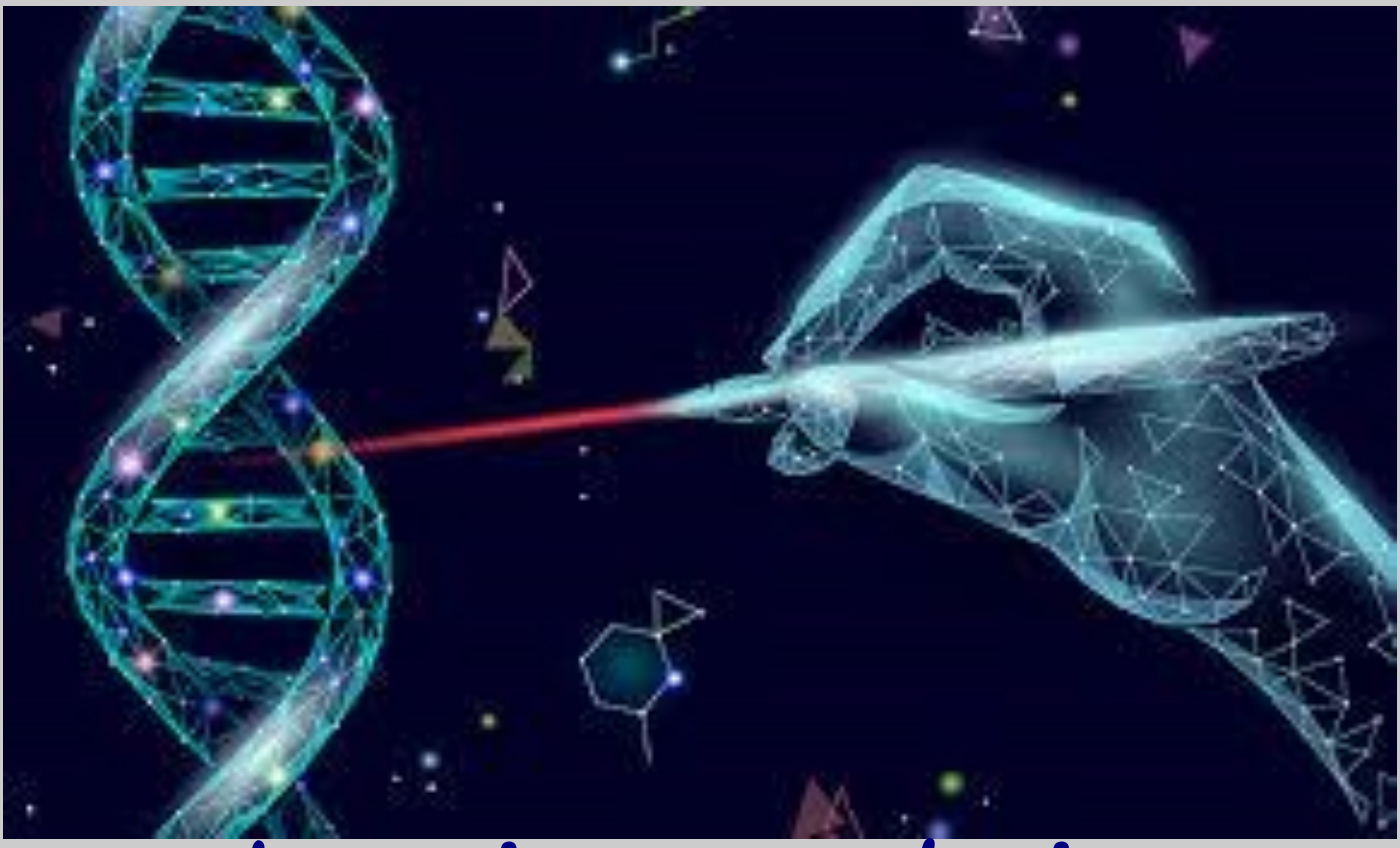
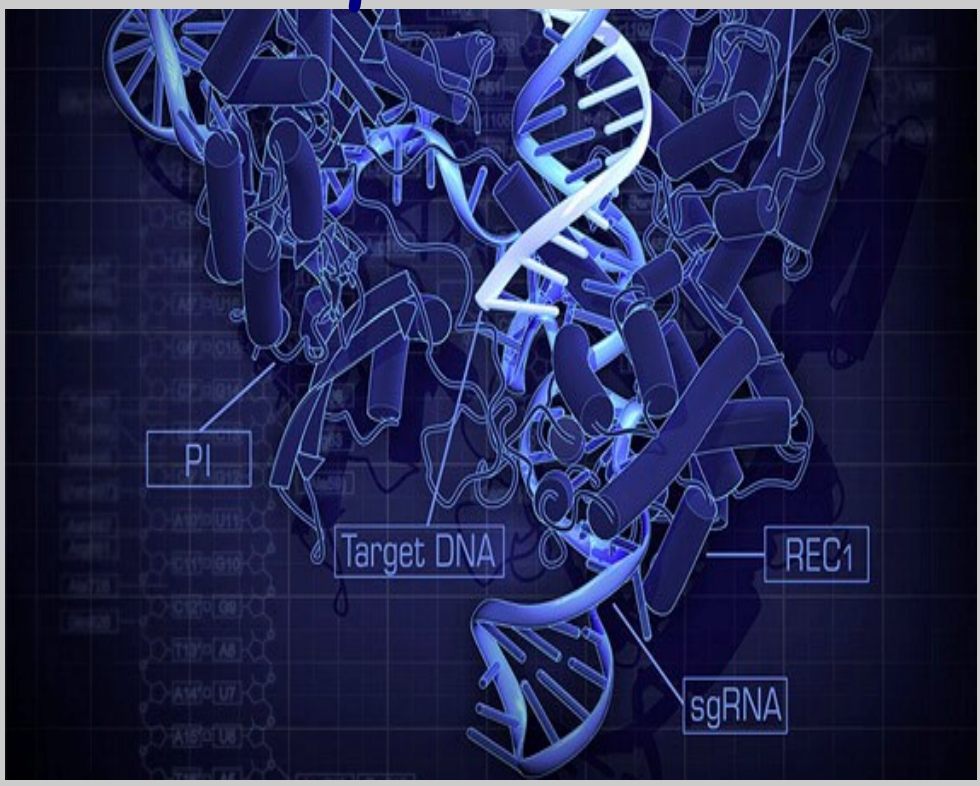
Kuwait Pharmacy Bulletin

DRUG INFORMATION FOR THE HEALTH PROFESSIONAL



the crispr revolution



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CRISPR- a new gene editing system

Clustered regularly interspaced short palindromic repeats (CRISPR) is a novel technology that promises to revolutionize gene editing. It was recognised initially in bacteria and archaea as a form of 'immune system' that protects these organisms from viruses. It works by incorporating sequences of the invading viral DNA into the prokaryotic genome to serve as a memory for subsequent attacks. This process has been adapted as a means of manipulating gene transfer in higher organisms to genetically edit their DNA using simple delivery mechanisms and reasonably few components, allowing the use of this technology therapeutically. This article presents an overview of this system as an adaptive immune response in prokaryotes and describes the different types of CRISPR/Cas systems and their adaptation for application in eukaryotes and compares this technique with other gene editing methods such as zinc finger nucleases (ZNF) and transcription activator-like effector nucleases (TALENs), showcasing examples of potential applications in human diseases including thalassemia, sickle cell anemia and hypercholesterolemia, along with genetically engineering malaria-carrying mosquitos and the ethical issues with implementing a process termed "gene drive".

I- What is CRISPR;how does it work?

Humans are equipped with protective immune defense systems, comprising many components, against pathogens. This system is categorized into an innate and an adaptive immune system, which usually work in succession. The first provides an immediate but non-specific response to pathogen infection. The second includes humoral and cell mediated immunity, which is conferred by various cells present in the circulation. The latter involves T killer cells, macrophages, and phagocytic cells that engulf pathogens or microorganisms. The former involves B cells that generate specific antibodies that remain circulating in the host. These antibodies form an "immunological memory", serving to protect against subsequent attacks on the host cells by same invading pathogen.

Clustered regularly interspaced short palindromic repeats, or CRISPR, is an acronym used to describe a process that constitutes *'a kind of adaptive immune/ protective system'* found in some prokaryotes, and approximately 40% of all bacteria and 90% of all archaea (Brodt, et al, 2011). Unlike the complex immune systems of eukaryotes, these single-celled organisms protect themselves from invading viruses by expanding their genome within the cell itself. This is primarily done by the CRISPR loci with the help of several helicases and nucleases within the family of proteins referred to as Cas proteins.

The CRISPR/Cas system depends on the CRISPR locus located in the chromosomal or plasmid DNA; it contains repeats and spacers placed alternatively in a regular manner. Both are short in length, as each spacer is about 26 - 72 bp, while each repeat is between 21 - 48 bp. The repeats are palindromic, meaning they are the same read in either direction, and they separate spacers from one another. The total number of spacers can vary between different CRISPR loci, ranging from a few spacers to several thousands, occupying a significant space within the genome (Jiang & Doudna, 2015). These non-repetitive spacers are acquired from the invading viral genome that previously infected the cell, which are cut by Cas proteins, mainly Cas1 and Cas2. These specific viral genome segments are recognized by their PAM sequence.

The PAM sequence, or the proto-spacer adjacent motif, is a crucial 3 bp site neighboring the viral genome sequence that will be eventually copied into the CRISPR locus, that has to be recognized by the Cas proteins in order for these proteins to identify the sequence that should be cleaved from the viral non-self-genome. Its main role is to protect the bacterial CRISPR locus from being cleaved by the same Cas proteins as it does not exist in the bacterial self-genome. However, self-targeting still occurs with the PAM recognition more than once out of 500 events, which usually end up in death for the bacterial host cell. (Gasiunas et al., 2014).

Once the PAM sequence is recognized and the viral genome is cleaved appropriately by Cas enzymes into stretches of protospacer bases, it is then placed at an area rich in A-T base pairs located at the start of the CRISPR locus; called the leader sequence. This event allows the visualization of the order in which spacers were acquired, showing a historical "time frame" record of the viral attacks upon one specific bacterium. After the first step of acquiring the viral genome and integrating it into the bacterial genome, known as spacer acquisition. The next

In this issue

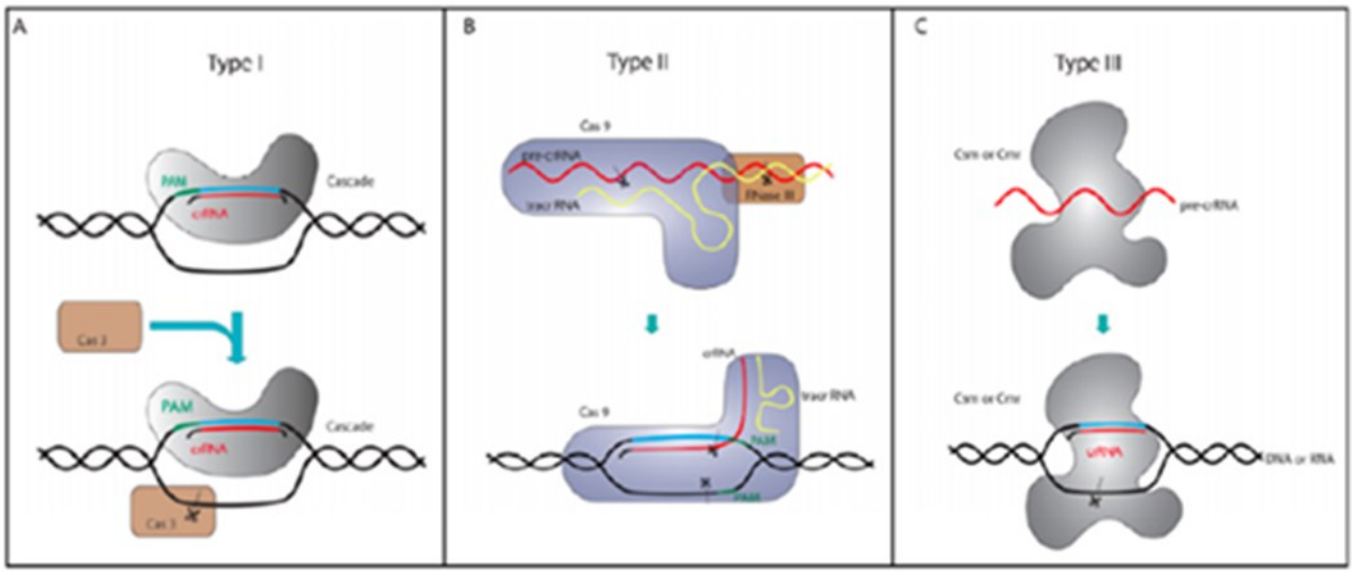

Fig 1. Interference step in the three types of CRISPR/Cas system, PAM, (green) is found in both type 1 and 2 while type 3 depends on strand complementarity.

step of expressing that protospacer sequence occurs upon subsequent viral infection so the CRISPR/Cas system can interfere with the genetic material of the virus and disable the start of its life cycle within the cell. CRISPR/Cas system works by three main steps (Fig 1).

i- Adaption

The first step in the CRISPR/Cas system is spacer acquisition or adaption of a spacer from the invading virus and it is the same in all the CRISPR/Cas systems. First, the virus attaches to the surface of the bacterial cell wall, inserting its genetic material which is in the form of either DNA or RNA. The Cas 1 and Cas2 proteins recognize the 3 bp PAM sequence and therefore cleave the adjacent viral sequence. This sequence is then placed into a spacer within the CRISPR locus to serve as memory. Cas 1 is considered a nuclease and an integrase that typically copies DNA, while Cas 2 is an endoribonuclease that copies RNA.

ii- Expression

The second step forms the CRISPR RNA, or "crRNA processing", which includes the viral genome and the CRISPR associated genes, both called the CRISPR locus. This happens upon subsequent attacks of the same virus. It involves copying the lower strand of the CRISPR locus, which was made in the adaption step, into crRNA.

iii- Interference

The third step in this adaptive immune response is disabling the virus by interfering with its life cycle. The mature crRNA is complexed with the appropriate Cas protein, which in turn targets and degrades the target viral genome upon subsequent attacks.

The last two steps differ in each CRISPR/Cas system type. The three types will be explained briefly with the focus on type 2, which is the most relevant in terms of applied application of CRISPR.

II- Types of CRISPR/Cas systems

i- Type1 CRISPR system

This is found in bacteria and archaea. It consists of six subtypes (IA-IF). The main player is Cas3 protein, which is a helicase to unwind the dsDNA helix and DNase that cleaves the formed ssDNA target.

The mature transcribed crRNA binds to a Cascade protein, which in turn changes conformation once it binds to the correct base pairs. This change recruits Cas3 to the site that should be degraded.

This system is a multi-protein complex. It also depends on the PAM sequence to distinguish between self and viral genome.

ii- Type2 CRISPR system

This system is found mostly in the bacterial genome; it consists of three subtypes (IIA-IIC). The main component is Cas9 protein, which contains two nuclease domains. However, it needs two RNA strands, the normal crRNA discussed previously, and a trans activating crRNA called tracerRNA. These two strands form the pre-crRNA complex. This complex is then cleaved by RNAse III into the final complex, termed single guided RNA (sgRNA).

This CRISPR system only requires two components. The first one is the result of combining crRNA and tracerRNA into sgRNA. The second component is the cas9 protein that has two loops which are responsible for two functions. The recognition loop recognizes the target sequence; it consists of an alpha helix with a REC1 and REC2 domains. Both of these two domains have an affinity for the repeat anti-repeat duplex on the target and are specific for Cas9, while the nuclease loop induces the double stranded break. This loop consists of three domains, which are RUvC, HNH and the PAM interacting

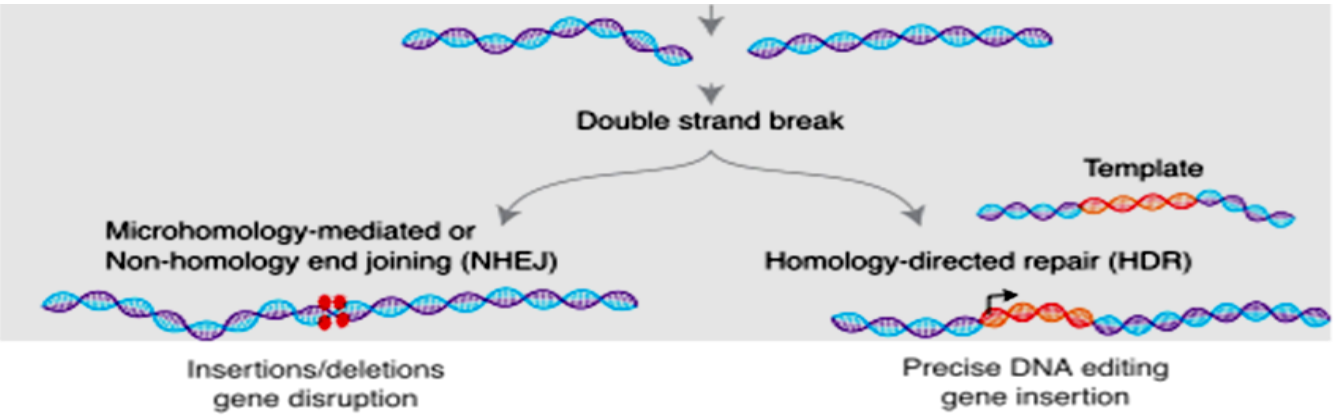

Figure2: DNA repair pathways via HDR and NHEJ pathways

domain or PI domain. The first two mentioned domains are named after similar structures that were found in the organisms from which they were discovered. RUvC is termed after a protein found in E. coli whereas HNH domain is found in T4 phage endonuclease VIII, which identifies and cleaves DNA.

Ultimately, the Cas9-sgRNA complex forms, which in turn recognizes the PAM sequence and attaches to the target forming a ternary complex and starts the process of DNA cleavage.

This system is a single-protein complex, which increases its utility to be further studied for therapeutic use due to its convenience.

iii- Type3 CRISPR system

This is found predominantly in the archaeal genome; it consists of two subtypes (IIIA & IIIB). The main component is Cas10 protein, which is of unknown function. However, it has been proposed that it has similar action to Cas3 as it has nuclease domain capable of target degradation.

This CRISPR system differs in its two subtypes. Type 3A cleaves DNA via crRNA-Csm protein complex formation, while type 3B can cleave RNA via the CMR protein, which is the only type known that can do so. Both subtypes require multiple other proteins for cleavage, many of which are still unknown. The targeting of DNA in this sequence does not require a PAM sequence but depends on the complementarity between the mature crRNA and the target.

III- Methods of dsDNA breaks repair

Harnessing the CRISPR/Cas for DNA editing requires methods to repair the cleaved sequence. There are two possible pathways of repair that can be applied, which also exist in nature: Non-homologous end-joining (NHEJ), and homologous directed repair, or HDR). A third pathway may be used in case NHEJ is inactivated, called alt-NHEJ. However, these two pathways are hard to distinguish from one another as they lead to similar outcomes (Mei et al., 2016).

These two pathways demonstrate competitive activity, as inhibition of NHEJ can improve the frequency of HDR. NHEJ can be produced via two pathways: the main canonical ligase IV-dependent NHEJ (C-NHEJ) and alt-NHEJ. Upon Scr7 treatment, the DNA ligase IV is suppressed, which ultimately inhibits C-NHEJ pathway and increases HDR-mediated gene editing.

The NHEJ pathway repairs DNA at 20-60% efficiency without a homologous sequence, which can lead to insertions, deletions, translocations or telomere fusion (Maruyama et al., 2015) and the loss of nucleotides is not corrected. The HDR pathway works by replicating a homologous template via strand invasion from the 3' side, resulting in "perfect repair". Prokaryotic cells depend mostly on HDR to repair DSBs, which makes their genome manipulation easier and more predictable. Eukaryotic cells have limited HDR and normally utilize the NHEJ pathway instead, which can be a significant hurdle when transitioning this technology to higher organisms. However, the CRISPR/Cas9 system only requires a repair template that is approximately 40-90 nucleotides long single-stranded DNA oligonucleotide (ssODN) after DNA cleavage.

The CRISPR pathway evolved to protect bacteria from viruses and is now being exploited to edit eukaryote genomes, in particular the human genome, for the purposes of both disabling existing genes, and for inserting new genes, or correcting defective ones. This "natural" method of genome editing may have far reaching therapeutic benefits. It also has significant advantages over current methods of genome editing using oligonucleotides, ribozymes or RNA interference technology. Some of these alternative methods are known as Zinc finger nucleases (ZFN) and transcription activator like-effector nucleases (TALENs) (Figure 3) and will be discussed below for comparison.

IV- Alternative methods of editing

i- Zinc finger nucleases

Zinc finger nucleases use a naturally occurring

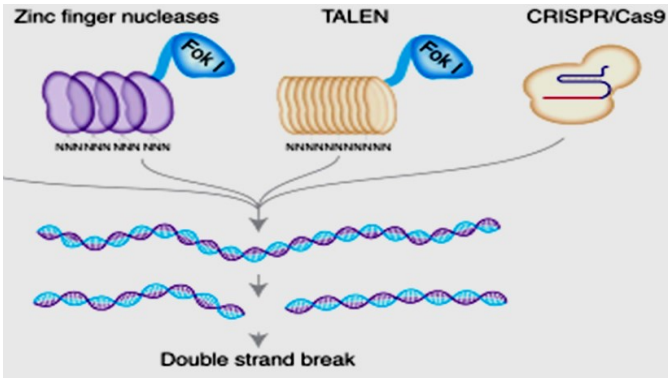

Fig 3. FokI is used in both ZNF and TALEN, however the type of protein ligand binding is different in each method.

restriction enzyme called fok 1. This enzyme was chosen because it functions as a homodimer, as two fok 1 proteins are needed at the same site for cleavage. It can also be linked to small protein motifs with a zinc ion responsible for stabilization of the complex. Each one of These protein ligands that resemble fingers can recognize a 3bp codon. These two properties increase fok 1 specificity. This method uses two halves of the fok 1 enzyme to be bound to two zinc fingers, one on each DNA strands, meaning two zinc fingers are needed for one DSB.

Zinc fingers can be chained as 6-7 proteins, each binding to 3bp codons and targeting 18-21 bp sequence in the DNA. Increasing the number of zinc fingers increases fok 1 specificity. However, adjacent inappropriate recognition may still occur.

ii- Transcription activator-like effector nucleases

TALENS work similarly to ZFN, as both utilize fok 1 enzyme for DNA cleavage. However, TALENS are naturally occurring proteins consisting of 33-35 amino acids; each one recognizes an individual nucleotide instead of a codon, making them easier to design and work with compared to ZFN.

iii- The upper hand of CRISPR/Cas9

The CRISPR/Cas system is considered a big breakthrough compared to the previously mentioned methods. It only requires two components to direct a very specific DSB, which are the sgRNA and Cas enzymes, that does not need further construction. Yet other modifications are developed constantly to adopt this technology in eukaryotic cells which will be discussed below.

V- CRISPR/Cas in eukaryotes

As mentioned previously, the CRISPR/Cas system was discovered in prokaryotes. So, several modifications were needed to transfer this technology to higher organisms such as eukaryotes. One of the main challenges is that eukaryotic cells have nuclei, whereas prokaryotic cells do not. This problem has been overcome by tagging nuclear localizing signal (NLS) sequences to Cas9 and codon-optimized for eukaryotic expression to move it into the mammalian genome successfully.

The components of CRISPR/Cas system can be delivered to living cells via viral or non-viral methods such as electroporation. One of the most common viruses used is adeno-associated virus. It has a non-integrating nature that protects the target DNA from viral genome invasion. It also lacks pathogenicity, so doesn't run risk of cell lysis.

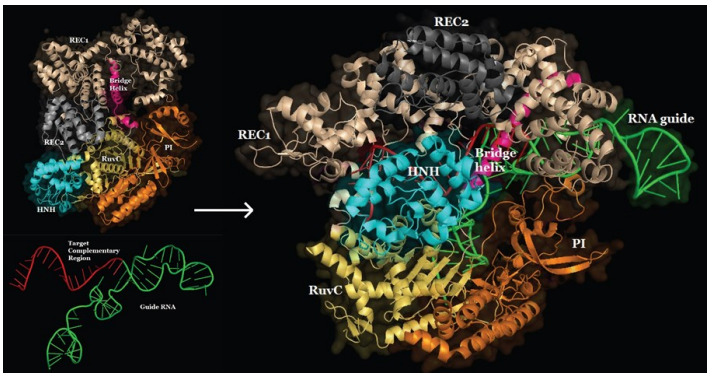

Fig 4. Crystal structure of Cas9 in complex with sgRNA and target DNA. The enzyme has a bi-lobed structure; upper recognition lobe contains REC1 and REC2, and the lower cleavage domain contains RuvC and HNH, which are the main focus of functional modifications in the different versions of Cas9 enzyme.

Functional modifications on the Cas9 enzyme

i- Cas9 nikase (Cas9n)

This enzyme variation consists of abolishing one of the nuclease cleavage domains. For instance, Cas9 with a D10A mutation can inactivate the endonuclease activity of RuvC, whereas a H847A can eliminate the function of HNH. Double nicking is combining Cas9n with two separate sgRNA to produce a staggered double stranded break. This method can activate the DNA repair pathway to increase the efficiency and specificity of the CRIPSR/Cas system.

ii- Dead Cas9 (dCas9)

This enzyme variation is catalytically inactive, meaning the activity of both RuvC and HNH domains are deactivated. It is useful in recognizing the target site and disturbing the transcription of the target genes without modifying the DNA, due to the physical hindrance of Cas9-sgRNA complex binding. This system is capable of two unique functions. First, the dCas9 fusion-mediated inhibition (CRISPRi) by binding to a repressor such as kruppel-associated box (KRAB) that blocks gene expression. Second, the dCas9 fusion-mediated activation (CRISPRa), which works by binding to an activator like VP16 and VP64 to activate gene expression.

iii- Light-activated Cas9

Two sites were discovered to have a role in this enzyme variation: the highly conserved K164, which is located

near sgRNA, indicating that it might play a rule in Cas9 -sgRNA interaction. The other site is K886, which could change the enzyme structure to expose lysine residue during sgRNA binding that is involved in positioning the non-target DNA strand.

This CRISPR/Cas system can be activated or deactivated by light stimulation to provide temporary genome editing. When CIBN (cryotochrome-interacting-basic-helix-loop-helix-1) and CRY2 (light-sensitive-cryptochrome 2) undergo conformational change and heterodimerization upon blue light simulation. The heterodimer recruits Cas9 to the target site and either activates or deactivates gene expression. This is done by binding CRY2 with an activator or a repressor protein, respectively (Polstein & Gersbach, 2015). (Fig 5).

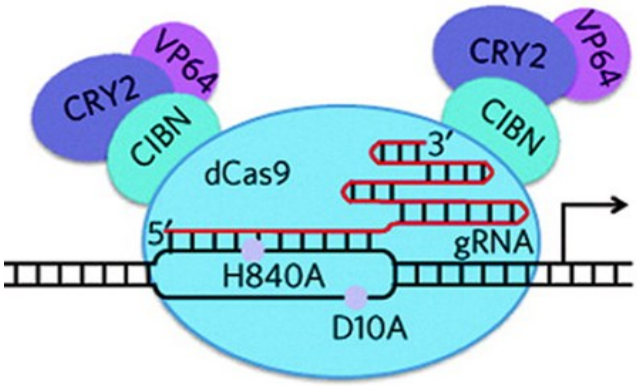

Fig 5. Binding of accessory molecules

VI- Overcoming limitations

The sgRNA contains a 20-nucleotide long sequence that determines the target cleavage site on the DNA. Hence, optimizing this sequence is crucial for sgRNA specificity and efficiency. This can be done by making sgRNA highly matched with the target sequence, as no more than 3 mismatches between sgRNA and the target site can be tolerated as the binding of these two components is structure dependent.

Another issue that needs to be minimized is the off-target effect. Increasing the length of the sgRNA region to more than 20 nucleotides does not improve cleavage specificity. However, truncating the 20 nucleotides to 17 -19 can improve specificity and efficiency at the target site, hence minimizing the off-target effect. PAM site recognition requires a 5'-NGG-3' sequence, but may also occur at 5'-NAG-3' and 5'-NGA -3' at a low frequency. Some Cas9 variants derived from mutations in the S. pyogens Cas9 (spCas9) such as VQR, shows affinity to 5'-NGAN-3' while VRER spCas9 variant recognizes 5'-NGCG-3'. Both of these variants can be used to increase specificity and decrease off-targeting of Cas9. Cas9n and double nicking also showed less off target effects as mentioned previously.

VII- Therapeutic applications

Hydrodynamic injections or adeno-associated viruses (AAV) vectors delivery of CRISPR/Cas9 technology provides a convenient and efficient way of genome editing.

i- Designing animal models of human diseases

The CRISPR/Cas system provides an easy and efficient way to genetically engineer mice models that possess null or mutated genes or tagged alleles compared to older methods such as transgenesis or gene targeting in embryonic stem cells (ESCs), which is costly and time consuming. This can be done by either hydrodynamic injection or site-directed mutagenesis (SDM).

SDM utilizes viruses and chemicals among other reagents to deliver the components of CRISPR/Cas system into specific neurons or immune cells.

In hydrodynamic injections, Cas9 mRNA, sgRNA, and donor oligonucleotides are injected into fertilized oocytes that are cultured *in-vitro* until the oocyte reaches the blastocyst stage (Wang et al., 2013). Once it is mature, the blastocyst can then be transferred into the uterus of a female mouse that will subsequently give birth to the offspring carrying the mutations. Tail-vein hydrodynamic injection has been used in mice with hereditary tyrosinemia to introduce a correction mutation, which ultimately resulted in rescuing 33.3% of liver cells (Yin et al., 2014).

Unlike studies that involve engineering embryonic cells, therapies based on manipulation of somatic cells are easier to translate into human therapies.

ii- Possible applications of CRISPR/Cas9 in human disease therapeutics

Non-multipotent somatic cells can be converted into induced pluripotent stem cells (iPSCs) with the same ability to regenerate as embryonic stem cells. iPSC is the ideal cell line for CRISPR therapeutics as it can be derived from the patient and genetically modified *in vitro*, cultured to differentiate into the desired cell type in the lab suitable for therapeutic purposes, then transplanted into the patient again. Some of the successful studies conducted using this method have been used for β-thalassemia and sickle cell disease.

a) β-thalassemia

The process of erythropoiesis, the production of red blood cells, depends on the formation of hemoglobin, which is comprised of four globulin chains each bound to a heme group. It comes in two main forms: fetal hemoglobin or HbF (two α and two gamma globulins) and adult hemoglobin or HbA (two α and two gamma globu-

lins). HbF comprises a small fraction of the total hemoglobin in the blood, which is mostly HbA. In β-thalassemia, a point mutation is inherited from the parents present on the human globulin β, or HBB gene. This mutation leads to a deficiency in the β-chains of HbA, resulting in the accumulation of alpha globulin chains within the cell, ultimately leading to hemolysis and anemia.

The treatment of this condition includes frequent blood transfusions (which can lead to iron toxicity) and allogenic hematopoietic stem cell transplantation (HSCT), which requires a suitable donor and comes with the risk of graft rejection or graft-versus-host reaction.

Gene therapy with CRISPR/Cas9 is currently being studied as a treatment alternative. The fibroblasts of β-thalassemia patients were reprogrammed by correcting the HBB gene via HDR to become transgene-free naïve-state iPSCs. This showed high targeting efficiency and minimal off-target effects (Yang et al., 2016)

b) Sickle cell anemia

Another disease caused by a single-point mutation in the HBB gene is sickle cell disease (SCD). This mutation leads to the formation of defective hemoglobin S, which carries oxygen well. However, upon deoxygenation it undergoes "sickling" where it changes shape and polymerizes with other HbS molecules that can clog arteries and or undergo hemolysis and anemia.

ZFN and TALENS were both employed in the treatment of SCD by correcting the HBB mutation in patient-derived iPSCs. One study aimed at mutation correction via CRISPR/Cas9 to target the endogenous HBB locus, which showed higher efficiency and less off-target effect compared to other gene editing methods (Huang et al.,2015)

Since elevated levels of HbF is beneficial in β-hemoglobinopathies an interesting approach that can be applied in the treatment of both thalassemia and SCD is the re-activation of HbF. The BCL11A locus contains an erythroid-enhancer region that is responsible for fetal-to-adult hemoglobin switch and HbF silencing. This locus was identified recently by CRISPR/Cas9 in *in situ* saturation mutagenesis, making it an excellent therapeutic target to be targeted for the treatment of these disorders (Canver et al., 2015).

c) Hypercholesterolemia

Normally, circulating low density lipoproteins (LDL) in the blood stream reach the liver where they bind to LDL-receptors (LDL-R) on the hepatocytes. These receptors internalize the LDL into the hepatocyte (Fig 5) where it is cleared then goes back to the surface of the hepatocyte. In hypercholesterolemia, the elevation of cholesterol levels in the blood can be attributed to a variety of reasons. One is that the LDL-R recycling is inhibited by proprotein convertase subtilisin/kexin type 9 (PCSK9) leading to the increase of circulating LDL and increasing the risk of cardiovascular diseases.

Many treatment options are available to halt the action of PCSK9, such as monoclonal antibodies: evolocumab and alirocumab that work by binding to the protein itself and ensuring the survival of LDL-R. Another proposed therapy that targets the same protein is using CRISPR/Cas9 technology to produce a loss-of-function mutation in the PCSK9 gene locus in mice liver, leading to low plasma cholesterol levels.

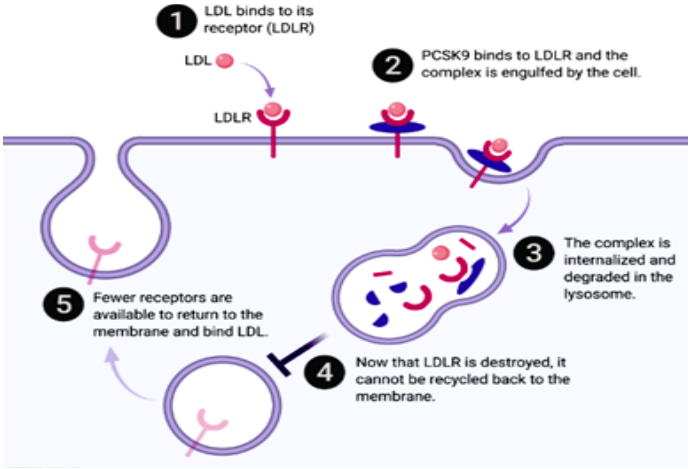

Fig 6. How PSCK9 raises cholesterol

VIII- CRISPR in combating malaria

Malaria is a vector-borne disease that kills approximately 2-3 million people each year, most of whom are children. This disease comes from a parasite of the species plasmodium that takes the anopheles mosquito as a vector or "parasite taxi". The transmission starts when a female mosquito, which is capable of biting humans, injects plasmodium sporozoites (immature form) via its saliva into the host's blood stream. These sporozoites

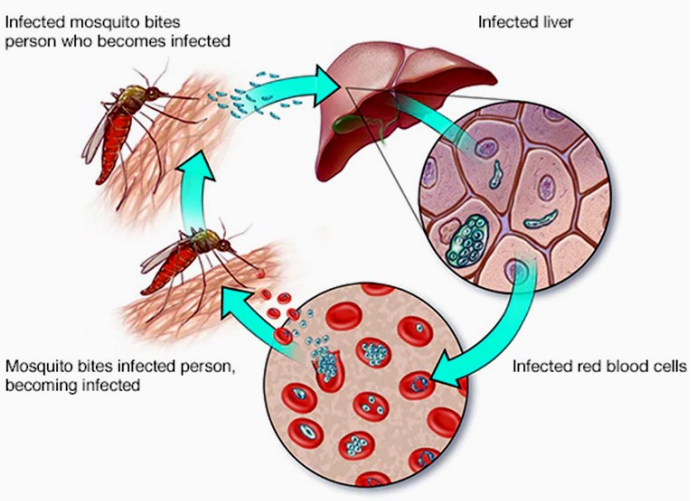

Fig 7. malaria parasite life cycle, from bite to transmission

travel in the blood until they reach the liver where they reside in the hepatocytes for asexual multiplication and maturation into merozoites. These are then released back into the blood where they invade red blood cells (RBC) where they are further replicated until the RBC bursts and releases the newly formed merozoites infecting other blood cells and the cycle continues. This process leads to the development of fever, diarrhea, and other flu like symptoms, eventually leading to depletion of RBC and death.

Many approaches have been used to combat malaria including the use of insecticides, which have the disadvantage of killing a wide range of insects and disturbing the ecosystem, and quinine drugs, which are useful but are facing the threat of drug resistance and suboptimal efficacy. Recently advancement in genome editing has opened new doors for possible ways to control this deadly disease. This can be done via multiple ways some of which include using a technique termed 'gene drive'.

Transgenic mosquitos that carry anti-parasite genes or genes that affect the fertility of the female mosquito to have always been a possibility. However, transgenic alterations are known to be lost upon reproduction in the subsequent generations rendering the transgenic mosquitos useless in the wider range of wild-type mosquitos upon releasing the former to mate with the latter in nature.

This issue could be solved by gene drive using the CRISPR/Cas9 system. The system is delivered into the germline DNA of the mosquitos using Lipofection, electroporation or microinjections. This system includes several gRNA genes, Cas9 genes and the coding genes that contain the instructions to guide Cas9 where to cut in the wild type gene. The cell then copies the transgenic sequence and repairs the damage in the wild type gene with that copy resulting in the mosquito having two copies of the transgenic sequence in each chromosome, one that was inserted that copied its genetic information to the other using CRISPR/Cas system to ensure the gene propagation to the rest of the subsequent generations.

The application of CRISPR/Cas9 in combating malaria using gene drive can be achieved by two methods, either inserting anti-plasmodium genes into the Anopheles mosquito rendering it resistant to the parasite or by interfering with the fertility and reproduction of the vector itself. The second method requires the identification of genes with specific requirements, such as genes that are spatially restricted to the germline. This requirement is to avoid interfering with somatic-wild type alleles, allowing the development of normal heterozygote mosquitos to transmit the mutated CRISPR allele to the next generation. Using orthology studies with drosophila melanogaster and sterility index, three genes were found to fulfill this requirement: AGAP005958, AGAP007280, which are both female fertility genes, and AGAP11377.

i- Knock-out experiment to confirm female fertility genes

The validation of the suitability of these three genes as candidates to "home" the CRISPR gene drive components to suppress female fertility was tested by a knock-out study via homologous recombination, adding a green fluorescent protein (GFP) transcription unit to indicate sterility by visual inspection. The first generation of mosquitos were fertile, which were intercrossed with the wild type to give their progeny. Depending on GFP intensity, the progeny was divided into two classes: strong intensity, indicating homozygous state that was sterile and intermediate intensity, indicating heterozygous state that was fertile. This study concluded that homozygous mosquitos carrying the knocked-out AGAP005958 or AGAP11377 alleles failed to lay eggs, while homozygous mosquitos carrying knocked-out AGAP007280 allele did laid eggs that did not hatch.

ii- Implementing fertility-genes knockout along with CRISPR gene drive

The same experiment was conducted with the anopheles mosquito, with the addition of the human codon-optimized spCas9 nuclease, vasa-promoter sequence, and sgRNA named p16503, p16505, p16501, targeting AGAP11377, AGAP005958, AGAP007280 respectively. All these components, along with GFP marker, were packaged into CRISPR plasmid and microinjected into the mosquito larvae. These modified mosquitos were crossed with the wild type and their progeny were screened showing the transmission rates to the subsequent generation were as high as 94.4-100%. The progeny that did not contain the CRISPR+ alleles were found to have evidence of DNA repair using NHEJ pathway, which is known to create off-target mutations. This finding, while indicating the powerful effect of gene drive in suppressing the mosquito population, also raises some serious concerns about the effect of this technology on the targeted species and the biosphere (Hammond et al., 2016).

iii- Gene drive issues and ethical considerations

Models showed that a few genetically modified mosquitos escaping the containment lab can modify a whole mosquito population. Also, gene drive may cross to a different species in the wild, e.g.: About 460 anopheles mosquitos species have been identified, of these only 30 -40 transmit the Plasmodium parasite, which is responsible for malaria endemics in humans. If gene drive transmits to all mosquitos it will lead to the extinction of species that are unintended targets, and ultimately

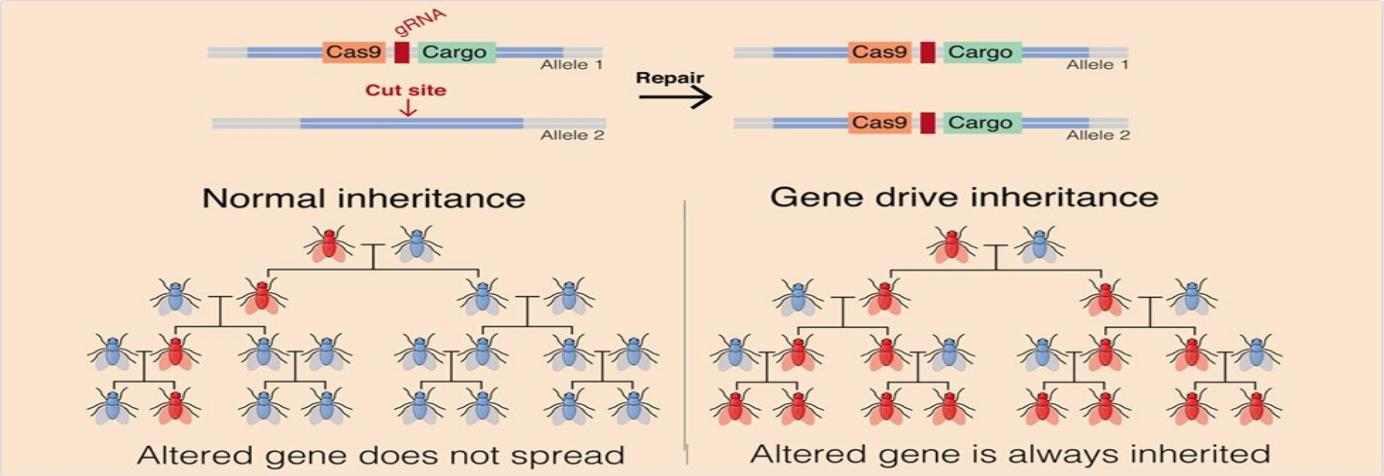

Fig 8. The powerful effect on CRISPR/cas9 gene drive compared to normal inheritance

affect the ecosystem. Mosquitos are food for many species, help filter debris and waste for plants to thrive, pollinate many species of flowers, and even affect the herding paths of certain animals. Disturbing the balance of nature by eradicating a species of animals or insects could likely lead to detrimental effects on crops and wild life.

Thus, this technology comes with a set of risks and benefits that should be compared before implementation, along with the ethical considerations that should be discussed. The benefit is clear as it's a revolutionary way that may solve the ongoing malaria crisis along with many other vector-borne diseases and will save plenty of lives. However, the fact is that this technology is relatively new and using it correctly requires more research. It may lead to undesirable consequences in the mosquito species, other related species (as mentioned above), or even new human diseases due to the possible adaption of the plasmodium parasite.

The main issue is the discussion and engagement of the African communities and other affected areas with endemics in implementing this gene drive. These communities tend to be run by dictatorships, has a high illiteracy rate, chronic poverty, and low education levels which affect the capacity of informed decision making and reaching a state of consensus between the affected countries.

Conclusions

CRISPR is a versatile gene editing tool that was first found in prokaryotes, such as bacteria and archaea, as a defense system against viruses. This system was later adapted for use in eukaryotic cells by the addition of functional modifications to the Cas nuclease, yielding Cas nikase, dead Cas and light-activated Cas, which are currently being studied to design animal models or treat human diseases, expanding the field of biotherapeutics. This technology only requires two components to direct a very specific DSB, giving it an upper-hand compared to older gene editing tools, ZFN and TALENS. Some of the applications discussed are thalassemia, sickle cell anemia, hypercholesterolemia, and genetically engineering malaria-carrying mosquitos that carry a gene drive with fertility-reducing genes. The wide applications of this technology raises many ethical concerns about the consequences of genetic engineering on the ecosystem and the genetically modified species along with the effect on human populations in which such mutated organisms are released. These issues can only be tackled by having nationwide discussion with the affected countries about the benefits and risks, further research and polishing of this technology's shortcomings.

References

Brodt A et al (2011). Biology Direct, 65: 1745-6150.
Canver MC et al (2015). Nature, 527: 192–197.
Eid A et al (2018.). Biochem J, 11: 1955–1964.
Gasiunas G et al (2014). Cellular Molec Life Sci, 71: 449–465.
Hammond A et al (2016). Nature Biotechnol, 34: 78–83.
Huang X et al (2015). Stem Cells, 33:1470–1479.
Jiang F & Doudna JA (2015). Curr Opin Struct Biol Sci, 30: 100-111.
Li et al (2015). Metabolic Engineering, 31: 13-21.
Maruyama T et al (2015). Nature Biotechnol, 33: 538–542.
Mei Y et al (2016). J Genet Genomics, 43: 63-75.
Polstei, LR et al (2015). Nature Chem Biol, 11: 198–200.
Ran FA et al (2013). Cell, 154: 1380–1389.
Rath D et al (2015). Biochimie. 177: 119-128.
UNESCO. Universal Declaration on Bioethics and Human Rights. http://portal.unesco.org/en/ev.php-URL_ID=31058&URL_DO=DO_ TOP-IC&URL_SECTION=201.html.
Xiao, A et al (2013). Nucl Acids Res. 41: e141.
Yang Y et al (2016). Stem Cells Transl Med, 51: 8-19.
Yin H et al (2014). Nature Biotechnol, 32: 551–553.

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TEST YOUR KNOWLEDGE

Answers on back page

a) ***The first step in the CRISPR/Cas system is***

1. crRNA processing
2. Spacer acquisition
3. Disabling the virus
4. None of the above

b) ***The Zinc finger nucleases use a naturally occurring restriction enzyme called***

1. Fok1
2. Lipase
3. HMG-CoA reductase
4. Trypsin

c) ***Possible applications of CRISPR/Cas9 are in***

1. β-thalassemia
2. Sickle cell anemia
3. Hypercholesterolemia
4. All of the above

d) The CRISPR/Cas system provides an easy and an efficient way to genetically engineer mice models that possess mutated genes by

1. Site-directed mutagenesis
2. Random-and-extensive mutagenesis
3. Transposon mutagenesis
4. Sequence saturation mutagenesis

Is there a problem?

A patient is given the prescription below to treat his diabetes. Is there any major error with the prescription?

GHC HOSPITAL

Patient Name: Ahmad Age: 48 years
Address: Street No: 453

Rx

Empagliflozin tablet 25mg
25 mg twice a day
Send one packet

Dr. Khalid
Signature Date: 17/6/21

Answer (Prescription Exercie)

The frequency is wrong.
Empagliflozin is a SGLT2 inhibitor given as 10 or 25mg once daily for diabetes.

TOPICAL ISSUES AND CONTROVERSIES

Strategies after chemotherapy failure

Chemotherapy is used either as a first-line therapy or in combination with other treatments, such as surgery. If chemotherapy does not work, a person may need to consider other treatment options.

Radiation therapy

Radiation therapy can serve as a primary treatment, but it also works well with other treatments, such as surgery. This therapy damages the DNA in cancer cells to the extent that they can no longer repair themselves. These damaged cancer cells will stop dividing and eventually die, at which point the body will break them down and remove them.

Advantages of radiation therapy

- causes only moderate pain
- minimal or no hair loss
- effectively kills large numbers of cancer cells within a tumor
- relatively safe for the individual as the radiation specifically targets the tumor
- minimal damage to organs near the tumor

Disadvantages of radiation therapy

- people who receive internal beam radiation therapy will be radioactive for a short period
- potential damage to vital organs if they are particularly close to the tumor
- may not kill all cancer cells if the tumor is very large
- inconvenient and time-consuming as people require treatment lasting from 5 days of the week to 2

months
- expensive, although the exact cost depends on the type and amount of treatment
- skin redness or soreness around the site of radiation
- site-specific side effects — for example, treating cancer in the esophagus or gastrointestinal tract can cause nausea or vomiting

Immunotherapy

Some types of cancer do not respond well to radiation or chemotherapy, so a person may need to try immunotherapy, which aims to help the immune system fight cancer by either stimulating the immune system in a general way or train it to attack cancer cells directly.

The main methods of delivering immunotherapy include:

Monoclonal antibodies With this method, a person receives synthetic antibodies that bind to specific proteins on cancer cells. This binding marks the cancer cell to help the immune system locate and destroy it.

Checkpoint inhibitors These are medications that stimulate T cells, which then identify and attack cancer cells more efficiently.

Cancer vaccines Vaccines stimulate the immune system to fight cancer. Some vaccines, such as the human papillomavirus (HPV) vaccine, can have protective effects. Specific types of HPV are known to cause certain cancers.

Adoptive cell transfer This involves removing T cells from a tumor and modifying them in a laboratory. The aim is to boost the ability of the T cells to detect and destroy cancer cells.

Advantages of immunotherapy

- effective against many types of cancer
- can improve the success of other treatments
- causes fewer side effects than treatments that target all the cells in the body, such as chemotherapy
- after learning to target cancer cells, the immune system remembers this response if cancer reappears

Disadvantages of immunotherapy

- risk of overstimulating the immune system and causing it to attack healthy organs, which can lead to severe complications in the lungs, intestines, kidneys, or other organs
- side effects, such as fatigue, cough, nausea, loss of appetite, skin rash, and flu-like symptoms

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Hormone therapy

Hormone therapy can treat some types of cancer, including prostate cancer and breast cancer, by taking advantage of the disease's dependence on hormones to grow.

Hormone therapy to treat breast cancer, or anti-estrogen therapy, focuses on lowering estrogen levels. The treatment may involve surgical procedures, such as removal of the ovaries, or medications that interrupt signals from the pituitary gland, a gland that stimulates estrogen production.

Hormone therapy to treat prostate cancer, or androgen-suppression therapy, lowers testosterone and dihydrotestosterone (DHT) production. Treatments include surgical procedures to remove one or both testicles and medications that prevent the production of testosterone and DHT.

Advantages of hormone therapy

- effective treatment for preventing the spread of cancer to other parts of the body
- can help reduce the risk of cancer returning after surgery

Disadvantages of hormone therapy

- only works on cancers that require hormones to grow
- side effects for females include headaches, hot flashes, weight gain, and vaginal dryness
- side effects for males include fatigue, hot flashes, breast sensitivity/enlargement, nausea, impotence, and lower sexual desire

Targeted therapy

Targeted therapy uses medication that specifically targets cancer cells, destroying them from the inside. Unlike chemotherapy, these medications do not affect healthy cells because they identify particular genetic abnormalities in cancer cells. Targeted therapy works best in combination with other treatments.

Targeted therapies may fight cancer by:

- blocking or turning off chemical signals that stimulate cancer cell growth
- changing proteins inside cancer cells, causing cell death
- preventing cancer cells from stimulating the growth of new blood vessels
- triggering an immune response to destroy cancer cells
- delivering toxic substances to cancer cells to kill them without affecting other cells

Advantages of targeted therapy

- specifically targets cancer cells
- not toxic to healthy cells
- the wide array of treatment options makes individual treatment plans possible

Disadvantages of targeted therapy

- only works for tumors with specific genetic mutations
- cancer cells may develop resistance
- side effects include diarrhea, skin rash, issues with blood clotting, high blood pressure, and liver problems, such as hepatitis

Conclusion

There are usually alternative treatments available if chemotherapy does not work. After considering alternative treatment options, some people choose to decline further treatment. If this is the case, an oncologist will focus on improving the individual's quality of life by developing a treatment plan to manage their cancer symptoms.

Reference

https://www.medicalnewstoday.com/articles/324158

Gene editing restores dystrophin in animal model of Duchenne and patient cells

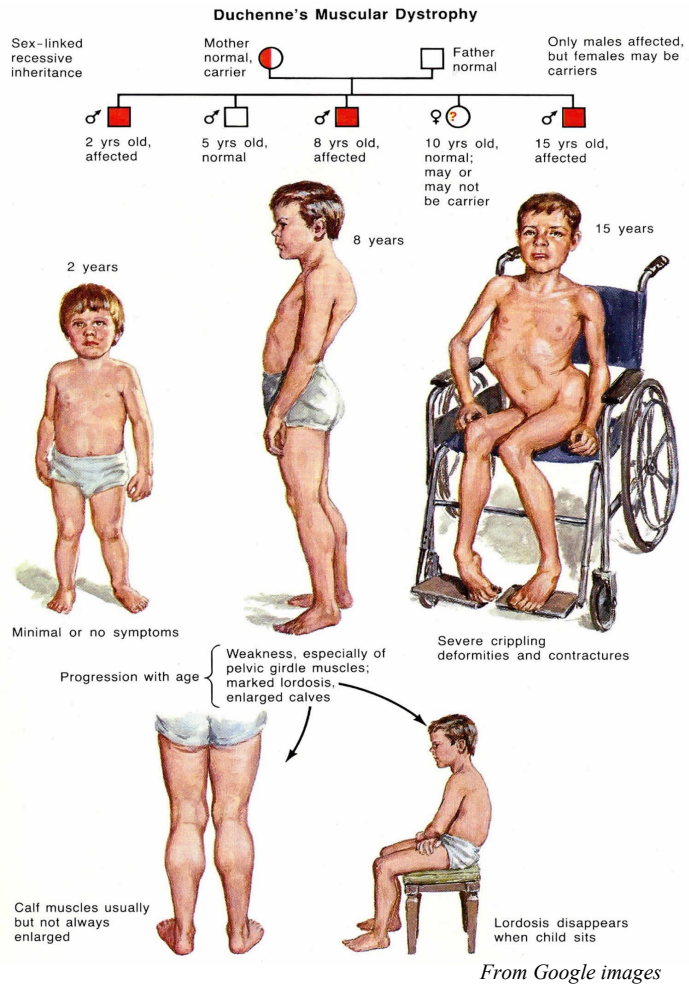
In a recent study published in Nature Medicine, researchers at the Technical University of Munich used CRISPR/Cas9 to correct defects in the *DMD* gene and restore dystrophin protein production, lengthening the lives of pigs in a model of Duchenne muscular dystrophy (DMD) and altering heart cells from a patient to make them less prone to irregular beats.

A type of gene therapy, CRISPR/Cas9 is an editing tool that can be used to remove, add, or change a portion of the DNA sequence of a gene of interest. It is based on a "cut-and-paste" mechanism, in which the enzyme Cas9 is guided toward DNA with the help of a small RNA molecule that specifically recognizes the DNA sequence selected for removal.

The group believe their work, the first to show "therapeutic success" in the large animal model of Duchenne they created, could be refined for use in people, and possibly be a long-lasting treatment.

CRISPR/Cas9 was used to remove a defective portion of the *DMD* gene sequence in their pig model of DMD, and in patient-derived induced pluripotent stem cells (iPSCs) carrying the same genetic defects.

DMD is the gene that encodes dystrophin, a protein essential for muscles to contract and relax without injury; mutations in this gene prevent its production. iPSCs are fully matured cells that can be



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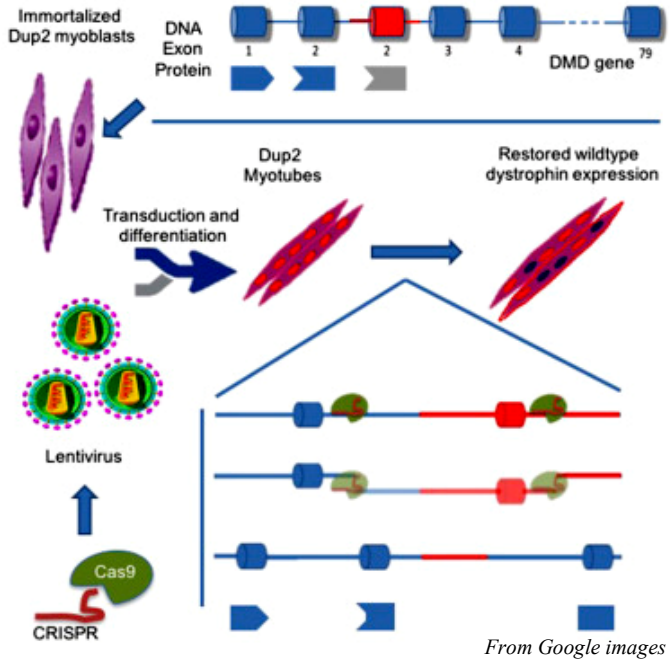

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Investigators believe their work may set the foundation for new ways of treating Duchenne muscular dystrophy. Unlike other treatments, gene correction mediated by CRISPR/Cas9 has the potential to offer a lifelong benefit. They claim that the ability of Cas9-mediated exon excision to improve DMD pathology in these translational models paves the way for new treatment approaches in patients.

Reference

https://musculardystrophynews.com/2020/02/04/crispr-cas9-gene-editing-restores-dystrophin-in-duchenne-pig-model-patient-cells-study/

Gut microbes: Key to treating ulcerative colitis

A team of scientists from Stanford University, California, has identified a gut microbe that is missing in some people, which may explain ulcerative colitis and open the door for its treatment. They hope that by replacing the function of this missing microbe, it may be possible to develop new and more effective treatments for ulcerative colitis.

Ulcerative colitis is a type of inflammatory bowel disease that causes inflammation and sores in a person's large intestine, which can result in abdominal pain, weight loss, diarrhea containing pus or blood, and other issues. The symptoms can range from mild to severe, and there is currently no cure. Instead, treatments focus on keeping the disease in remission for as long as possible. Treatment usually begins with medications, but if these do not work, surgery may be necessary.

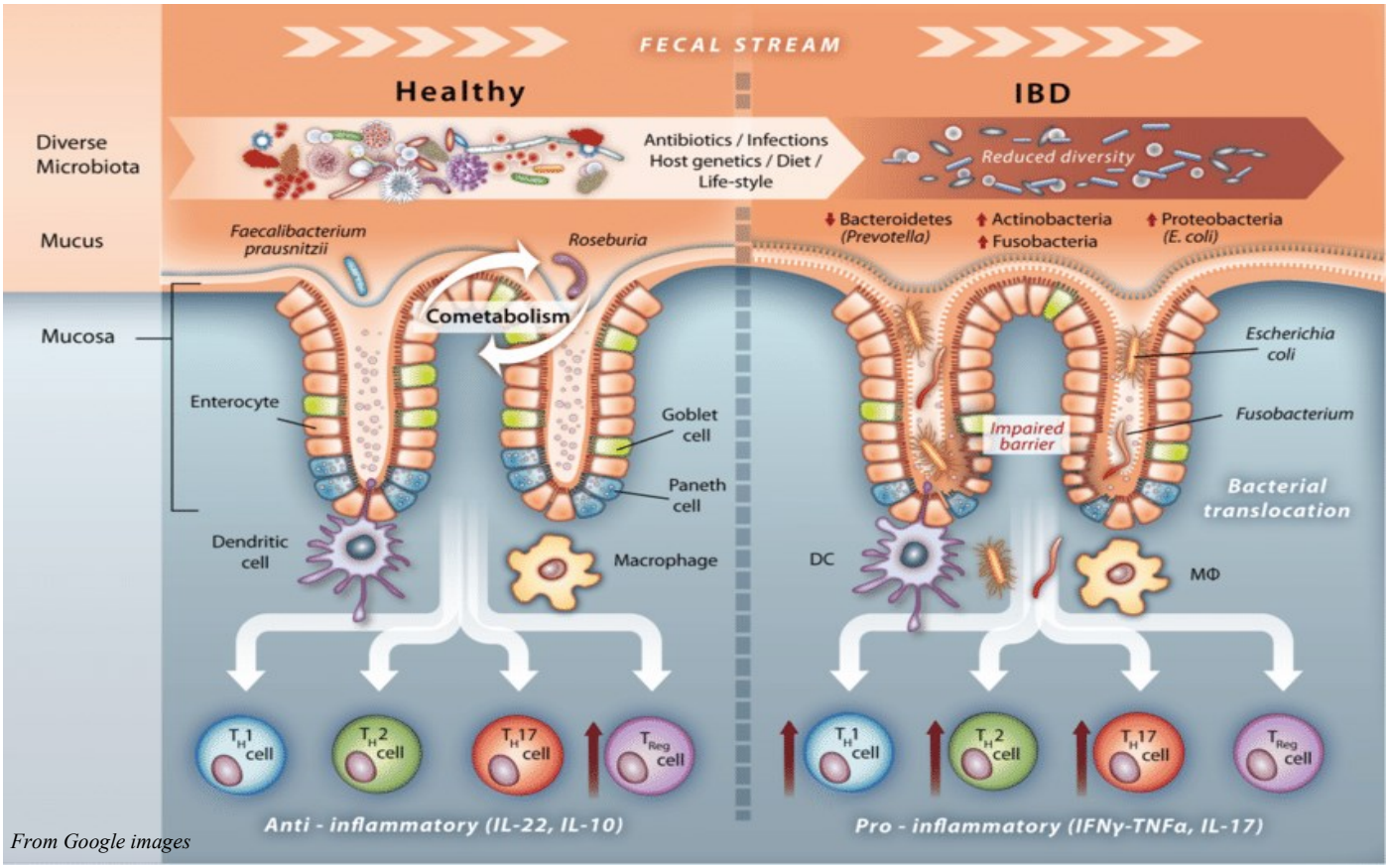
According to the Crohn's and Colitis Foundation of America, 23–45% of people with ulcerative colitis will eventually need to have surgery which involves complete removal of a person's colon and rectum. The surgeon will then create either a stoma, which acts as an external pouch to collect intestinal contents, or an ileoanal reservoir, which is a J-shaped pouch at the end of the small intestine that does the same job. It is unclear why ulcerative colitis affects some people and not others; the new research from the Stanford team suggests that a key reason may be the lack of gut microbes.

Bile acids

Some people who have surgery to create the J-shaped pouch for their ulcerative colitis will then find that inflammation and the associated symptoms return. Interestingly, people who have the genetic condition familial adenomatous polyposis (FAP), which also requires the creation of a J-shaped pouch, never experience any inflammatory symptoms. The researchers wanted to work out why this was the case. To do so, they compared two groups of participants, one with FAP and the other with ulcerative colitis, looking for any significant differences between them. They found that a key difference was the presence of a type of bile acid in the intestines, which was in far greater quantities in those with FAP than in those with ulcerative colitis. These bile acids are a natural part of a healthy gut and help break down fats.

Gut microbes

In the intestines, bacteria convert these bile acids to secondary bile acids. The researchers were able to identify a specific bacterial family called Ruminococcaceae that was under-represented in those with ulcerative colitis. Ruminococcaceae bacteria are the main type of microbe that converts primary bile acids into secondary bile acids. All healthy people have Ruminococcaceae in their intestines. But in the ulcerative colitis pouch patients, members of this family were significantly depleted. To explore the role of BAs in intestinal inflammation, they did metabolomic, microbiome, metagenomic, and transcriptomic profiling of stool from ileal pouches in patients with ulcerative colitis (UC) who were colectomy-treated patients versus controls (FAP). They demonstrated that relative to FAP, UC pouches have reduced levels



From Google images

of lithocholic acid and deoxycholic acid (normally the most abundant gut SBAs), Ruminococcaceae, and genes required to convert PBAs to SBAs.

The team then gave acid supplements to mice who had ulcerative colitis to replace any missing secondary bile acids. This reduced inflammation as well as the normal symptoms of colitis in mice.

This anti-inflammatory effect is in part dependent on the TGR5 bile acid receptor. These data suggest that dysbiosis induces SBA deficiency in inflammatory-prone UC patients, which promotes a pro-inflammatory state within the intestine that may be treated by SBA restoration. Researchers hope this study will lead to treatment with a naturally produced metabolite that's already present in high amounts in a healthy gut.

To get to this point, the team is now conducting a clinical trial to discover whether an acid supplement can help people with ulcerative colitis. In this study the researchers will investigate if ursodeoxycholic acid (UDCA) will reduce markers of inflammation and improve quality of life in UC pouch patients (colectomy-treated patients with ulcerative colitis) with active antibiotic refractory pouchitis.

References:

1. *https://www.medicalnewstoday.com/articles/gut-microbes-could-be-key-to-treating-ulcerative-colitis*
2. *Sinha et al. Dysbiosis-Induced Secondary Bile Acid Deficiency Promotes Intestinal Inflammation. Cell Host & Microbe. 2020. [In press]*
3. *https://www.sciencedirect.com/science/article/abs/pii/S1931312820300627?via%3Dihub*
4. *https://clinicaltrials.gov/ct2/show/NCT03724175*

The first long-acting HIV drug combo monthly shots

U.S regulators have approved the first long-acting drug combo for HIV, monthly shots that can replace daily pills. Approval of the two-shot combo called Cabenuva is expected to make it easier for people to stay on track with their HIV medicines and to do so with more privacy. It's a huge change from not long ago, when patients had to take multiple pills several times a day, carefully timed around meals.

Cabenuva combines rilpivirine, sold as Edurant by Johnson & Johnson's Janssen unit, and a new drug-cabotegravir, from ViiV Healthcare. They're packaged together and given as separate shots once a month. Dosing every two months is also being tested.

The FDA approved Cabenuva for use in adults who have had their disease well controlled by conventional HIV medicines and who have not shown signs of viral

resistance to the two drugs in Cabenuva.

The agency also approved a pill version of cabotegravir to be taken with rilpivarine for a month before switching to the shots to be sure the drugs are well tolerated.

The shot combo in the US would cost $5,940 for an initial, higher dose and $3,960 per month afterward. The company said that is "within the range" of what one-a-day pill combos cost now. Studies found that patients greatly preferred the shots.

Even people who are taking one pill once a day just reported improvement in their quality of life to switch to an injection, according to an HIV specialist at the University of California who also added that long-acting shots also give hope of reaching groups that have a hard time sticking to treatment, including people with mental illness or substance abuse problems.

Separately, ViiV plans to seek approval for cabotegravir for HIV prevention. Two recent studies found that cabotegravir shots every two months were better than daily Truvada pills for keeping uninfected people from catching the virus from an infected sex partner.

Source:

www.independent.co.uk/news/fda-approves-1st-longacting-hiv-drug-combo-monthly-shots-shots-people-shots-pills-infection-b1790984.html

Cure of Type 1 diabetes in mice using stem cells

In type 1 diabetes, a faulty autoimmune response causes the immune system to attack and destroy insulin-producing beta cells within the pancreas. As many as 187,000 children and adolescents in the US were living with type 1 diabetes in 2018. An additional 1.4 million people aged over 20 years have the condition and manage it with insulin, according to the same statistics from the Centres for Disease Control and Prevention (CDC).

New research at Washington University School of Medicine in St. Louis uses an innovative technique to convert human stem cells into insulin-producing beta cells much more effectively.

The insulin-producing cells created rapidly cured type 1 diabetes in mice, and the benefits lasted for 9 months. Transplanting billions of such cells may soon cure type 1 diabetes. Previous research has pointed to human pluripotent stem cells (hPSCs) as a potential therapeutic avenue for type 1 diabetes. Pluripotent stem cells are an attractive option for researchers from a therapeutic standpoint because they can self-renew in lab cultures and can differentiate into a variety of cell types. Researchers have previously used hPSCs to create insulin-producing beta cells. However, they were not able to do so effectively enough to cure type 1 diabetes.

A common problem when one tries to transform a human stem cell into an insulin-producing beta cell, or a neurone or a heart cell, is that other unwanted cells are also produced. For example, in the case of beta cells, we might get other types of pancreas cells or liver cells. While implanting these unnecessary or "off-target" cells does not cause any harm, creating more of them offsets the number of therapeutically useful cells.

A billion beta cells are needed to cure a person of diabetes. But if a quarter of the cells made are actually liver cells or other pancreas cells, instead of needing a billion cells, 1.25 billion cells are needed which makes curing the disease 25% more difficult. However, the new research used an innovative technique that bypassed this problem.

The new *stem cell* technique targets the cytoskeleton, or inner "scaffolding", of the hPSC to direct their differentiation into pancreatic cells. The cytoskeleton is a structure that helps cells keep their shape and offers the mechanical support that allows cells to move, divide, and multiply. Targeting this structure allows the researchers to create fewer irrelevant cells and better functioning beta cells that helped control blood sugar.

The team transplanted the islet-sized aggregates of beta cells differentiated from hPSC into mice with type 1 diabetes. Pancreatic islets are groups of cells located in the pancreas. Some of these cells are insulin-producing beta cells. This transplantation procedure rapidly reversed severe pre-existing diabetes in mice. The reversal occurred at a rate similar to that of human islets, and normal blood sugar control was maintained for at least 9 months. These mice had very severe diabetes with blood sugar readings of more than 500 mg dL of blood , levels that could be fatal for a person, and when they gave the mice the insulin-secreting cells, within 2 weeks their blood glucose levels had returned to normal and stayed that way for many months.

However, there are a few more steps to follow before this can help humans. First, researchers must test the cells in larger animals and then find a way to automate the new technique to produce the billions of cells required for the millions of people that have type 1 diabetes.

References

1. *https://www.medicalnewstoday.com/articles/type-1-diabetes-cured-in-mice-using-stem-cells*
2. *Hogrebe N.J. et al. Nat Biotechnol (2020). doi.org/10.1038/s41587-020-0430-6*

Outgoing Editorial

The Kuwait Pharmacy Bulletin was initiated in 1997 by the founding Dean of the Faculty of Pharmacy, David Biggs, with Nancy MacCullum as the Managing editor. She was succeeded by Ladislav Novotny in the summer of 1998 who ran it with the assistance of Sam Kombian and Margaret Peterson Biggs until 2000.

The objective of this publication was to disseminate information on various pharmaceutical topics to be distributed around the university, hospitals, polyclinics and private pharmacies as an educational initiative by the newly established Faculty of Pharmacy.

Previously, there had been a general newsletter published by the Faculty of Medicine but this had been discontinued and therefore this publication has remained the sole scientific output of its kind from the Health Sciences Centre of Kuwait University.

When I was asked to take over in 2000, I extended the scope of the bulletin to include a wider range of medical subjects to broaden its appeal to a more general audience, and to make it more attractive and readable. We also started distributing it free of charge to other universities around the local region. Since 2009 the Bulletin has also been made available in electronic format on the university HSC website.

For brief periods, I was assisted by Ahmed El Hashim and Lloyd Matowe and then by Douglas Ball. From 2005 I was joined by Leyla Hassan Sharaf and Abdel-moneim Awad. The latter resigned in 2010 and since then I have been most ably assisted till the current day by Leyla and Sam Koshy. I am truly grateful to them for their expertise in helping to select, adapt and edit articles for the Bulletin.

During the past 20 years we have printed modified articles taken from many different sources on a great variety of topics, with particular emphasis on disease conditions that are prevalent in Kuwait, and have greater interest for the local readership.

The general format has been to have one major article and then other smaller ones. Since 2009 we adopted the practice of procuring the main article from our final year undergraduate students, to highlight and promote their work. Articles have been selected not only on mainstream advances but also on provocative themes. Two of the most recent issues have, perhaps not surprisingly, been focused on Covid-19. We have also been publishing lists of drugs as they are registered by the Ministry of Health authorities in Kuwait; this information has been supplied by the Quality Assurance department, and we express our appreciation for their kind assistance over the years.

It is hoped that the information we have disseminated has been informative and useful to our readers, and stimulated their interest in learning more about our general biology, our health, and what we do to maintain it.

Yunus Luqmani

Leyla Sharaf

Sam Koshy

Answers to: Test your knowledge

Correct answers: a-2; b-1; c-4: d-1

The *Kuwait Pharmacy Bulletin* (ISSN 1028-0480) is published quarterly by the Faculty of Pharmacy, Kuwait University, and includes a list of recently approved drugs from the MOH or FDA. It aims to provide instructive reviews and topical news items on a range of drug related issues. It is widely distributed free within the university, to hospitals, polyclinics & private pharmacies as well as to other universities within the Gulf & Middle East region.

***Executive Editor*: Yunus Luqmani. *Assistant Editors*: Leyla Hasan Sharaf, Samuel Koshy**

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