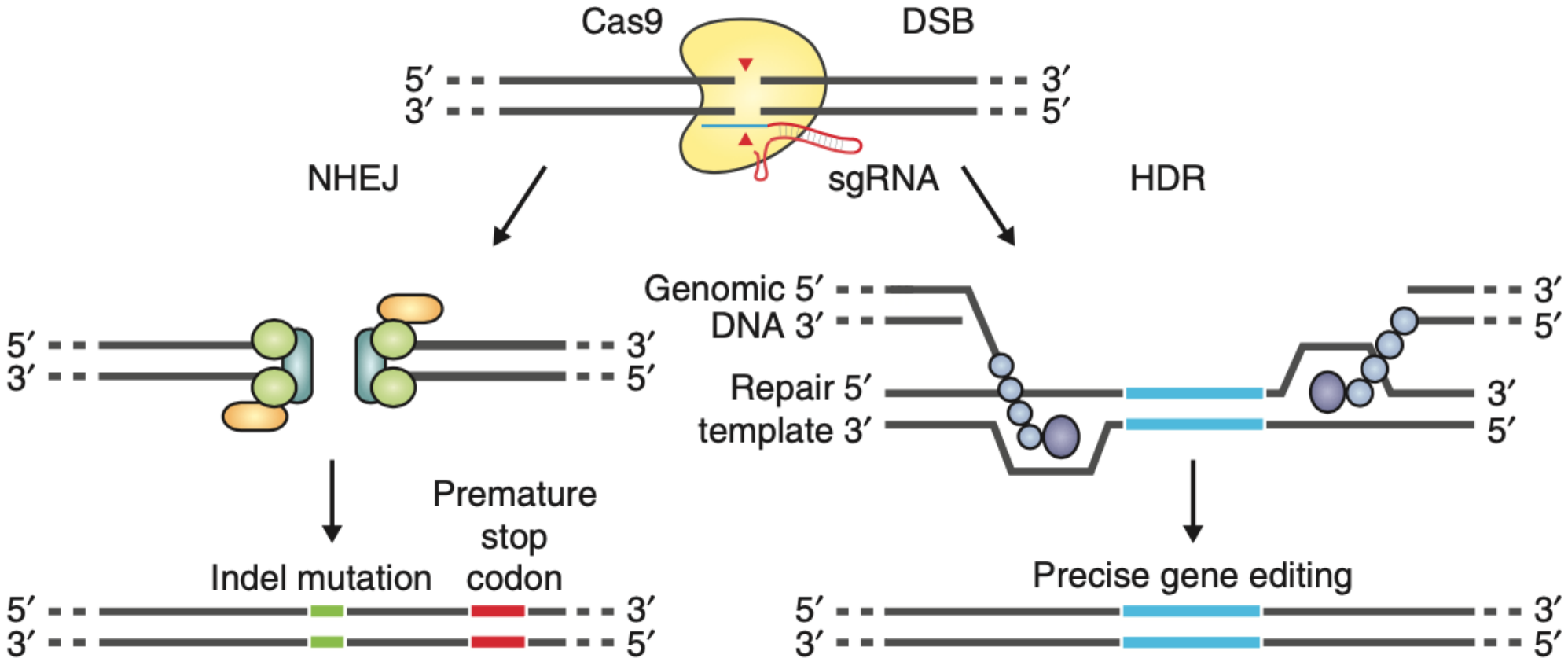
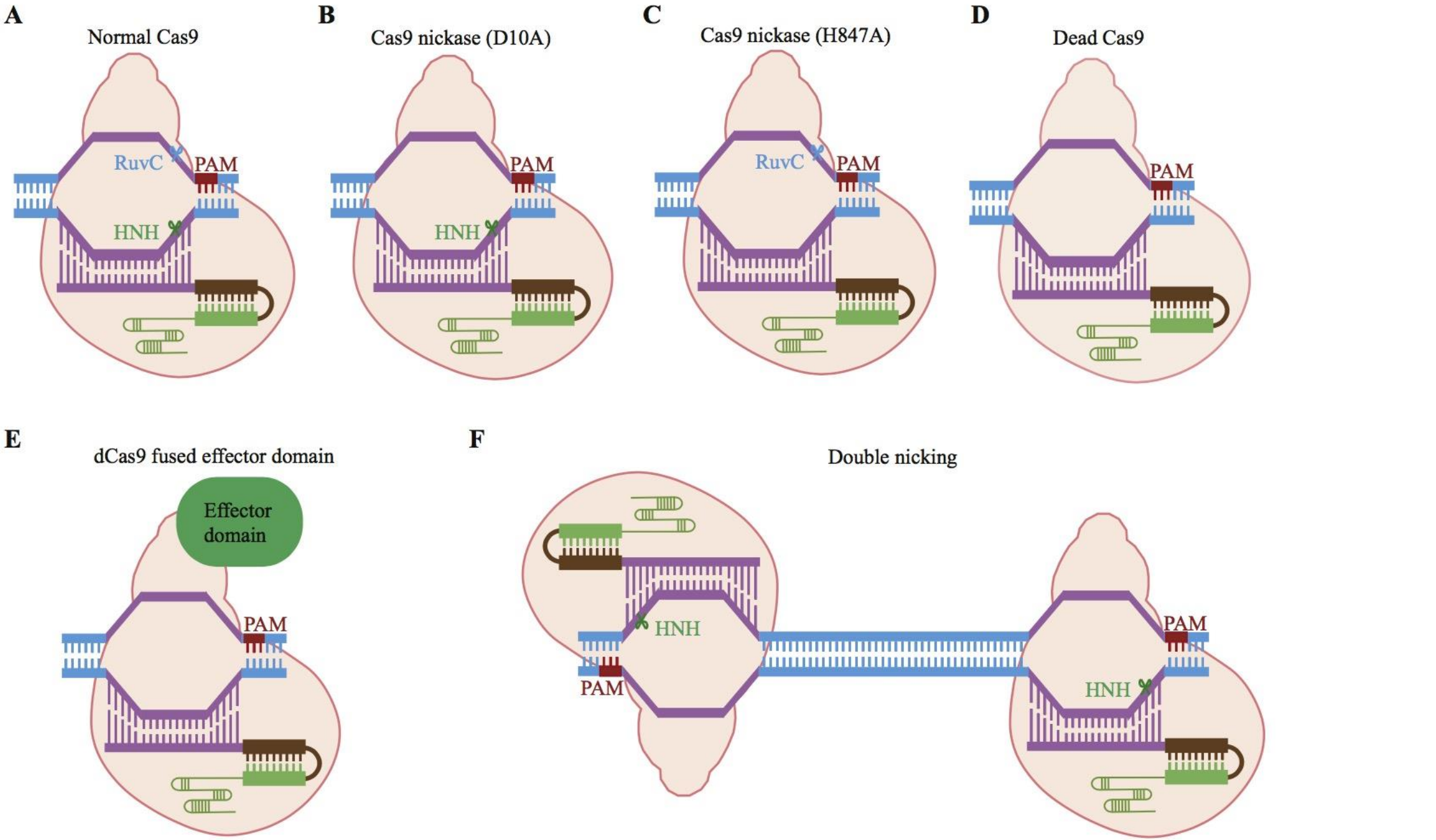
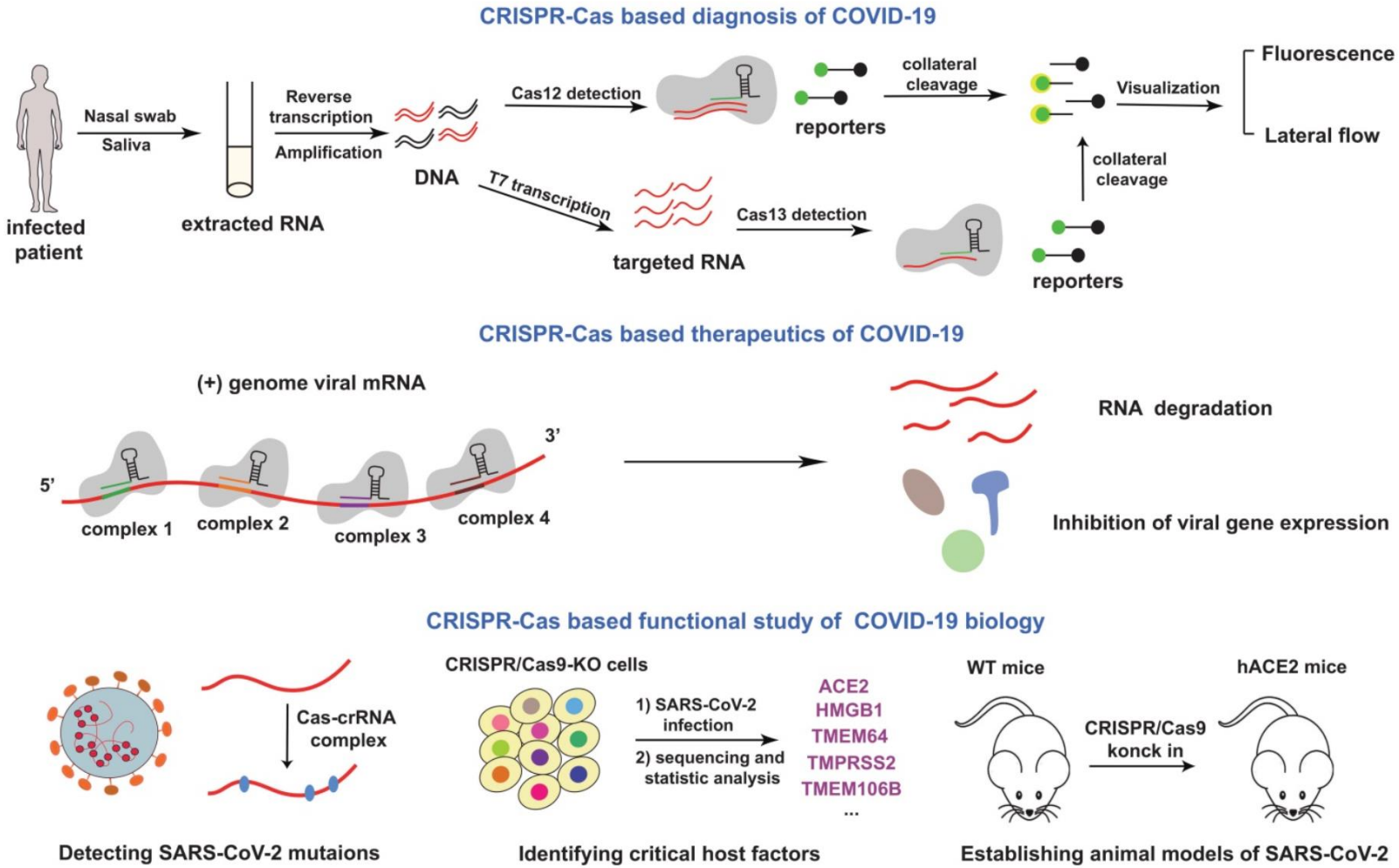


DSB repair promotes gene editing. DSBs induced by Cas9 (yellow) can be repaired in one of two ways. In the error-prone NHEJ pathway, the ends of a DSB are processed by endogenous DNA repair machinery and rejoined, which can result in random indel mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frameshifts and the creation of a premature stop codon, resulting in gene knockout. Alternatively, a repair template in the form of a plasmid or ssODN can be supplied to leverage the HDR pathway, which allows high fidelity and precise editing. Single-stranded nicks to the DNA can also induce HDR.

The modifications of Cas9 based on its functional domains



Y. Mei et al. / Journal of Genetics and Genomics 43 (2016) 63–75



The Zhang lab has adapted natural RNase activity of the Cas13 protein to develop and optimize the method termed Specific High Sensitivity Enzymatic Reporter UnLOCKING (***SHERLOCK*** and SHERLOCKv2) (Gootenberg et al., 2017 and 2018). While the Doudna lab has used Cas12a's non-specific ssDNA degradation to develop the method termed DNA Endonuclease Targeted CRISPR Trans Reporter (***DETECTR***) (Chen et al., 2018).

Both SHERLOCK and DETECTR harness the promiscuous cleavage and degradation of neighboring ssRNA and ssDNA by Cas13 and Cas12a, respectively, to cleave and activate a reporter. The detectable signal from this reporter can be measured and quantified to determine the presence and quantity of DNA, RNA or a mutation of interest. Together SHERLOCK and DETECTR demonstrate the power of ***CRISPR-based diagnostics***.

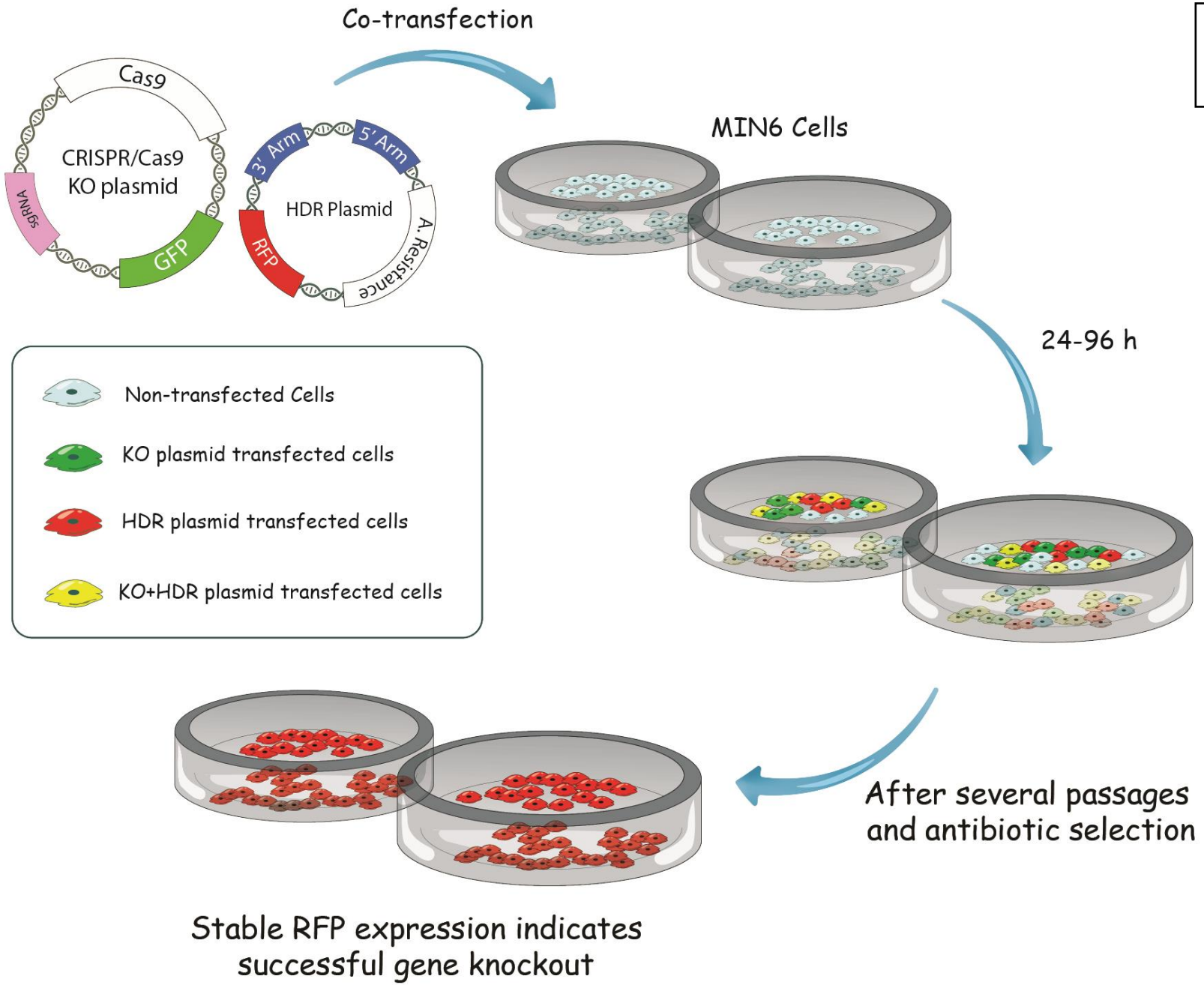
Schematics for CRISPR-Cas based applications in COVID-19. In the top of the picture, a cardinal principle of CRISPR-based diagnosis was shown. Researchers extracted patient sample RNA from nasopharyngeal or oropharyngeal swabs, and transformed RNA into cDNA with the process of reverse transcription. Then amplified virus cDNA, and use Cas protein targets predefined coronavirus sequences. The collateral cleavage of ssDNA probe confirmed detection of the virus. In the middle of the picture, the mechanism of therapeutics was depicted. Genome viral mRNA was degradation after CRISPR-Cas system cleavage, and the viral gene expression have also been inhibited. In the bottom of the picture, potential utilization of CRISPR in COVID-19 mechanism research was exhibited.

Table 2 | **Comparison of three classes of programmable nucleases***

	ZFNs	TALENs	RGENs
DNA targeting specificity determinant	Zinc-finger proteins	Transcription activator-like effectors	crRNA or sgRNA
Nuclease	*Fok*I	*Fok*I	Cas9
Success rate‡	Low (~24%)	High (>99%)	High (~90%)
Average mutation rate§	Low or variable (~10%)	High (~20%)	High (~20%)
Specificity-determining length of target site	18–36 bp	30–40 bp	22 bp (total length 23 bp)
Restriction in target site	G-rich	Start with T and end with A (owing to the heterodimer structure)	End with an NGG or NAG (lower activity) sequence (that is, PAM)
Design density	One per ~100 bp	At least one per base pair	One per 8 bp (NGG PAM) or 4 bp (NGG and NAG PAM)
Off-target effects	High	Low	Variable
Cytotoxicity	Variable to high	Low	Low
Size	~1 kb×2	~3 kb×2	4.2 kb (Cas9 from *Streptococcus pyogenes*) + 0.1 kb (sgRNA)

Cas9, CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 9; crRNA, CRISPR RNA; N, any nucleotide; PAM, protospacer adjacent motif; RGEN, RNA-guided engineered nuclease; sgRNA, single-chain guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease. *A wide range of success rates and mutation rates (which depend on factors such as the methods used to construct these nucleases, delivery methods and cell lines or organisms) have been reported. The numbers given here are based on our own studies using HEK293 cells[5,15,54,62,124,192]. Mutation frequencies are higher in K562 cells and HeLa cells than in HEK293 cells. ‡The success rate is defined as the proportion of nucleases that induce mutations at frequencies >0.5% in HEK293 cells. §The average mutation rate is based on the frequency of non-homologous end-joining-mediated insertions and deletions obtained at the nuclease target site.

A Preclinical Study



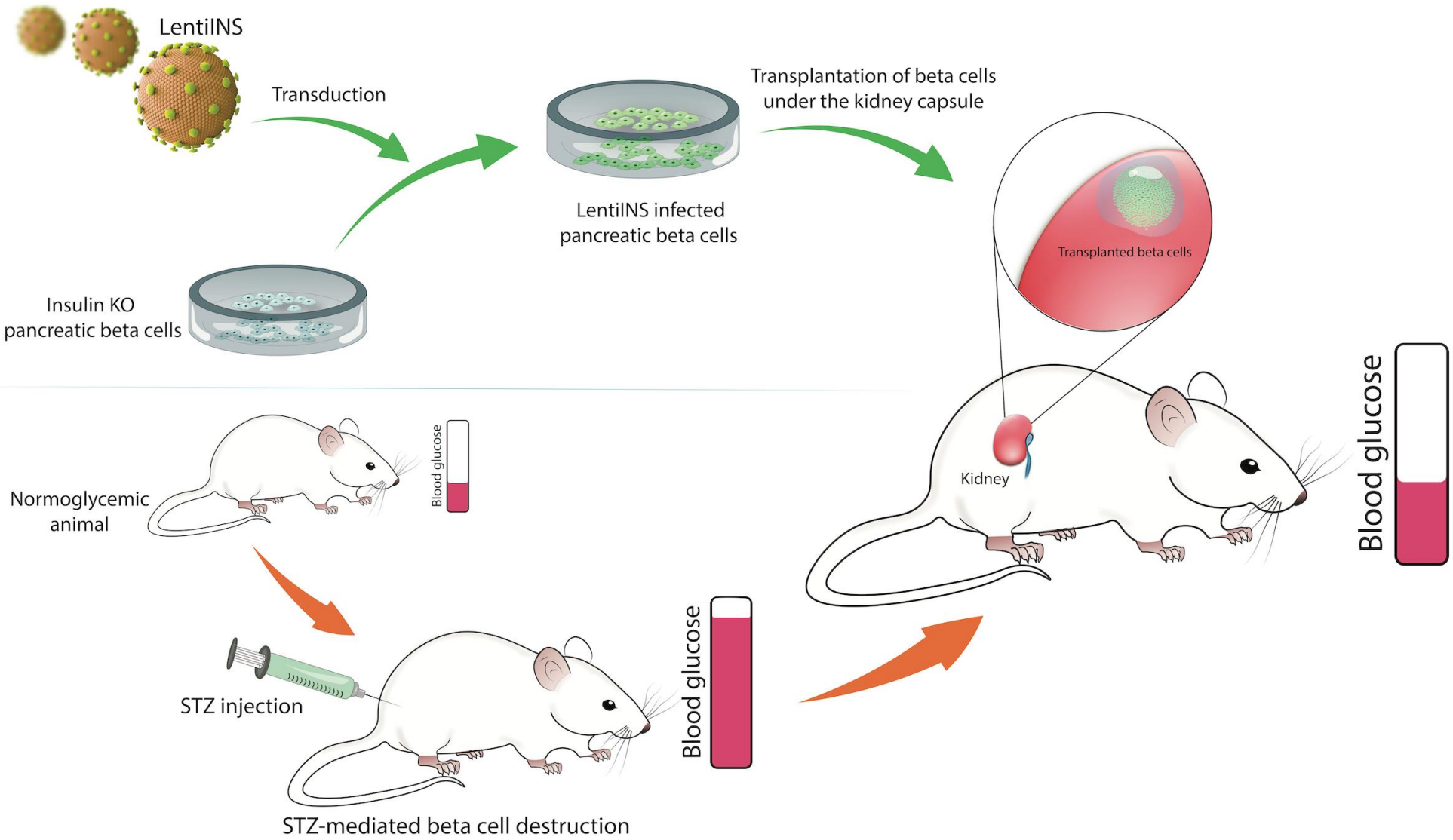
Production of CRISPR/Cas9-mediated insulin-deficient pancreatic beta cell line for testing the therapeutic efficacy of insulin gene therapy vectors.

In this scenario, the CRISPR/Cas9 knockout plasmid carries a Cas9 protein-encoding sequence, sgRNA, and green fluorescein protein as a reporter. Homology-directed repair (HDR) plasmid contains two genomic fragments of DNA complementary to insulin gene (3' Arm and 5' Arm) and red fluorescein protein-encoding DNA sequences.

Both CRISPR/Cas9 knockout plasmid and HDR plasmid are transfected into **a pancreatic beta-cell line** such as **MIN6** cells. After several passages and antibiotic selection, beta cells expressing red fluorescent protein remain alive suggesting **successful knockout** (KO) of the insulin (INS) gene.

INS KO cells can be cloned by limited dilution and further expanded in cell culture. INS KO cells can be used in in vitro complementation assays as well as transplanted under the kidney capsule of diabetic rats to assess the therapeutic efficacy of gene therapy vectors. KO: Knockout; HDR: Homology-directed repair.

Citation: Eksi YE, Sanlioglu AD, Akkaya B, Ozturk BE, Sanlioglu S. Genome engineering and disease modeling *via* programmable nucleases for insulin gene therapy; promises of CRISPR/Cas9 technology. *World J Stem Cells* 2021; 13(6): 485-502



Testing the therapeutic efficacy of the lentivirus vector carrying insulin gene (LentiINS).

The chemotherapeutic agent **Streptozotocin** can be used to destroy endogenous pancreatic beta cells to induce an experimental animal model of diabetes.

Insulin knockout (INS KO) pancreatic beta cells after transduction with the LentiINS can be transplanted under the kidney capsule of diabetic animals.

Diabetic animals transplanted with the LentiINS-transduced INS KO pancreatic beta cells are expected to lower the blood glucose.

On the other hand, hyperglycemia is anticipated in diabetic animals transplanted with INS KO pancreatic beta cells alone.

Citation: Eksi YE, Sanlioglu AD, Akkaya B, Ozturk BE, Sanlioglu S. Genome engineering and disease modeling *via* programmable nucleases for insulin gene therapy; promises of CRISPR/Cas9 technology. *World J Stem Cells* 2021; 13(6): 485-502

CRISPR Clinical Trials
A 2022 Update

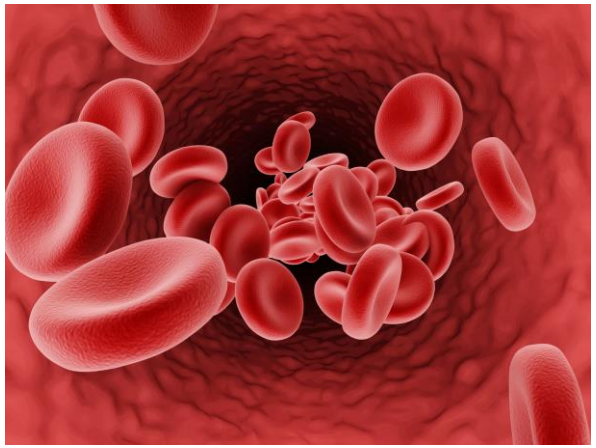
CRISPR Clinical Trial Overview

This year marks the 10th anniversary of the development of CRISPR as a genome-editing tool, an achievement that earned ***Jennifer Doudna and Emmanuelle Charpentier the 2020 Nobel Prize in Chemistry***. In the first five years, the field focused on refining how CRISPR works in different cell types, improving its efficiency at cutting DNA, and developing CRISPR towards clinical applications for the first time. In the following five years, the discovery and engineering of new CRISPR proteins with different capabilities expanded the CRISPR toolbox and the first CRISPR clinical trials began, yielding sometimes stunning results.

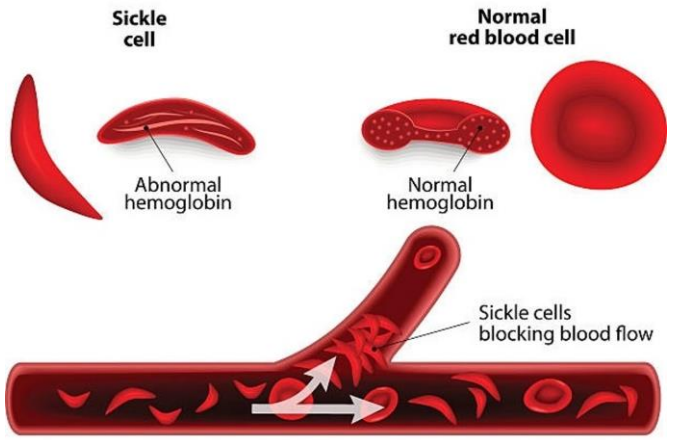
In the United States, the Food and Drug Administration (FDA) assesses new disease treatments for safety and efficacy through clinical trials on patient volunteers. Early trials (phase 1) evaluate safety and treatment side effects. Later trials (phase 2 and 3) evaluate how effective treatments are and compare new therapies to standard treatments. While the number of CRISPR clinical trials is growing each year, ***most of the current trials using CRISPR-based treatments are still in early stages***. That means that even if the treatments are safe and effective, they're likely still a few years away from a possible FDA approval and being available to patients in the US.

The development of CRISPR genome editing opens up new possibilities in precision medicine. Current trials are underway in seven treatment areas: ***blood disorders, cancers, inherited eye disease, diabetes, infectious disease, inflammatory disease, and protein-folding disorders***.

Innovative Genomics Institute



Blood Disorders



The disorders of β-hemoglobin, sickle cell disease (SCD) and β-thalassemia, are major sources of morbidity and mortality worldwide. These diseases are ***the most common genetic disorders*** in the world. It is currently estimated that at least 300,000 children with these diseases are born every year, giving rise to a worldwide population of tens of millions of patients with these diseases. Red blood cells use the protein hemoglobin to carry oxygen from the air we breathe to the rest of the body. Variations in a gene that encodes part of the hemoglobin molecule cause two genetic disorders: ***sickle cell disease (SCD) and beta thalassemia***.

In SCD, a single base substitution causes **a missense mutation** of **a valine for glutamic acid** at **amino acid six** of the **β-globin** protein chain, which leads to a propensity of the sickle hemoglobin to polymerize. This in turn results in deformation of erythrocytes containing this hemoglobin, which **can block small blood vessels**, leading to impaired oxygen delivery to tissues. This can result in significant clinical complications including **pain crises**, respiratory complications, and organ damage. In β-thalassemia, **insufficient production of the β-globin** molecule results in an excess of **unpaired α-globin chains** that can **precipitate** within **erythroid precursors**. The precipitation of these free α-globin chains **impairs the maturation** and leads to death of these precursors, causing **ineffective production of erythroid cells**. As a result, **a significant anemia occurs** and the consequent expansion of erythroid precursors can lead to secondary problems in bones and other organs.

There are some treatments available for SCD and beta thalassemia, but patients often have severe symptoms and complications even with treatment. ***Bone marrow transplant*** can be curative; however, this can only be done when a healthy, matching donor can be found. Bone marrow transplant is not an option for most SCD or beta thalassemia patients. Patients with more severe cases of either condition need frequent ***blood transfusions***.

The approach taken to treat these blood disorders with CRISPR technology in the most advanced trial doesn't directly correct the gene variants that cause disease. It uses a clever workaround: instead of restoring healthy adult hemoglobin, the goal is to ***increase levels of fetal hemoglobin***. This is a form of hemoglobin that fetuses make in the womb, but children and adults don't make. We don't know yet why humans switch from one form of hemoglobin to the other after birth, but fetal hemoglobin is not affected by the sickle cell mutation and can take the place of defective adult hemoglobin in red blood cells. This treatment can be used for both SCD and beta thalassemia.

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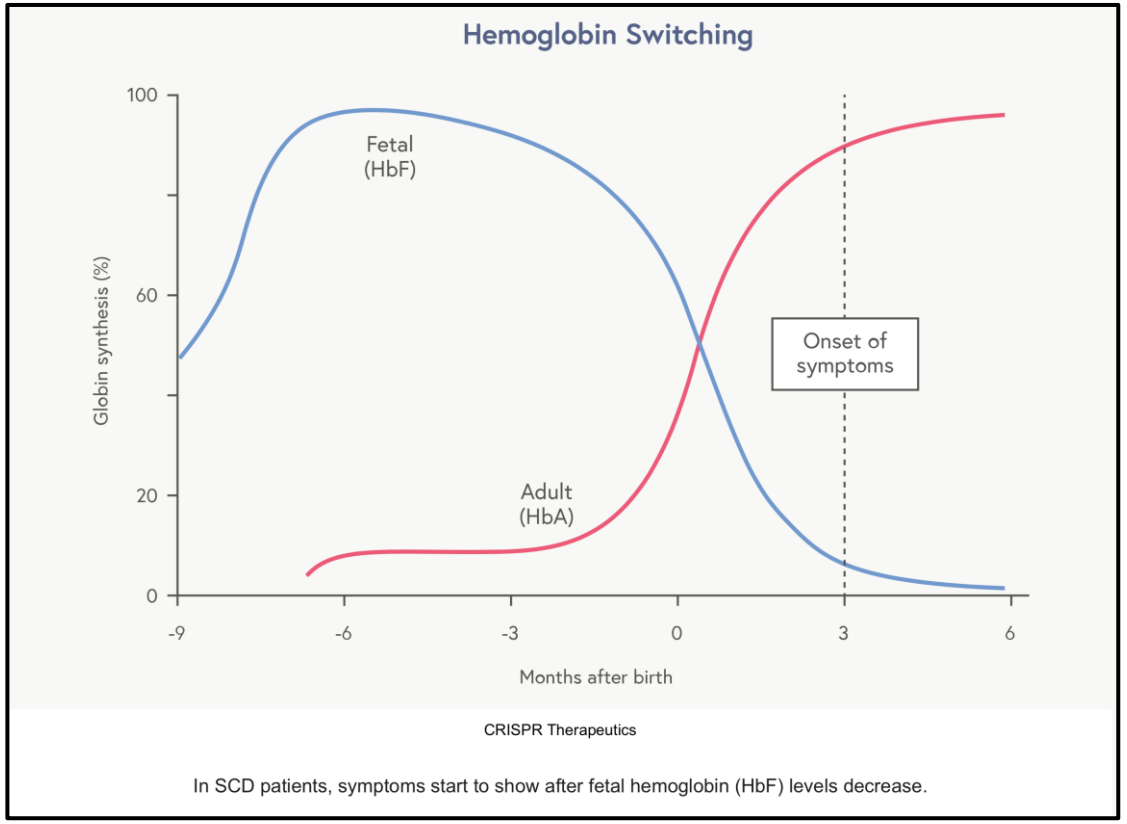
Manipulation of Gamma-Globin Gene Expression an Approach for Healing Hemoglobinopathies

Beta-hemoglobinopathies, particularly, sickle cell disease (SCD) and beta-thalassemia, are a group of inherited monogenic recessive disorders marked by defective or decreased production of beta-globin chains, respectively. Globally, b-hemoglobinopathies are highly prevalent in the Mediterranean region, Africa, Middle East, South and Southeast Asia, and the Pacific Islands.

The fetal-to-adult hemoglobin switch and silencing of fetal hemoglobin (HbF) have been areas of long-standing interest among hematologists, given the fact that ***clinical induction of HbF production holds tremendous promise to ameliorate the clinical symptoms of sickle cell disease (SCD) and b-thalassemia***.

Persistent production of gamma-globin by gene-modified autologous hematopoietic stem cells could be **an effective gene therapy strategy to alleviate the severity of beta-hemoglobinopathies**.



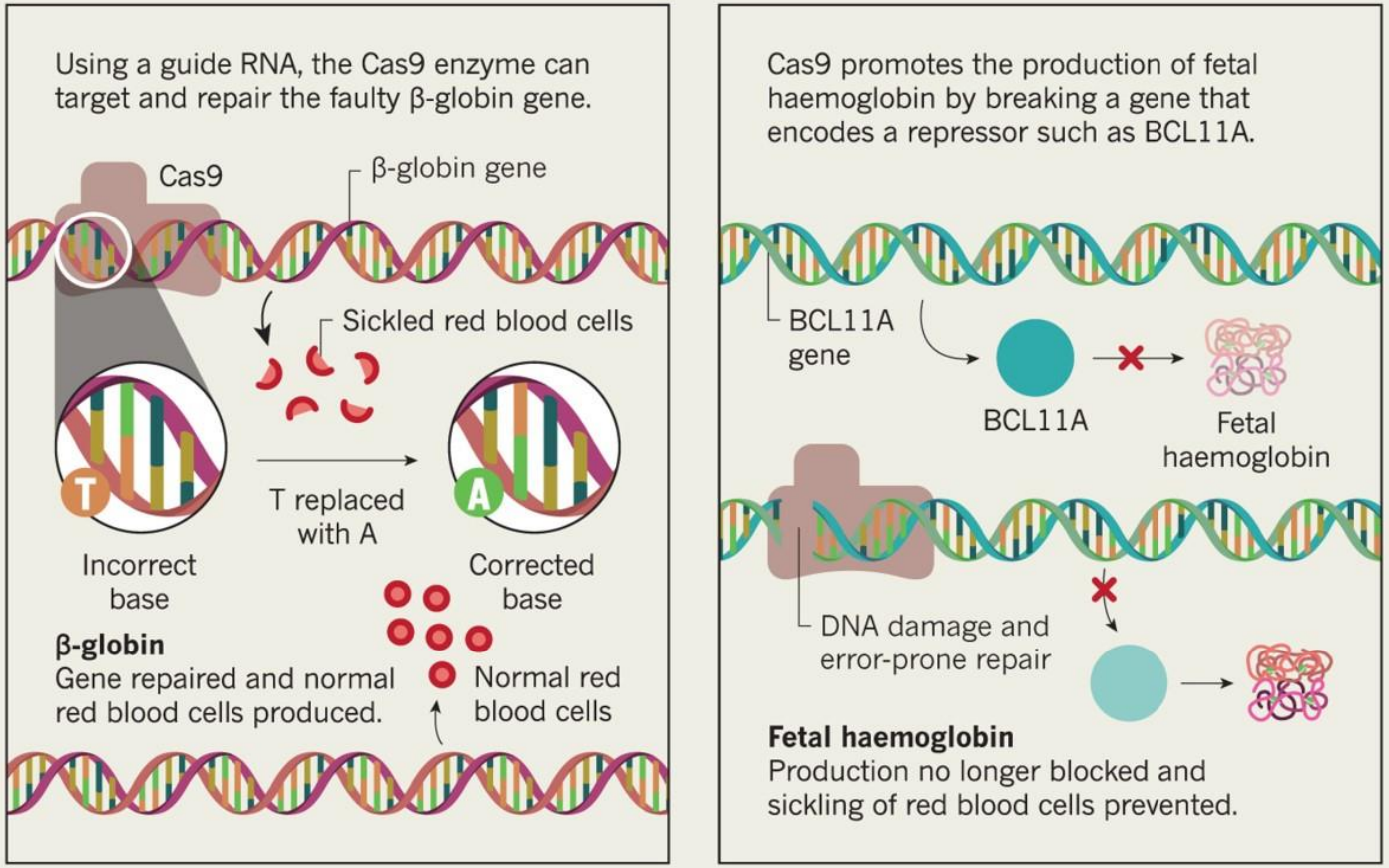
In SCD patients, symptoms start to show after fetal hemoglobin (HbF) levels decrease.

A variety of clinical observations have shown that the severity of SCD and β-thalassemia is ameliorated via increased production of HbF. Children with SCD were noted to be asymptomatic until after infancy, which was postulated to be attributable to elevated fetal hemoglobin (HbF) levels (Watson 1948). This notion was substantiated by observations of rare patients with **compound heterozygosity** for sickle cell disease and **hereditary persistence of fetal hemoglobin (HPFH)** mutations, who were **largely asymptomatic** (Weatherall and Clegg 2001). These observations were subsequently confirmed with larger epidemiological studies in SCD, which showed that increased HbF levels can significantly and quantitatively ameliorate the clinical severity and decrease mortality in SCD (Platt et al. 1991, 1994; Castro et al. 1994).

Similar observations have been made in patients with β-thalassemia. Clinical observations in rare β-thalassemia patients with elevated production of HbF showed that these increased levels resulted in a milder clinical course and infants with β-thalassemia only begin to show symptoms after the expression of HbF declines in the months following birth (Weatherall 2001; Weatherall and Clegg 2001). Larger epidemiological studies of thalassemia populations have confirmed such findings (Premawardhena et al. 2005; Galanello et al. 2009; Nuinoon et al. 2010).

Cold Spring Harb Perspect Med. 2013 Jan; 3(1): a011643.

Treatment Strategy in Blood Disorders



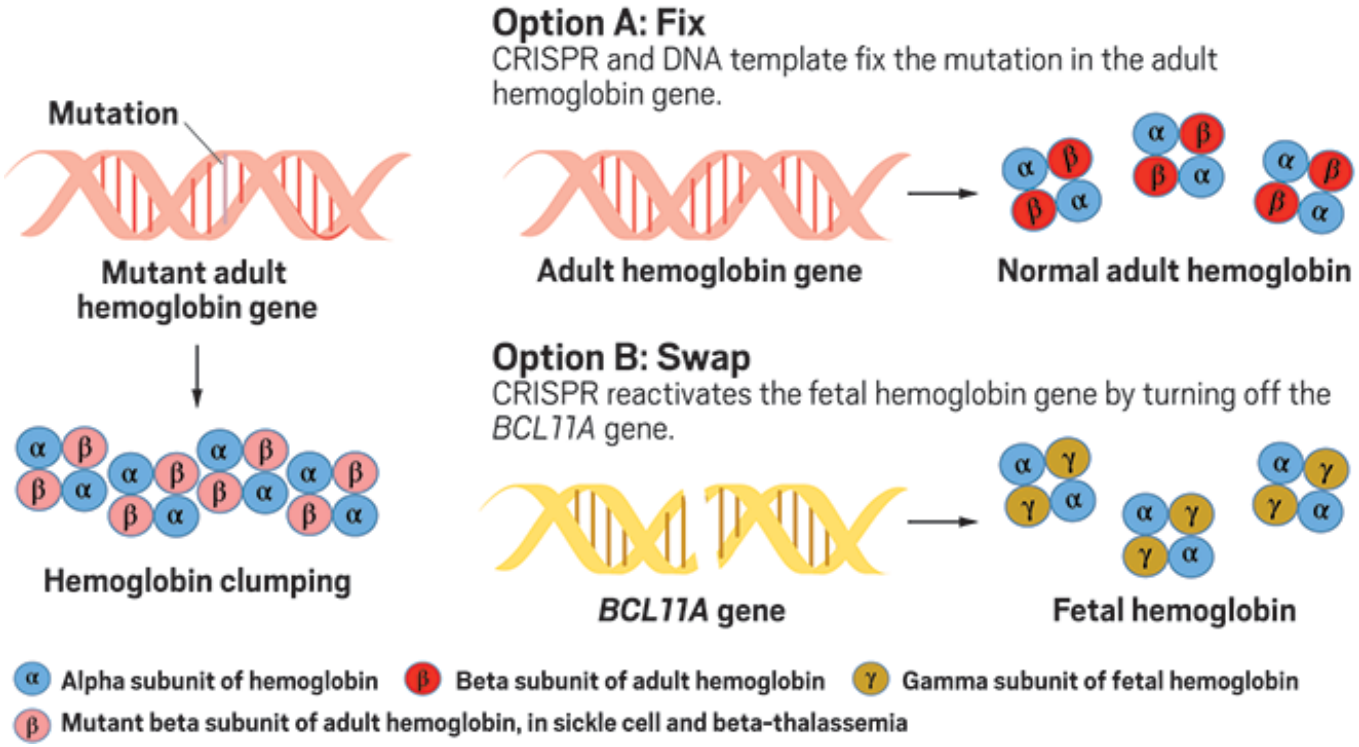
In individuals with Sickle Cell Disease, symptoms start to show during infancy, after fetal hemoglobin (HbF) levels decrease. The first step of treatment is to harvest a patient's blood stem cells directly from their blood. Next, scientists edit the genomes of these cells to turn the fetal hemoglobin gene on. Then, chemotherapy eliminates the disease-causing blood stem cells from the patient's body. Finally, billions of genome-edited stem cells are put back into their bloodstream. These genome-edited blood stem cells are administered by IV. If it works as intended, these cells will take up residence in the bone marrow, creating a new blood stem cell population which will make edited red blood cells that produce fetal hemoglobin.

This treatment approach is called **ex vivo genome editing**, because the editing occurs outside of the patient's body. The advantage of ex vivo editing is ensuring that genome-editing tools only come in contact with **the right target cells**. It also **avoids the risk** of **long-term presence** of CRISPR components in the body, like unwanted edits or immune reactions.

Other trials that are just beginning or will begin soon also use an ex vivo approach, but are different on a molecular level. One uses base editing rather than conventional CRISPR, to turn on fetal hemoglobin. Two other trials aim to directly correct the mutation that causes sickle cell disease using CRISPR Cas-9, restoring healthy, adult hemoglobin.

S30 | NATURE | VOL 549 | 28 SEPTEMBER 2017

Treatment Strategy in Blood Disorders



Scalability making enough of a treatment to get it to the many people who need it will be **a major challenge** for CRISPR-based treatments for blood disorders, both because of the technical challenges of creating the individualized product and administering the treatment protocol, and the cost. **The cost** of the current CRISPR-based therapy is in t**he $1–2 million** range, and can **only be performed at a small number of medical facilities** worldwide, putting it well out of reach of the vast majority of people with SCD or beta thalassemia.

In the first use of an ex vivo CRISPR-based therapy to treat a genetic disease, researchers treated an individual with **beta thalassemia in Germany in 2019**. CRISPR Therapeutics and Vertex Pharmaceuticals are running this trial in Europe and Canada. According to company press releases, at least 14 more individuals have since been treated and followed for at least three months, with five followed for over a year. The first individual with SCD was treated with the same therapy **in Nashville, Tennessee in 2019**. At least six more individuals with SCD have been treated since then and followed for at least three months, with two followed for over a year.

So far, patient volunteers with both conditions have made remarkable recoveries.

- Patients treated for SCD or beta thalassemia show ***normal to near-normal hemoglobin levels***, where at least 30% (SCD) or 40% (beta thalassemia) of hemoglobin is fetal hemoglobin.
- Patients with beta thalassemia are free from needing blood transfusions. Patients with SCD are **free from transfusions** and disabling pain crises.
- Molecular tests on bone marrow from each of six patients **a year or more** after treatment show **the continued presence of genome-edited cells**.

CRISPR Therapeutics and Vertex Pharmaceuticals are jointly running these combined phase 1, 2, and 3 trials in the US, Canada, and Europe. In Europe and the US, this treatment has been given special status to fast-track approval. If trial data continue to be so positive, ***the treatment (Exa-cel) could be approved as soon as 2023***.

Cancers

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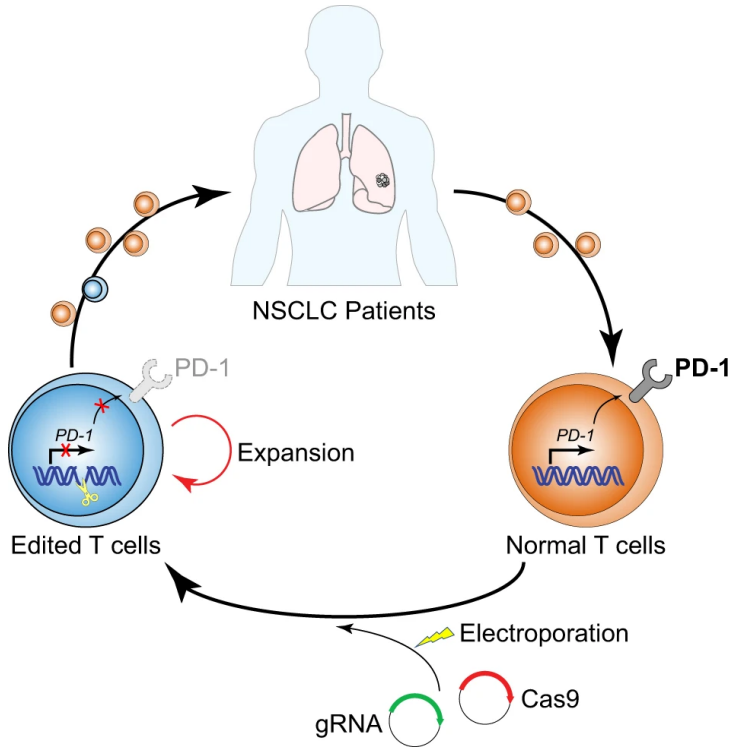
Cancer refers to diseases that are caused by uncontrolled cell growth. Right now, CRISPR-based therapies are mainly aimed at treating blood cancers like ***leukemia*** and ***lymphoma***.

T cells are a type of white blood cell that have a central role in immune system response. T cells are covered in receptors that recognize other cells as safe or threatening. They patrol the body, killing foreign or dangerous cells, or recruiting other cells to assist. In CAR-T immunotherapy, researchers genetically engineer an individual's T cells to have a receptor that recognizes their cancer cells, telling the T cells to attack.

The immune system is highly regulated to avoid attacking healthy cells. Some T cell receptors work as "checkpoints" that determine whether an immune response occurs. When a T cell ***PD-1 receptor*** comes in contact with a molecule called ***PD-L1*** on another cell, it communicates that it is a ***"safe" cell*** and the T cell leaves it alone.

Cancer cells often cloak themselves in these safety signals, tricking the patrolling T cells into ignoring them. Researchers are using ***CRISPR to edit the PD-1 gene in T cells to stop them from making functional PD-1 receptors*** so they can't be tricked by cancer cells. This immunotherapy approach is known as ***checkpoint inhibition***, and it is often used in conjunction with CAR-T engineering to give T cells the greatest possible chance of eliminating cancer.

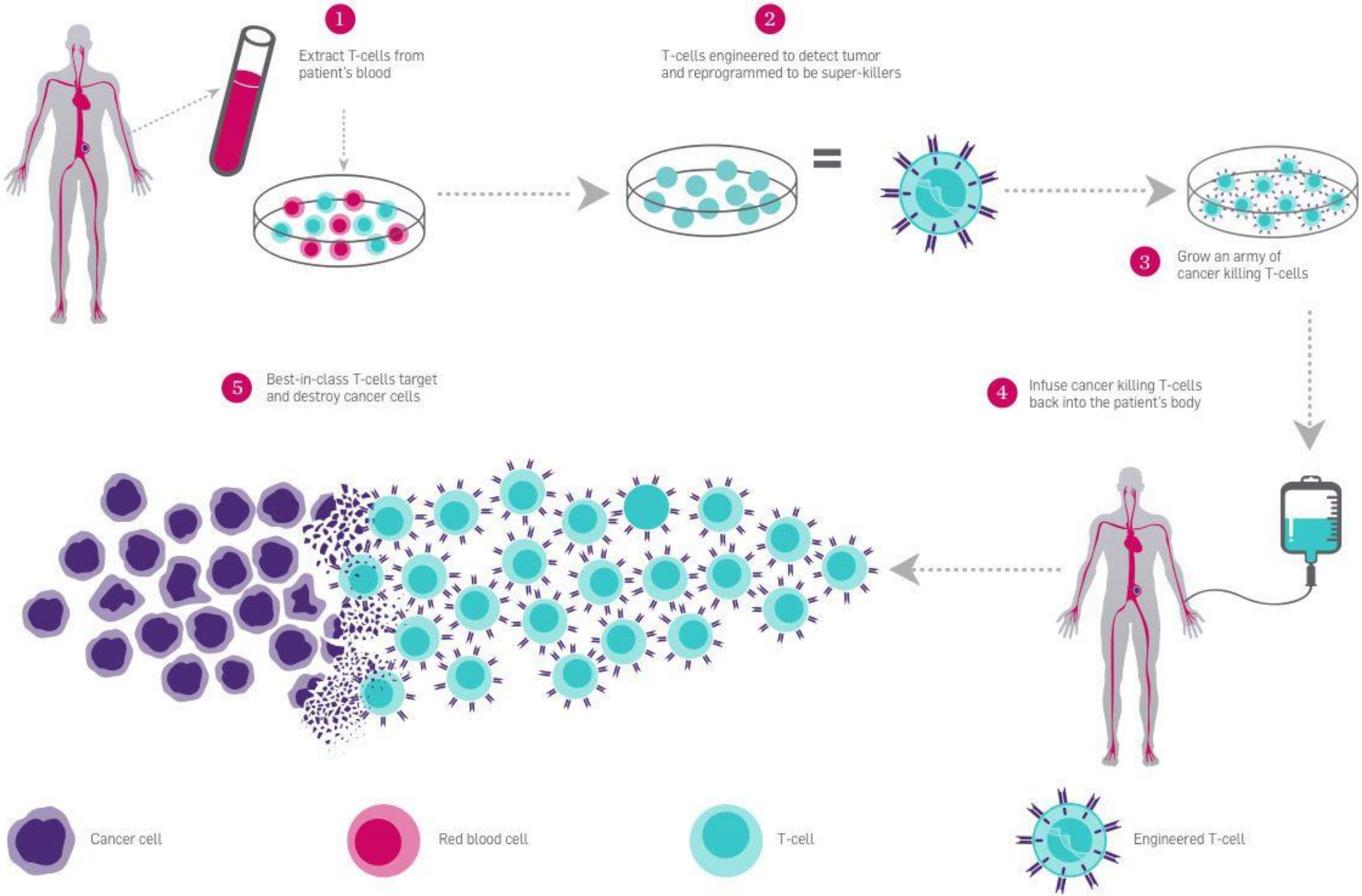
For these treatments, researchers harvest T cells from a patient's blood and engineer them in a lab. Then, they put them back into the patient's bloodstream by IV. Because this treatment relies on ***ex vivo editing***, it is easy to deliver the genome-editing tools to the target cells. CAR-T therapy was approved for use in treating blood cancers in 2017.



PBMCs were isolated from late-stage NSCLC patients and electroporated with plasmids encoding Cas9 and a pair of gRNAs targeting the second exon of PD-1 gene. The edited T cells were expanded in vitro for 17–40 days before being reinfused back into patients. Treated patients were monitored for up to 96 weeks for in vivo persistence of edited T cells, treatment-related AEs, and disease progression.

Signal Transduction and Targeted Therapy (2020)5:168

Overview of the immunotherapy process



First Trial of CRISPR- Edited T cells in Lung Cancer

In 2016, an individual with lung cancer became the first person in the world to be treated with a CRISPR-based therapy: this patient was injected with PD-1 edited T cells in a Chinese clinical trial. This and an American clinical trial using CRISPR-based immunotherapies for cancer have been completed. Several other clinical trials using CRISPR-based immunotherapies, mainly to treat blood cancers, are ongoing.

In the Chinese trial, researchers at Sichuan University treated **12 patients with non-small-cell lung cancer with PD-1 edited T cells**. This approach did not include CAR-T, as it is not currently an option for lung cancers. Like early stage trials in the US, the main goal was to assess **safety** and **side effects** rather than efficacy.

In April 2020, the researchers reported that the treatment was safe to administer and had minor side effects like fever, rash, and fatigue. **The intended edit** was found with **a low efficiency**: a median of **6% of T cells**/patient before infusion back into the patient. Off-target effects — unintended changes at various places in the genome — also occurred at a low frequency and were mostly in parts of the genome that don't code for proteins. On-target effects — unintended changes at the target site — were more common (median of 1.69%). 11 out of 12 patient volunteers had edited T cells two months after the infusion, although at low levels. ***Patients with higher levels of edited cells had less disease progression***.

In conclusion, success in cellular therapy for NSCLC is likely to await strategies using **the infusion of sufficient numbers of tumor-reactive T cells** augmented by resistance to immunosuppression via **efficient gene editing and/or engineering** secretion of active molecules or expression of dominant-negative receptors.

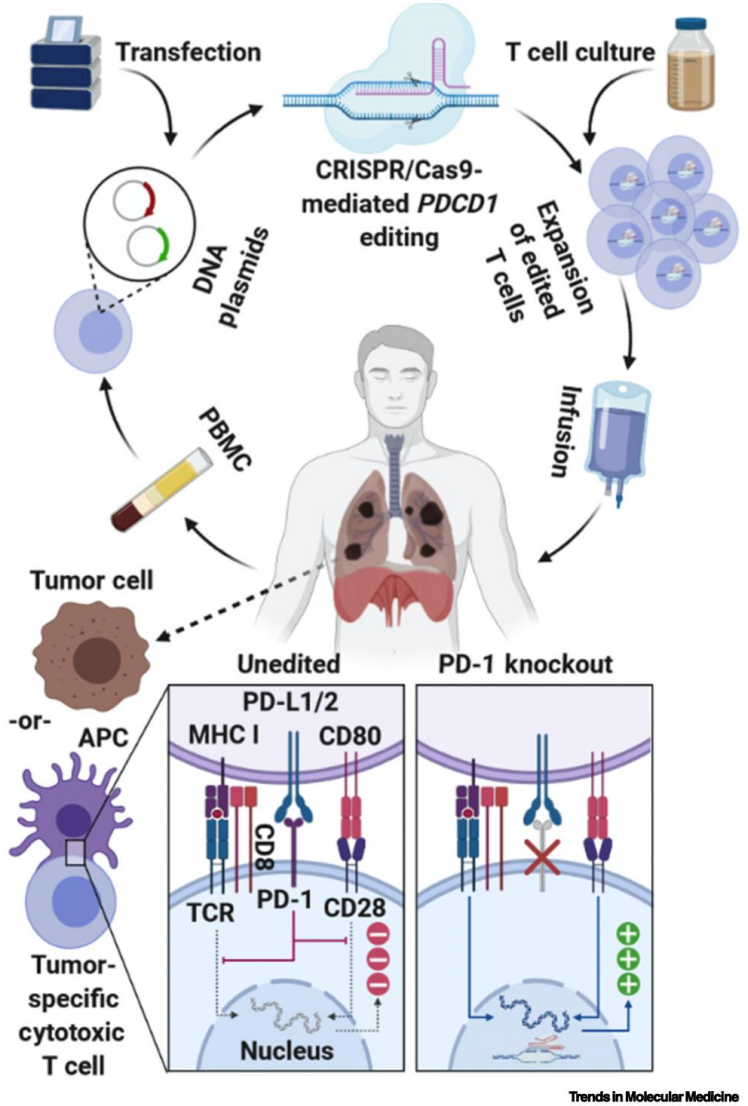


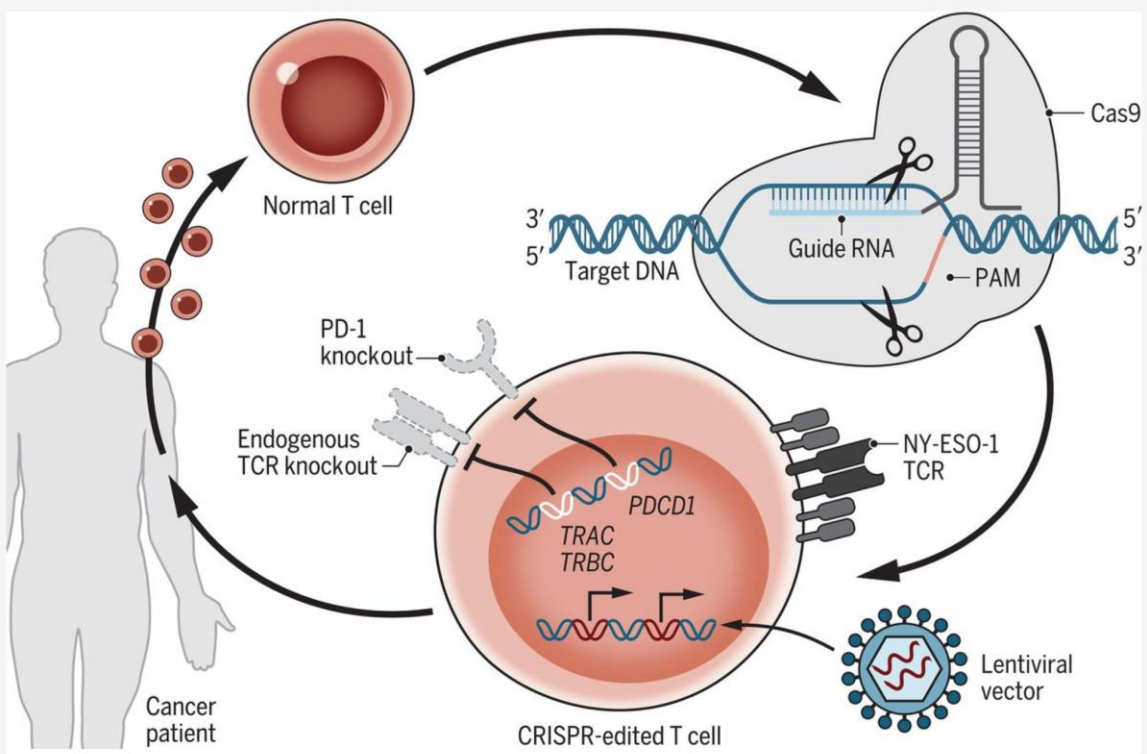
Figure 1. CRISPR-Cas9-Mediated PD-1 Gene (*PDCD1*) Editing to Create an Autologous T Cell Therapy Product for Nonsmall Cell Lung Cancer (NSCLC). Peripheral blood mononuclear cells (PBMC) were isolated from patients with advanced NSCLC, followed by transfection via electroporation of DNA plasmids encoding Cas9 protein and single guide RNAs targeting *PDCD1*. After transfection, cells were resuspended in a T cell culture media and expanded for 20–28 days. Each patient underwent two cycles of infusion with gene-edited autologous T cells. PD-1-edited T cells (e.g., cytotoxic T cells) are presumably capable of recognizing neoantigens presented by tumor cells or antigen-presenting cells (APCs) via the major histocompatibility complex (MHC). Unedited T cells are susceptible to inhibition (– – –) through the PD-1/PD-L1/2 axis, whereas T cells with PD-1 knocked out via CRISPR/Cas9 can be activated (+ + +) to elicit antitumor activity.

Trends in Molecular Medicine, August 2020, Vol. 26, No. 8

The first CRISPR-based therapy trial in the US ***combined CAR-T and PD-1 immunotherapy approaches***, using CRISPR to edit three genes in total. This **phase 1** study, run by **the University of Pennsylvania** in collaboration with the Parker Institute, was completed **in 2020**. Like the Chinese trial, *the goals were to determine if the treatment was* ***safe*** *and had* ***acceptable side-effects****, not to cure patients*. **Two patient volunteers** with advanced white blood cell cancer (***myeloma***) and one with metastatic bone cancer (***sarcoma***) were treated. Researchers reported that the treatment was ***safe to administer*** *and* ***had acceptable side effects***.

The edited T cells took up residency in the **bone marrow** and remained at stable levels for the **nine months** of the study. Biopsies on the patient with bone cancer showed that **T cells** were able to find and **infiltrate tumors**. Off-target effects were rarely observed. However, unintended edits at the target site were observed frequently, with **70% of cells** showing at least one mutation at or near **the target site** during the T cell manufacturing process. After infusion and over time in patients, the percentage of cells with mutations decreased.

This **phase I first-in-human pilot study** demonstrates the initial **safety** and **feasibility** of **multiplex CRISPR-Cas9 T cell human genome engineering** in patients with advanced, refractory **cancer**.



CRISPR-Cas9 engineering of T cells in cancer patients.
T cells (center) were isolated from the blood of a patient with cancer. CRISPR-Cas9 ribonuclear protein complexes loaded with three sgRNAs were electroporated into the normal T cells, resulting in gene editing of the *TRAC*, *TRBC1*, *TRBC2*, and *PDCD1* (encoding PD-1) loci. The cells were then transduced with a lentiviral vector to express a TCR specific for the cancer-testis antigens NY-ESO-1 and LAGE-1 (right). The engineered T cells were then returned to the patient by intravenous infusion, and patients were monitored to determine safety and feasibility. PAM, protospacer adjacent motif.

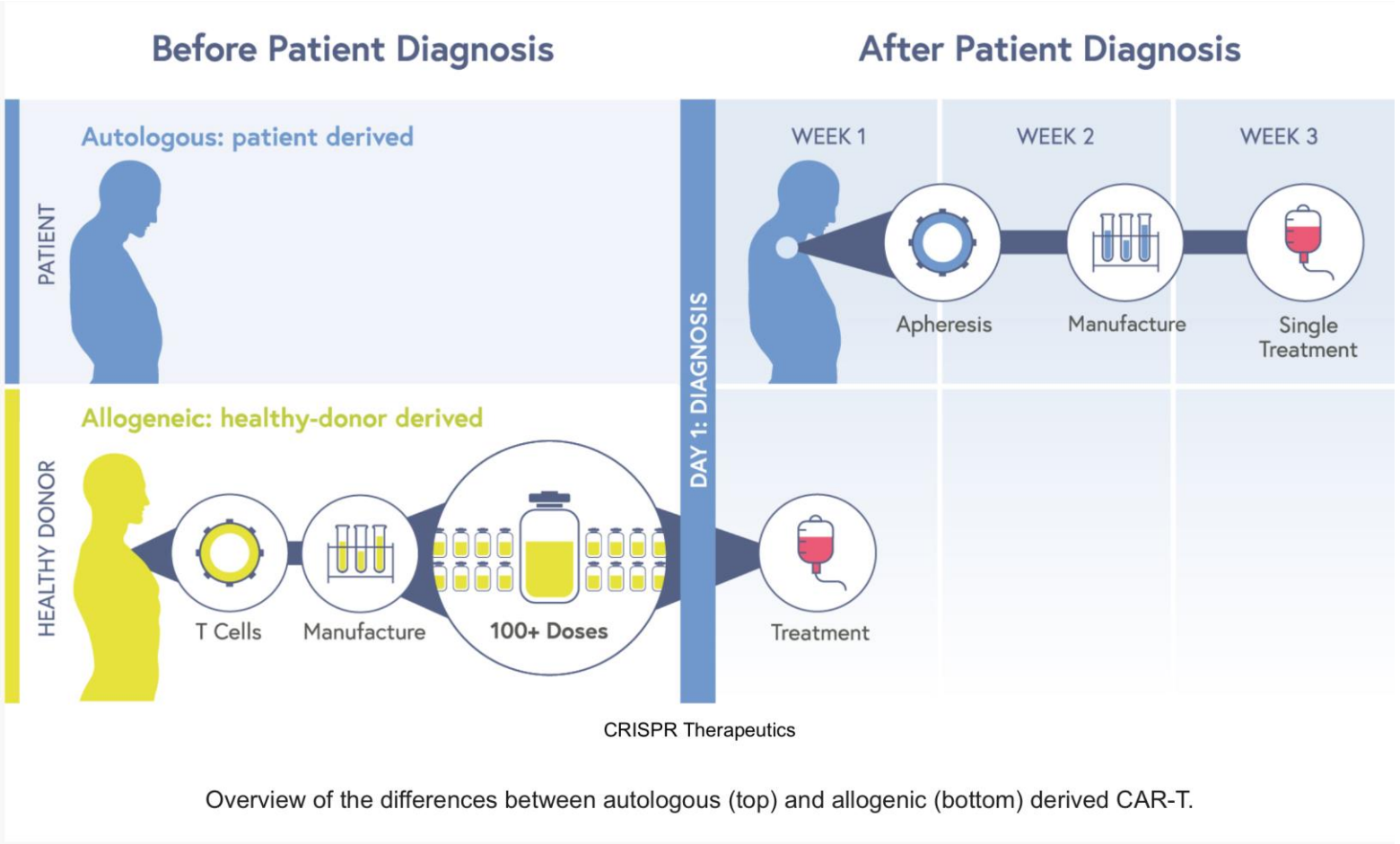
Cite as: E. A. Stadtmauer *et al.*, *Science* 10.1126/science.aba7365 (2020).

The therapies in the two trials described above are **autologous**: cells are taken from each patient, edited, multiplied, and then put back into the same patient. This process is **expensive, time-consuming**, and **few facilities** can do it. Sometimes the manufacturing process — which is starting with **cells from a sick patient** — just doesn't work, **produces low potency cells**, or individuals die of their disease while waiting for the manufacturing process to be completed.

Allogeneic therapies are made from **cells from a healthy donor**. These cells are edited to **attack cancer cells** and **avoid being seen** as a threat by the recipient's immune system, and then **multiplied into huge batches** which can be given to **large numbers of recipients**. Allogeneic products **reduce cost, time** until treatment, and **potentially provide more consistently potent cells**. Allogeneic therapies are sometimes referred to as "**off-the-shelf**."

In October **2021**, **CRISPR Therapeutics** announced results from their ongoing **US-based Phase 1 trial** for **an allogeneic T cell therapy**. The press release from CRISPR Therapeutics gave preliminary results for individuals with **lymphomas** who had been treated and followed for at least four weeks after treatment. **Side effects were not severe**, and **the safety profile was superior to other CAR-T products**.

In these patients, almost **60% showed a positive response** to treatment, with **21% showing no sign of disease** for six months after a single treatment. This is similar to approved autologous CAR-T therapies made without CRISPR technology.



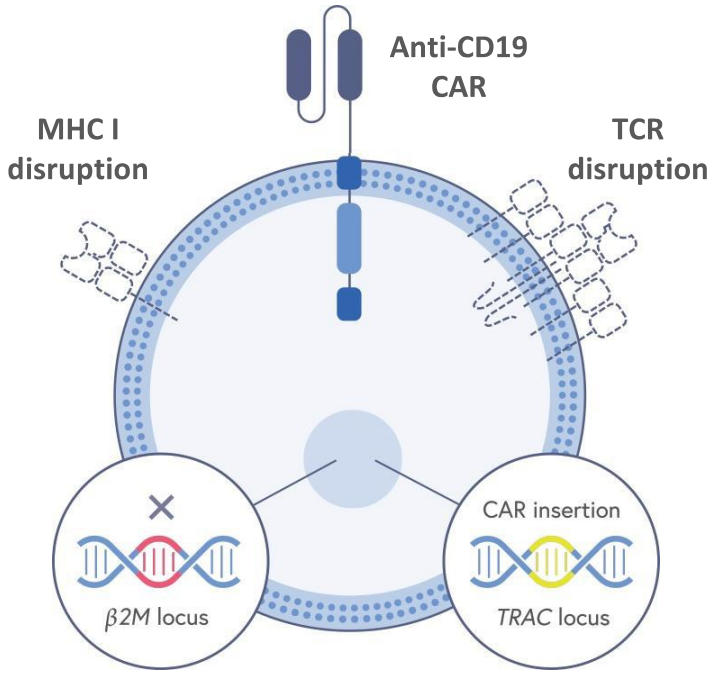
Overview of the differences between autologous (top) and allogenic (bottom) derived CAR-T.

Together, these studies indicate that **CRISPR-engineered CAR-T therapy** may be a promising line of treatment: they appear to be **fairly safe**, **the side effects are tolerable**, and the treatment **does not tend to induce a strong immune reaction**.

CTX110: Differentiated CRISPR-edited Allogeneic CAR-T Design

Multiplex CRISPR gene editing in one step designed to:

- **Improve persistence in the allo setting** via β2M knock-out to eliminate MHC I expression
- **Avoid need** for more toxic lymphodepletion regimens



- **Prevent GvHD** via TCR disruption
- **Improve consistency and safety by precise insertion** of CAR construct into *TRAC* locus without using lentivirus or retrovirus

*CTX120™ and CTX130™ utilize the **same CRISPR-edited allogeneic T cell design**, but with different CAR targets, as well as additional editing in the case of CTX130*

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Points to Consider

The FDA has already approved CAR-T therapies and PD-1 pathway inhibitors that don't use genome editing. In other words, the proof-of-principle work for these therapies has already been done successfully.

The efficiency of editing — meaning, the percentage of cells that actually got edits — was poor in both autologous trials. But these trials were done using technology from 2016, and there have been significant improvements over the last six years. These trials are an important proof of concept about the immediate safety and tolerability of the treatment, but hopefully new trials will show improved editing efficiency.

If researchers get **better editing efficiency**, will genetic checkpoint inhibition work as well or better than checkpoint-blocking drugs? Will PD-1 editing be as or more effective than antibody treatments that disable PD-1? Future research will have to answer these questions. And while **right now CRISPR-based CAR-T does not provide an advantage over conventional CAR-T**, CRISPR provides options to develop T cell therapies in ways that are not possible with conventional gene therapy. Researchers are working on **CRISPR-editing T cell therapies** where genes are added at specific locations in the genome, or using base editing to make changes to multiple genes at once.

The push towards **allogeneic**, or **off-the-shelf treatments** is particularly interesting, given the possibility for **quicker** and **broader access**. We will be sure to keep an eye on the development of this and other allogeneic cancer immunotherapy products.

There are two more big areas where CRISPR-based immunotherapies for cancer are heading. The first is **targeting solid tumors**, with at least three early stage trials going on currently. Solid tumors are a tougher challenge than blood cancers. First, in blood cancers, the cancerous cells are easier for immune cells to reach. In solid tumors, immune cells have **to infiltrate a solid mass** that isn't friendly to T cells. Second, scientists are still trying to find ways to send T cells specifically to solid tumors. And finally, when T cell therapy is effective, it kills cancer cells. When a high number of cells are killed at once — from a big tumor, or multiple smaller tumors — the dead cells can cause **a dangerous inflammation reaction**. We'll definitely be keeping an eye out for safety and side effect data from the new trials.

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GENETIC BLINDNESS

Leber Congenital Amaurosis (***LCA***) is the most common cause of inherited childhood blindness, and ***LCA10 (CEP290)*** is the most common form of LCA. This disease is caused by **a single nucleotide mutation in a photoreceptor gene**, leading to serious vision loss or blindness within the first few months of life.

Photoreceptor cells in the eye convert light into nerve signals that travel to the brain. In LCA10, the **photoreceptor gene has a mutation**, leading the cells to make a shortened, faulty version of a crucial protein. This **faulty protein** makes the photoreceptor cells **dysfunctional**. When patients with LCA10 receive light to the eye, the dysfunctional photoreceptor cells can't send all of the necessary messages to the brain. Without good communication between the eyes and the brain, ***patients experience vision loss or blindness***.

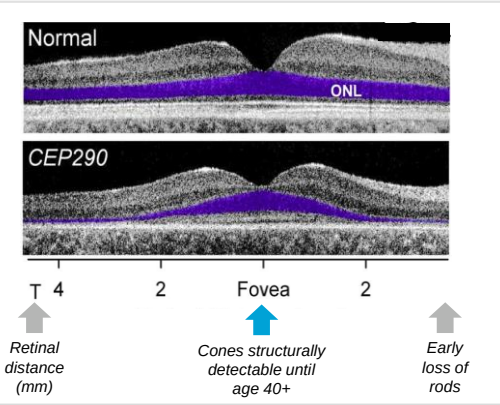
The CRISPR treatment for LCA10 makes a change to the patient's defective photoreceptor gene so that it makes a full-size, functional protein instead of the short, broken version of the protein. If enough cells are edited to make the healthy protein, the hope is that patients will regain vision.

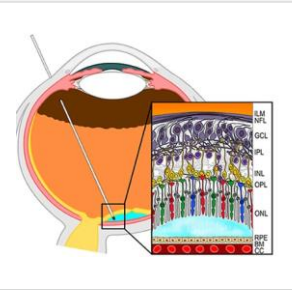
brilliance

CEP290-Related Retinal Degeneration
A Rare Cause of Early Onset Loss of Vision

Currently No Approved Treatments for CEP290-related Retinal Degeneration

- CEP290-related retinal degeneration causes progressive vision loss/ blindness in children within the first decade of life[1,2]
- Autosomal recessive disease
- Disease characterized by early loss of photoreceptors in the eye
- Focal cone rich area of the retina in the area of the fovea however remains intact until adulthood, which provides the opportunity for gene correction





Cross-sectional OCT scans along the horizontal meridian through the fovea in a normal subject, a *CEP290*-LCA patient, ONL (outer nuclear layer) is highlighted in purple

editas MEDICINE | CEP290: centrosomal protein 290

1. Kumaran N, et al. Br J Opthalmol 2017
2. Weleber RG. LCA Gene Reviews 2013

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Impact of CEP290-Related Degeneration on Patients

DISEASE SYMPTOMS

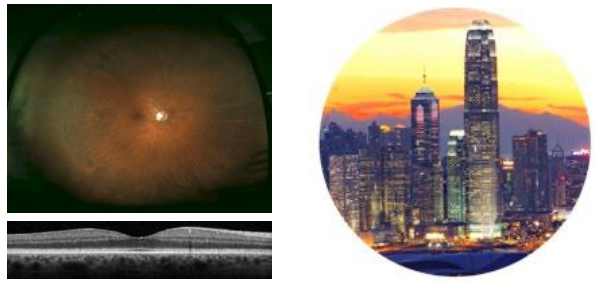
- Blindness usually diagnosed in infancy or early childhood
- Severely impaired visual acuity
- Loss of peripheral vision
- Night blindness
- Rapid, involuntary eye movements (nystagmus)

PATIENT IMPACT

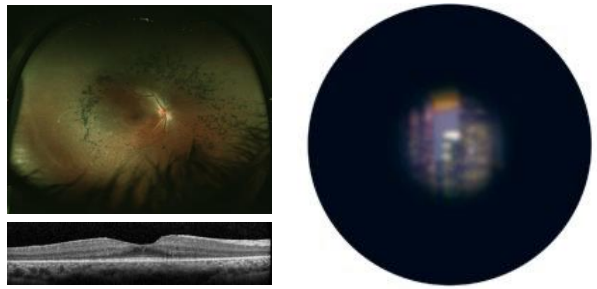
- Inability to adequately navigate enclosed spaces
- Risk of falls and injury
- Inability to be mobile or independently use public transportation
- Constrained social function
- Impaired academic performance
- Challenges with employment

PATIENT RETINA & VISION

NORMAL



CEP290



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LCA10 Photoreceptor

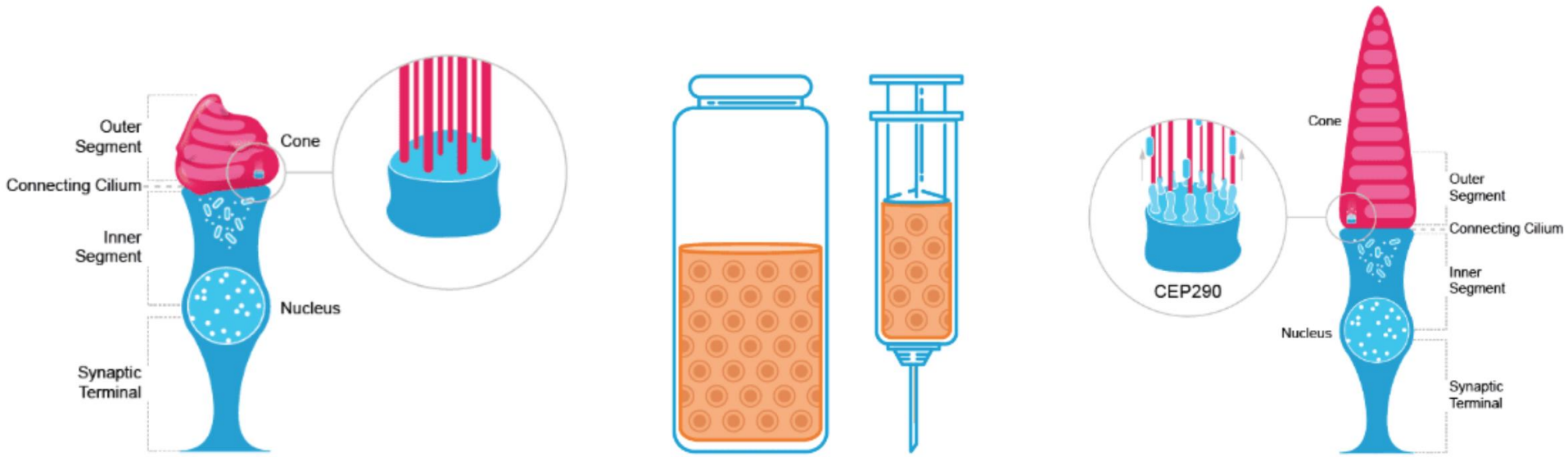
Degenerates because CEP290 lacking

EDIT-101

Removes disease-causing mutation

Rescued Photoreceptor

By correcting CEP290 protein



Degeneration of outer segment but cell body remains intact

EDIT-101 subretinal injection to remove disease-causing mutation

Restoration of full-length protein and rebuilding of outer segment

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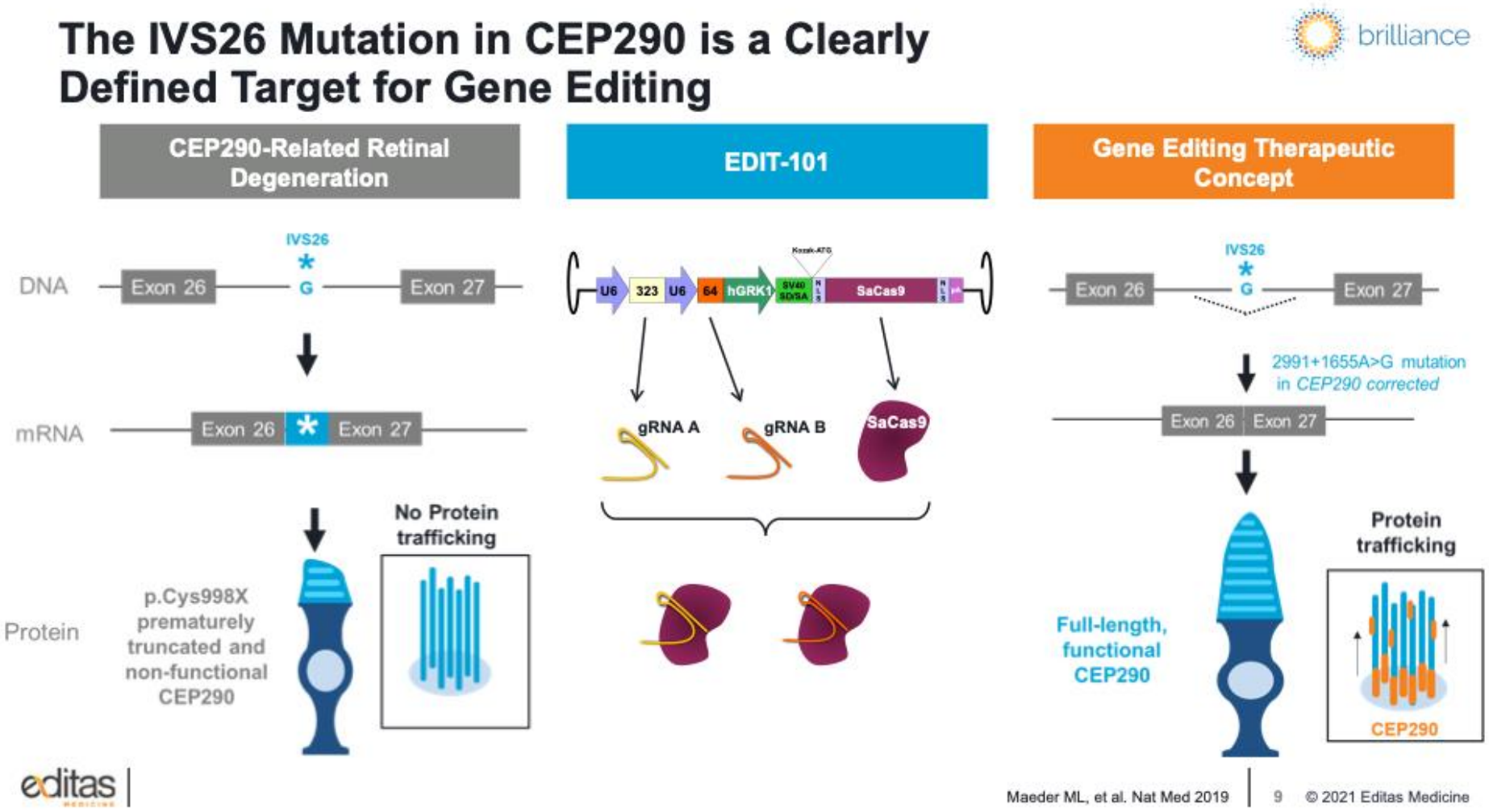
After successful editing, photoreceptor cells regain their standard shape.



EDIT-101 is an CRISPR/Cas9-based experimental medicine (genome-editing medicine) designed to remove **the abnormal splice donor site** caused by the IVS26 mutation in CEP290 (which most commonly causes LCA10), and thereby restore normal CEP290 expression. An upstream sgRNA directs the first Cas9 cleavage to a site located upstream of the IVS26 mutation, and a downstream sgRNA directs the second Cas9 cleavage to a site downstream of the mutation. The two cleavage ends are directly ligated through the NHEJ process, resulting in **the excision of the intronic fragment** flanking the IVS26 mutation. The truncated intron 26 is removed during mRNA processing by the RNA splicing machinery.

EDIT-101 is administered via a subretinal injection to reach and deliver the gene editing machinery directly to photoreceptor cells. EDIT-101 has been granted Rare Pediatric Disease and Orphan Drug designations from the U.S. Food and Drug Administration (FDA) and Orphan Medicinal Product designation from the European Medicines Agency (EMA).

EDIT-101 Allows for Local Delivery of the Editing Complex to Specifically Target the Photoreceptors to be Corrected

brilliance

Patient volunteers receive a single dose of the CRISPR therapy by injection directly into the eye. The injection contains a nonpathogenic virus (AAV) carrying the Cas9 protein and its guide RNA. Viruses are often used in gene therapy and genome editing because they have a natural ability to get into cells. For the LCA10 treatment, the viral vector is engineered so the therapy is active only in photoreceptor cells.

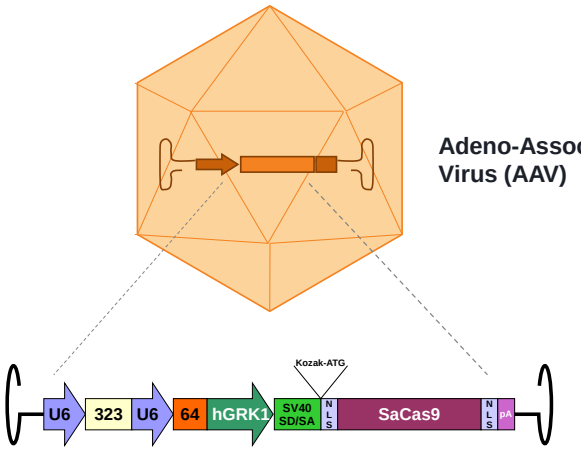
This approach is an in vivo treatment, meaning the genome editing occurs inside the patient's body. Compared to ex vivo editing, **in vivo editing** has more challenges and different risks. One of the biggest risks is that viral delivery tools or genome editing components will provoke **dangerous immune reactions** in a patient. Another big challenge is finding ways to stop **the CRISPR enzymes from sticking around for too long**, since that would give them a greater chance of making unwanted cuts in the DNA.

The eye is actually an ideal organ for **in vivo editing**. It is **small**, so it only requires **a single-dose**, **small-volume** treatment. The eye has **less immune reactivity** than most tissues, making a dangerous immune reaction less likely. And because the eye is **relatively contained**, the CRISPR components aren't likely to travel to other parts of the body, so there is a lower risk of unwanted genome editing or immune responses in other tissues.

AAV5 encoding two gRNAs and SaCas9 delivered subretinally as a single administration

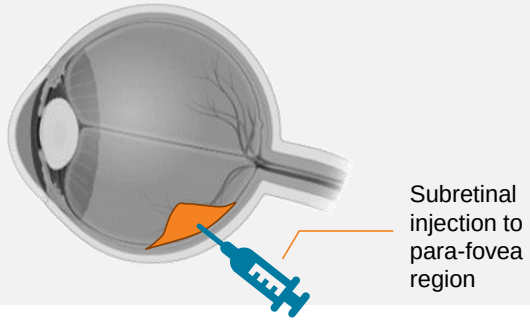
EDIT-101

CRISPR-Cas9 gene editing



EDIT-101 specifically targets the part of the retina where viable photoreceptors are found

- **Photoreceptor-tropic** AAV5 vector
- **Highly specific** Guide RNAs
- **Restricted** Cas9 expression in **Photoreceptor Cells**
- **Local delivery to subretinal space** limits the risk of biodistribution outside of the eye



editas MEDICINE

Maeder ML, et al. Nat Med 2019 | 10 © 2021 Editas Medicine

The human **rhodopsin kinase promoter** in an AAV5 vector confers **rod- and cone-specific expression** in the primate retina. In experiments on a mouse model of LCA with the same mutation, researchers found that ~10% of cells showed the desired edit — this is thought to be the minimal percent needed to get some vision restoration.

The treatment showed few side-effects in animal models, and studies in human retinal cells showed no off-target effects at over 100 sites with similar sequences.

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Carlene Knight, who participated in a gene-editing trial for inherited blindness, can now see more light, and even discern objects and doorways.

Current CRISPR Clinical Trial for LCA10

This is the first in vivo CRISPR therapy trial, meaning, the first time CRISPR is being used to edit someone's genes within their own body. The first patient volunteer in this US-based study, sponsored by ***Editas Medicine***, was given a low-dose of the treatment in March 2020. Dosing of **two patient** volunteers in the first, ***low-dose cohort*** was completed by November 2020 and dosing of **four patient** volunteers in ***an adult mid-dose cohort*** followed, completed by June 2021. Starting with a low dose reduces the risk of dangerous side effects throughout the trial. Enrollment for ***a high-dose adult cohort*** (**four patient** volunteers) and ***a pediatric cohort*** (**four patients**) began in June 2021, after safety was established from dosing adult patients with low and mid doses. Dosing of the new cohorts is expected to be completed by July 2022. ***Patients are dosed in a single eye, with the other eye serving as a control to test the vision of the treated eye against.***

No papers have been published sharing trial data, but **Editas** has issued press releases and presented data at two conferences in fall 2021. According to press release materials, ***no serious adverse events*** or ***dose-limiting toxicities*** had been ***observed***.

Efficacy is challenging to evaluate in these individuals. Because their vision is so reduced, the classic line-by-line letter reading eye test you may be familiar with cannot be used. A number of other tests, including ***mobility*** (e.g., ability to navigate around objects in one's path) and ability to ***detect light*** were used. Researchers are still honing in on the best way to assess vision change in these patients. In two of the three mid-dose subjects who were followed for at least three months, ***there were improvements in some of the vision assessments***.

More patients need to be treated and followed longer to confirm the approach is safe and to determine just how much it can help patients. But the current results are so promising that the researchers have gotten the go-ahead to move on to the next group of patients. ***The treatment is far from curing the patients in the trial, but the changes some experienced are significant enough to have a meaningful impact on their daily lives.*** The procedure didn't work for all of the patients, who have been followed for between three and nine months. The reasons it didn't work might have been because their dose was too low or perhaps because their vision was too damaged.

An exemplary story: Carlene Knight's vision was so bad that she couldn't even maneuver around the call center where she works using her cane. "I was bumping into the cubicles and really scaring people that were sitting at them," says Knight, who was born with a rare genetic eye disease. But that's changed as a result of volunteering for a landmark medical experiment. Her vision has improved enough for her to make out doorways, navigate hallways, spot objects and even see colors.

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Points to Consider

The FDA is satisfied with the initial safety data, but ***long-term safety is unknown***. Because this treatment is delivered with a viral vector, ***there will be ongoing expression of CRISPR-Cas components in the eye***. Ongoing expression presents a higher risk of **unwanted DNA edits**, and, perhaps more crucially, of **immune reactions to the viral vector and/or the Cas protein** over the long-term. It will be necessary to follow these patient volunteers for years to come to see how they fare.

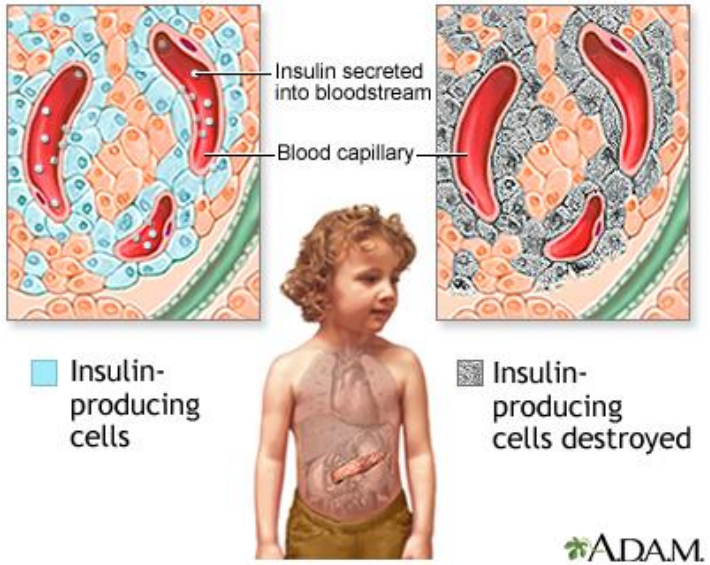
In terms of **efficiency** and **off-target effects**, there is currently no way to directly assess what percentage of cells are being edited or whether there are unwanted edits in living patient volunteers. Inferences about the efficiency of editing can only be made relative to h**ow much vision improvement there is** among patient volunteers. Researchers are following individuals who have already been dosed to track whether improvements in vision are stable over time, improve, or are lost.

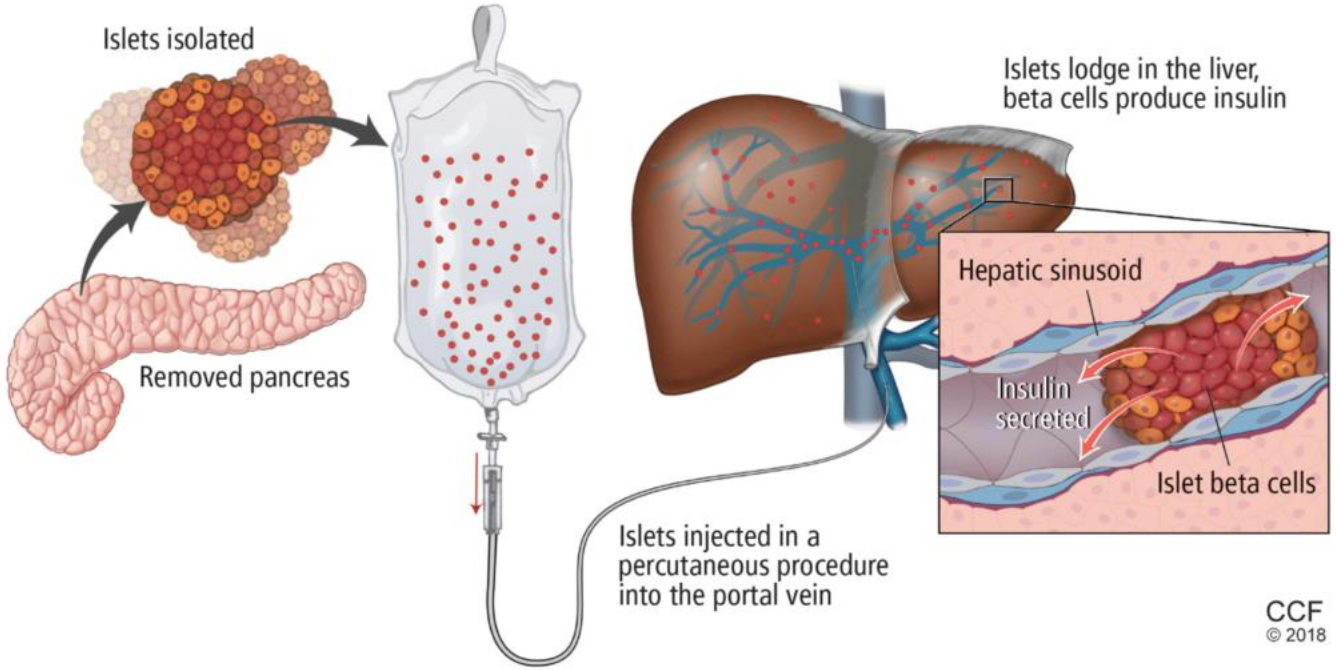
This trial came on the heels of the FDA approval of Luxturna, a gene therapy product for a different inherited retinal disease that causes vision loss. “The retina is a promising place for molecular therapies. In fact gene augmentation [for Luxturna patients] had such dramatic improvement that the FDA rapidly approved it,” says Bruce Conklin, M.D., IGI Deputy Director and Gladstone Institutes Investigator. ***“But LCA10 was a much tougher target, since the vision loss is so great that the brain actually loses its functional writing to the eye. So, even if the therapy is successful in the retina, the brain can not process the information.”*** In other words, because the extent of vision loss is more severe in LCA10 patients, and because vision loss occurs earlier in development, it is likely that the part of the brain that processes visual information has not developed all the necessary connections for vision. ***So, even if the retina issue is corrected, the brain of individuals with LCA10 may not have the ability to process much visual information.***

The company reported their data of improvement on some measures in two patients in an optimistic light. That said, the reception by the scientific community was mixed. Some saw the results as a step in the right direction; others saw it as a deep disappointment. Hopefully the ***next steps of the trial — treating individuals with LCA10 at a younger age (pediatric cohort) and/or with higher doses (adult high-dose cohort) — will yield more dramatic and definitive improvements.*** Higher dose treatments may prove more effective, although they are also more likely to provoke stronger immune reactions against the treatment. In all scenarios, the surgery necessary to administer the therapy is complex and may prove to be the major risk of this approach.

Innovative Genomics Institute

DIABETES

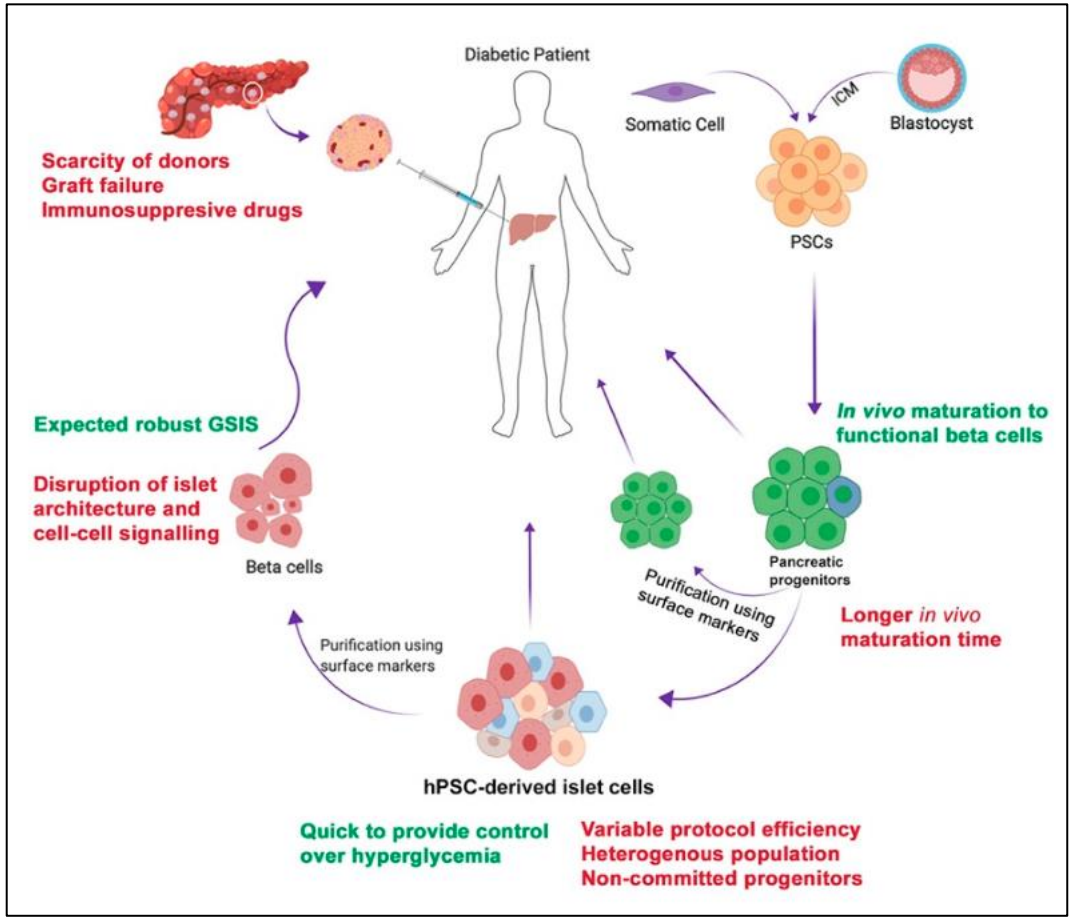




Type 1 diabetes (T1D) is an endocrine disorder that occurs when pancreatic ***beta cells are destroyed***, usually by an ***autoimmune reaction***. Without enough beta cells, the body cannot make enough insulin. Individuals with T1D must carefully monitor blood sugar and insulin levels for their entire lives, avoiding life-threatening blood sugar highs and lows by carefully timing meals, exercise, and self-dosing of insulin. T1D is not caused by diet, exercise, or weight, and cannot be controlled through lifestyle changes alone. Kidney damage, nerve pain, damage to blood vessels and the heart, vision loss, and limb amputation are common complications of T1D.

Researchers have long been interested in transplanting healthy pancreatic cells into individuals with T1D. While ongoing clinical trials in this area show that pancreatic cell transplantation can greatly benefit individuals with T1D, ***individuals who receive conventional pancreatic cell transplants must take drugs that suppress the immune system on an ongoing basis so that their body does not attack the transplanted cells***. Immunosuppressant drugs can have serious side effects, including increased risk of dangerous **infections** and **cancers**.

In the new ***CRISPR treatment strategy***, pancreatic cells will be made from stem cells. CRISPR will be used to edit the immune-related genes of these cells so that the patient's immune system does not attack them. These cells will be implanted into the patient's body in a special pouch. Blood vessels will grow along the outside of the pouch, bringing the cells oxygen and vital nutrients from the blood, and taking up insulin from the cells. The aim is for patients to have **healthy new pancreas cells** to help **control** or **even cure** their T1D **without** having to take **immunosuppressants**.



Schematic representation showing the potential use of human pluripotent stem cell (hPSC) for diabetes treatment. Human pluripotent stem cells (hPSCs) serve as a valuable tool for cell therapy and disease modeling. Further, hPSCs can be classified into two types-embryonic stem cells (hESCs) that are derived from the inner cell mass of the embryo and induced PSCs (hiPSCs) that are generated by somatic cell reprogramming. ***hPSCs can be expanded in vitro to provide an unlimited starting source for beta cell generation and can be differentiated into any cell type under appropriate cues***.

hPSCs derived from the inner cell mass (ICM) of the blastocyst and hiPSCs generated from patient somatic cells can be differentiated into **pancreatic progenitors** that mature in vivo into **glucose-responsive beta cells** following transplantation. These pancreatic progenitors can be **purified** as well as **encapsulated** prior to transplantation. Alternatively, hPSC-derived pancreatic progenitors can be differentiated into pancreatic beta cells in vitro and then transplanted in diabetic patient. In vitro differentiation to beta cells yields non-committed progenitors or polyhormonal and other endocrine cells.

Therefore, these hPSC-derived beta cells can be purified using specific cell surface markers, that could disrupt the islet architecture recapitulated during differentiation, that may result in loss of cellular contact-conferred functional properties.

Cells **2020**, *9*, 283; doi:10.3390/cells9020283

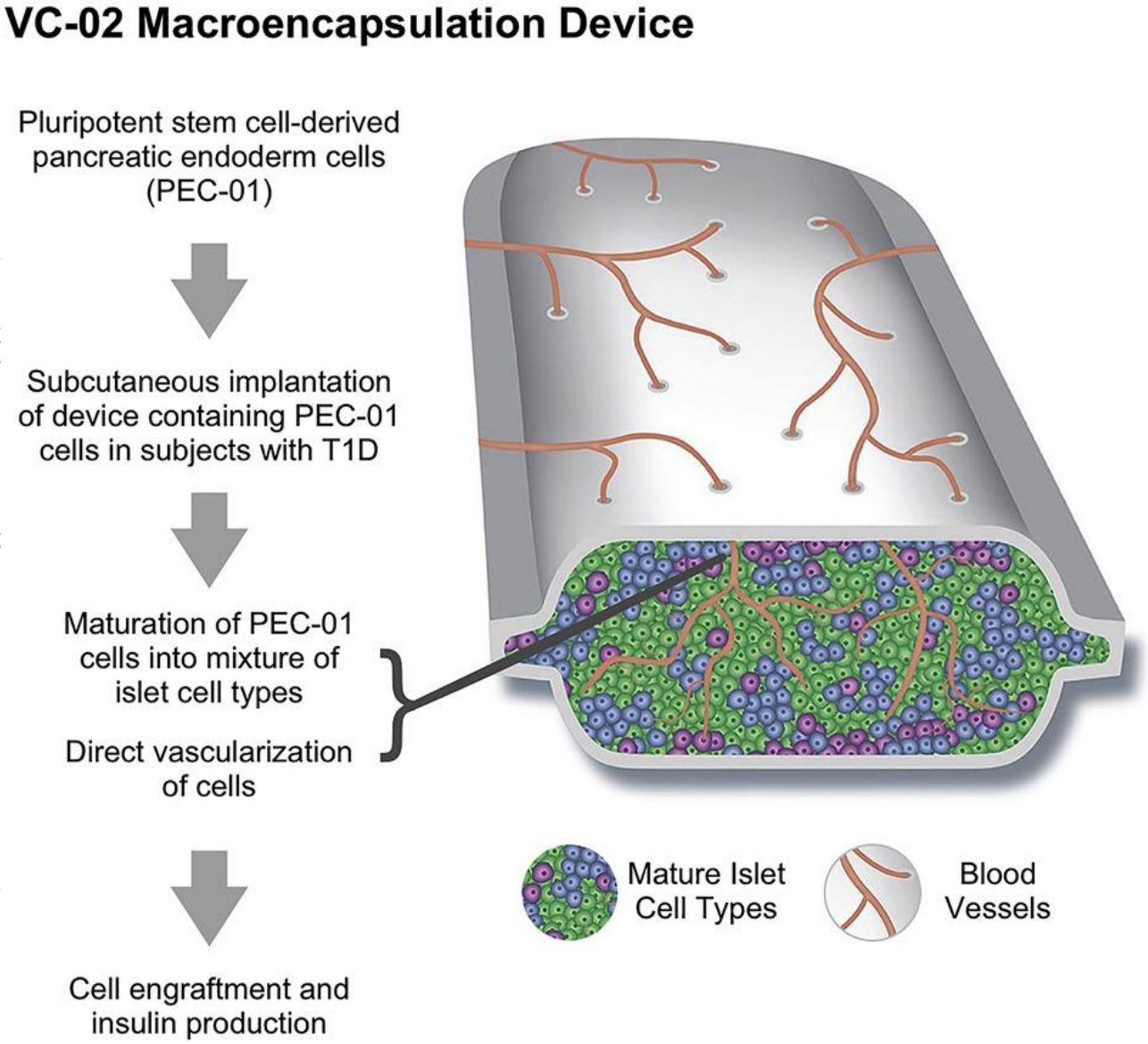
Insulin expression and **C-peptide** in type 1 diabetes subjects implanted with **stem cell-derived pancreatic endoderm cells** in an encapsulation device

Islet transplants have been used to successfully treat patients with **unstable**, **high-risk type 1 diabetes**, but the procedure has limitations, including a very **limited supply of donor organs** and challenges in obtaining **reliable and consistent islet preparations**. An effective stem cell-derived islet replacement therapy would solve these issues and has the potential to help a greater number of people.

These preliminary data from an ongoing first-in-human phase 1/2, open-label study provide proof-of-concept that **pluripotent stem cell-derived pancreatic endoderm cells** (PEC-01) engrafted in type 1 diabetes patients **become islet cells** releasing insulin in a **physiologically regulated** fashion. Participants also received **immunosuppression medications** to help the cells grow and to prevent the body's immune system from rejecting the implanted VC-02^{TM} units.

In this study of 17 subjects aged 22- 57 with type 1 diabetes, PEC-01 cells were implanted subcutaneously in VC-02 macro-encapsulation devices, allowing for direct vascularization of the cells. Preliminary clinical results have shown that, following implant and when effectively engrafted, ViaCyte's PEC-01 pancreatic progenitor cells **mature into human islet tissue**, including **glucose-responsive insulin-secreting beta cells** and other cells of the islet responsible for regulating blood glucose.

In clinical trials to date, ViaCyte's product candidates have been **well-tolerated** with **minimal product-related side effects**. Clinical evidence, both histological and measurements of C-peptide (insulin) production, shows that **PEC-01 cells** are **functioning as intended** when appropriately engrafted.

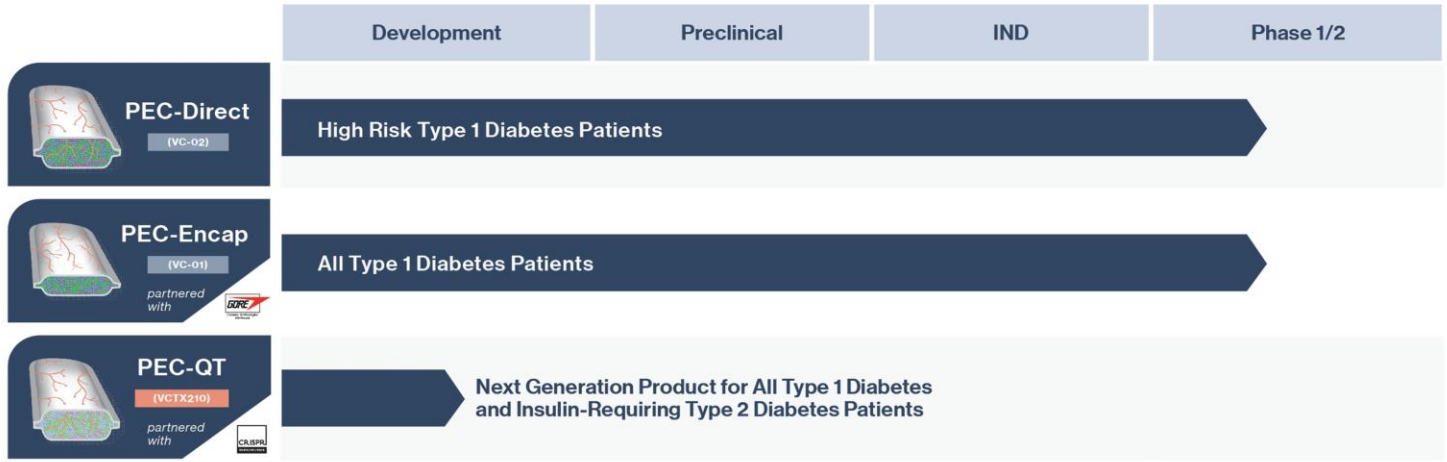


Cell Reports Medicine *2*, 100466, December 21, 2021

ViaCyte's Product Pipeline

	Active Cell Component	Device	Vascularization	Long-term Immunosuppression	Patient Target
PEC-Direct (VC-02)	PEC-01 Cells	Open Device	Direct	Required	High Risk Type 1 Diabetes
PEC-Encap (VC-01) partnered with GORE	PEC-01 Cells	Encaptra Device	Surface - Diffusion	None	All Type 1 Diabetes
PEC-QT (VCTX210) partnered with CRISPR	Immune-Evasive PEC-01	Open Device	Direct	Not Expected	All Type 1 Diabetes and Insulin-Requiring Type 2 Diabetes

ViaCyte's Product Pipeline



PEC-Direct (VC-02)

For PEC-Direct, the pouch is designed to allow blood vessels to enter the device and directly interact with the implanted PEC-01 cells. The direct vascularization of the implanted cells is intended to allow for robust and consistent engraftment but **will necessitate** the use of **immune suppression** therapy because the implanted cells are not hidden from the immune system.

PEC-Encap (VC-01)

This device is designed to **prevent immune cells** from **directly contacting the implanted cells** so they may thrive and function without provoking an immune response or being destroyed. In the case of PEC-Encap, the Encaptra® system has generally prevented immune rejection and immune sensitization and effectively protects implanted cells from the patient's adaptive immune system.

PEC-QT (VCTX210)

For PEC-QT, ViaCyte's proprietary CyT49 pluripotent human stem cell line will be **specifically engineered to avoid destruction** by the patient's immune system, potentially eliminating the need for immunosuppressants. The cells are designed to be immune-evasive, not expected to be rejected by the immune system.

Innovative Genomics Institute

Current CRISPR Clinical Trial for Diabetes

Conventional pancreatic cell transplantation is already being tested in clinical trials with some strong signs of improvement in T1D patients: some are able to stop self-administering insulin altogether and those who are not still report improvement in managing blood sugar.

There is currently one clinical trial for T1D, sponsored by ***CRISPR Therapeutics*** and ***ViaCyte, Inc***. The first patient volunteer was treated in Canada earlier this year. This is a phase 1 trial, which will assess safety, side effects, and whether the cells are able to avoid attack by the immune system. This trial represents the first use of CRISPR to treat an endocrine disease.

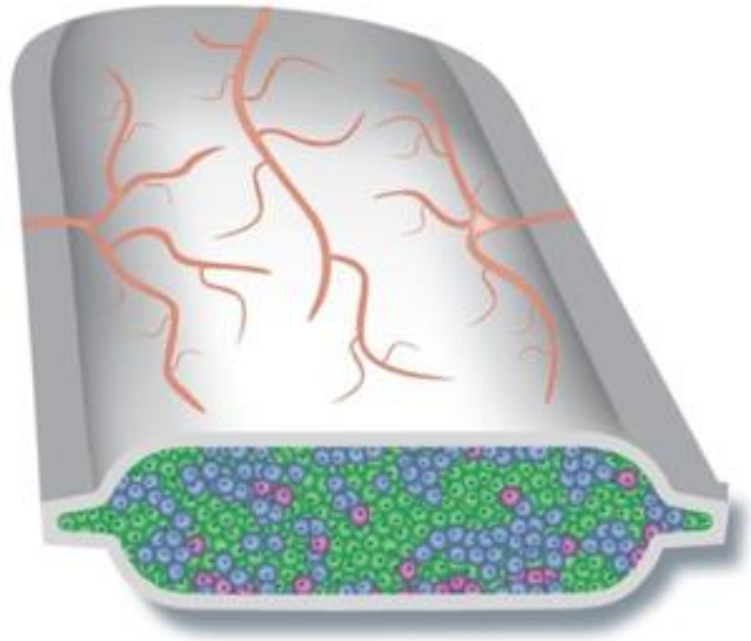
There are two main differences in this trial:

1) ***The transplanted cells are derived from stem cells*** and
2) ***CRISPR is used to edit the cells to avoid detection by the patient's immune system***.

If this works, patients could have the **benefit of transplantation** — **improvement** or **even a cure** for T1D — **without** the risks and side effects of **immunosuppressants**. Early data from ViaCyte's other trials of implanted stem cells sans CRISPR edits show positive indications that ***the cells are safe and turn into mature insulin-producing cells***. Whether the cells in this trial can successfully evade detection by the immune system will be the most crucial outcome to watch.

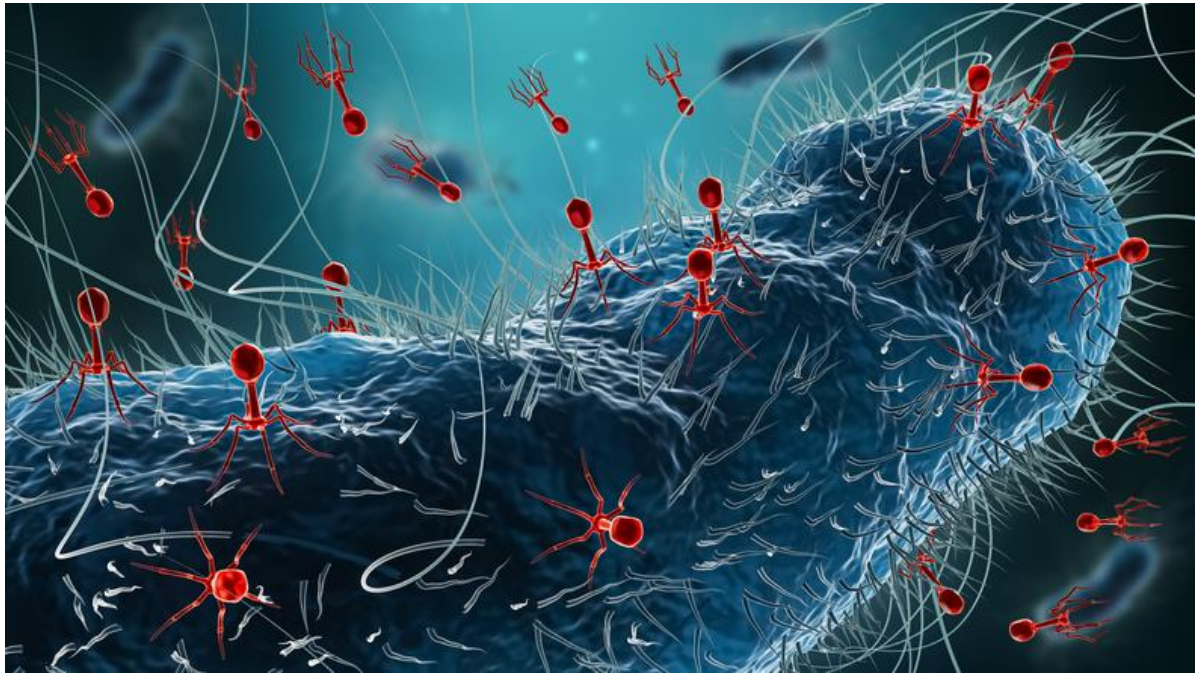
If this treatment is successful, it has a **scalability advantage over conventional transplants**. In **conventional transplants**, cells come from a **deceased donor** on an individually-matched basis or the **patient's own cells** are harvested and edited, **a highly technical process** that **few facilities** can do.

In this trial, **the pancreatic cells** are made from **a stem cell source** that could be **given to any patient** in need. In other words, the pouch of edited cells could be **an off-the-shelf** type product, rather than an **individually manufactured** product. Off-the-shelf products are **quicker**, **easier**, and **cheaper** to make than personalized products and could increase the accessibility of this therapy.



ViaCyte
The implantable pouch is meant to encourage surrounding blood vessel growth

INFECTIOUS DISEASE
CHRONIC UTI



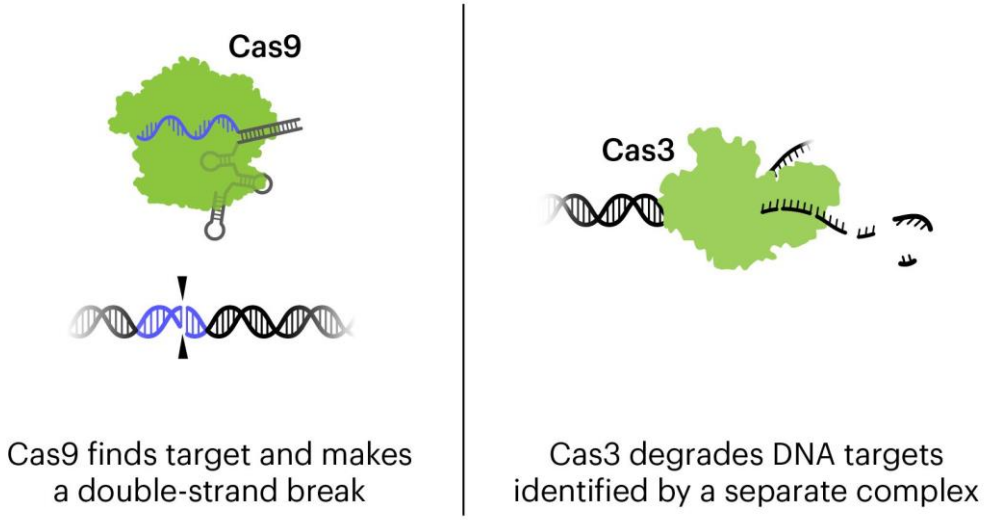
Urinary tract infections (UTIs) are a common infection causing over 8 million visits to health care providers every year. E. coli, a common fecal bacteria, is usually the culprit. UTIs cause a burning sensation during urination and the need to urinate frequently. Beyond discomfort, they can become dangerous if they affect the kidneys or if bacteria enter the bloodstream. Most UTIs are easily treated with a short course of antibiotics, but sometimes antibiotics are ineffective or the infection keeps recurring, known as chronic UTI.

Bacteriophages, or phages for short, are viruses that attack bacteria. They usually work by injecting their genetic material into bacteria and using the bacteria as a factory to make more bacteriophages. Eventually, the bacteria will burst, dying as they release more copies of the phage. Phages are being developed for use against bacterial infections, and have gotten more attention recently as antibiotic resistance has become a major public health threat.

Phages have been considered a possible antibacterial therapy since they were first identified about 100 years ago, but the discovery of antibiotics like penicillin, as well as the difficulty of patenting phages, limited the development of ***phage therapies***. Over the past decades, phages have occasionally been used by doctors for what is known as "***compassionate treatment.***" Compassionate treatment is when an unapproved drug or therapy is used to treat a seriously ill individual when no other treatments exist. At least 25 case reports of compassionate phage therapy have been published in the last 20 years. Some reports claim success at healing patients, but under compassionate treatment, clinicians use different phages in different amounts for different conditions — ***clinical trials are necessary to really evaluate the safety and efficacy of phage treatments***.

As resistance to traditional antibiotics like penicillin becomes an increasing public health threat, there is renewed interest in developing and testing ***phage therapies***. ***Phages could even be preferable to effective antibiotics, because each phage usually only kills a specific kind of bacteria. Antibiotics are destructive to healthy bacteria as well, whereas phage therapy has the potential to be much more targeted and precise***.

CURRENT CRISPR CLINICAL TRIAL



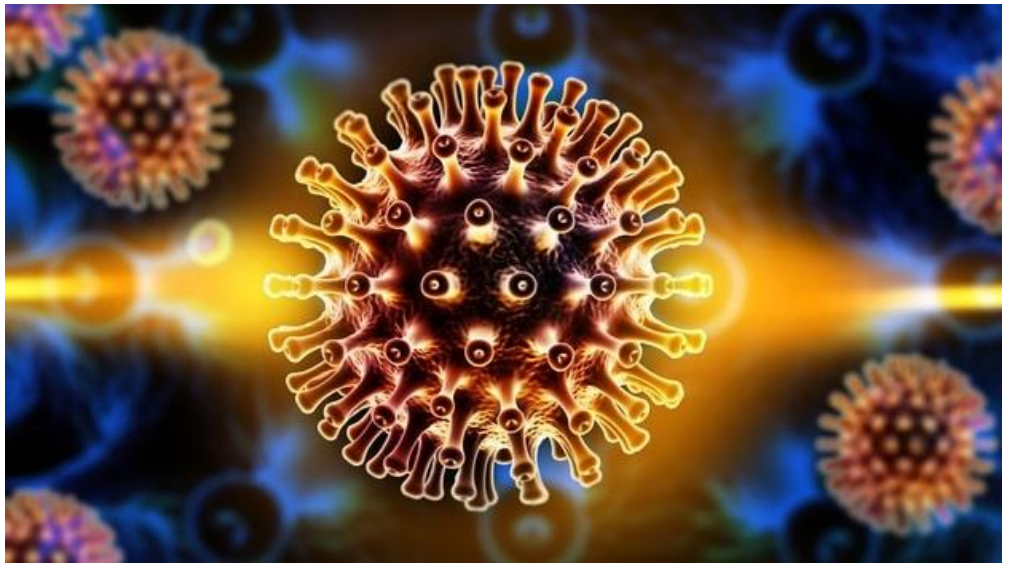
In addition to innovations using CRISPR technology, this trial is significant because it is one of the first few well-controlled clinical trials for phage therapy, and the first to combine the CRISPR system with phage therapy. In this treatment, phages have been engineered to be an even more powerful tool against E. coli. In addition *to the natural action of phages that kills bacteria*, ***these bacteriophages contain CRISPR-Cas3 in their genome***. *While the more-famous Cas protein Cas9 makes a precise cut at a single location, Cas3 shreds DNA at the gene regions it is targeted to find.* In this treatment, the CRISPR-Cas3 system is made to target the genomes of the targeted E. coli strains and damage them by shredding stretches of DNA. In experiments on isolated cells and in animals with urinary tract and other infections, the addition of CRISPR-Cas3 makes phages much more effective at killing E. coli.

The treatment currently in clinical trials is a cocktail of three bacteriophages combined with CRISPR-Cas3, designed to attack the genome of the three strains of E. coli responsible for about 95% of UTIs. The destruction of the genome kills the bacteria. **Locus Biosciences** delivered the treatment directly to the bladder by catheter in the phase 1 trial. This is the first trial using a CRISPR-based therapy to treat infection. ***It is also the first trial to use the Cas3 protein, which targets longer stretches of DNA for destruction, rather than Cas9, which makes a precise cut at one location***.

Locus Biosciences completed their US-based Phase 1b trial in February 2021. In press releases, they reported that results of ***the trial supported the safety and tolerability of the new therapy, with no drug-related adverse effects***. No data have been published yet, but Locus says ***the initial results show a decrease in the level of E. coli in the bladder of patient volunteers given the CRISPR-based treatment***. A representative of Locus Biosciences has confirmed that the company is moving forward with a phase 2/3 trial, expected to begin recruiting patients by June 2022.

EXCISION BIOTHERAPEUTICS

INFECTIOUS DISEASE HIV/AIDS



Human immunodeficiency virus, commonly referred to as HIV, is a virus that attacks the body's immune system. HIV infects ***CD4 T lymphocytes***, a type of immune cell that is important for fighting infections. HIV makes copies of itself inside the CD4 cell and then kills the cell, releasing more copies of the virus to infect and kill other CD4 cells. If HIV is untreated, it can lead to ***acquired immunodeficiency syndrome (AIDS)***, a condition where the immune system is severely damaged and an individual can get very sick or die from common infections. Individuals with AIDS are also vulnerable to rare infections and cancers that are not seen in people with healthy immune systems.

EBT-101 is a unique, ***clinical-stage in vivo CRISPR-based therapeutic designed to cure HIV infections after a single intravenous infusion***. EBT-101 employs ***an adeno-associated virus-9 (AAV9) to deliver CRISPR-Cas9 and dual guide RNAs***, enabling a multiplex editing approach that simultaneously targets ***three distinct sites within the HIV genome***. This allows for the excision of large portions of the HIV genome, thereby **minimizing potential viral escape**. It is the first time a CRISPR-based therapy targeting an infectious disease has been administered to a patient and is expected to enable the first ever clinical assessment of a multiplexed, in vivo gene editing approach.

Investigators described using **COTANA** (CRISPR-Off-Target Nomination and Analysis) to guide CRISPR-Cas9 editing to produce pairs of guide RNAs (gRNAs) that cleave HIV, with **minimal similarity** to sites in the **human genome**. The subsequent application of multiplex amplicon sequencing identified high levels of viral excision without "indels" (unintended insertion/deletion of nucleotides into genomic DNA).

This is **the first trial** targeting a retrovirus, and it is sponsored by ***Excision Biotherapeutics***. It is **currently open** for enrollment, with an aim of enrolling ~**9 patient** volunteers at locations across the US. As a phase 1/2 trial, the goals are to evaluate **safety** and **side effects**, **correct dosage**, and efficacy of the treatment at excising the virus out of cells.

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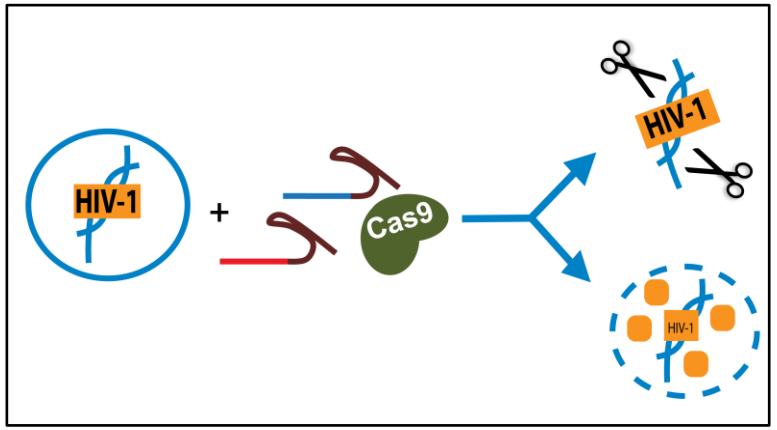
Points to Consider

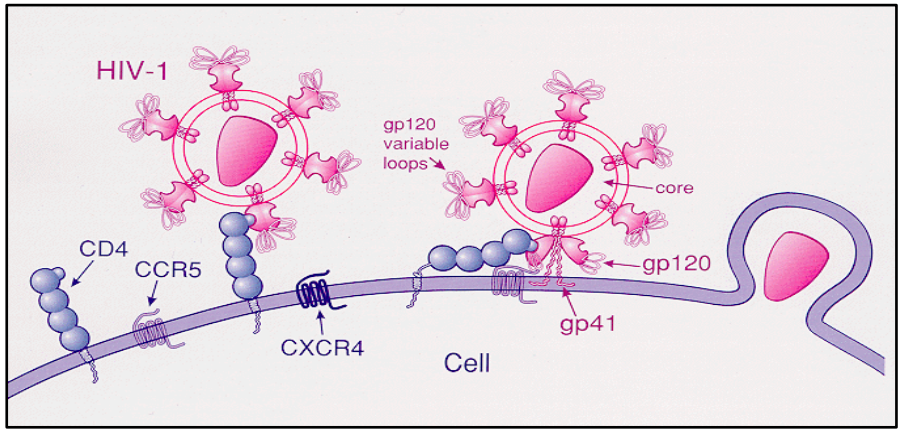


Since researchers have learned about the HIV “reservoir,” their goal has been to eliminate it to cure patients. In terms of efficacy, CRISPR will be able to disrupt and cut the HIV genetic material in its hiding place in the human genome. But the big question is ***what percentage of cells will it be able to reach***? ***Can it edit enough cells to eliminate the infection***? This is a much tougher problem than treating conditions like blood disorders where getting enough cells making enough healthy protein is sufficient for a cure. Editing less than half of blood stem cells seems to be enough to effectively cure sickle cell disease or beta thalassemia. ***But for the HIV treatment to be curative, a CRISPR therapy needs to eliminate the HIV genetic material from almost every cell where it is hiding.*** Many in the field are **highly skeptical** that this approach can work.

One common concern about genome-editing therapies is ***off-target effects***: unwanted edits made by the genome-editing components. ***This trial is only the second systemic administration of a CRISPR treatment***. In most other treatments, specific types of cells are edited outside of the body, and then put back in after editing (like treatments for blood disorders, cancer, or T1D) or genome-editing therapies are delivered to self-contained organs (like treatment for genetic blindness and chronic UTI). Systemic delivery exposes a wide variety of body tissues to genome-editing components, making the risk of off-target effects higher than for non-systemic approaches.

This is ***a viral-delivered Cas9 — a highly immunogenic protein*** — with what, as best as we can tell, ***is a ubiquitous promoter***. It’s different than an in vivo editor making cuts in the eye, which is an immunoprivileged, self-contained setting. In other words, ***when CRISPR-based treatments are delivered by a virus and with this particular genetic sequence, the genome-editing components can persist in many parts of the body for a long time***. The longer they are in the body, and the more widely spread, the greater the opportunity for unwanted edits and immune reactions against the CRISPR components. ***The individuals in this trial will be followed for years to come to monitor for any long-term health effects that could be associated with unwanted changes to the DNA.***

Berlin and London Patients



Targeting CCR5 as a Component of an HIV-1 Therapeutic Strategy

Attachment of HIV to a CD4+ T-helper cell:

- The gp120 viral protein attaches to CD4.
- gp120 variable loop attaches to a coreceptor, either CCR5 or CXCR4.
- HIV enters the cell.

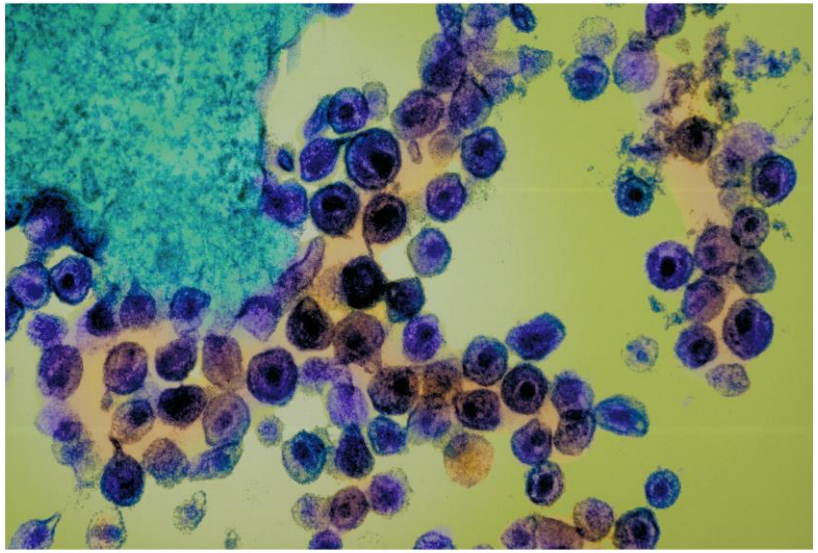
This isn't the first time scientists have tried to use gene editing in the hope of curing people with HIV, but other efforts have focused on a **protective mutation** in a gene called **CCR5**. In the 1990s, scientists found that people with this naturally occurring mutation **didn't get HIV** when exposed to it.
The mutation—known as ***delta 32***—thwarts the virus's ability to get inside immune cells.

But scientists have been trying to find ways to genetically disable CCR5 for more than a decade now. It all started in 2007, when a German doctor took a 41-year-old man with ***HIV/AIDS*** and **leukemia** off of his antiretroviral drugs and hooked a thin tube up to a vein in his chest. Through it, the so-called ***Berlin Patient*** received blood cells from a bone marrow donor who had ***a naturally occurring mutation called CCR5 Δ32***. He was ***missing a chunk of DNA that ultimately allows an HIV virus to enter immune cells.*** The patient survived his cancer and became the first (though no longer the only) person considered to be fully cured of HIV/AIDS. The first person cured of HIV - Timothy Ray Brown - has died from cancer in 2020.

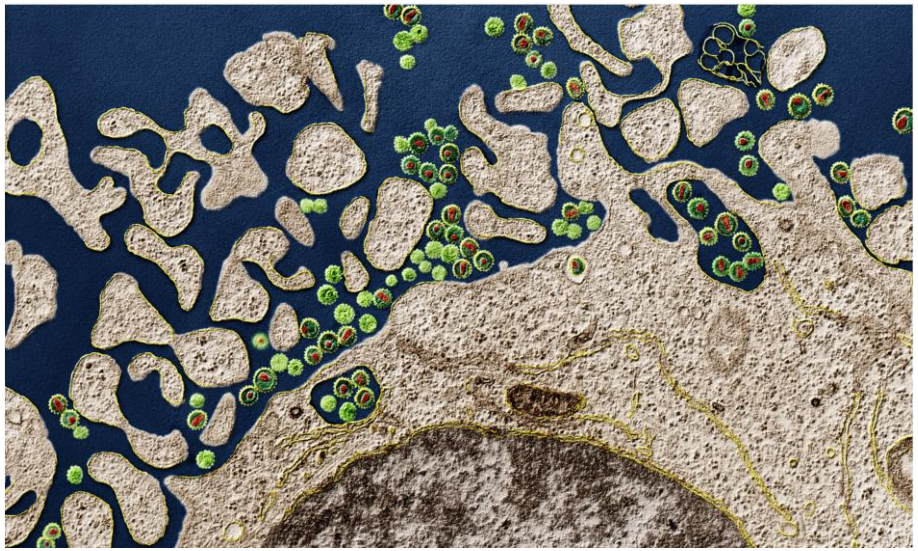
In March 2019, a team of British scientists from the University of Cambridge claimed to have successfully treated an ***HIV-positive man*** (Adam Castillejo) with ***lymphoma*** from London with the same stem-cell technique that Brown's doctors used a decade ago. It involved transplanting the patient with bone marrow from a donor who had a naturally occurring mutation in a gene called CCR5. HIV uses the CCR5 protein to invade certain immune cells. Without it, the virus is locked out. The scientists report that their "**London patient**" has been free and clear of the virus.

As of 2022, two people have now been **cured of HIV** after receiving **bone marrow transplants** from donors with the CCR5. Known as the Berlin patient and the London patient, both had cancer and received transplants to treat their disease. But ***these transplants aren't a viable option for most people—they're highly risky, and donors with the delta 32 mutation are scarce.***

Chinese Scientists Try to Cure One Man's HIV With Crispr



Immune cells infected with HIV PHOTOGRAPH: BIOPHOTO ASSOCIATES/SCIENCE SOURCE



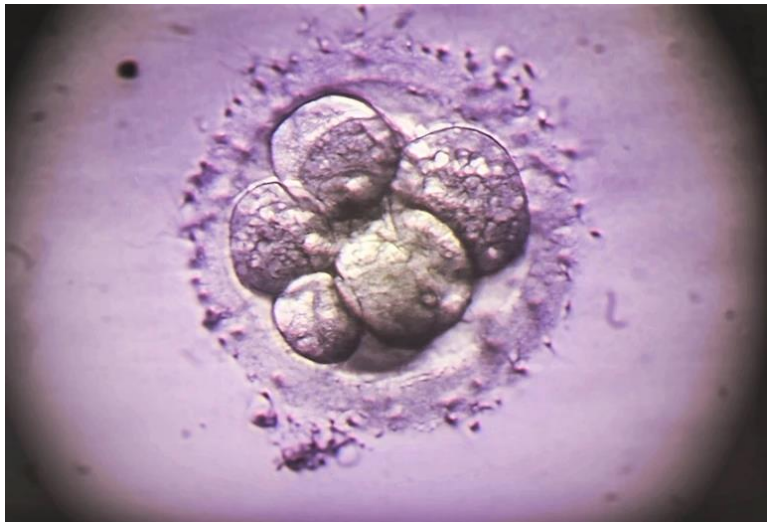
In July of **2017**, doctors in Beijing blasted a **leukemia patient** with chemicals and radiation to wipe out his bone marrow, making space for millions of stem cells they then pumped into his body through an IV. These new stem cells, donated by a healthy fellow countryman, would replace the patient's unhealthy ones, hopefully resolving his cancer. But unlike any other routine bone marrow transplant, this time researchers edited those stem cells with Crispr **to cripple** a gene called ***CCR5***, without which HIV can't infiltrate immune cells.

Now, more than two years later, the patient is in good health, his cancer in full remission, as researchers report today in the New England Journal of Medicine. ***The edited stem cells survived*** and are still keeping his body supplied with all the necessary blood and immune cells, and a small percentage of them continue to carry the protective CCR5 mutation. ***Not enough to have cured him of HIV, though—he remains infected and on antiretroviral drugs to keep the virus in check***. Still, experts say the new case study shows this ***use of Crispr appears to be safe*** in humans and moves the field one step closer toward creating drug-free HIV treatments.

In summary, ***Chinese scientists combined Crispr with a bone marrow transplant in an attempt to cure a patient with HIV and leukemia***. In a typical transplant, donor stem cells are transferred to a recipient to replace their cancerous blood cells. These cells go on to form new, healthy blood cells. ***To also address the patient's HIV, researchers edited the donor stem cells with Crispr to disable CCR5***. ***But after the transplant, only a small percentage of the patient's bone marrow cells ended up with the desired edit***.

N Engl J Med 2019;381:1240-7.

CRISPR baby scientist He Jiankui sentenced to 3 years in prison



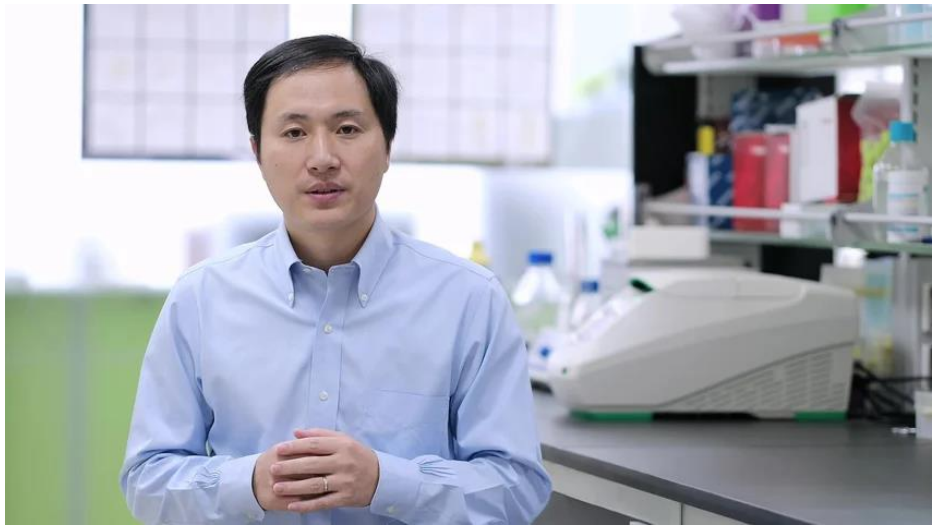
In November **2018**, media from all over the world reported that **two twin girls** had been born **with modified genes** to make them HIV immune. Their birth was the result of an 'experiment' (presently it can only be called that) conducted by ***He Jiankui*** with couples in which the males were HIV carriers. Using CRISPR technology to immunize the babies against the HIV virus, He Jiankui managed to disable the CCR5 gene that enables the HIV infection (although he still did not present complete evidence of this achievement).

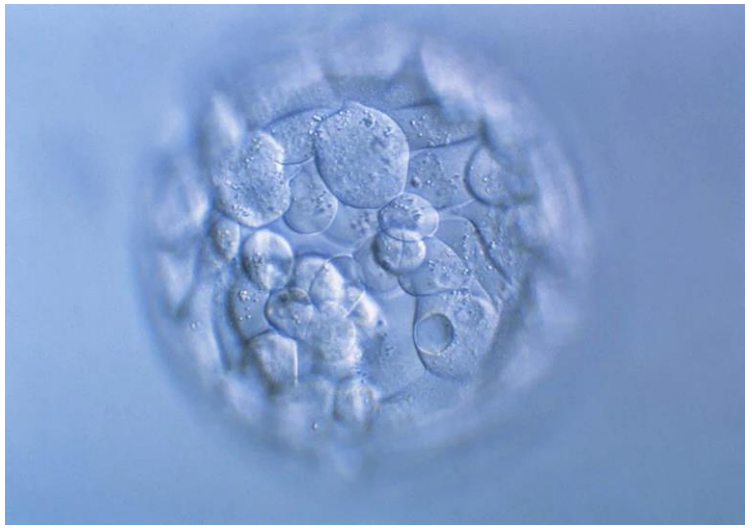
Researcher He Jiankui said his CRISPR-Cas9 procedure could help babies resist future HIV infections, but the germline edits spurred a global backlash over whether today's research is capable of accurately and ethically inserting genetic traits that could be passed down to future offspring. (Wikimedia Commons/The He Lab)

However, Chinese existing regulation, thought not very detailed, does not provide legal basis for the experiment carried out by He Jiankui and his team (Nie, 2018; Nie & Cheung, 2019). In particular, the 2003 "Ethical Guiding Principles for Research on Embryonic Stem Cell" issued by China's Ministry of Science and Technology and then Ministry of Health (now National Health Commission), ***very clearly bans research to be performed on human in vitro embryos after the 14th day of existence***, ***and its subsequent implantation into a human uterus***. Furthermore, in spite of the alleged reason for the genetic intervention related with the prevention of HIV, the scientific community also knows that the CCR5 gene is related with major brain functions. He Jiankui might have done some kind of human enhancement by created two especially intelligent human beings, with better memory and higher IQ (Joy et al., 2016).

The First Chinese Edited Babies: A Leap of Faith in Science
JBRA Assist Reprod. 2019 Jul-Sep; 23(3): 197–199.

The creator of the CRISPR babies has been released from a Chinese prison but *banned for life from participating in reproductive medicine and research.*





The problem with the Chinese episode is not so much the use of gene editing, but ***its untimely use, without scientific evidence supporting the safety of CRISPR-Cas9***. According with analysis done to Jiankui's work, “neither Lulu nor Nana possessed the 32-base pair deletion desired in the CCR5 gene, and each embryo instead expressed variants of various lengths. ***These novel mutations have not been previously shown to prevent HIV infection and may even be harmful***. Some of He's data also suggest the presence of both edited and unedited cells, leading to a phenomenon called ***mosaicism***, as well as ***off-target effects*** of the edit that could cause ***other unanticipated changes in the genome***” (Nie & Cheung, 2019).

Following international condemnation of the experiment, He was placed under home arrest and then detained. In December 2019, he was convicted by a Chinese court, which said the researcher had “***deliberately violated” medical regulations*** and had “***rashly applied gene editing technology to human assisted reproductive medicine***.” He Jiankui and his other two colleagues pleaded guilty to the charges and have been ***banned for life from participating in reproductive medicine and research. He has been sentenced to three years in prison***, according to a report from China’s state news agency, Xinhua. He’s colleagues Zhang Renli and Qin Jinzhou received sentences of 24 and 18 months in prison, respectively. He was also fined 3 million yuan, or ***about $430,000***. A court in Shenzhen found that He, an associate professor at the nearby Southern University of Science and Technology, and two embryologist colleagues were guilty of an “***illegal medical practice***” that resulted in the birth of twin girls with altered genomes.

The daring Chinese biophysicist who created the world’s first gene-edited children has been set free after three years in a Chinese prison (April 4, 2022). He Jiankui created the first gene-edited children. ***The price was his career. And his freedom.***

The First Chinese Edited Babies: A Leap of Faith in Science

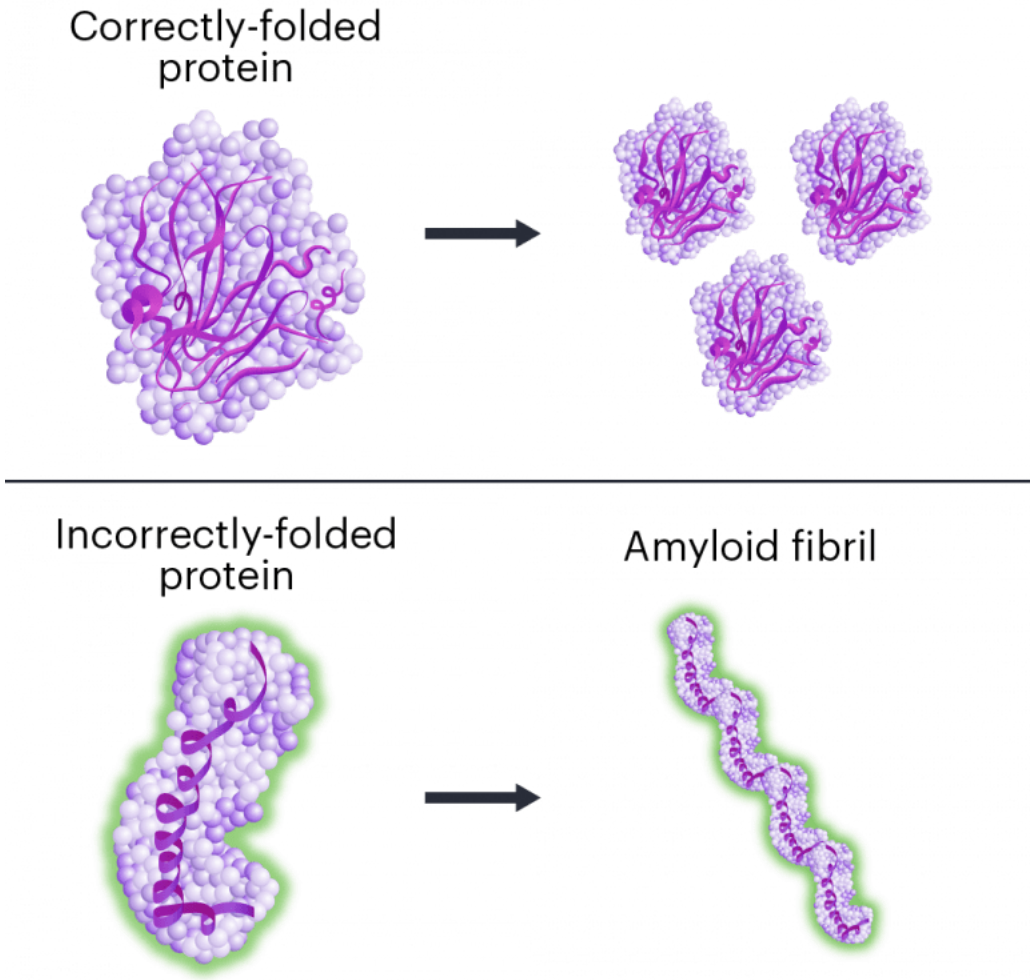
JBRA Assist Reprod. 2019 Jul-Sep; 23(3): 197–199.

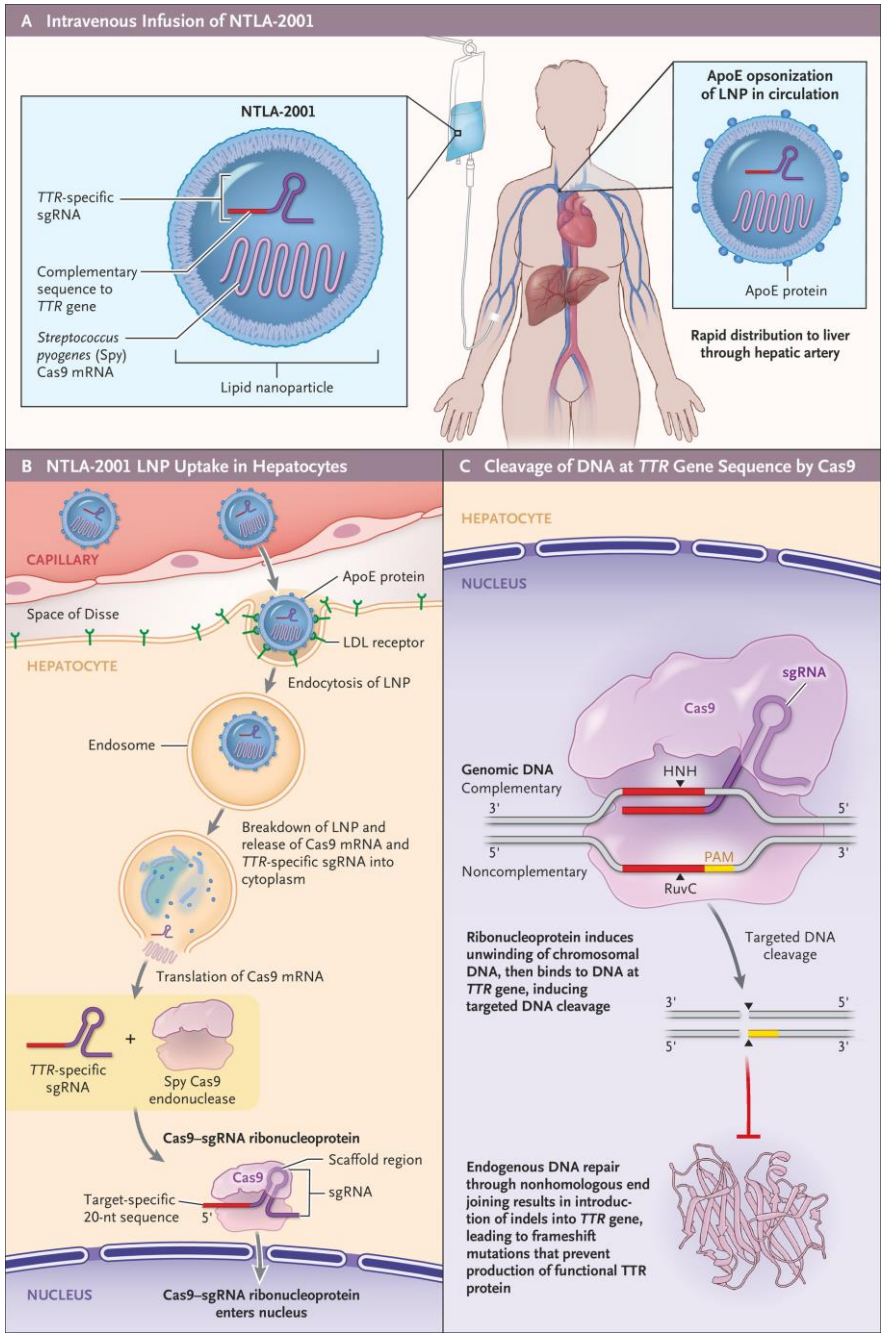
PROTEIN-FOLDING DISEASE – HEREDITARY TRANSTHYRETIN AMYLOIDOSIS

Transthyretin (**TTR** or TBPA) is a **transport protein** in the plasma and cerebrospinal fluid that transports the thyroid hormone **thyroxine** (T4) and **retinol** to the liver. This is how transthyretin gained its name: **trans**ports **thy**roxine and **retin**ol. The liver secretes TTR into the blood, and the choroid plexus secretes TTR into the cerebrospinal fluid. ***Transthyretin amyloidosis***, or ATTR amyloidosis, is a rare, progressive and fatal disease. ***Hereditary ATTR (ATTRv) amyloidosis*** occurs when a person is born with mutations in the TTR gene, which causes the liver to produce structurally abnormal ***transthyretin (TTR)*** protein with a propensity to misfold. These damaged proteins build up as amyloid in the body, causing serious complications in multiple tissues, including ***the heart, nerves and digestive system***. ATTRv amyloidosis predominantly manifests as ***polyneuropathy*** (ATTRv-PN), which can lead to nerve damage, or ***cardiomyopathy*** (ATTRv-CM), which can lead to heart failure.

Some individuals without the genetic mutation produce non-mutated, or wild-type TTR proteins that become unstable over time, misfolding and aggregating in disease-causing amyloid deposits. This condition, called ***wild-type ATTR*** (ATTRwt) amyloidosis, primarily affects the heart. There are an estimated 50,000 people worldwide living with ATTRv amyloidosis and between 200,000 and 500,000 people with ATTRwt amyloidosis.

Based on Nobel Prize-winning CRISPR/Cas9 technology, NTLA-2001 could potentially be the first single-dose treatment for ATTR amyloidosis. NTLA-2001 is the ***first investigational CRISPR therapy candidate to be administered systemically***, or through a vein, to edit genes inside the human body. Intellia's proprietary non-viral platform deploys lipid nanoparticles to deliver to the liver a two-part genome editing system: ***guide RNA specific to the disease-causing gene*** and ***messenger RNA that encodes the Cas9 enzyme***, which carries out the precision editing. Robust preclinical data, showing deep and long-lasting transthyretin (TTR) reduction following in vivo inactivation of the target gene, supports NTLA-2001's potential as a single-administration therapeutic.





Mechanism of Action of NTLA-2001

Panel A shows the primary components of NTLA-2001. The carrier system for NTLA-2001 is a ***lipid nanoparticle (LNP)***. The LNP is based on a proprietary ionizable lipid, combined with a phospholipid, a pegylated lipid, and cholesterol, formulated in an aqueous buffer for intravenous administration. ***The active components of NTLA-2001 are a human-optimized messenger RNA (mRNA) molecule encoding Streptococcus pyogenes (Spy) Cas9 protein and a single guide RNA (sgRNA) molecule specific to the human gene encoding transthyretin (TTR)***. These components form the cargo of the LNP for drug administration. After intravenous administration of NTLA-2001 and entry into the circulation, the LNP is opsonized by apolipoprotein E (ApoE) and transported through the systemic circulation directly ***into the liver***, where it is preferentially distributed.

Panel B shows transport of the NTLA-2001 LNP into the capillaries of the hepatic sinusoids inside the liver. As with other clinically approved LNPs, NTLA-2001 is then expected to undergo uptake by ***the low-density lipoprotein (LDL) receptor*** expressed on the surface of the hepatocytes, followed by endocytosis and endosome formation. After breakdown of the LNP and disruption of the endosomal membrane, the active components (***the TTR-specific sgRNA and the mRNA encoding Cas9***) are released into the cytoplasm. The Cas9 mRNA molecule is translated through the native ribosomal process, producing the Cas9 endonuclease enzyme. The TTR-specific sgRNA interacts with the Cas9 endonuclease, forming a ***(CRISPR)–Cas9 ribonucleoprotein complex***.

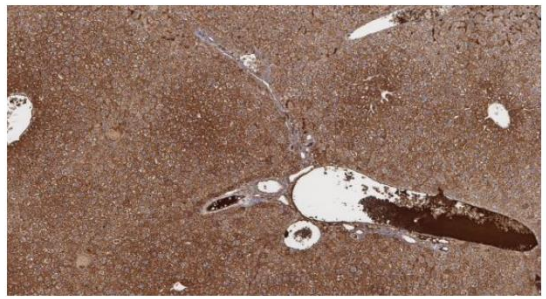
Panel C shows that the Cas9 ribonucleoprotein complex is targeted for nuclear import and enters the nucleus, where it recognizes the protospacer-adjacent motif (PAM) on the noncomplementary DNA strand in TTR. *A target-specific 20-nucleotide sequence at the 5′ end of the sgRNA binds to the DNA double helix at the target site, allowing the CRISPR-Cas9 complex to unwind the helix and access the target gene. Cas9 undergoes a series of conformational changes and nuclease domain activation (HNH and RuvC domains), resulting in DNA cleavage that is precisely targeted to the TTR sequence, as defined by the sgRNA complementary sequence.* Endogenous DNA-repair mechanisms ligate the ends of the cut, potentially introducing insertions or deletions of bases (**indels**). The generation of an indel may result in the reduction of functional target-gene mRNA levels as a result of **missense** or **nonsense** mutations decreasing the amount of full-length mRNA, ultimately resulting in decreased levels of the target protein.

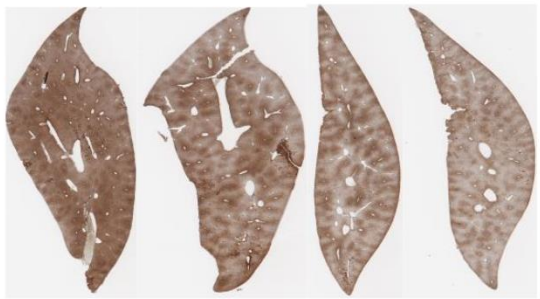
N Engl J Med 2021; 385:493-502

Effective Transthyretin (TTR) Liver Knockout (KO) in Mice After Single LNP Dose

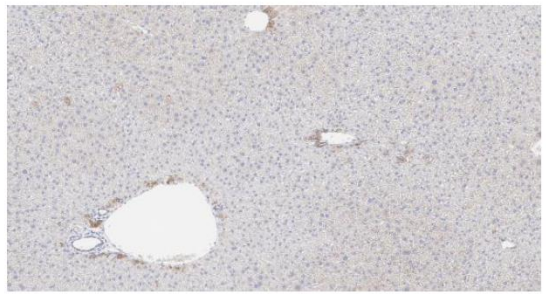
Mouse TTR Immunohistochemistry (IHC)

Vehicle
1 week

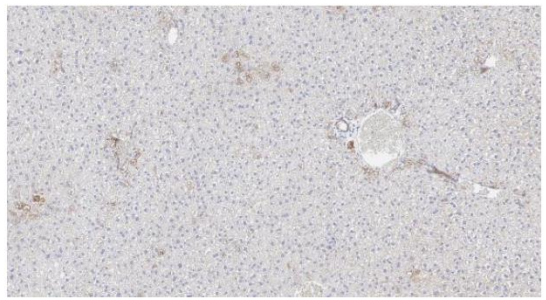


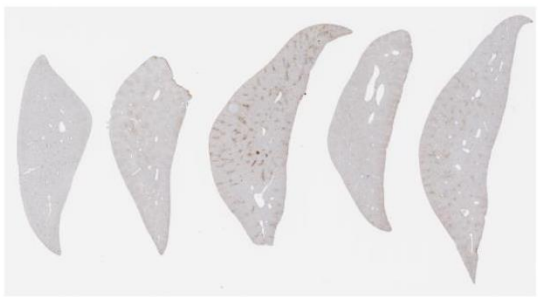


TTR Knockout
1 week

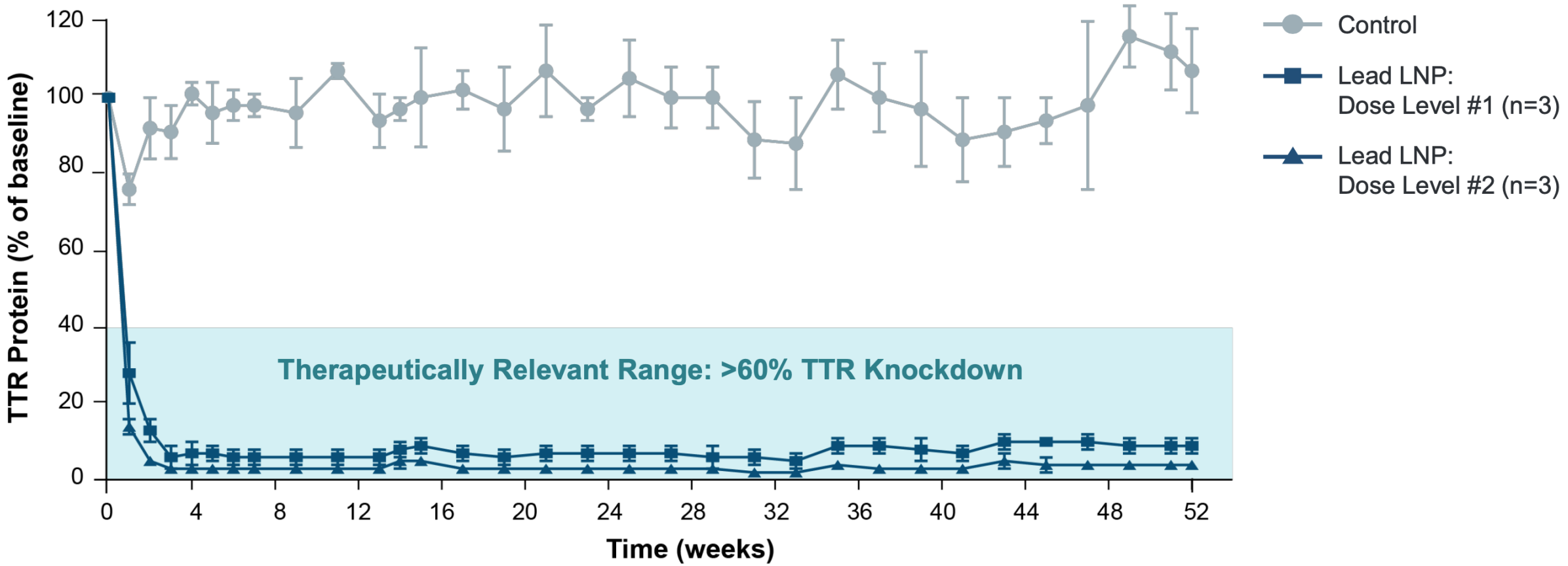


TTR Knockout
6 months





Year-Long, >95% Serum TTR KO After a Single Dose in NHPs

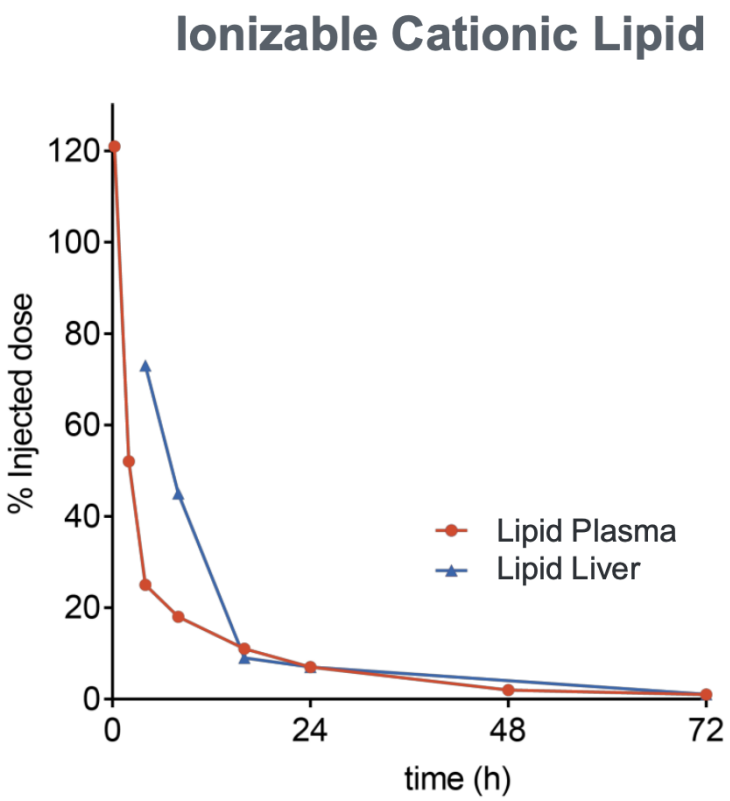


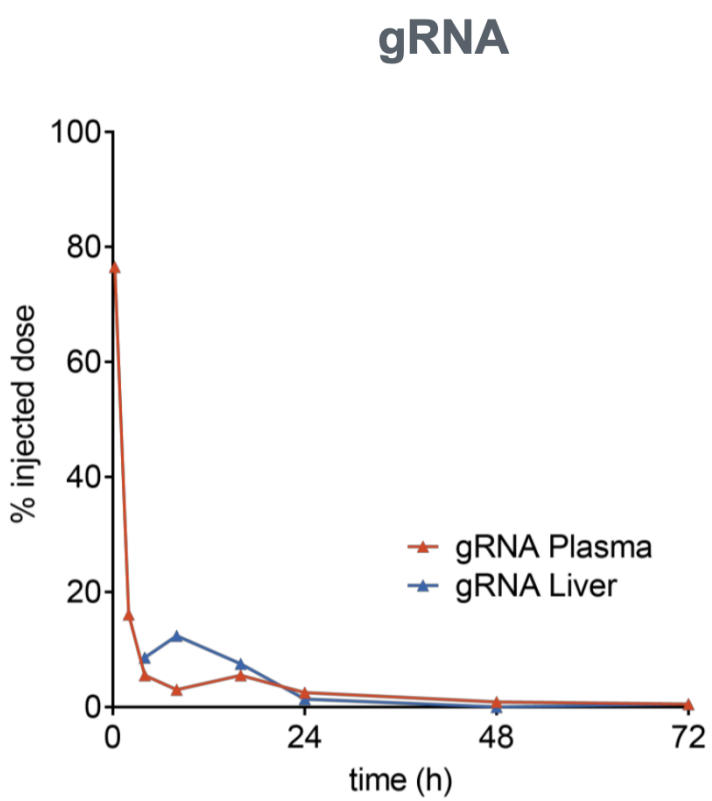
REGENERON

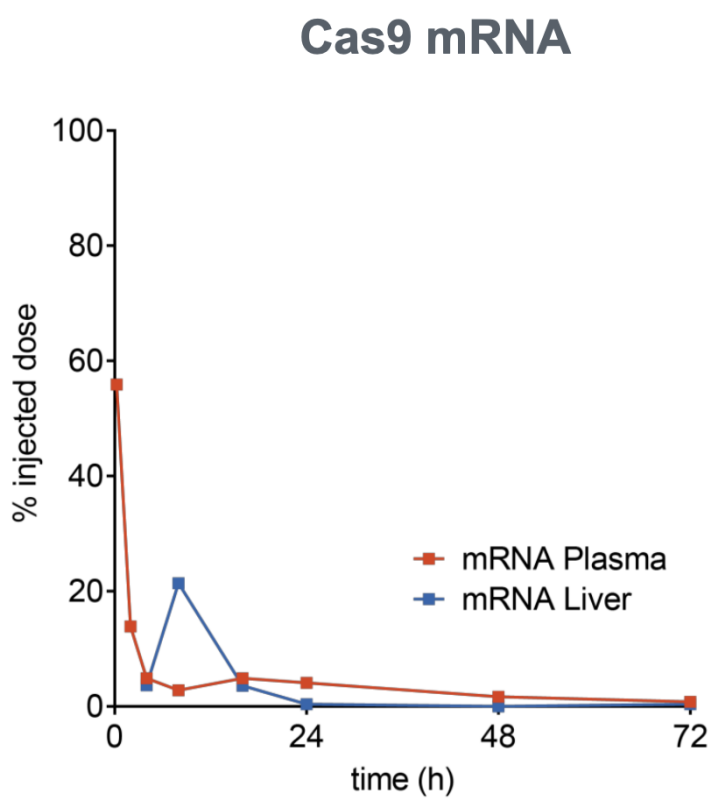
Intellia THERAPEUTICS

NHP: Non-Human Primates

Transient Exposure to LNP and RNA Cargo After Single Administration in NHPs





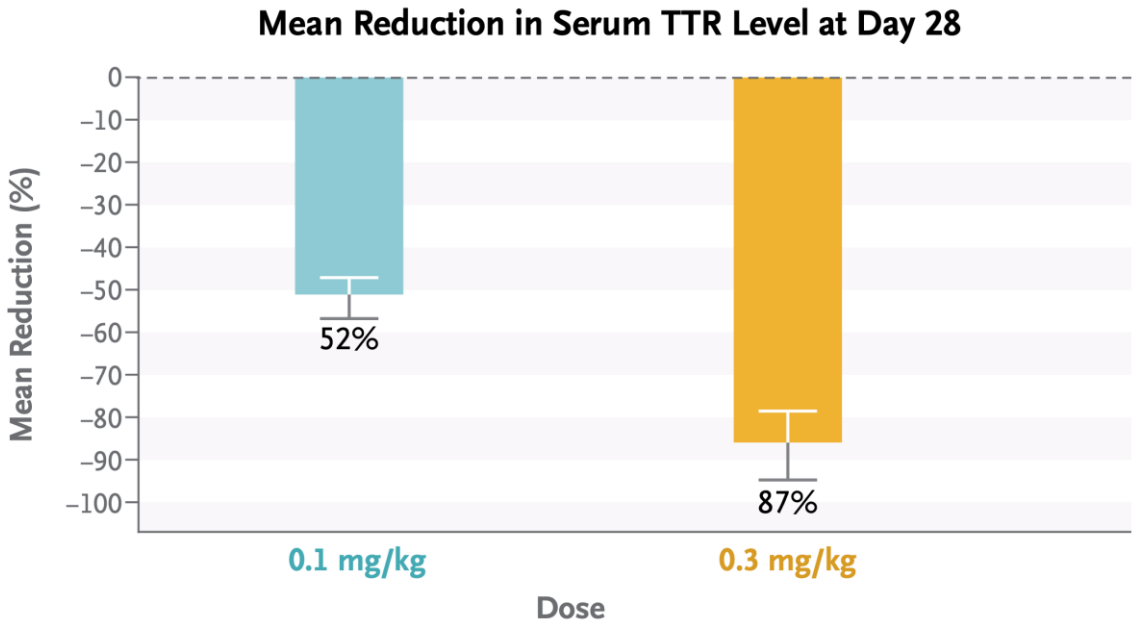


CURRENT CRISPR CLINICAL TRIAL

This is the first trial that uses ***lipid nanoparticles*** to deliver the genome-editing treatment. It is also the first trial ***to deliver genome-editing components systemically***, that is, to the whole body rather than to one specific type of cell or tissue.

The phase 1 trial is sponsored by ***Intellia Therapeutics*** in collaboration with ***Regeneron Pharmaceuticals***. The first patient volunteer was dosed in November **2020** in the United Kingdom. In total, data has been shared on **15 patient volunteers**, who received **one of four potential dosages** of the gene-editing reagents. Most adverse events were mild. ***All patient volunteers showed a reduction in TTR protein levels, with higher doses leading to greater reductions in the protein.*** The protein level was reduced by an average of 87% in individuals who received the highest dose.

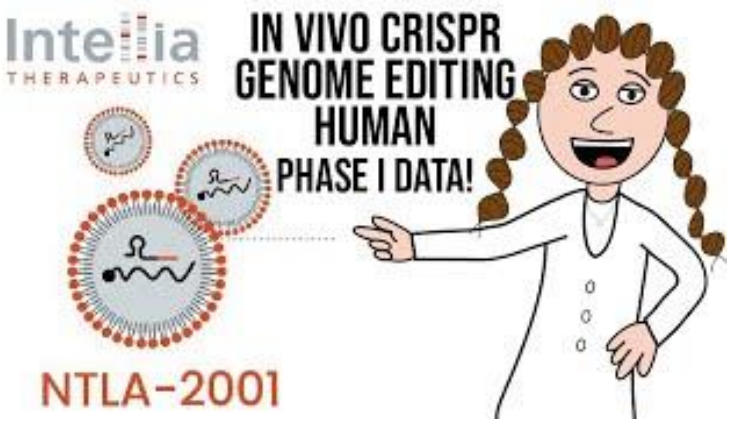
In amyloid disorders like TTR, the level of precursor proteins is related to clinical outcomes. In other words, ***if the patient volunteers continue to produce less TTR protein, it is very likely that they will have less severe disease.*** Intellia CEO John Leonard hopes that with dramatic reductions in toxic proteins being produced, people's bodies will be able to clear out the toxic protein and even reverse damage done by the disease, but it's too soon to tell. Currently, more patient volunteers are being enrolled in the phase 1 study.



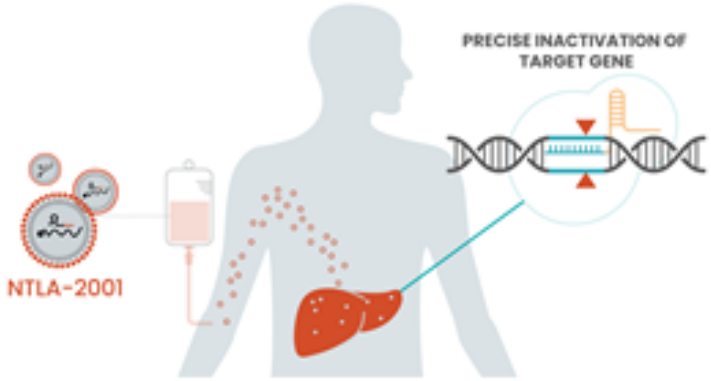
CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis

This trial involving a small number of patients with hereditary transthyretin amyloidosis provides proof-of-concept evidence that CRISPR-Cas9–based gene editing with NTLA- 2001 greatly reduces TTR levels after a single infusion, with only mild adverse events.

N Engl J Med 2021; 385:493-502



Points to Consider



This is the first experimental CRISPR therapy to be ***administered systemically to edit genes*** inside the human body. In other treatments, specific types of cells are edited outside of the body, and then put back in after editing (like treatments for blood disorders and cancer) or genome-editing therapies are delivered to self-contained organs (like treatment for genetic blindness and chronic UTI). These strategies help ensure that only the cells or tissue of interest are edited.

One common concern about genome-editing therapies is unwanted edits made by the genome-editing components. This is particularly a concern with CRISPR treatments delivered by viruses, since the genome-editing components may persist in the cell for a long time, giving them more opportunity to make editing errors. Avoiding systemic delivery helps reduce the overall risk.

In this trial, risks are reduced because 1) ***Lipid nanoparticles tend to aggregate in the liver, which is the tissue being targeted in ATTR treatment*** and 2) ***No viruses are used.*** In animal studies of the hATTR treatment delivered by lipid nanoparticles, the genome-editing components were cleared from the body in less than a week, dramatically reducing the chance of unwanted edits. Another risk of systemic administration of editing reagents ***is the potential to trigger a dangerous immune reaction***: so far, the outlook is sunny as no trial participants have experienced any serious side effects.

Efficiency — meaning, ***what percentage of cells are edited*** — is a big question. In nonhuman primates, only 35–40% of liver cells need to be edited to reduce TTR levels enough to have a therapeutic benefit. *There are no reports yet on what percentage of cells are edited in these patients, but* ***the strong reductions in TTR protein are extremely encouraging as indicators of efficiency of editing and efficacy of the treatment****.* The early positive indicators from this trial are encouraging for treating other disorders where editing liver tissue may be beneficial.

INFLAMMATORY DISEASE – HEREDITARY ANGIOEDEMA

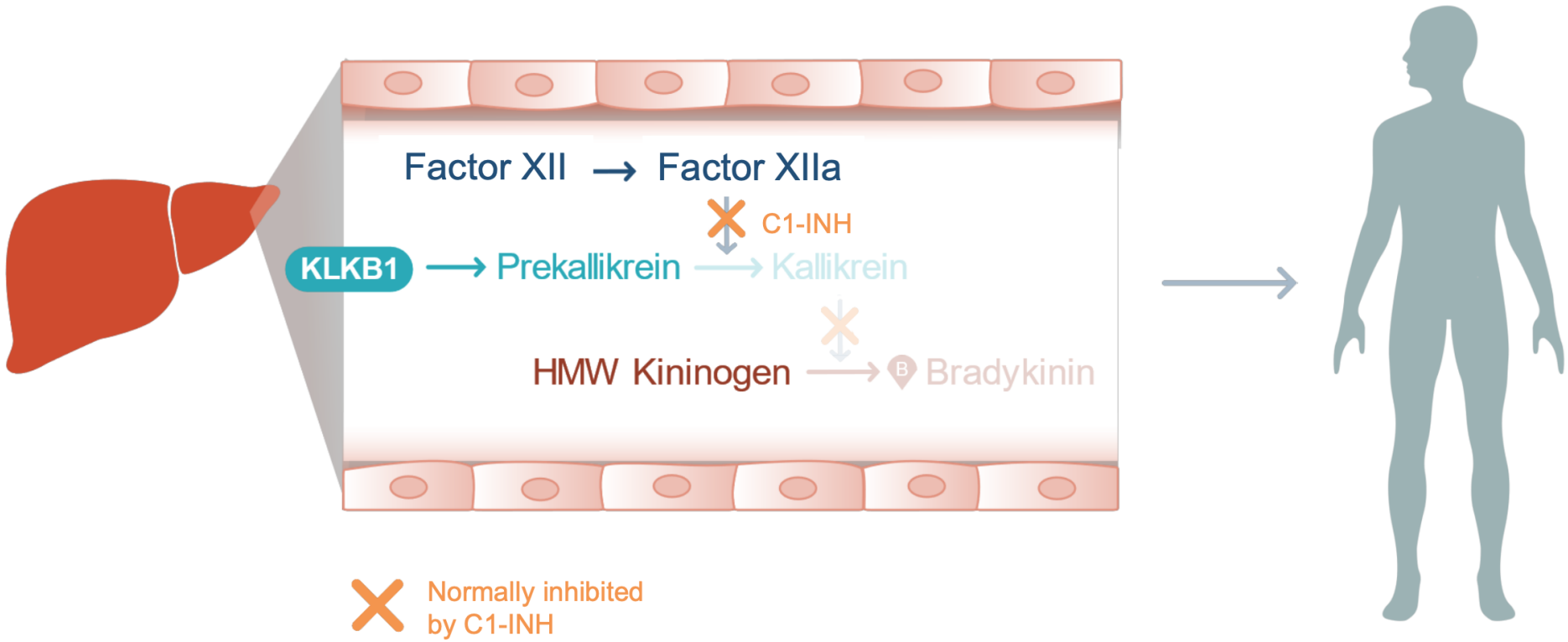
In **hereditary angioedema (HAE),** an individual has severe attacks of **inflammation**, leading to **swelling**. Swelling usually happens in the **arms** and **legs**, **face**, **intestines**, or **airway**. Swelling of the **intestines** can lead to severe pain, nausea, and vomiting, and swelling of the **airways** can be life-threatening. Individuals with HAE usually begin to **get attacks** in **childhood**. Without treatment, attacks occur **every 1–2 weeks**, lasting 3–4 days each. HAE affects about **1 in every 50,000 people**.

There are three known types of HAE. Types I and II are caused by **mutations** in the gene that makes the **C1 inhibitor protein**. In healthy individuals, proteins that increase and decrease inflammation are in a careful balance, helping the body respond to threats and injuries to just the right degree. The C1 inhibitor protein helps reduce inflammation. In **HAE**, mutations lead to **lower levels** of **C1 inhibitor protein**. Without enough C1 inhibitor protein, the protein **bradykinin** accumulates in the **blood**. Bradykinin makes fluid **leak from the blood vessels** into body tissues. When this happens excessively, it leads to HAE **swelling attacks**.

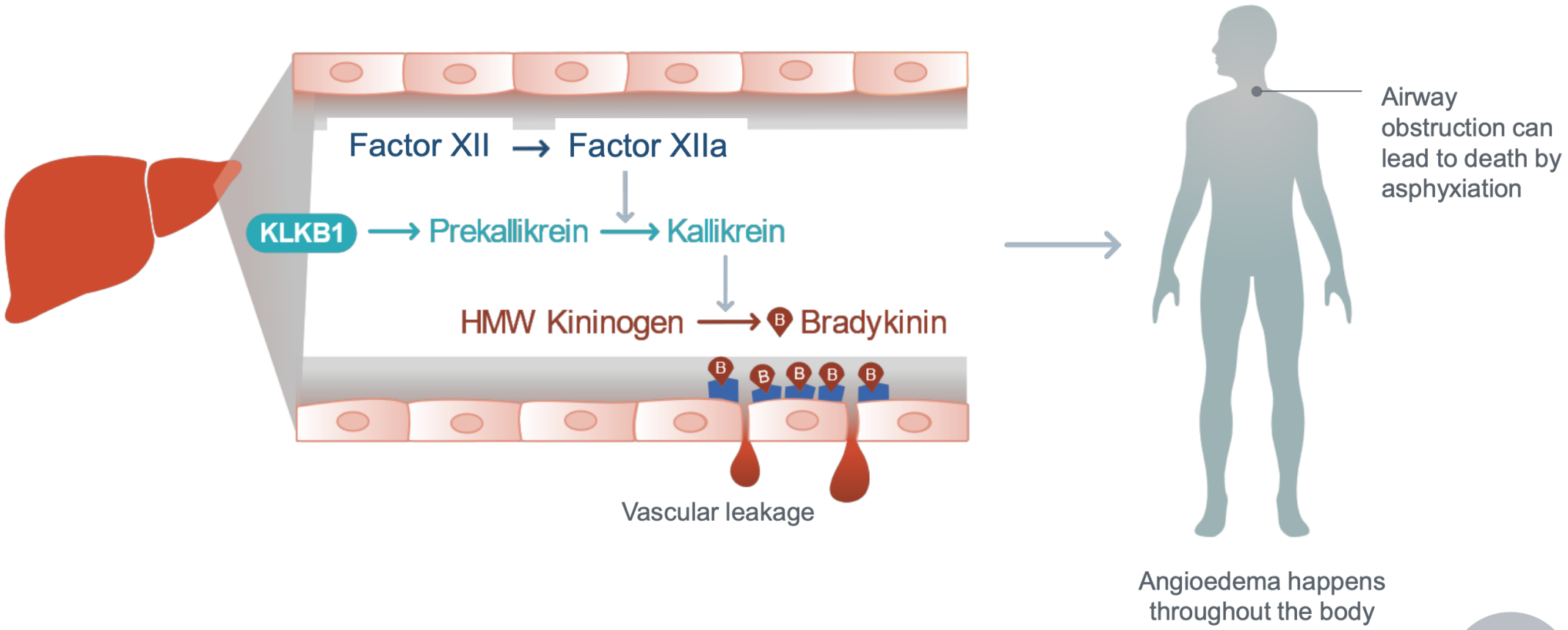
Current treatment options require daily pills or intravenous (IV) or injection administration as often as **twice per week**. Even when administered regularly, individuals with HAE may still experience occasional attacks.

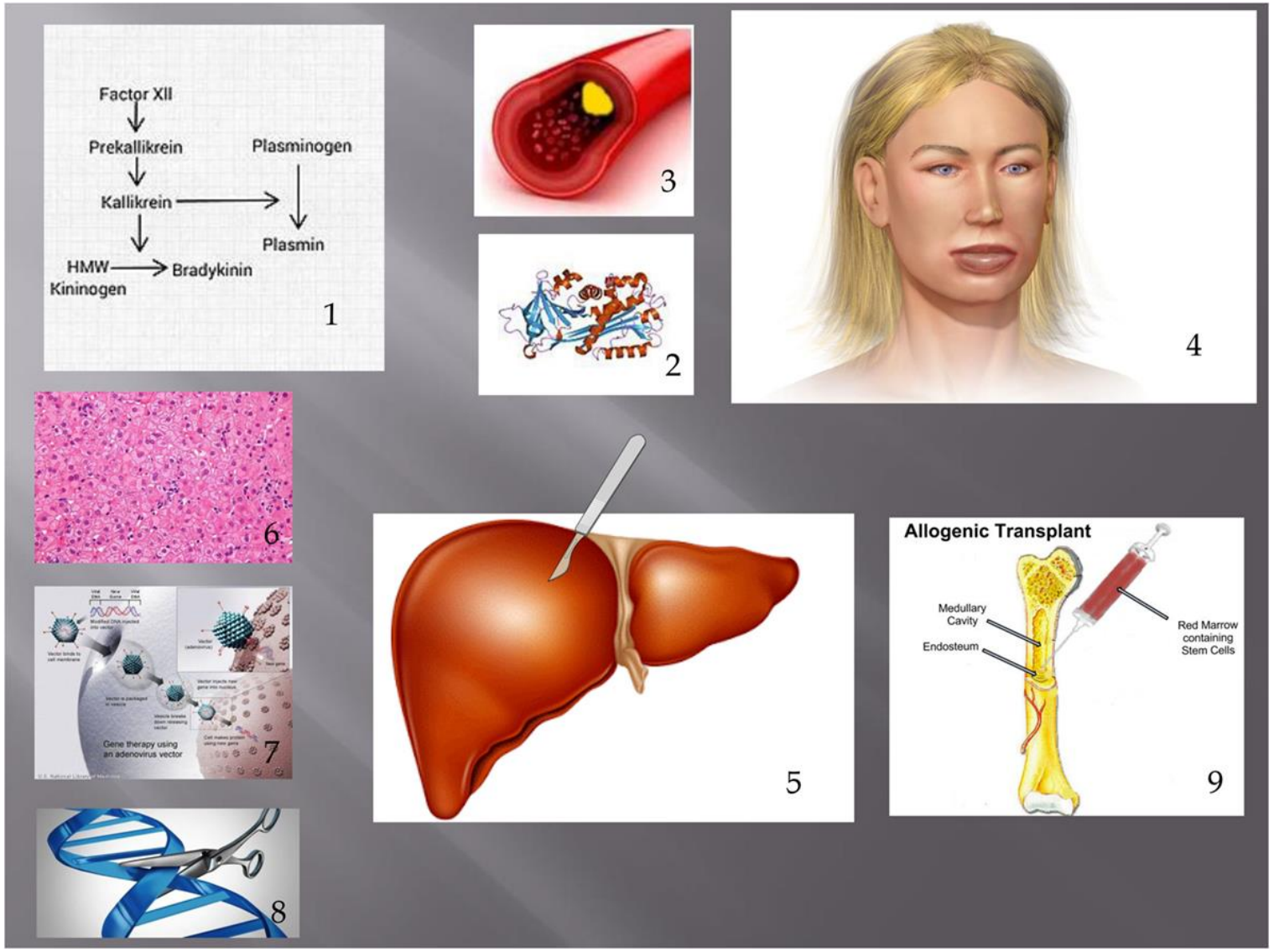
Like hATTR, angioedema can happen spontaneously, but for some individuals, the mutated gene is passed down from their biological parents. This leads to hereditary angioedema. HAE is a rare disease, affecting about **150,000 people** worldwide.

C1 Esterase Inhibitor (C1-INH) Regulates the Release and Buildup of Bradykinin



C1-INH Deficiency Results in Unregulated Release and Buildup of Bradykinin, Activating Endothelial Cells and Leading to Angioedema





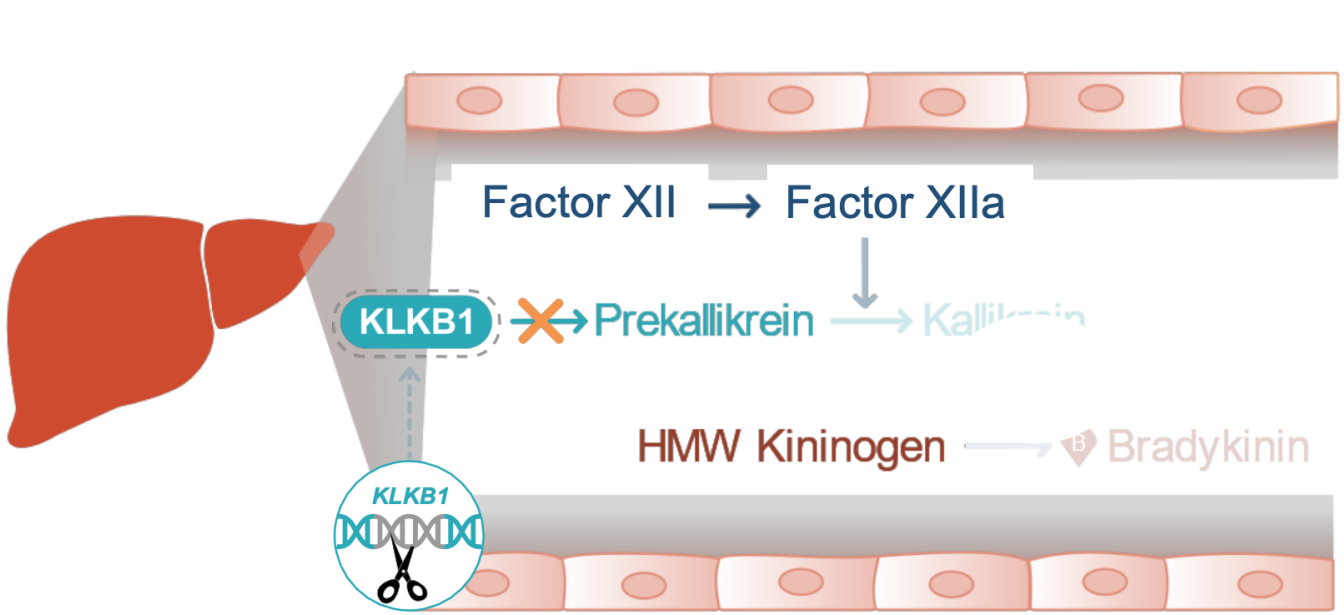
Treatment Strategy:

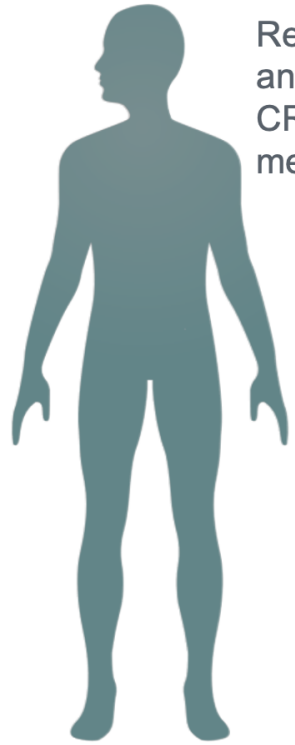
The protein prekallikrein is processed into kallikrein, which is crucial to making bradykinin. In this treatment, the aim is to make the gene that codes for prekallikrein nonfunctional (knock-out), reducing the amount of kallikrein, and ultimately reducing the amount of bradykinin. CRISPR-Cas9 is used to make a cut in the prekallikrein gene and when the body tries to repair the DNA break, it renders the gene nonfunctional. If this works as intended, it will reduce the amount of bradykinin, bringing it back into balance with C1 inhibitor protein to prevent inflammation attacks.

Previous research shows how much kallikrein needs to be reduced to prevent attacks. In Intellia's work in nonhuman primate models, a single dose of the CRISPR-based treatment can reduce kallikrein protein to this level, and the reduction was sustained for more than a year.

Pathogenesis of HAE and treatment options: (1) Contact phase. In HAE, an excess of bradykinin is present as a result of impaired C1 INH function (2). This leads to extravasation of fluid from blood vessels (3) leading to angioedema (4). Possible treatments discussed include liver transplantation (including auxiliary partial orthotopic liver transplantation (APOLT) (5), hepatocyte transplantation (6), liver-based gene therapy (7) , and genome editing (8). Bone marrow transplantation has also led to a cure of HAE (9).

CRISPR/Cas9-Mediated KO of *KLKB1* Reduces the Undesired Bradykinin Activity in People with HAE





Reduced angioedema anticipated following CRISPR/Cas9-mediated KO of *KLKB1*

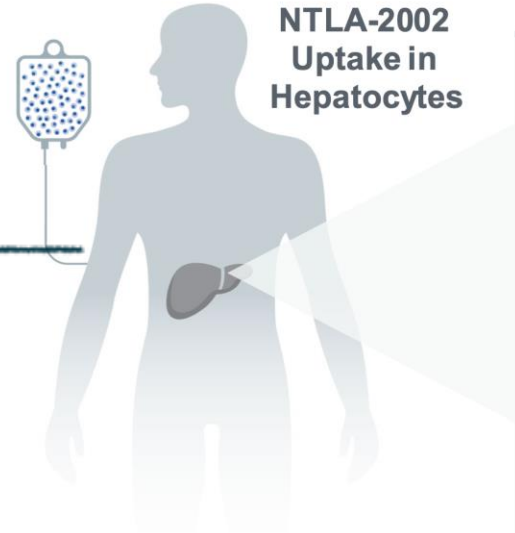
- **Prekallikrein inhibitors are clinically validated in preventing HAE attacks**
- ***KLKB1* KO is expected to be safe, as human nulls show no associated pathology***

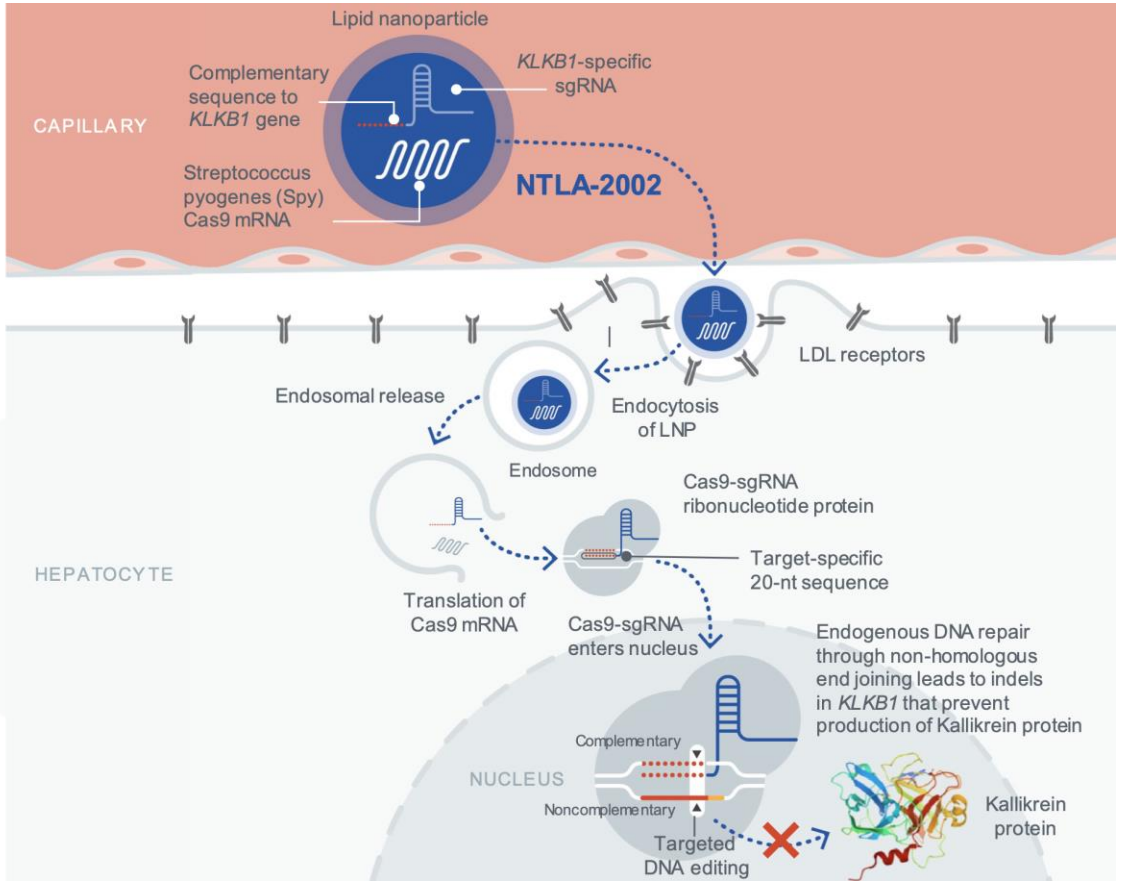
*Fletcher Syndrome, PMID 20424433

In vivo CRISPR/Cas9 editing of KLKB1 in patients with HAE

Session V: Bradykinin Symposium, Berlin, Germany
16 September 2022

NTLA-2002 is a novel, investigational CRISPR/Cas9-based *in vivo* gene editing therapy





The treatment that is currently in clinical trials uses **CRISPR-Cas9** tools **to reduce** the amount of **bradykinin** protein the body makes. Less bradykinin means less inflammation and swelling. The treatment is delivered in a single dose by IV. As in the hATTR treatment, the aim isn't to fix a gene, but to break a gene to stop the disease process. The CRISPR components cut the gene of interest, creating a double-stranded break in the DNA. As the cell tries to repair the DNA without a corrected template, the repair attempts mutate the gene even more. And when a gene is too badly damaged, a cell will sometimes stop making the protein it codes for.

In this trial, the CRISPR-Cas9 reagents are delivered by lipid nanoparticles. The aim is **to edit cells in the liver**. Lipid nanoparticles have a **natural tendency** to **accumulate** in the **liver**, so researchers are taking advantage of this to get the treatment to where it should be. This is an in vivo, systemic treatment, administered intravenously. This **phase 1/2 study** is being sponsored by **Intellia Therapeutics**, enrolling up to **55 patient** volunteers **in New Zealand**. The first phase will test two different doses of the treatment, looking at safety and side effects. The second phase will compare the efficacy of the treatment relative to placebo.

NTLA-2002 global Phase 1/2 study design: Two-part, multi-center study of NTLA-2002 in adults with HAE Types I and II

Today's interim data cover the first six patients
(Data cut-off: 27 July 2022)

Intervention:
Single dose administered via an intravenous (IV) infusion

PHASE 1
Open-label, single-ascending dose

- 75 mg (n=3)
- 25 mg (n=3)
- 50 mg* (dosing complete)

PHASE 2
Expansion study to confirm recommended dose

Randomized

- Dose 1 (n=10)
- Dose 2 (n=10)
- Placebo Arm (n=5)

PRE-TREATMENT REGIMEN
Day -1: Oral dexamethasone 8 mg (or equivalent)

Day 1: IV dexamethasone 10 mg (or equivalent), IV or oral H1 and H2 blocker, C1-INH

PRIMARY OBJECTIVES
Evaluate safety & tolerability

OTHER OBJECTIVES
PK, PD, clinical efficacy (attacks)

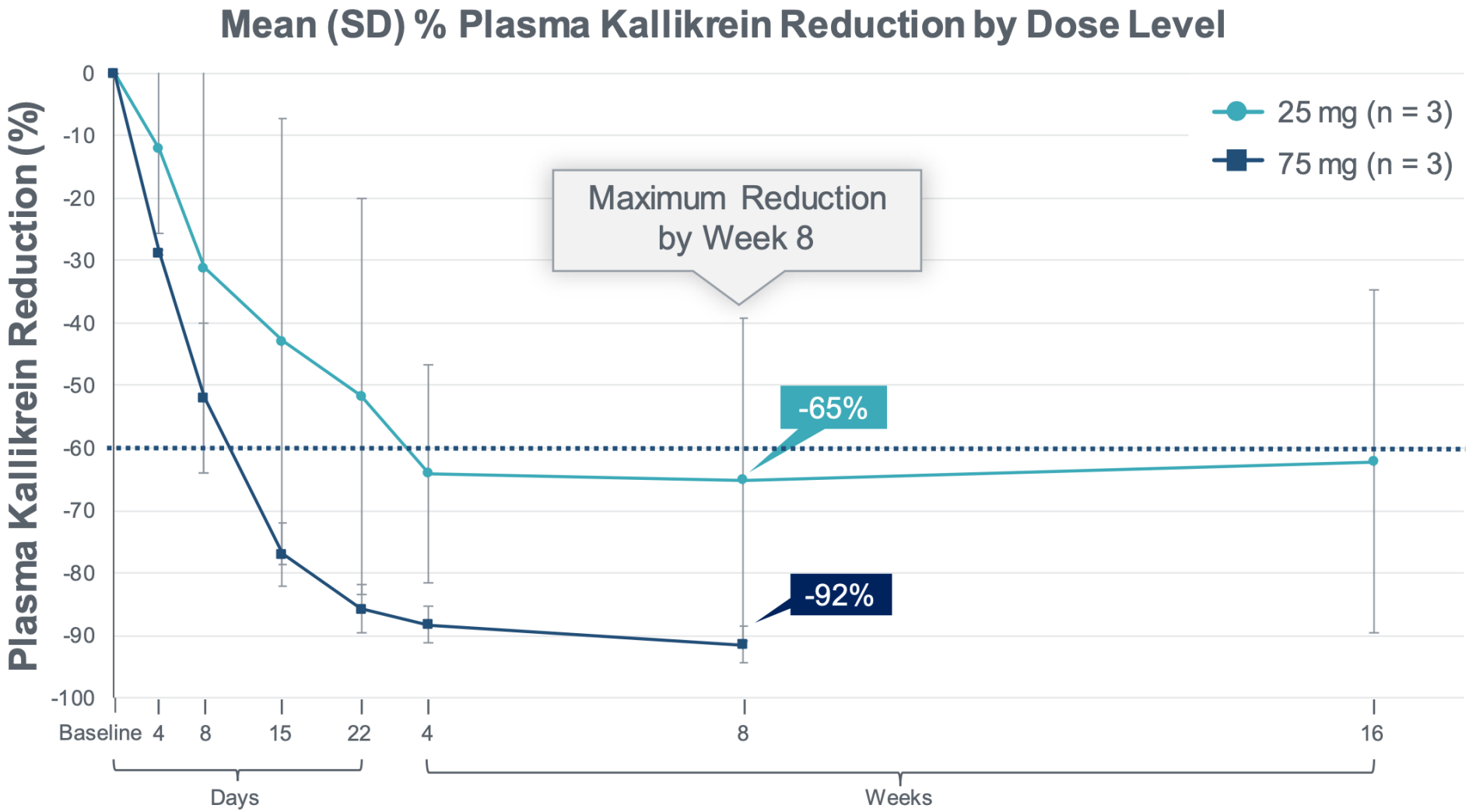
PRIMARY OBJECTIVES
Clinical efficacy (attacks through week 16)

OTHER OBJECTIVES
PD, safety & tolerability, PK, QoL

This presentation includes data for an investigational product not yet approved by regulatory authorities

*Minimum of 3 subjects and maximum of 6 patients per cohort
H1, Histamine Receptor 1; **H2,** Histamine Receptor 2;
C1-INH, C1 Esterase Inhibitor; **PK,** Pharmacokinetics; **PD**, Pharmacodynamics

NTLA-2002 resulted in rapid and deep plasma kallikrein reduction at both dose levels



This presentation includes data for an investigational product not yet approved by regulatory authorities

Dashed line represents targeted minimum reduction
SD, Standard Deviation

A single dose of NTLA-2002 led to robust, dose-dependent and durable reductions in total plasma kallikrein levels

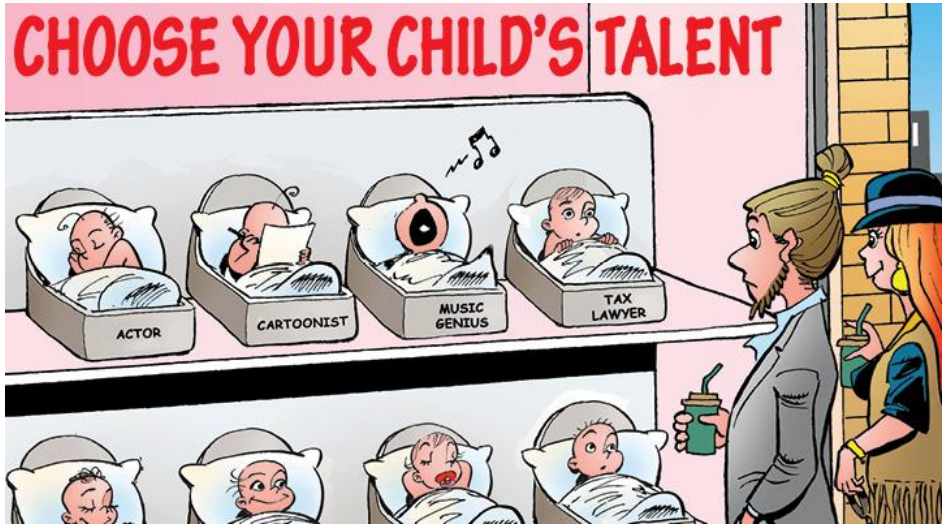
- **Mean plasma kallikrein reductions of 65% (25 mg) and 92% (75 mg) achieved at week 8**
- **Mean >90% reduction in HAE attacks in the 25 mg cohort through week 16**
  - All patients in the 25 mg cohort achieved complete attack control
  - Patients on prior prophylactic therapy were able to discontinue and remain attack free
- **NTLA-2002 was generally well-tolerated across both dose levels; all AEs were of mild or moderate severity**
- **Based on these observations of robust pharmacodynamic responses and preliminary evidence of efficacy, no further dose escalation is planned**
  - A 50 mg cohort has been enrolled to further inform phase 2 dose selection

These data support the promise of CRISPR-based *in vivo* genome editing in humans

This presentation includes data for an investigational product not yet approved by regulatory authorities

Innovative Genomics Institute

Conclusions



CRISPR genome editing is only 10 years old, but we are already seeing remarkable progress. Each year, more trials are launched and therapies expanded into new disease areas. Taken together, these CRISPR clinical trials are helping scientists learn about the types of DNA changes CRISPR enzymes make in different cells, (including unwanted off-target changes and problematic on-target changes), the way the immune system reacts to CRISPR-Cas tools, and how well different delivery and administration methods work.

Over the past couple of years, there has been encouraging news: Many patients seem to be **functionally cured of sickle cell disease or beta thalassemia**, and the edited cells have taken up residence in the bone marrow, indicating the potential for a long-lasting cure. Trials for **cancer immunotherapies** are at early stages, but the **safety** and **tolerability** of the treatments looks promising for moving forward with more newer versions of editing technology, **off-the-shelf products**, for moving towards new cancer targets, and even developing new cell types for immunotherapy. The **initial safety** results for treating **LCA10** and **chronic UTI** are **positive** and we hope to get more efficacy data over the next year. The preliminary results from the hATTR trial are particularly encouraging for this disease and a wide range of diseases with liver pathology, including the new HAE trial. New trials started this year also widen the scope of CRISPR applications to include more common diseases: HIV/AIDS and type 1 diabetes.

All of the treatments are relatively new. Positive results still require long-term follow-up to see if the treatment remains effective, whether patients suffer ill effects from unwanted edits, and whether there are immune reactions in patients with virally delivered Cas proteins.

Innovative Genomics Institute

Future Directions

While the current CRISPR clinical trials are exciting, they focus on the basic capabilities of CRISPR-Cas enzymes and offer only a glimpse of their therapeutic potential. Future milestones will help us learn more about CRISPR's ability to treat or even prevent diseases:

- A CRISPR treatment that involves inserting DNA to repair or replace a harmful DNA sequence, in essence "pasting" in new material, is still coming. The **Graphite Bio sickle cell disease trial** will be the first to attempt to **directly correct** a mutation back to the healthy variant.

- A CRISPR therapy that edits multiple genes at the same time, also known as ***multiplex editing***. Researchers have achieved impressive feats in isolated cells and animal models, and multiplex editing for cancer immunotherapy is currently being developed.

- A treatment that uses ***base editing***. Base editing uses CRISPR components to directly change single DNA letters without making double-stranded breaks in the DNA. For diseases caused by single-letter changes to DNA, base editors may be a safer editing option than conventional CRISPR. Several base-editing treatments are being developed for clinical trials, including for sickle cell disease.

- A treatment that uses ***prime editing***. Prime editing, like base editing, uses CRISPR components to make changes to DNA without making double-stranded breaks. Prime editors can potentially change both single bases and longer stretches of DNA, but has not yet been applied therapeutically.

- A trial where CRISPR tools are used to *turn genes on and off* without changing the DNA sequence. These strategies, known as ***CRISPR activation and CRISPR inhibition***, don't require making breaks in a patient's DNA, so they might be a safer option than conventional CRISPR. ***CRISPRi*** and ***CRISPRa*** may be reversible — another potential advantage for some applications.

Thanks for your patience

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