CRISPRs and Cancer

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We examine CRISPRs and Cas9 as a means to treat cancers on a genetic basis. This is a new and highly effective tool for genetic engineering and holds some promise for cancer therapy.

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1 INTRODUCTION

CRISPRs, specifically "clustered regularly interspaced short palindromic repeats"[1], are portions of a cell's DNA which contain a particular type of short repetitions.. These specific repetitions are then followed by additional short segments of DNA which have been collected from some prior exposure to a virus phage. Namely CRISPRS are selective DNA snippets which have been garnered from viral phages which in some past period tried to attack the prior lineage of this cell. They are used to create Cas ("CRISPR associated" genes) which in turn have the capability of cleaving genes and inserting new ones.

CRISPR-Cas systems are now a useful toolkit for engineering eukaryotic cells, and especially human cells. They are also used in plant cells and that is a second tale but one worth examining as well.

As Jinek et al have recently said (Jinek et al 2014):

Although type I and III CRISPR-Cas systems rely on multiprotein complexes for crRNA-guided DNA targeting, type II systems use a single RNA-guided endonuclease, Cas9, that requires both a mature crRNA and a trans-activating crRNA (tracrRNA) for target DNA recognition and cleavage (8, 9). Both a seed sequence in the crRNA and conserved protospacer adjacent motif (PAM) sequence in the target are crucial for Cas9-mediated cleavage.

The use of the crRNA and the tracrRNA are the two key elements which we shall discuss in this process. Also the Type II CRISPR-Cas system is the one which has received the most attention.

Cas9 proteins are abundant across the bacterial kingdom, but vary widely in both sequence and size. All known Cas9 enzymes contain an HNH domain that cleaves the DNA strand complementary to the guide RNA sequence (target strand), and a RuvC nuclease domain required for cleaving the noncomplementary strand (nontarget strand), yielding double-strand DNA breaks (DSBs).

These DSB open up the DNA at a desired location. Thus if one has a specific gene to be spliced out, and to be replaced, the first step is to open the DNA at the site of that desired gene. Thus the above step is a critical first step.

In addition, Cas9 enzymes contain a highly conserved arginine-rich (Arg-rich) region previously suggested to mediate nucleic acid binding. On the basis of CRISPR-Cas locus architecture and protein sequence phylogeny, Cas9 genes cluster into three subfamilies: types II-A, II-B, and II-C. Cas9 proteins found in II-A and II-C subfamilies typically contain ~1400 and ~1100 amino acids, respectively.

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 . When directed to target loci in

[1] Recall that a palindrome is a collection of letters which can be read the same forwards of backwards. For example; GCATTACG.

*eukaryotes by either dual **crRNA:tracrRNA** guides or **chimeric single-guide RNAs**, Cas9 generates site-specific DSBs that are repaired either by nonhomologous end joining or by homologous recombination, which can be exploited to modify genomic sequences in the vicinity of the Cas9-generated DSBs.*

The opened DNA then can be targeted by crRNA:tracrRNA segments that remove and replace the targeted DNA or by a chimeric single-guide RNA which accomplishes this all in one step. This is the second step in CRISPR gene targeting and re-engineering. We shall discuss this a bit more later.

Furthermore, catalytically inactive Cas9 alone or fused to transcriptional activation or repression domains can be used to control transcription at sites defined by guide RNAs. Both type II-A and type II-C Cas9 proteins have been used in eukaryotic genome editing. Smaller Cas9 proteins, encoded by more compact genes, are potentially advantageous for cellular delivery using vectors that have limited size such as adeno-associated virus and lentivirus.

CRISPR, those collections of small sets of palindromic DNA inserted in the hosts original DNA, can be collectively called a process that is naturally occurring in nature and it is also a procedure that can then be implemented across a wide selection of cell types. In a sense it has been called the lower organism's immune system, a means of remembering previous attackers to the organisms such as bacteria, and a way to use that memory as a defense mechanism against future attacks. The mechanism can then be used in higher level organisms as a reverse process, a means of attacking bad genes and then inactivating them. It is in effect a trick to take what lower organisms have developed for protection and employ in higher level organisms for therapeutic purposes.

In a recent paper by Villion and Moineau the authors examine the two sides of CRISPR, the side that adds segments of foreign DNA to enable an immune type system and the side that deletes selected DNA.

To cope with this never-ending threat, microorganisms have developed a wide range of defense mechanisms.

Among them, CRISPR-Cas system is the new kid on the block as its silencing role was reported only five years ago. An outburst of articles, meetings, and reviews has since followed, arguably making it one of the hottest topics in microbiology.

CRISPR (clustered regulatory interspaced short palindromic repeats) loci are found in approximately 45% of sequenced bacterial genomes as well as 90% of archaeal ones and one genome can contain multiple CRISPR loci. Variable short regions, called spacers, separate each of the short repeats. The spacers are mainly homologous to viral or plasmid sequences. CRISPR-associated (cas) genes are often located adjacent to the CRISPR locus. The diversity and specificity of the cas operons has led to the identification of signature cas genes and to a polythetic classification scheme for CRISPR-Cas systems (types I to III, with several subtypes).

Notwithstanding their particularities, CRISPR-Cas systems operate through three general steps to provide immunity. In the adaptation stage, some cells will respond to the invasion of a phage or a plasmid by adding a new repeat-spacer unit into the CRISPR array, mostly polarized at the 5′ end. Strikingly, the spacer sequence comes from the invading nucleic acid while the newly added repeat derives from another repeat of the array.

The mechanistic details on how this adaptation/immunization occurs are still unknown but some Cas proteins are involved. The unique spacer content is now considered a sign of past challenges and can serve as a marker for strain typing. In the second step, small non-coding CRISPR RNAs (crRNAs) are generated. A long precursor CRISPR RNA is first produced from an AT-rich leader/promoter region, which is then processed within the repeats and mature into crRNAs.

Several Cas proteins participate into the biogenesis of crRNAs. Finally, in the interference stage, the crRNACas protein complex will bind to the invading nucleic acid target and cleave it, providing a defense system to the host microbe. Therefore, CRISPRCas systems are RNA-based adaptive microbial immune systems that target nucleic acid intruders.

They end with the following, the double edged sword portion:

Although already outstanding in bridging gaps in our understanding of CRISPR-Cas systems, this fascinating story does not end here. The authors investigated the possibility of using this dual-RNA system to program Cas9 to specifically cleave any desired DNA molecules. Minimal requirements to have an efficient single chimeric RNA molecule mimicking the dual RNA structure were defined and led to site specific DNA cleavage by Cas9. In fact, several different chimeric guide RNAs were engineered and used to cleave a plasmid containing the specific target and a PAM. These findings coupled to the previous observations that CRISPRCas systems can be functionally transferred from one organism to another open up exciting possibilities for gene targeting and genome editing of microbes and even higher organisms.

That is they have developed a way to reverse the process, using the mechanism now, not to add a piece of DNA, but to cleave a piece of DNA. This opens the door for many types of treatment of cancers where we may know the genetic defect and then can cut it out, cell by cell.

We examine briefly herein some of the recently discovered uses of CRISPR technology to address cancers of various types. The CRISPR approach is another tool in the toolbox of biologists which can become a means for medical application.

As Cain and Boinett state:

The CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR associated proteins) adaptive immune system is widespread in bacteria and archaea and provides heritable protection against disruptive mobile genetic elements (MGEs), such as bacteriophages and plasmids. CRISPR loci contain a series of repetitive DNA motifs separated by spacer sequences; these spacers are derived from MGEs and incorporated after exposure to each new foreign element. The CRISPR transcript is processed into small CRISPR RNAs, which are displayed on Cas protein complexes, enabling RNA-guided degradation of the foreign DNA

by Cas nucleases…. The flexibility and specificity of genome editing using CRISPR loci enables the efficient generation of mutated genotypes in diverse species. Furthermore, as CRISPR loci show strain-specific conservation at the nucleotide level, they are proving to be valuable markers for typing studies and, in conjunction with whole-genome sequencing, can provide insights into the phylogenetic relationships between different bacteria.

As reported in The Independent[2]:

The Crispr process was first identified as a natural immune defence used by bacteria against invading viruses. Last year, however, scientists led by Jennifer Doudna at the University of California, Berkeley, published a seminal study showing that Crispr can be used to target any region of a genome with extreme precision with the aid of a DNA-cutting enzyme called CAS9.

Since then, several teams of scientists showed that the Crispr-CAS9 system used by Professor Doudna could be adapted to work on a range of life forms, from plants and nematode worms to fruit flies and laboratory mice.

Earlier this year, several teams of scientists demonstrated that it can also be used accurately to engineer the DNA of mouse embryos and even human stem cells grown in culture. Geneticists were astounded by how easy, accurate and effective it is at altering the genetic code of any life form – and they immediately realized the therapeutic potential for medicine.

"The efficiency and ease of use is completely unprecedented. I'm jumping out of my skin with excitement," said George Church, a geneticist at Harvard University who led one of the teams that used Crispr to edit the human genome for the first time.

"The new technology should permit alterations of serious genetic disorders. This could be done, in principle, at any stage of development from sperm and egg cells and IVF embryos up to the irreversible stages of the disease," Professor Church said.

David Adams, a DNA scientist at the Wellcome Trust Sanger Institute in Cambridge, said that the technique has the potential to transform the way scientists are able to manipulate the genes of all living organisms, especially patients with inherited diseases, cancer or lifelong HIV infection.

"This is the first time we've been able to edit the genome efficiently and precisely and at a scale that means individual patient mutations can be corrected," Dr Adams said.

"There have been other technologies for editing the genome but they all leave a 'scar' behind or foreign DNA in the genome. This leaves no scars behind and you can change the individual nucleotides of DNA – the 'letters' of the genetic textbook – without any other unwanted changes," he said.

[2] http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html

The essence of the above is twofold. First it is the use of CRISPR as a mechanism in prokaryotes and possibly in eukaryotes. The second is an important observation that we now have another tool for the genetic engineering tool box. The observation that in genetic engineering that many of the "tools" are artifacts of nature should not be overlooked.

2 WHAT IS A CRISPR?

We will now examine in more detail what a CRISPR is and how it functions. Let us begin by examining it in a bit more detail. As Randow et al state:

In archaea and bacteria, for example, even adaptive forms of resistance—long considered the hallmark of vertebrates—contribute to cell autonomous immunity, as exemplified by the clustered regularly interspaced short palindromic repeats (CRISPR) system, which recognizes foreign DNA in a sequence-specific manner. In metazoans, cellular self-defense synergizes with the whole-body protection provided by traditional immunity to confer pathogen resistance. Here, professional immune cells patrol their environment in search of pathogens, whereas cell-autonomous immunity guards both individual immune and non-immune cells against the immediate threat of infection.

Cellular self-defense thus has the potential to confer antimicrobial protection on most, if not all, cells….In bacteria, foreign DNA is sensed and destroyed by the CRISPR system and restriction endonucleases. Because recognition motifs for most restriction endonucleases occur frequently in the host's own genome, these enzymes are paired with matching methyltransferases, which modify host DNA to demarcate it as "self." In eukaryotic cells, rather than being modified, DNA is largely sequestered inside the nucleus, which fosters the detection of foreign DNA in other compartments and allows the deployment of enzymes that mutate and/or degrade DNA without risk to the host genome.

Thus as noted above, the original understanding was as a bacterial self-defense system. Now as Horvath and Barrangou state also concerning the original understanding:

Microbes have devised various strategies that allow them to survive exposure to foreign genetic elements. Although out-populated and preyed upon by abundant and ubiquitous viruses, microbes routinely survive, persist, and occasionally thrive in hostile and competitive environments. The constant exposure to exogenous DNA via transduction, conjugation, and transformation have forced microbes to establish an array of defense mechanisms that allow the cell to recognize and distinguish incoming "foreign" DNA, from "self " DNA and to survive exposure to invasive elements. These systems maintain genetic integrity, yet occasionally allow exogenous DNA uptake and conservation of genetic material advantageous for adaptation to the environment.

Certain strategies, such as prevention of adsorption, blocking of injection, and abortive infection, are effective against viruses; other defense systems specifically target invading nucleic acid, such as the restriction-modification system (R-M) and the use of sugar-nonspecific nucleases. Recently, an adaptive microbial immune system, clustered regularly interspaced short palindromic repeats (CRISPR) has been identified that provides acquired immunity against viruses and plasmids.

They also state:

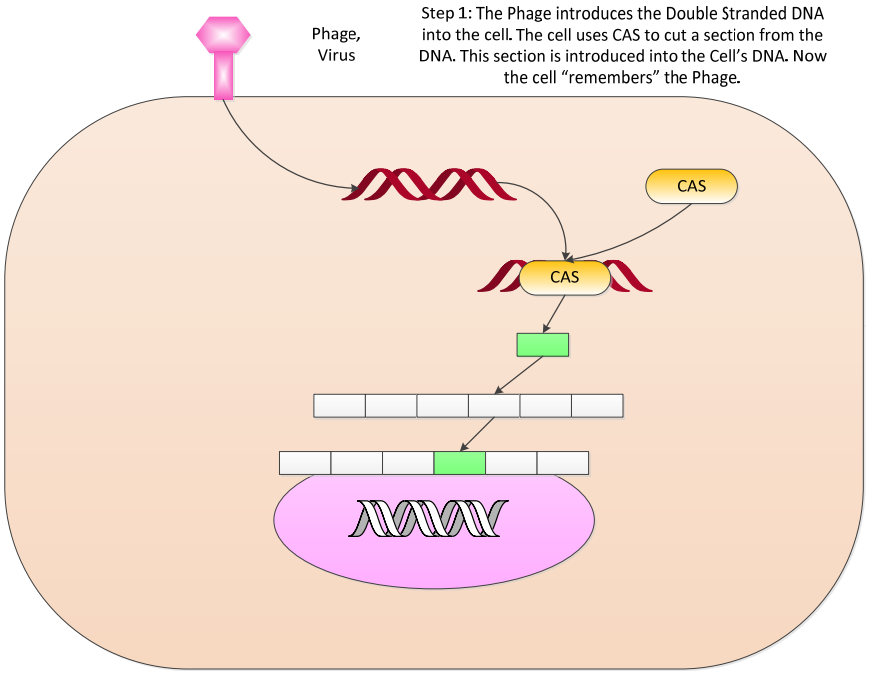
Microbes rely on diverse defense mechanisms that allow them to withstand viral predation and exposure to invading nucleic acid. In many Bacteria and most Archaea, clustered regularly interspaced short palindromic repeats (CRISPR) form peculiar genetic loci, which provide acquired immunity against viruses and plasmids by targeting nucleic acid in a sequence-specific manner.

These hypervariable loci take up genetic material from invasive elements and build up inheritable DNA-encoded immunity over time. Conversely, viruses have devised mutational escape strategies that allow them to circumvent the CRISPR/Cas system, albeit at a cost. CRISPR features may be exploited for typing purposes, epidemiological studies, host-virus ecological surveys, building specific immunity against undesirable genetic elements, and enhancing viral resistance in domesticated microbes.

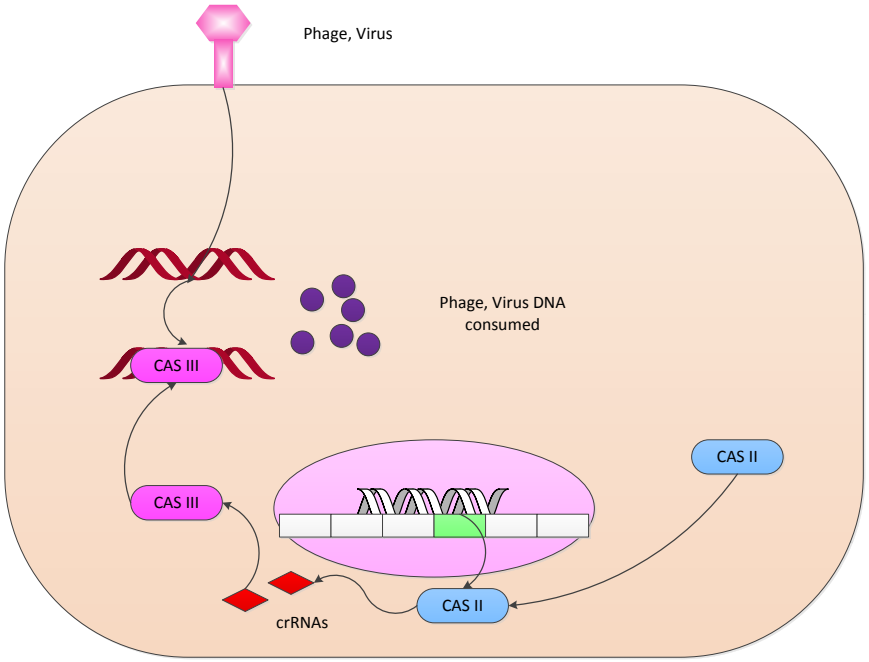
Thus we first examine how CRISPR-Cas functions in its primal environment and then we take this to human environments where we can use it as an added tool in our genetic engineering toolkit.

2.1 CRISPR DYNAMICS

We now examine some of the dynamics of the CRISPR system. We start with the use of CRISPR in a bacterial cell. We assume the cell is attacked by some viral phage and the phage sends its RNA/DNA into the cell in anticipation of replication within the host. Now from Horvath and Barrangou (as modified) we have the following description for this initial portion of the process as shown below:



The Cas protein recognizes the invading DNA and transports a portion of it to the nuclear DNA and inserts it into the cells DNA. How specifically Cas does this task is not yet well understood. The when another phage with the same or frankly similar DNA invades again, then Cas II is activated and the section of the DNA activates a Cas II which then consumes the invading DNA.



Now the above process is a natural part of the day to day activities of bacteria. But it also is a paradigm for deal with eukaryotic cells, namely cutting and pasting genes into cells.

2.2 TYPES OF CRISPR

From Jinek et al, they discuss the three types of CRISPR systems:

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 pre-crRNAs 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.

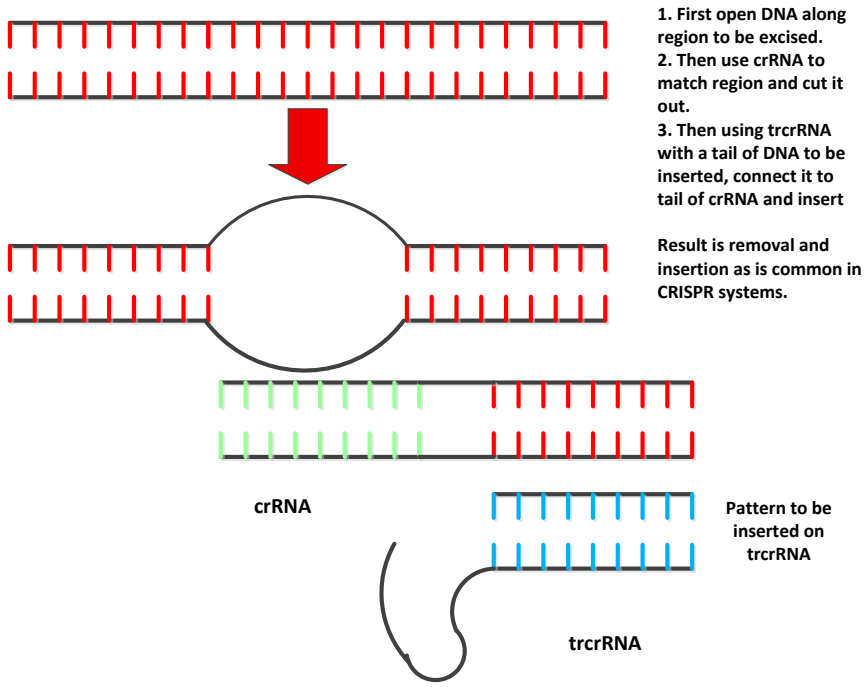
…in type II systems, Cas9 proteins constitute a family of enzymes that require a base-paired structure formed between the activating tracrRNA and the targeting crRNA to cleave target dsDNA.

Site-specific cleavage occurs at locations determined by both base-pairing complementarity between the crRNA and the target protospacer DNA and a short motif [referred to as the protospacer adjacent motif (PAM)] juxtaposed to the complementary region in the target DNA.

Thus the heart of the CRISPR engineering tool is the Type II system and specifically the Cas9 proteins used in the placement and extraction. In addition we also have just introduced above the crRNA and the tracrRNA which are used as part of the cleavage process. The crRNA provides a site specific cleavage targeting whereas the tracrRNA allows for insertion.

2.3 CRISPR INSERTION

We now examine briefly the Jinek et al approach to a cut and paste using CRISPR. This is a Type II system and it allows for the removal of a targeted section of DNA and the replacement with another crafted piece. We show the overall construct below. This is a simplified version of Jinek et al.



We use the above as a very simplified example of how we can insert specific DNA in an excised targeted region.

3 CRISPR AND CANCER

Now one would think that perhaps this could become a therapeutic as applied to various cancers. Consider it use as a kinase inhibitor in CML. Would it work there by targeting the aberrant kinases? What of an application in melanoma with a BRAF V400 mutation? Can we cut and paste back the proper genetic sequence? If so, how do we deliver the elements of the process, especially in a metastatic case? Furthermore, how do we determine what genes must be modified, and does that mean that we not only customize it for a patients but also for cells? Finally how do we know that there are not some deleterious sequellae from this cutting and pasting process, what if we "miss" the gene in some cell and start an secondary malignancy?

These are all reasonable questions that lead us to examine the CRISPR process in further detail

As recently stated by Stephen et al:

We are also optimistic that completely different approaches to treating cancer will contribute to eliminating Ras cancers, including new ways of knocking down/out genes using RNAi and CRISPR technologies and delivering these payloads to tumors, as well as new ways of deploying the immune system.

In this respect, it is noteworthy that anti-CTLA-4 therapy appears to be equally effective in treating melanoma driven by N-Ras or B-Raf; therefore, Ras cancers may not be excluded from these approaches as they have been from others. All of these considerations lead us to be optimistic about future prospects of finally delivering the knockout punch.

As Way et al state:

Synthetic biology is a young discipline with the declared goal of rationally engineering biological systems through approaches similar to those used by engineers to build bridges and send people to the moon. This field has rapidly developed over the past 15 years from its initial conceptualization by a few academics and government program managers into a sizeable field whose meetings attract large numbers of participants. Recently, new tools have emerged that should allow specific integration at desired sites in the genome. For example, methods based on zinc-finger, TALE, and CRISPR fusions to nucleases can be used to generate double-strand breaks at specific sites in the genome. The questions remain—where should we integrate, and how can we avoid effects of adjacent sequences?

4 CRISPR AND CANCER TREATMENT

Thus one may ask if one knows that some gene has been the cause of a cancer, can we then treat the cells with a CRISPR-Cas system to delete the gene and replace it with a normal wild type. If we have a procedure to do this then perhaps this is a therapeutic approach. It does, of course beg the question of how this is accomplished even if we have the chimeric Cas delivery system. We also must ask if we have identified all the genes. There are also many other such questions. Yet this has become a focal point of interest.

In a recent paper by Yin et al the authors discuss the delivery of a CRISPR-Cas9 mediated cutting and reintroduction of a gene into liver cells by means of an injection process. The result was conversion of the errant gene cells into normal wild type cells. They utilized the backwards flow of the CRISPER-Cas9 approach for cutting and injection. This potentially paves the way for substantial progress in alternative targeted gene replacement and return to normal states. As Yin et al state:

We demonstrate CRISPR-Cas9–mediated correction of a Fah mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia. Delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells. Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype. Our study indicates that CRISPR-Cas9–mediated genome editing is possible in adult animals and has potential for correction of human genetic diseases.

From Gene News[3] we have a more detailed discussion worthy of note regarding the above recent report:

MIT scientists report the use of a CRISPR methodology to cure mice of a rare liver disorder caused by a single genetic mutation. They say their study … offers the first evidence that this gene-editing technique can reverse disease symptoms in living animals. CRISPR, which provides a way to snip out mutated DNA and replace it with the correct sequence, holds potential for treating many genetic disorders, according to the research team.

"What's exciting about this approach is that we can actually correct a defective gene in a living adult animal," says Daniel Anderson, Ph.D., the Samuel A. Goldblith associate professor of chemical engineering at MIT, a member of the Koch Institute for Integrative Cancer Research, and the senior author of the paper.

The recently developed CRISPR system relies on cellular machinery that bacteria use to defend themselves from viral infection. Researchers have copied this cellular system to create gene-editing complexes that include a DNA-cutting enzyme called Cas9 bound to a short RNA guide strand that is programmed to bind to a specific genome sequence, telling Cas9 where to make its cut.

[3] http://www.genengnews.com/gen-news-highlights/crispr-reverses-disease-symptoms-in-living-animals-for-first-time/81249682/

At the same time, the researchers also deliver a DNA template strand. When the cell repairs the damage produced by Cas9, it copies from the template, introducing new genetic material into the genome. Scientists envision that this kind of genome editing could one day help treat diseases such as hemophilia, Huntington's disease, and others that are caused by single mutations.

For this study, the researchers designed three guide RNA strands that target different DNA sequences near the mutation that causes type I tyrosinemia, in a gene that codes for an enzyme called FAH. Patients with this disease, which affects about 1 in 100,000 people, cannot break down the amino acid tyrosine, which accumulates and can lead to liver failure. Current treatments include a low-protein diet and a drug called NTCB, which disrupts tyrosine production.

In experiments with adult mice carrying the mutated form of the FAH enzyme, the researchers delivered RNA guide strands along with the gene for Cas9 and a 199-nucleotide DNA template that includes the correct sequence of the mutated FAH gene.

"Delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells," wrote the investigators. "Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype."

While the team used a high pressure injection to deliver the CRISPR components, Dr. Anderson envisions that better delivery approaches are possible. His lab is now working on methods that may be safer and more efficient, including targeted nanoparticles.

The above described an interesting I vivo approach to the editing and insertion of a specific gene in a specific location. Although this is of interest, it is limited to a very specific site and also using a difficult delivery mechanism.

As to more extensive editing capabilities we examine Zhang et al who state:

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and multiplexable genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases. CRISPR/Cas9 comprises of a non-specific Cas9 nuclease and a set of programmable sequence-specific CRISPR RNA (crRNA), which can guide Cas9 to cleave DNA and generate double-strand breaks at target sites. Subsequent cellular DNA repair process leads to desired insertions, deletions or substitutions at target sites.

The specificity of CRISPR/Cas9-mediated DNA cleavage requires target sequences matching crRNA and a protospacer adjacent motif (PAM) locating at downstream of target sequences. Here, we review the molecular mechanism, applications and challenges of CRISPR/Cas9-mediated genome editing and clinical therapeutic potential of CRISPR/Cas9 in future.

The above stresses the strong point of CRISPR-Cas9, namely its specificity. It can target specific DNA, assuming we know what to target. It can then replace that with a substitute, assuming we

know that the substitute does no harm and in fact is positively therapeutic. From Pandika we have[4] the following lengthy discussion regarding the evolution of this specific result:

Because a Nobel Prize winner says this breakthough is better than his breakthrough. Jennifer Doudna has always had an explorer's spirit. It's what led the UC Berkeley molecular and cell biology professor to engineer a cheaper, easier way to correct DNA defects. Her game-changing technology takes a mysterious bacterial genetic code and transforms it into a powerful tool for cutting and pasting bits of genetic material – meaning not only could the entire field of gene therapy be revived, but her genome-editing tool could one day be used to treat a range of diseases, from cancer and AIDS to hereditary disorders like Down syndrome and Huntington disease.

Every time we see some new tool for the toolkit the immediate tendency is to label it as a cure fr cancer.

Most scientists weren't even aware of these so-called CRISPRs, much less their function. But Doudna suspected they hid a crucial purpose….The bacterial enzyme Cas9 is the engine of RNA-programmed genome engineering in human cells. Doudna unearthed the first clue when she found that a protein called Cas9 acts like a pair of molecular scissors…

"I wasn't actively trying to go in any particular direction," she said. That willingness to wander, to maybe even get a little lost, could be how she was able to make a creative break from earlier genome-editing technologies. Doudna "certainly didn't set out to discover a genome editing tool by any stretch of the imagination." It all began with a puzzle she couldn't resist solving, thanks largely to her father. When Doudna was growing up, the literature professor got her hooked on one of his favorite pastimes —decoding short pieces of encrypted text, or cryptograms.

In 2005, a colleague presented Doudna with a genetic cryptogram — weird repetitive RNA sequences tucked in the genomes of many of the bacteria she studied. Most scientists weren't even aware of these so-called CRISPRs, much less their function. But Doudna suspected they hid a crucial purpose.

Sure enough, scientists discovered that CRISPRs played an important role in immunity: they recognize the DNA of viral invaders for the bacteria to chop up and fight off. But how did this search-and-destroy mechanism work? Teaming up with Umea University molecular biologist Emmanuelle Charpentier, Doudna unearthed the first clue when she found that a protein called Cas9 acts like a pair of molecular scissors. A CRISPR RNA fragment hooks up with Cas9 to precisely target the DNA of an invading virus, which it then cuts and destroys.

Here's where it gets really complicated. Martin Jinek, a postdoctoral researcher in Doudna's lab, found that Cas9 in bacteria needs two RNA guide strands – this sent the gears in their heads turning. What if they could engineer the system to require only a single, programmable RNA strand? Then biologists could use it to easily target and cut any DNA sequence. Doudna felt "a chill of excitement." Maybe they could link the two RNA strands into one, and loop it in on

[4] http://www.ozy.com/rising-stars-and-provocateurs/jennifer-doudna/4690.article

itself—mimicking a double-stranded structure. Those chills were warranted: Doudna's lab and other groups successfully used this simplified CRISPR system to modify genes in bacteria, plant and animal cells.

One early form of CRISPR-based gene therapy could involve editing the genes responsible for blood disorders like sickle-cell anemia in bone marrow cells, growing them into mature blood cells and injecting them back into patients.

However, the application needs a more effective insertion system. It also needs to demonstrate that it does not wander and affect other genes.

Little more than a year after Doudna first described CRISPR in the journal Science, the cut-and-paste technology has yielded promising results in labs around the world. Last month, researchers from the Netherland's Utrecht institute reported in Cell Stem Cell that CRISPR corrected the gene mutation responsible for cystic fibrosis in stem cells developed from two children with the life-threatening disease. Doudna believes a clinical trial of CRISPR-based gene therapy could begin in less than a decade.

As is all too often the case, any prediction of clinical application may be much too speculative. Single gene targeting may become the first step, albeit even there one must be cautious.

Doudna experienced "many frustrations" getting CRISPR to work in human cells. But she knew if she succeeded, CRISPR would be "a profound discovery" — and maybe even a powerful gene therapy technique.

We knew if the system could be made to work in human cells, it would be a really profound discovery.

"I hope you're sitting down," an excited colleague told Doudna in an unexpected phone call. "CRISPR is turning out to be absolutely spectacular in [Harvard geneticist] George Church's hands." He had even gotten it to work in human cells. Thrilled, Doudna immediately contacted Church. They shared their results, and both published studies in January 2013 showing that CRISPR can cut, delete and replace genes in human cells. University of Massachusetts biologist Craig Mello, who shared the 2006 Nobel Prize for another genome editing tool, hails Doudna's CRISPR technique as a "tremendous breakthrough," even admitting that "in many ways it's better" than his own technique.

Other techniques can also edit genes at specific DNA regions. But they require scientists to engineer a separate protein for each target site. In contrast, CRISPR only needs the Cas9 protein, allowing it to correct multiple defects at once. Besides being cheaper and easier to use, CRISPR is also much more precise, reducing the risk of off-target modifications introducing dangerous mutations. As a result, it could help revive the gene therapy field, whose early clinical failures — including patient deaths — led some to dismiss it as overhyped.

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene. Understanding

and exploiting how Cas9 avoids these close matches "is an active area of investigation," Doudna said. Still, CRISPR is "a real game-changer," Mello told the Independent. "It's incredibly powerful."[5]

Indeed the observations above detail some of the powers of CRISPR-Cas9 complex.

[5] http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html

5 OBSERVATIONS

The CRISPR-Cas9 system has proven to be a workable in vivo editing mechanism for specific gene cut and paste situations. However there are several key questions that seem to hang over it. None are so severe as to cause substantial concern but in toto they clearly indicate potential but substantial work is still required, especially in the area of cancer therapeutics.

We thus present and discuss several such questions:

1. Can the CRISPR-Cas9 system target the correct sets of aberrant cancer genes?

The issue here is that in many cancers we have a multiple set of genes which are aberrant. To make it even more complex, there are cancer cells with different mixtures of mutated or inoperable genes. How thus does one target this broad and varying complex. A single genetic mutation is one thing an broad complex set of changes is another.

2. Can CRISPER-Cas9 system be delivered in vivo in a more effective manner?

The current delivery mechanism is targeted at specific cells in a specific location. What does one do with a metastatic cancer. Oftentimes you do not even know where the cells may be. Then again one also faces the issue of the stem cell and it special characteristic. This delivery most likely be difficult.

3. Is CRISPR-Cas9 a dose related system approach rather than an all-encompassing curative approach? Namely does it cut-and-paste a large set of genes but perhaps not all?

Is the delivery system akin to dosing in normal pharmacokinetics or is it a totally different mechanism.

4. How does CRISPR-Cas deal with metastatic cells wherein there are multiple sets of genetic alterations?

The multiplicity of gene breakdowns and the process in which this happens becomes a complex driver for applying this technology. Is there a single key to solving the problem or must one continue to track changes and chase the shadows of the genetic changes?

5. What are the potential deleterious sequellae possible from a CRISPR approach and how can they best be avoided?

The ultimate question will be what else can this process do. The unintended consequences may be significant. As was noted above:

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene.

What then are those mistakes which can occur, especially when targeting multiple genes?

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