CRISPR Cas9: A New Genetic Tool

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CRISPR Cas9 has received an immense amount of attention since it has been considered for use for various potential human diseases. We have discussed it before as applied to cancer but in this paper we examine it as a tool in the tool kit of genome engineers. This is not a paper for bench people who will deal with this in various organisms but its purpose is directed at systems analysts who want to grasp the CRISPR Cas9 from a system engineering perspective in the context of genomic engineering.

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1 INTRODUCTION

Current day biotechnology is in many ways a set of tools in a large tool box that handle the what and how of manipulating genes and their products. The tool and tool box metaphor is quite powerful and descriptive. The problem oftentimes is the why and also the integration of all of these elements from a technique to a technology.

In this brief paper we examine the CRISPR element less from that of a bench technique than as a technology that can be used in gene engineering. There is a mindset being explored that differs from that of the bench biologist. As an engineering approach one asks how can this technique be moved to a useful technology, and how deeply does one have to understand the underpinnings to use it effectively and safely.

One of the challenges of genetic engineering is the ability to select a specific gene and alter it, or add another gene or delete a gene. A key step in all of these is the ability to cut and paste at specific sites, at very specific sites. Now that one can read a gene in detail and when one knows what the desired result should be, then the cut and paste side is critical. Pasting is somewhat well known, especially if we have cut at the right location. CRISPR is a tool that does just that, it is a very accurate, fast, and low cost gene cutting tool.

In this note we examine its structure from a systematic perspective. This will help understand what factors are the key factors and what elements should be understood. This is not a note for a bench biologist, it is not meant to be comprehensive. Yet unlike many of the simplified descriptions in the media I try to provide adequate depth with breath of applications.

We also try to establish the "gene engineering" tools that this mechanism can support. Finally we discuss some of the concerns which have arisen in the use of CRISPRs.

To summarize, I refer to Mali et al who state:

Functioning of the type II CRISPR-Cas systems in bacteria.

Phase 1: in the immunization phase, the CRISPR system stores the molecular signature of a previous infection by integrating fragments of invading phage or plasmid DNA into the CRISPR locus as 'spacers'.

Phase 2: in the immunity phase, the bacterium uses this stored information to defend against invading pathogens by transcribing the locus and processing the resulting transcript to produce CRISPR RNAs (crRNAs) that guide effector nucleases to locate and cleave nucleic acids complementary to the spacer.

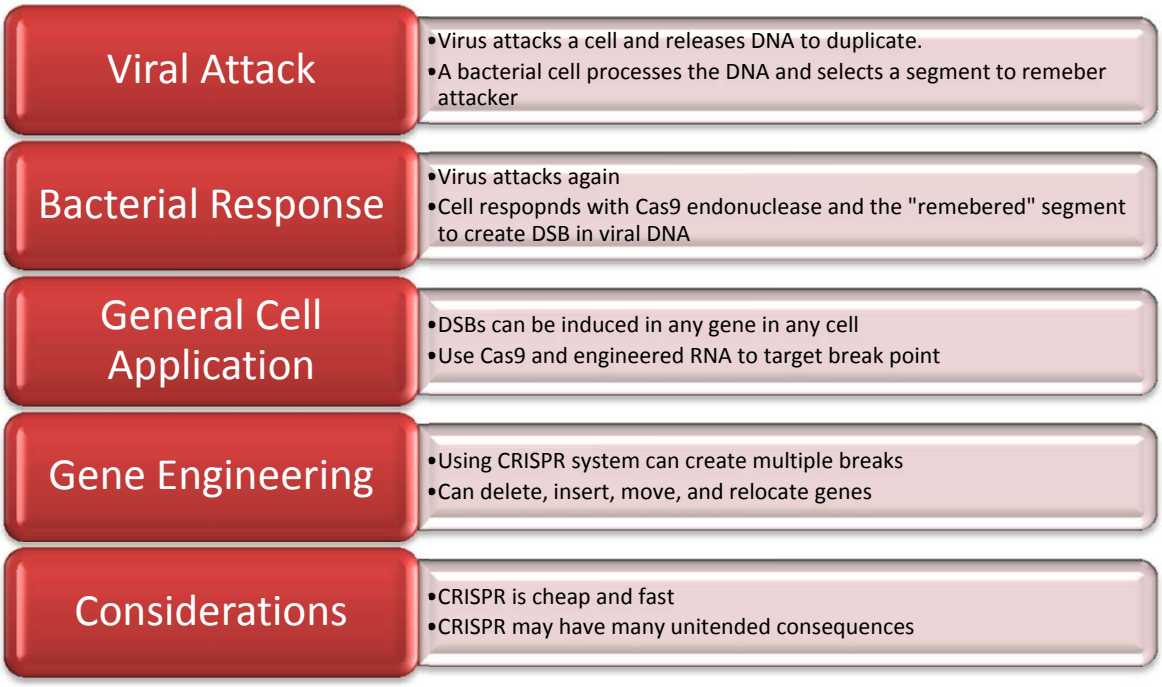
First, tracrRNAs hybridize to repeat regions of the pre-crRNA.

Second, endogenous RNase III cleaves the hybridized crRNAtracrRNA, and a second event removes the 5' end of the spacer, yielding mature crRNAs that remain associated with the tracrRNA and Cas9.

The complex cleaves complementary 'protospacer' sequences only if a PAM sequence is present.

Namely, this tool was seen developed in bacteria. The bacterium notes a section of the invading viral DNA, and then records that segment in its own DNA. Then when the virus attacks a second time, using the Cas9 nuclease protein produced by the bacteria then uses the RNA generated by the "remembered" sequences to attack the virus, and cut it so that it is made inoperable and it is digested.

Thus in the report we follow the following considerations:



2 BACTERIAL IMMUNOLOGY

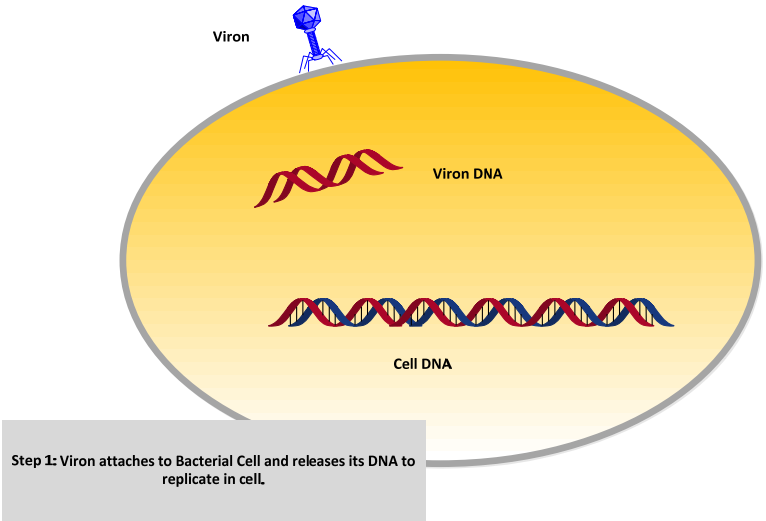
The CRISPR phenomenon comes from examining bacteria and their quasi immune response to viral attacks. Simply stated;

Bacteria have developed a technique where they can recognize a foreign viral DNA segment and then "attack" is with an enzyme and a targeted RNA segment that results in the foreign DNA being broken and becoming ineffective. This bacterial process effectively kills the DNA of the invader, stops its reproduction and induces an autophagy.

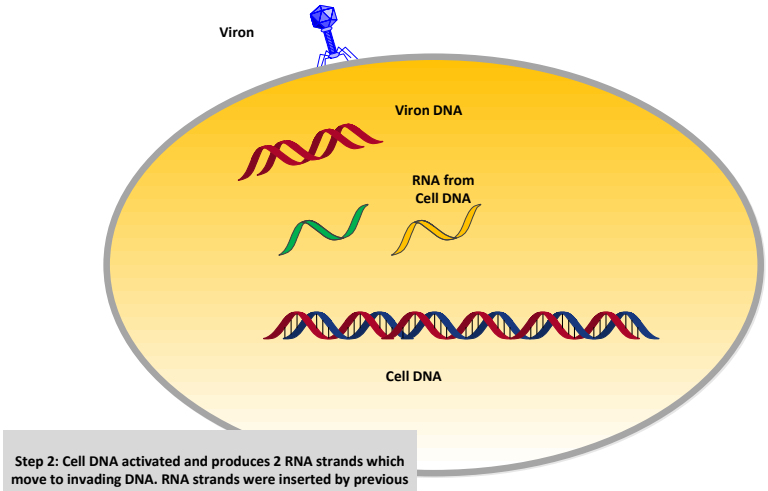
Now in discovering this process one then can take this same enzyme and modify the RNA that comes with it to match a location on some DNA we may be considering to manipulate and using this combo we can then cut DNA at a precise point anywhere we so desire. It is a powerful tool to cut DNA at a unique location. From there we can then add or delete DNA segments in a gene, in a somatic cell or in a germ line cell. It is fast and inexpensive and can be done in almost any lab.

Let us now begin with a viron attaching itself to a bacterium. We will assume that at some prior time some process has occurred where the bacteria had seen this for the first time. At this time that process is still a work in progress. But let us assume that this is a subsequent encounter and that in the process the bacteria has managed to record this prior encounter with a strand of DNA from that viron so that it can produce an RNA which is a map of some small segment of the viron's DNA.

This is a lot of assumptions but it is generally where we start with the tool. We just want to know what the tool does not how it was made or even how in any detail it does what it does. In many ways we are looking at tools as a technician, namely use this tool this way and get this result. Leave the details for someone else.

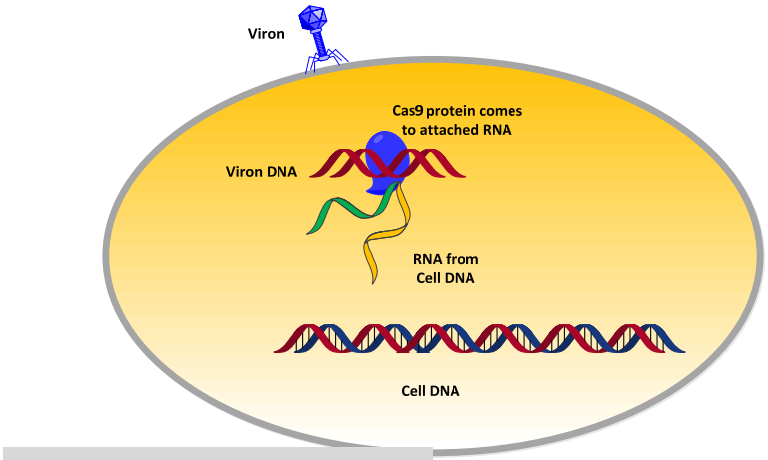


Now when the viron gets into the cell there is produced RNA from the bacteria that was RNA based upon a prior encounter with this viron. Namely this RNA released matches a segment of DNA in the viron. Also remember that a virus just wants to use a cell, any cell, to reproduce itself, which frankly is just reproducing its DNA (or RNA). If the bacteria can use this knowledge of the attacker then what can it do to stop the reproduction, and potentially the organism's death.



Step 2: Cell DNA activated and produces 2 RNA strands which move to invading DNA. RNA strands were inserted by previous attack by a viron with such DNA.

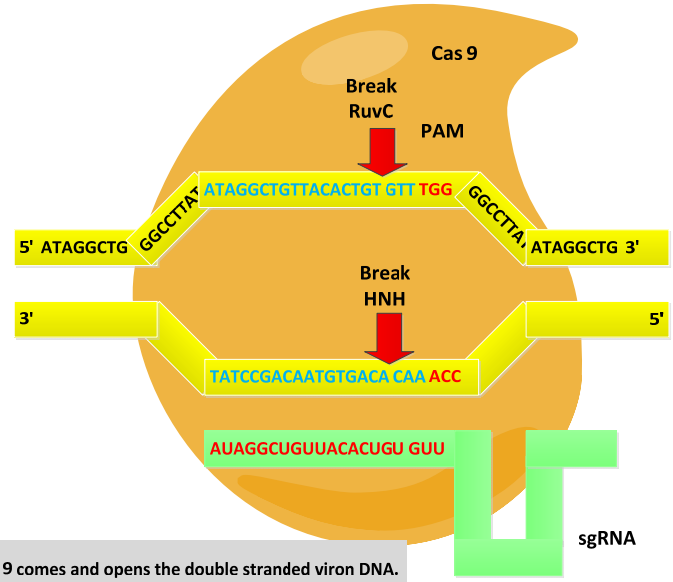
Now the RNA segments migrate to the viron DNA and along with a protein called cas9. The cas9 protein is the secret sauce of this tool.



Step 3: Cas9 protein is released and combines with viron DNA, combined RNA to create a CRISPR Cas9 cut of the viron DNA to be shown next in detail.

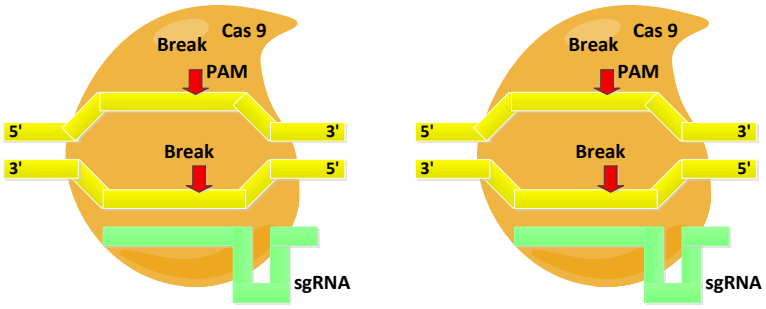
The details of the operation are depicted in the graphic below. One must recall that this tool works but its operation is not fully understood. The Cas9 protein surrounds the desired site which has been selected by a combination of two factors. The first is the PAM sequence, in this case 3 nucleotides, nt, which act as a marker and then a 20 nt long matching strand down from the PAM. This key determines where the break occurs. In a bacteria's immune like response it needs

both, the PAM to be certain it does not kill itself and the 20 nt strand which gives a good marker for a specific site. In effect we have 23 nt for specific targeting. In genetic engineering cases we select the PAM as specified by the Cas9 source and then engineer the sgRNA element. That yields a specific break site at 3 nt down from the end of the PAM. The two Cas9 fragments, RuvC and HNH are what cause the break.



Step 4: Cas 9 comes and opens the double stranded viron DNA. The sgRNA searches down the DNA looking for a match AND for the PAM sequence here being TGG or ACC. Then the sgRNA comes and binds to the bottom DNA. When binding complete two elements of Cas 9 break the DNA strands 3 nucleotides down from the PAM sequence. Then the viron DNA is defective and is dismembered by some form of autophagy. The viron DNA has been defeated.

The example below extends the above example to a double stranded break.



3 CRRNA AND TRACRRNA

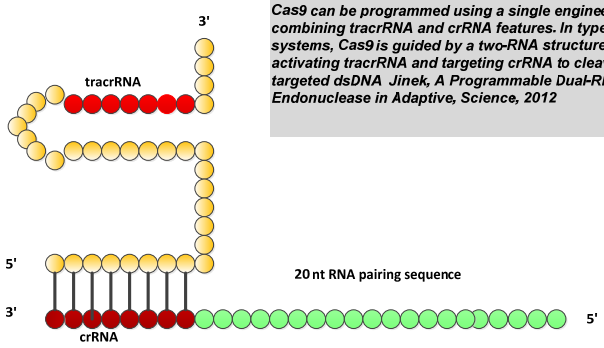
The two RNA segments, crRNA and tracrRNA can be configured in several ways. But they are the targets elements that are used to select where the break is to be. And once selected it is usually a double strand break. However single strand breaks can be accomplished as well.

As Jinek et al state:

In the expression and interference phases, transcription of the repeat-spacer element into precursor CRISPR RNA (pre-crRNA) molecules followed by enzymatic cleavage yields the short ***crRNAs that can pair with complementary protospacer sequences of invading viral or plasmid targets****. Target recognition by crRNAs directs the silencing of the foreign sequences by means of Cas proteins that function in complex with the crRNAs ….*

There are three types of CRISPR/Cas systems. The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA. In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA)*** *complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA*

We demonstrate one variation of this below. Note the tracrRNA and its binding with crRNA and the 20 nucleotide ("nt") sequence which will select out the point at which we desire a break to be made.



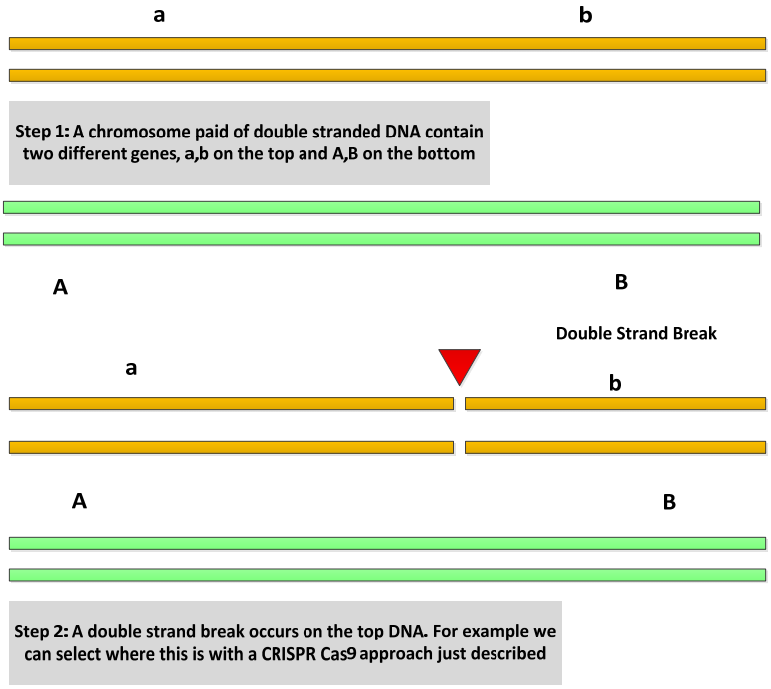
Cas9 can be programmed using a single engineered RNA molecule combining tracrRNA and crRNA features. In type II CRISPR/Cas systems, Cas9 is guided by a two-RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically–targeted dsDNA Jinek, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive, Science, 2012

4 GENE ENGINEERING

No we ask; given a break at the right point what do we do next? That is the beginning of gene engineering. We briefly examine homologous repair, a somewhat well understood process, which uses the other chromosome as a template. The use of templates may also be done to insert new genes as well.

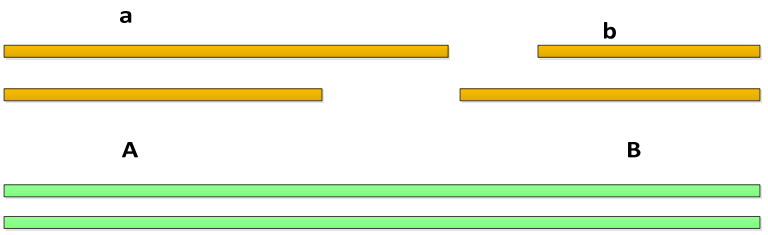
1. Let us start with a chromosome pair, one from each parent. We show this below.

2. Now we assume a double strand break, DSB, occurs on the top chromosome pair. We show this below:

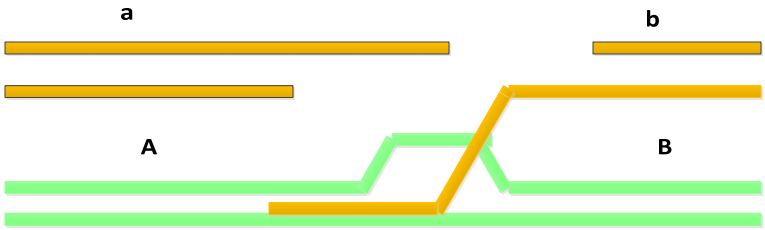


3. Next we see a shortening of strands as shown below;

4. Then we see and elongation and use of the strand in the uncut DNA as a template. This can be used for other templates as insertion mechanisms.



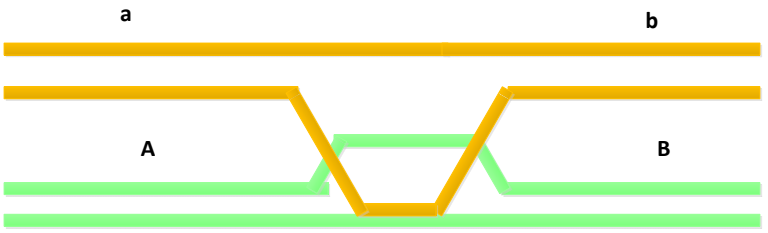
Step 3: The cut strands are shortened in top and bottom branches



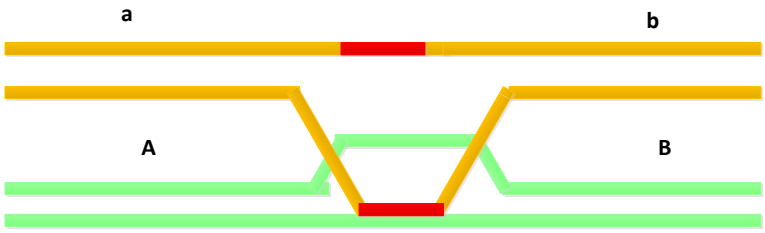
Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

5. We see both top and bottom expanding and a crossover occurring.

6. We can see this also as an insertion mechanism.



Step 5: Bottom completes and top finishes



Step 6: A selected gene can be inserted and added, or it can equally well be removed by a two cut process.

Now this is a simple reconstruction of the process. Details are in Watson et al.

5 APPLICATIONS

There are a large set of applications for this tool. We consider some here but it is anticipated that there will be many more. There is a balance between correcting gene defects in such disorders as muscular dystrophy, thalassemia, sickle cell anemia, or cystic fibrosis, and cancers such as those involving BRCA genes. There are also applications in the field of plant genetics which are extensive. Plants are pluripotent; that is, a single cell can regenerate almost any plant. Thus adding or extracting a gene can dramatically change a plant's characteristics. We have examined some of these opportunities elsewhere for horticultural plants.

5.1 GENE EXTRACTION

The simplest application is gene extraction. Using two DSBs at the desired locations we can accurately extract a gene.

5.2 GENE INSERTION

Gene insertion is a major step. A template must be available and an insertion point specified. It is also important to understand the location of any promoter genes or suppressor genes. Just inserting may not always work. There is also the issue of methylation and acetylation as well as miRNA interference.

5.3 SOMATIC APPLICATIONS

As the body matures or as a result of a genetic defect, we often see genetic changes which result in less than benign results.

5.4 GERMLINE APPLICATIONS

This is the most concerning application. Recently a group of researchers have indicated their concern and we shall discuss it later. However, one can take a sperm and ovum, cut-and-paste a new set of genes, and then allow them to combine and we have developed putatively a new species.

5.5 TYPES OF APPLICATIONS

There have been a multiplicity of papers on various applications We have indicated some general ones above but the Zhang Lab at MIT has performed a great deal worth examining

From the work of Cox et al,

The specific type of genome editing therapy depends on the nature of the mutation causing disease.

(a) In gene disruption, the pathogenic function of a protein is silenced by targeting the locus with NHEJ. Formation of indels in the gene of interest often results in frameshift mutations that create premature stop codons resulting in a nonfunctional protein product or nonsense-mediate decay of transcripts, suppressing gene function. Gene disruption may also be used to introduce protective loss of- function mutations into wild-type genes to generate a therapeutic effect (Box 1).

(b) In NHEJ gene correction, two DSBs targeted to both sides of a pathogenic expansion or insertion may be resolved by NHEJ, causing a therapeutic deletion of the intervening sequences. This form of treatment would require multiplexed targeting of disease-causing mutations.

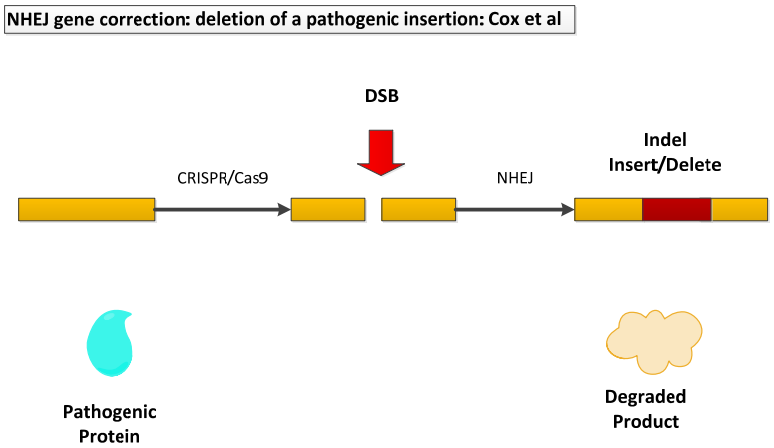
(c) HDR gene correction can be used to correct a deleterious mutation. A DSB is induced near the mutation site in the presence of an exogenously provided, corrective HDR template. HDR repair of the break site with the exogenous template corrects the mutation, restoring gene function.

(d) An alternative to gene correction is HDR gene addition, which introduces a therapeutic transgene into a predetermined locus. This may be the native locus, a safe harbor locus or a non-native locus. A DSB is induced at the desired locus, and an HDR template containing sequence similarity to the break site, a promoter, a transgene and a polyadenylation sequence is introduced to the nucleus. HDR repair restores gene function in the target locus, albeit without true physiological control over gene expression.

We graphically demonstrate some of these below:

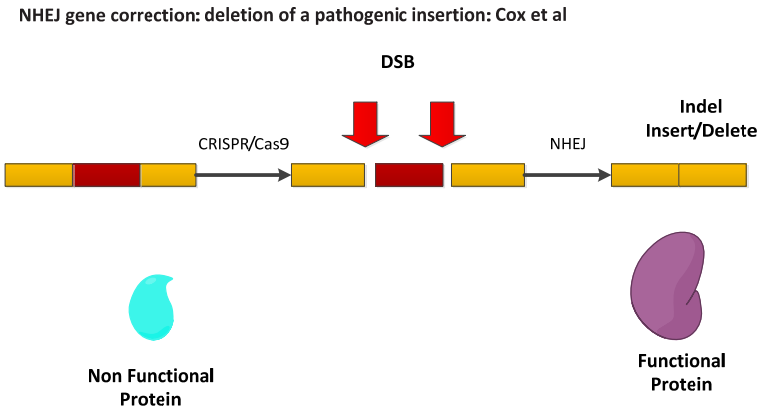
5.5.1 Deletion

The first cases below are a non-homologous break and joining of some insertion/deletion ("indel") to degrade the production of a protein. This is a simple double stranded break.



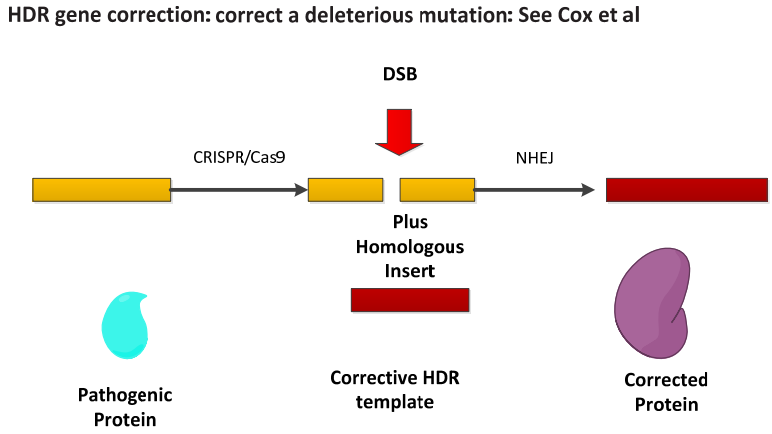
5.5.2 Correction

The second is again one using a DSB but this time a specific section is deleted and it requires two DSBs to be initiated. This may be an application where we seek a functional protein. It may be possible to employ this in a fusion gene process as well.



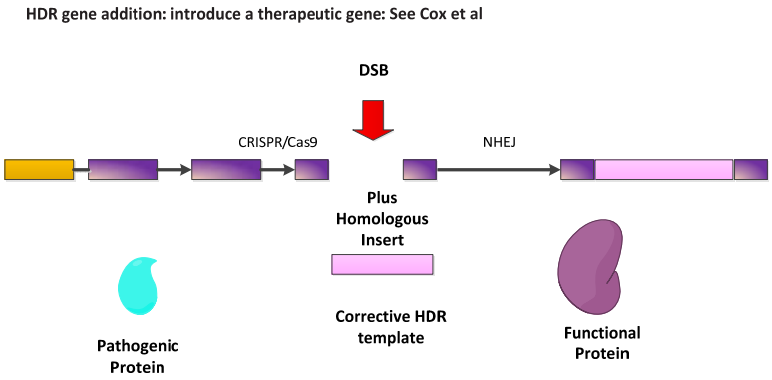
5.5.3 Homologous Correction

The next two applications are for homologous changes where we have a template to reconstruct the gene of the type we have previously discussed. The example below is a simple correction process with a single DSB.



5.5.4 Homologous Insertion

The final example below is for a complicate deletion and insertion. Again it uses a template and a homologous rebinding.



5.5.5 Ex Vivo and In Vivo

There are several dimensions in applying this tool to humans. They can be inserted ex vivo or in vivo. As Cox et al state:

… in ex vivo editing therapy, cells are removed from a patient being treated, edited and then re-engrafted. For this mode of therapy to be successful, the target cells must be capable of surviving outside the body and homing back to target tissues after transplantation.

… in vivo therapy involves genome editing of cells in situ. For in vivo systemic therapy, delivery agents that are relatively agnostic to cell identity or state would be used to effect editing in a wide range of tissue types. Alternatively, targeted in vivo therapy may also be achieved through targeted local injection of viral vectors to the affected tissue or through the systemic injection of viral vectors with inherent tropism for specific diseased tissues, such as the eye brain, or muscle.

Now we can see this as applied in somatic or germ line cells.

	Ex Vivo	***In Vivo***
Somatic	One may consider this approach in certain hematopoietic cell lines.	Insertion into targeted somatic cells of modified genes may produce or delete expressions of genes. Various means of insertion are possible.
Germ Line	This is a somewhat routine procedure for certain reproductive processes. However the viability and sustainability of such cells may have issues.	Each of the germ line cells may be separately dealt with insertions or deletions and then the two combined. This method also permits the insertion of CRISPR Cas9 genes themselves to assure continual propagation of the desired change. However this may be a very complex approach since it deals with in vivo germ line cells where injecting the cells may not function completely.

6 OBSERVATIONS

Having given a high level description of this tool we can make several key observations. Amongst them is the recent concern as to the potential abuse of the process.

CRISPR Cas 9 is a new technique to cut and splice genes. We had written about it about a year ago regarding its use in cancer treatment and also regarding the patent so quickly issues. Now David Baltimore, a highly respected scientist, and colleagues have in Science suggested a prudent set of steps as to its use in humans. It is reminiscent of the concerns some 40 years ago regarding recombinant DNA.

6.1 CONTROLS TO USE

Recently several groups of researchers have become concerned regarding the unintended consequences of CRISPRs. For example David Baltimore et al recommend:

In the near term, we recommend that steps be taken to:

1) Strongly discourage, even in those countries with lax jurisdictions where it might be permitted, any attempts at germline genome modification for clinical application in humans, while societal, environmental, and ethical implications of such activity are discussed among scientific and governmental organizations. (In countries with a highly developed bioscience capacity, germline genome modification in humans is currently illegal or tightly regulated.) This will enable pathways to responsible uses of this technology, if any, to be identified.

2) Create forums in which experts from the scientific and bioethics communities can provide information and education about this new era of human biology, the issues accompanying the risks and rewards of using such powerful technology for a wide variety of applications including the potential to treat or cure human genetic disease, and the attendant ethical, social, and legal implications of genome modification.

3) Encourage and support transparent research to evaluate the efficacy and specificity of CRISPR-Cas9 genome engineering technology in human and nonhuman model systems relevant to its potential applications for germline gene therapy. Such research is essential to inform deliberations about what clinical applications, if any, might in the future be deemed permissible.

4) Convene a globally representative group of developers and users of genome engineering technology and experts in genetics, law, and bioethics, as well as members of the scientific community, the public, and relevant government agencies and interest groups—to further consider these important issues, and where appropriate, recommend policies.

Baltimore et al have a significant point. Not only can this be significant on a person by person basis but it also has the potential to be weaponized. The technology is out there, thousands are now proficient in it, the cost is low and the means for distribution is high.

Clearly a sensible effort in collaboration with others is essential. The problem is that with much of science, the genie is out of the box.

The system used by bacteria to defend against a virus attacking is the CRISPR Cas 9 system. An interesting use of a protein, enzyme, and a DNA segment that can open DNA at desired locations and cut and insert new segments of DNA. We have been discussing this for well over a year now and have discussed its potential and its risks.

Now along come researchers who instead of doing this in somatic cells do it in germline cells, thus changing the potentially maturing entity. Thus each cell has this changed gene or genes.

In a recent Nature article the authors state[1]:

There are grave concerns regarding the ethical and safety implications of this research. There is also fear of the negative impact it could have on important work involving the use of genome-editing techniques in somatic (non-reproductive) cells....In our view, genome editing in human embryos using current technologies could have unpredictable effects on future generations. This makes it dangerous and ethically unacceptable. Such research could be exploited for non-therapeutic modifications. We are concerned that a public outcry about such an ethical breach could hinder a promising area of therapeutic development, namely making genetic changes that cannot be inherited. At this early stage, scientists should agree not to modify the DNA of human reproductive cells. Should a truly compelling case ever arise for the therapeutic benefit of germ-line modification, we encourage an open discussion around the appropriate course of action.

Now this point is well made. Germline cell changes introduce all sorts of issues. Not only is there the issue of what this new gene will do, we hardly have begun to understand gene interactions, but the issues of epigenetic factors such as methylation dramatically change the risks.

Then again you do have the techno-advocates in Technology Review, who state[2]:

When I visited the lab last June ... proposed that I speak to a young postdoctoral scientist named ..., a Harvard recruit from Beijing who'd been a key player in developing a new, powerful technology for editing DNA, called CRISPR-Cas9. With ...had founded a small company to engineer the genomes of pigs and cattle, sliding in beneficial genes and editing away bad ones. As I listened to ..., I waited for a chance to ask my real questions: Can any of this be done to human beings? Can we improve the human gene pool? The position of much of mainstream science has been that such meddling would be unsafe, irresponsible, and even impossible. But ... didn't hesitate. Yes, of course, she said. In fact, the Harvard laboratory had a project to determine how it could be achieved.

[1] http://www.nature.com/news/don-t-edit-the-human-germ-line-1.17111

[2] http://www.technologyreview.com/featuredstory/535661/engineering-the-perfect-baby/

She flipped open her laptop to a PowerPoint slide titled "Germline Editing Meeting." Here it was: a technical proposal to alter human heredity. "Germ line" is biologists' jargon for the egg and sperm, which combine to form an embryo. By editing the DNA of these cells or the embryo itself, it could be possible to correct disease genes and to pass those genetic fixes on to future generations. Such a technology could be used to rid families of scourges like cystic fibrosis. It might also be possible to install genes that offer lifelong protection against infection, Alzheimer's, and ... told me, maybe the effects of aging.

These would be history-making medical advances that could be as important to this century as vaccines were to the last.

The problem is, as the writers in Nature and in Science led by David Baltimore, has noted, the germ line modifications could be unwieldy.

Just because we have a new technology is no reason to let is loose. The problem with this technology is that it not only can be weaponized but that it can be done in a basement lab. This not building a nuclear weapon. This is potentially setting the world afire.

The again there is the issue of Government regulation. In an interesting piece in Xconomy the author remarks[3]:

But researchers' and investors' fear that a patchwork of regulation would cripple biotechnology in the United States did not disappear right away. Biologist Thomas Maniatis of Harvard left his home lab to work on the techniques in tighter-security conditions at Cold Spring Harbor Laboratory in New York.

Others went abroad. Biogen, founded in 1978, put its first major lab in Geneva, Switzerland. This was a time of intense concern about environmental dangers from the chemical industry in particular and science in general. It took some years for biologists to gain respect among local state, and federal officials for their sense of responsibility in the recombinant DNA maelstrom of the mid-1970s. But politicians did accept that biotechnology was a significant new industry that other countries, like Japan, might seize if America dropped the ball.

A valid point, but in the 70s we worried about errant scientists. Now we are terrified about terrorist post docs! One wonders what would be worse; the Government Regulators or the Terrorist?

6.2 CURRENT APPLICATIONS

There are many areas where this technology may have immediate use. There is a report in Genome Research of CRISPR being used to correct β-Thalassemia. They state[4]:

[3] http://www.xconomy.com/boston/2015/03/26/amid-gene-editing-worry-a-return-to-biotechs-1st-asilomar-moment/2/

[4] http://genome.cshlp.org/content/early/2014/07/30/gr.173427.114.abstract

β-thalassemia, one of the most common genetic diseases worldwide, is caused by mutations in the human hemoglobin beta (HBB) gene. Creation of human induced pluripotent stem cells (iPSCs) from β-thalassemia patients could offer an approach to cure this disease. Correction of the disease-causing mutations in iPSCs could restore normal function and provide a rich source of cells for transplantation.

In this study, we used the latest gene-editing tool, CRISPR/Cas9 technology, combined with the piggyBac transposon to efficiently correct the HBB mutations in patient-derived iPSCs without leaving any residual footprint.

No off-target effects were detected in the corrected iPSCs, and the cells retain full pluripotency and exhibit normal karyotypes. When differentiated into erythroblasts using a monolayer culture, gene-corrected iPSCs restored expression of HBB compared to the parental iPSCs line.

Our study provides an effective approach to correct HBB mutations without leaving any genetic footprint in patient-derived iPSCs, thereby demonstrating a critical step toward the future application of stem cell-based gene therapy to monogenic diseases.

We have considered Cancer applications in a separate note in 2014. Cox et al present the following summary Table:

Disease type	*Nuclease platform*	*Therapeutic strategy*
Hemophilia B	ZFN	HDR-mediated insertion of correct gene sequence
HIV	ZFN and CRISPR	NHEJ-mediated inactivation of CCR5
Duchene muscular dystrophy (DMD)	CRISPR and TALEN	NHEJ-mediated removal of stop codon, and HDR-mediated gene correction
Hepatitis B virus (HBV)	TALEN and CRISPR	NHEJ-mediated depletion of viral DNA
SCID	ZFN	HDR-mediated insertion of correct gene sequence
Cataracts	CRISPR	HDR-mediated correction of mutation in mouse zygote
Cystic fibrosis	CRISPR	HDR-mediated correction of CFTR in intestinal stem cell organoid
Hereditary tyrosinemia	CRISPR	HDR-mediated correction of mutation in liver

6.3 ISSUE OF CONCERN

As noted there may be many unintended consequences which have yet to be explored. We examine a few of them here and we are certain many more will arise in the future. This is a

powerful technology, one that is inexpensive and fast acting, and one which if in th wrong hands can be used for less than benign purposes.

6.3.1 Accuracy of Cutting

How accurate are these breaks? There are two elements. One is the actual targeting and that seem to be excellent. The other would be the equivalent of a false targeting. Namely targeting an identical string at the wrong place.

6.3.2 Promoters and Other Interactions

We know that just having the right gene does not mean that it is expressed. Thus promoters and similar interactions must be considered. Gene expression is oftentimes a complicated process.

6.3.3 Methylation Factors

Methylation is well known to play active roles in gene expression. In the cut and paste mode we may change methylation profiles. This could dramatically change gene expression, since one gene product may be another gene promoter.

6.3.4 Other Epigenetic Factors

There are many other epigenetic factors including acetylation, miRNA, lncRNAs and the like. It is uncertain how these factors can be influenced in this process.

7 APPENDIX: TERMS AND DEFINITIONS

Term	*Definition*
Cas9[5]	CRISPR-associated (Cas) proteins to direct degradation of complementary sequences present within invading viral and plasmid DNA. The *Streptococcus pyogenes* SF370 type II CRISPR locus consists of four genes, including the Cas9 nuclease, as well as two noncoding CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs)
CRISPR	Clustered regularly interspaced short palindromic repeats.
crRNA	CRISPR RNA
tracrRNA[6]	In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded*** (ds) NAspecific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein
sgRNA[7]	Type II CRISPR–Cas systems have been adapted as a genome-engineering tool. In this system, crRNA teams up with a second RNA, called trans-acting CRISPR RNA (tracrRNA), which is critical for crRNA maturation and recruiting the Cas9 nuclease to DNA. The RNA that guides Cas9 uses a short (20-nt) sequence to identify its genomic target. ***This three-component system was simplified by fusing together crRNA and tracrRNA, creating a single chimeric ‘‘guide’’ RNA abbreviated as sgRNA or simply gRNA.***
PAM[8]	The CRISPR locus is transcribed and processed into short CRISPR RNAs (crRNAs) that guide the Cas to the complementary genomic target sequence. There are at least eleven different CRISPR– Cas systems, which have been grouped into three major types (I–III). In the type I and II systems, nucleotides adjacent to the protospacer in the targeted genome comprise the protospacer adjacent motif (PAM). The PAM is essential for Cas to cleave its target DNA, enabling the CRISPR–Cas system to differentiate between the invading viral genome and the CRISPR locus in the host genome, which does not incorporate the PAM. For additional details on this fascinating prokaryotic adaptive immune response.
Homologous	Homologous chromosomes are a set of one maternal chromosome and one paternal chromosome that pair up with each other inside a cell during meiosis.

[5] Jinek et al
[6] Jinek et al
[7] Harrison et al
[8] Harrison et al

Term	*Definition*
RuvC[9]	RuvC is the resolvase, which cleaves the Holliday junction. It is thought to bind either on the open, DNA exposed face of a single RuvA tetramer, or to replace one of the two tetramers. Binding is proposed to be mediated by an unstructured loop on RuvC, which becomes structured on binding RuvA. RuvC can be bound to the complex in either orientation, therefore resolving Holliday junctions in either a horizontal or vertical manner. Cas9 contains domains homologous to both HNH and RuvC endonucleases.
HNH[10]	The domain HNHc (SMART ID: SM00507, SCOP nomenclature: HNH family) is associated with a range of DNAbinding proteins, performing a variety of binding and cutting functions. Several of the proteins are hypothetical or putative proteins of no well-defined function. The ones with known function are involved in a range of cellular processes including bacterial toxicity, homing functions in groups I and II introns and inteins, recombination, developmentally controlled DNA rearrangement, phage packaging, and restriction endonuclease activity Cas9 contains domains homologous to both HNH and RuvC endonucleases

[9] Bennett and West
[10] Mehta et al

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