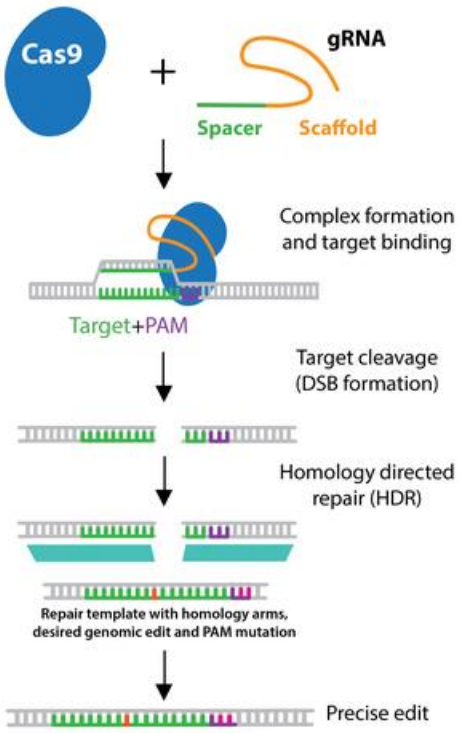

EVOLUTION IN HUMAN HANDS: THE PAST, PRESENT AND FUTURE OF CRISPR-CAS

Abstract

All the information of the composition of an organism is stored inside the DNA in a unique sequence of four nucleotides. For thousands of years, humans have altered the genetic composition of their environment in various ways. The discovery of gene specific targeting approaches like ZFN, TALE and especially CRISPR made genome editing more precise, effective and efficient. Which crucial developments preceded the breakthrough of the CRISPR-Cas technique? What are the applications of CRISPR at this moment? And what are the prospects? CRISPR-Cas is the adaptive immune system of bacteria. In bacteria genomic information of pathogens is stored between spacers. CRISPR proteins actively scan the genome, when a match is found the Cas protein cleaves out the threat. CRISPR-Cas can be reprogrammed to target any specific target of interest. Depending on the function domains CRISPR has many applications: introduction of knockouts, knock-ins, BE, epigenome modifiers, and transcriptional regulators. Due to this toolbox CRISPR has the ability to alter genes and gene activity in plants, animals and humans. CRISPR has application in clinical research, agriculture, cancer treatment, diagnostics, infectious disease and germline editing. The future perspective of CRISPR is promising. More research is necessary to increase the safety and efficiency and minimalize side effects and negative consequences. In addition, ethical issues should be fully considered and strict regulation should be implied. As a result, CRISPR theoretically has the ability to eradicate inheritable diseases, enhance human features and reverse aging.

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Contents

1 Introduction

1.1 Benefits of targeted approach

Deoxyribonucleic acid (DNA) is a polymer which forms two chains that coil around each other and form a double helix. Within this helix, all genetic instructions are being stored in a sequence of four nucleotides (adenine, guanine, thymine or cytosine). These sequences hold the information for the development, growth, functioning and reproduction of all known organisms. When DNA replicates, errors may occur leading to mutations. Mutations can also arise from DNA damage from external factors and erroneous repair mechanisms. Often these mutations lead to diseases or other negative influences for the individual. A gene targeted approach at the specific mutation site could alter or reverse it, and as a result cure diseases. Such a technique could induce specific mutations that created, for example; animal models of human diseases for fundamental research and drug development. Furthermore, specific genes in plants and animals can be modified to make strains that have desirable phenotypes which for example grow faster. A very promising technique for a targeted approach is CRISPR-Cas. Preceding CRIPSR's discovery some other techniques to alter the sequence of DNA were developed over time, each with a variety of specificity and efficiency.

1.2 History of genome editing

The genome of a eukaryotic organism exists out of billions of DNA bases. In these compositions, mutations can spontaneously occur overtime. By selective breeding of organisms with beneficial mutations, humankind has been able to shape them to their needs for thousands of years (Nolte et al., 2019). A better understanding of the sciences of genetics and selective breeding came with Mendel's discoveries roughly 150 years ago (Domoney et al., 2016). Human induced mutations were invented in the second half of the twentieth century. Auerbach and Muller have demonstrated that mutations could be triggered by chemical or radiation treatment (Auerbach et al., 1947). Mutagenesis increased the rate of the occurrence of mutations. However, the disadvantage of this technique is that the genome is changed at random sides. To deflect these negative side-effects recombinations between exogenous DNA and a homologous chromosome were introduced, which made genome editing substantially more precise. Still, recombination techniques are very inefficient and it requires strong selection, especially in mouse models (Neal et al., 1999).
One of the essential developments was the realisation that a double-strand break (DSB) initiation can increase the integration of a target gene at the target side (Jasin, 1994). The majority of DSB is repaired by a DNA repair mechanism called non-homologous end joining (NHEJ). A DSB is introduced by using a natural occurring meganuclease. Every meganuclease has its own specificity, which made brought applications expensive and time-consuming (Adli, 2018). Therefore, meganucleases were redesigned to modify their specificity, resulting in an improved manner to target sequences of interest but only available for a small section of the genome (Sussman et al., 2004). Repair by NHEJ introduces some possible complications. Firstly, NHEJ can result in excluding of the exogenous DNA templates at the DSB site. Secondly, NHEJ can lead to randomly including and excluding DNA base-pairs at the break site (Jeggo, 1998). Meganuclease can introduce a DSB for integration of foreign DNA; however, meganuclease lack specificity and broad applicational properties.

1.3 Zinc finger nucleases and transcription activator like effector

The discovery of zinc finger protein led to a higher plasticity and broader application than meganucleases. Zinc fingers are small ion-regulated protein motifs that can find and attach to a specific sequence in DNA. Each zinc finger consists of a module with the ability to recognize a DNA sequence of 3 base pairs (Carroll, 2011).

In order to achieve a higher specificity of DNA binding, multiply zinc finger modules connect into a big complex. The zinc fingers are combined with a nonspecific cleavage domain called Fok I endonucleases. The combination of zinc fingers and Fok I results in an artificial nuclease that can cut DNA at a predetermined site (Chandrasegaran, 1996). The zinc finger nucleases (ZFNs) can tremendously enhance the targeted homologous recombination (HR). ZFNs can be used in animal models and human cells increasing the therapeutic value of this technique (Bibikova et al., 2001). There are 64 unique fingers, consisting out of a three base-pair DNA code, up to 7 zinc fingers can be assembled in a complex (Urnov et al., 2010). Zinc fingers have an enormous number of combinations. However, due to the set combinations of pairs of three, the combinations of sequences are limited.

An alternative for ZFN to overcome its limitations is Transcription activator-like effector (TALE). TALE is a protein originating from the plant pathogen *Xanthomonas* bacteria (Joung & Sander, 2013). The TALE protein consists of various tandem repeats. The difference between TALE and ZFN is that the repeats of TALE specifically recognize a single base-pair (Boch et al., 2009). TALE alone cannot make a desired cut, likewise ZFN, it is fused with the non -specific endonuclease Fok I creating transcription activator-like effector nuclease (TALEN) (Ousterout & Gersbach, 2017). Two separate monomers will each bind to opposing sites of a DNA strand and form a tail-to-tail dimer and make a site-specific DSB (Figure 1) (Miller et al., 2011).

Artificially designed meganucleases, shortly accompanied by precise techniques like ZFN and TALEN introduced new methods in genome-editing, increasing the specificity and efficacy. However, for each different target side a new set of proteins has to be designed and engineered (Adli, 2018). Making these techniques not ideal and leaves room for a more accessible one.

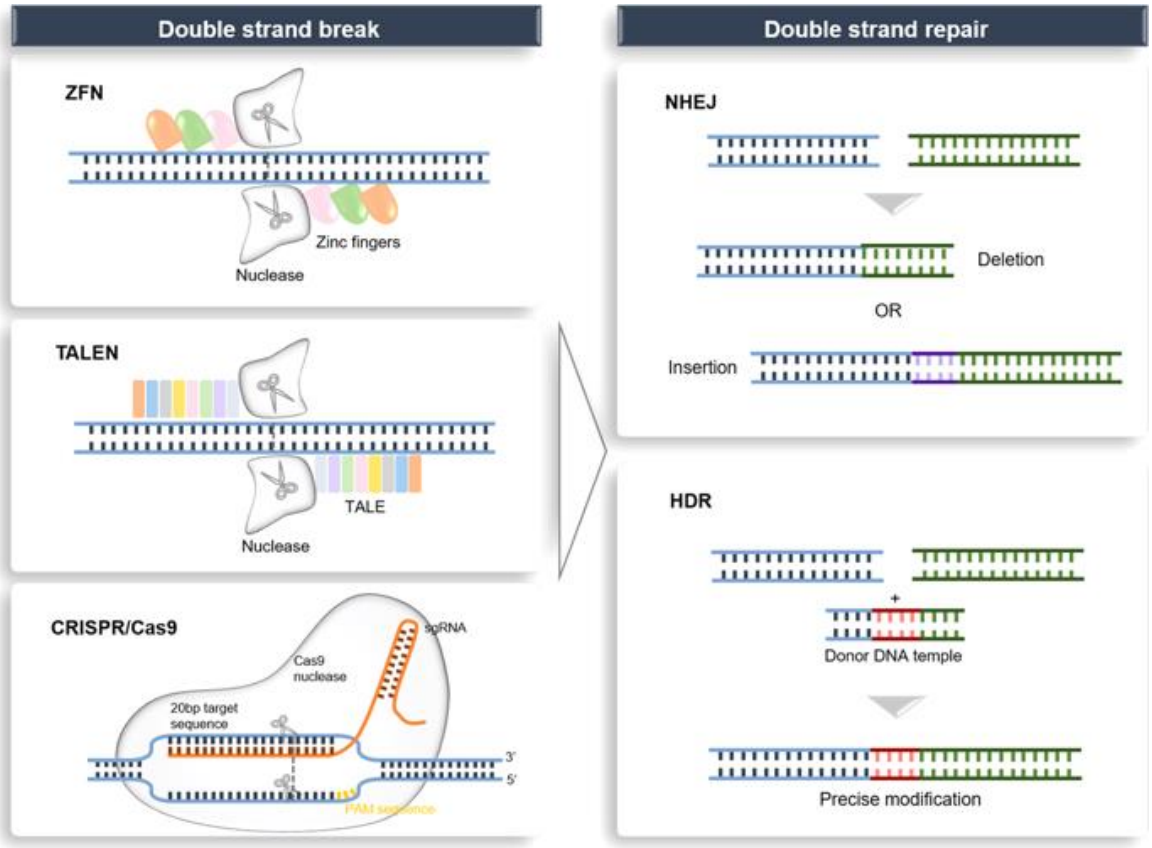

Figure 1|Genome editing nucleases ZFNs, TALENs and CRISPR-Cas9. Introduction of DSB at targeted site. DSBs can be repaired by nonhomologous end-joining (NHEJ) or, if a donor template is present, homology-directed repair (HDR). NHEJ targeted gene disruption leads to the formation of indels. When two DSBs target both sides of a target sequence, a deletion of the sequence can be created. When a donor DNA temple is present HDR can introduce a precise gene modification at the DSB site (Li et al., 2020)**.**

1.4 Rise of CRISPR-Cas

CRISPR-Cas is a revolutionary technology, it is highly efficient and applicable. The DNA-targeting sequence is relatively easier to adjust, this makes it more flexible and user-friendly than ZFN and TALEN. The applications of CRISPR have a brought range: being a promising approach in the fight against inherited diseases and cancer; creating custom-made animal models; genome-wide screening and diagnosing of humans; switching genes on or off; artificially modify plant genetically.
Which crucial developments preceded the breakthrough of the CRISPR-Cas technique? What are the applications of CRISPR at this moment? And what are the prospects?

2 The Discovery of CRISPR

2.1 Repeats, spacers and cas9 in microbial immunity

Repeats of a specific sequence were first recognized in *Holoferax mediterranei, an archaeon*. The way this microbe's genome is cut by restriction enzymes depends on the salt concentration of its surroundings. The modified genome fragments are characterized by multiple copies of almost flawless, near palindromic repeats of a 30-base sequence. Spacers separate repeats with approximately 36 bases (Mojica et al., 1993). The same repeats were also found in *Escherichia coli* prior to *Holoferax mediterranei* (figure 2) (Ishino et al., 1987). However, the significance of these highly preserved repeats in such distant microbes were yet to be considered. With the rapid discovery of other microbes with these similar repeats, they went under the name of dubbed short regularly spaced repeats (SRSRs). This name quickly changed in the familiar name; clustered regularly interspaced short palindromic repeats (CRISPR) (Jansen et al., 2002). Further research into the spacers leads to the discovery that spacers match pre-existing sequences with transmissible genetics deriving, such as; plasmid conjugation or bacteriophages (Soria et al., 2005). These pre-existing sequences hinted in the importance of CRISPR for microbial immunity.
The spacer on its own is not enough for phage resistance. Investigation in the role of CRISPR-associated protein (Cas) started. Two distinct proteins, Cas7 and Cas9, were first examined. A bacterium requires cas7 for the ability to gain resistance against a phage. However, a bacterium which already has a phage-derived spacer does not need cas7 to remain resistant — suggesting that cas7 is responsible for the generation of spacers (Bolotin et al., 2019). Cas9 has two nuclease sites essential for cutting out foreign DNA. In contrast to Cas7, Cas9 is necessary for a bacterium to remain immune (Makarova et al., 2006). The cooperation of repeats, spacers and Cas are key elements of the adaptive immune system of microbes. The underlying mechanism of these elements needed further investigation.

2.2 The components that regulate CRISPR-Cas

By individually knocking out each component, scientist showed a cascade is necessary for cleaving CRISPR locus precursor RNA. The cleavage, results in a 61 -nucleotide-long CRISPR RNA (crRNA). They found that all crRNAs started with the preceding eight base-pairs of a repeat, followed with the complete sequence of a spacer and ended with the beginning of the next repeat. They were thereby confirming the earlier hypothesis that palindromic repeats influence the secondary structure (Sorek, Kunin, & Hugenholtz, 2008.). crRNA binds to complementary foreign DNA and leads to recognition of the invading sequences by the Cas protein. Recognition by Cas is not enough to make a cut.
The Cas protein will not cleave or bind successfully to the target DNA sequence if it is not followed by a the proto-spacer adjacent motif (PAM) sequence. PAM is a 2-6 base pair DNA sequence directly following the DNA sequence targeted by the Cas protein. PAM is not a component of the bacterial CRISPR locus but a component of the invading virus or plasmid. The Cas protein cuts a DNA blunt end three base pairs upstream of PAM. Thereby proofing that Cas9 cuts invading DNA at the specific position that is guided by the CRISPR RNA sequence (Barrangou, 2007).

Another small RNA transcribed from a sequence directly next to the CRISPR locus called trans-activating CRISPR RNA (tracRNA), consists out of 24 base-pairs. These pairs are almost in perfect complementary with the CRISPR repeats. Leading to the conclusion that tracRNA is essential for the alterations and maturation of crRNAs and in the end for the function of CRISPR (Deltcheva et al., 2011). In later studies, tracrRNA showed to have also a vital role in the ability of Cas9 to cleave DNA (Jinek et al., 2012). A better understanding of the underlying mechanism of CRISPR-Cas is necessary for the application in vitro and effectually in vivo.

2.3 CRISPR-Cas as custom gene editor

Siksnys and his colleagues showed it was possible to reprogram Cas9 with a spacer custom-designed sequence that can target a DNA site of their choosing (Deltcheva et al., 2011). Around the same time, Jinek and Charpentier came to the same results. Also, they demonstrated that crRNA and tracRNA could be fused to a single-guide RNA(sgRNA) (Jinek et al., 2012). These sgRNAs would become broadly used in the genome-editing field, because it made CRISPR efficiently in vivo. Many in vitro and some in vivo experiments were conducted at this moment, but mammalian cells were yet to be altered by CRISPR-Cas. In contrast to bacteria and other microbes, eukaryotic cells have a less accessible genome and a different internal environment. Certain boundaries had to overcome. Firstly, A more reliable and better-distributed Cas9 in *streptococcus pyogenes* than other *streptococci'* derived Cas9 was found. Secondly, it was verified that mammalian cells could process crRNA. Finally, the testing of a broad range of tracrRNA isoforms to find one that was stable in human cells. Combining these findings, scientist were able to target 16 sites in the mouse and human genome (Cong et al., 2013). These findings had a tremendous impact and demonstrated the ability to mutate specific genes with high accuracy and efficiency in mammalian cells.

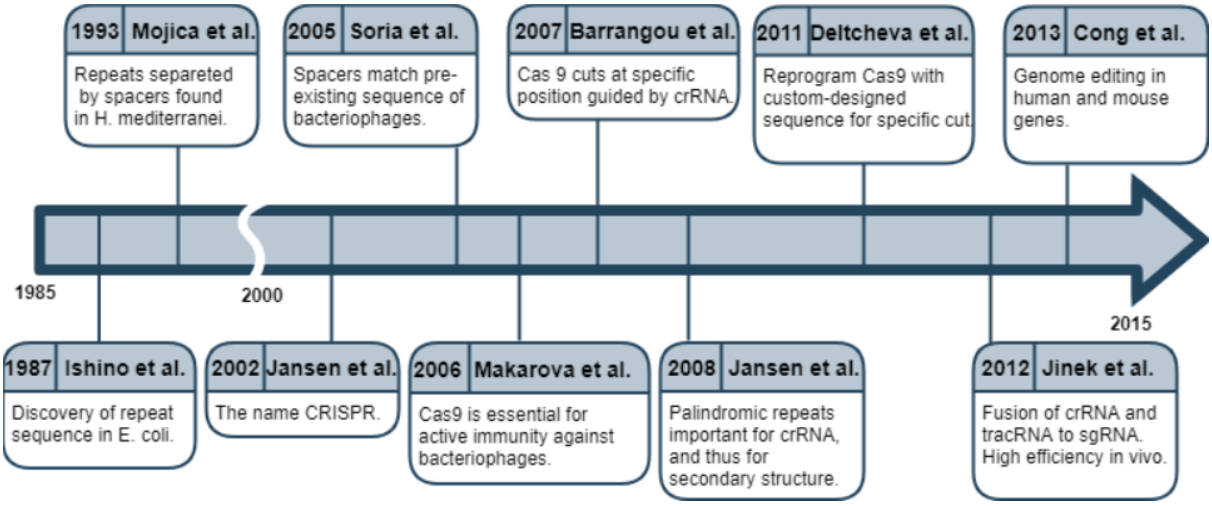

Figure 2| Overview timeline of important discoveries of CRISPR.

3 Applications of CRISPR-Cas

3.1 Mechanisms of programable CRISPR-Cas

CRISPR-Cas equips microbes with an immunological memory of prior infections by mobile genetic entities and bacteriophages. The genetic information lays in spacers between repeating segments. Immunity rises from transcription and processing of crRNAs containing the spacer's genetic information of the pathogen, this Cas-type RNA-guided nucleases (RGNs) will bind to the corresponding sequence of the invader. The complex binds, the target site is cleaved by two Cas proteins that form a trans-acting (Figure 3a) (Hille & Charpentier, 2016).

Two major classes of CRISPR-Cas systems have been identified in prokaryotes. The division is based on the organization of the effector's components. Class 1 CRISPR-Cas systems utilize multi-protein effector complexes consisting of 3 types (I, III, and IV). Class 2 systems employ single protein effectors consisting of 3 types (II, V, and VI). Each type has its own specific corresponding Cas protein and target (Table 1) (Brokowski et al., 2019). RGNs are the first easily adjustable nuclease system that behaves as ribonucleoprotein particle s(RNPs), a complex formed between RNA and RNA binding protein, and uses Watson-Crick base paring for sequence recognition (Gasiunas et al., 2012). CRISPR Cas is a basis for a new range of DNA modifiers by combining additional functional domains to the RNP components (Patsali et al., 2019). The current molecules include introduction of NHEJ for knockouts, knock-in, epigenome modifiers, base editors (BEs) and transcriptional regulators (Patsali et al., 2019).

		n of subtypes	Cas endonuclease	Target
Class I	type I	7	Cas 3	DNA
	type III	4	Cas 10	DNA/RNA
	type IV	1	--	--
Class II	type II	3	Cas9	DNA
	type V	3	Cas12	DNA
	type VI	3	Cas 13	RNA

Table 1| Class I and Class 2 CRISPR-Cas systems.

3.2 Knockout and knock-in

CRISPR-Cas is an effective way to generate targeted loss-of-function mutations at a specific gene site. With gRNA Cas9 can be programmed to pair at any genomic sequence and introduce a DSB. The only requirement is the presence of PAM at the 3' end of the target sequence on the loci. A DSB in mammalian cells are predominant repaired by NHEJ. NHEJ is error-prone and can lead to base deletion and insertions (indels). Therefore, CRISPR-Cas induced NHEJ repair is useful in generating gene knockouts through the initiation of frameshifting indels into protein-coding sequence, leading to non-functional protein products and premature translation termination or to the deterioration of mRNA through the activation of nonsense-mediated decay pathway (Spiegel et al., 2019).

DSB error-prone repair can generate indels but can also lead to the insertion of exogenous DNA molecules either randomly or using homology arms. CRISPR-Cas can introduce DSB and is therefore a tool to knock-in genetic elements and to allow in frame insertions, with as purpose to fuse a tag to the open reading frame (ORF) of interest or to introduce a desired mutation at a precise location. The most abundant used technique for in frame knock-in is to trigger the insertion of a donor DNA flanked by homologous sequence to both sides of the DSB. CRISPR-Cas induced knock-ins are routinely used for homology directed repair (HDR) in cell culture, because of the advantage gained by antibiotic selection of correctly edited cells. HDR-based integrations are significantly less efficient in model animals like zebrafish or mouse (Albadri et al., 2017). CRISPR induced knock-in can also be used, for example, to tag endogenous proteins with a luminescent peptide to monitor the production of an introduced protein for example (Madsen & Semple, 2019).

3.3 Deactivated Cas

The introduction of a point mutation on the nuclease domain can render Cas inactive, leading to a deactivated Cas (dCas). The dCas molecules is deprived of the ability to cleave target DNA but retains the ability to bind. dCas can act as an epigenome modifier and can adjust the accessibility of a gene of interest. A CRISPR epigenome modifier consists out of a fusion of dCas9 with binding or functional enzyme domains, with the ability to recruit such enzymes, including histone acetyltransferase, histone and DNA methylase, and histone and DNA demethylase (Figure 3c) (Choudhury et al., 2016).
When dCas9 or other sgRNA fuses to a transcriptional activator or repressor domain CRISPR can be used as a transcriptional regulator of specific genes or a network of genes (Figure 3d) (Zetsche et al., 2016). CRISPR-dCas9 can facilitate to repression of transcription of genes by interfering at the elongation stage or by disturbing the correct formation of the RNA polymerase complex in the beginning step, both of them by steric hindrance (Lo & Qi, 2017). Regulated modulation of transcription created by an alteration of protein or RNA segment of the RGN, multiple genes can even get regulated by differential ligand-induction (Ferry et al., 2017). Such small artificial transcriptional regulators can be delivered in vivo by Adeno-associated virus (AAV) vector, with an activity of 24 weeks in target cells (Thakore et al., 2018). However, Actions of these altered CRISPR-Cas RNP molecules are not restricted to DNA, they can affect RNA as well. dCas9 are used as epigenome modifiers and transcription regulator. dCas9 is an example of other types of Cas broaden the applications of CRISPR.

3.4 Other types of Cas

Cas12a/Cpf1 has been recently harnessed for genome editing. Cas12a has distinctive features from Cas9. First, Cas12a recognizes a 5' T-rich PAM, different from the 3' G-rich PAM used by Cas9. Second, Cas12a is a single crRNA-guided endonuclease, in contrast with Cas9 which is guided by a dual-RNA system consisting of a crRNA and tracrRNA. Third, after cleavage Cas9 introduces blunt ends within the PAM-proximal target site, whereas Cas12a generates staggered ends distal to the PAM site. The diverse properties of Cas12a system provides an expansion of the CRISPR toolbox. However, more research is necessary to improve Cas12a efficiency (Teng et al., 2019).
Cas13 is a recent discovery and is used in applications for posttranscriptional RNA-editing. In addition, the recent CasRx engineered protein shows promising results in specific RNA cleavage in human cells (Figure 3e). Cas13 is relatively small in size and is therefore deliverable by AAV vectors (Hsu et al., 2019).
dCasRx can regulate pre-mRNA splicing (exon splicing) and operate as a splice effector(Figure 3f). (Hsu et al., 2019). It can edit RNA to change adenosine into guanosine (Cox et al., 2018). Modifications of Cas13-type molecules can also be exploited as BEs (Figure 3g).

3.5 Base editors

BE makes use of a CRISPR-Cas9 complex with nucleotide specific chemical modifiers; additional helper-components are added to accomplish chemical alteration of DNA bases (Rees et al., 2017). A mutated Cas9 with a nickase activity, the ability to introduce a single-strand cut with the same specificity as a regular CRISPR-Cas9, is made more efficient by inhibition of the repair mechanism of the altered base.

This modification will be copied by the opposing strand, resulting in a double-stranded sequence change without the need of a DSB and therefore minimalizing the change of a recombination event (Figure 3b) (Kim et al., 2018). Scientists are able to (without a DSB) precisely convert four codons (CAG, CAA, TGG and CGA) into a stop codon, what leads to the ability to turn 97%-99% of known protein-coding genes in the human genome into functional knockouts (Billon et al., 2017). BEs have a very high therapeutic potential for altering a single-base mutation. In particular, adenine-deaminase BEs with the ability to correct G > A mutation, these mutations are accountable for almost half of all known single nucleotide pathogenic polymorphisms in humans (Gaudelli et al., 2018). However, these BEs are only able to catalyse base transition, In other words; conversion of a pyrimidine (a heterocycles aromatic ring structure) to another pyrimidine of a purine (a double heterocycles aromatic ring structure) to another purine. BEs cannot catalyse base transversion, substitutions of pyrimidines with purines and the other way around, combined with a limited editing window and PAM restrictions make it only available for a small portion of single-nucleotide mutations (Patsali et al., 2019).

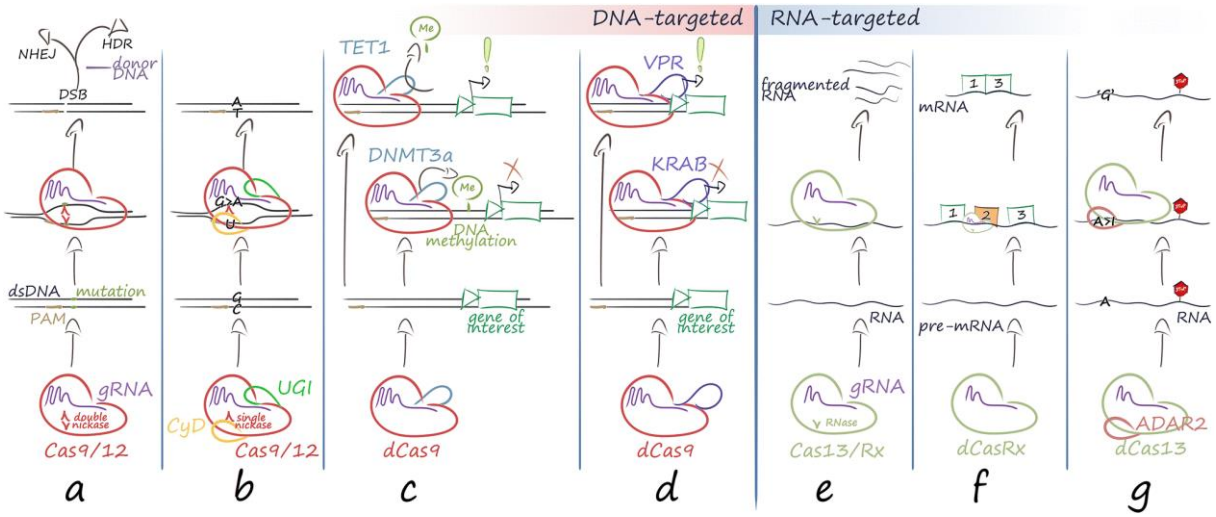

Figure 3| CRISPR-Cas9 based techniques tools for selective DNA/RNA editing. Types of modification depend on functional domains to the RNP components. (a) RNA-guided nucleases introduce a DSB at the PAM site of the sequence specified by the spacer. DSB trigger repair by NHEJ or HDR. (b) BEs introduce a permanent sequence change without DSB. First the conversion of C to U is shown. Resulting in the establishment of a T-A base pair after DNA replication. (c) Epigenome regulators dCas9 can recruit histone-modifying enzymes, such as TET1 DNA demethylase or DNMT3 DNA methyltransferase, which remove or add a methyl group. Resulting in an activation or deactivation respectively. (d) dCas9 Transcriptional regulators can recruit transcriptional activator or repressor domains to non-permanent modulate gene expression. (e) Posttranscriptional regulators Cas13 can execute targeted RNA cleavage without restriction of a PAM site. (f) dCasRx operates as a pre-messenger RNA splicer. (g) RNA BE functionates analogue to DNA BE it can convert adenosine to inosine, resulting in a A>G during translation (Patsali et al., 2019).

4 CRISPR-Cas out in the field

4.1 CRISPR-Chip for diagnostic and clinical application

A promising prospect for diagnostics is the so-called CRISPR-Chip. Consisting out of enhanced graphene-based field-effect transistor (gFET) termed CRISPR-Chip. It functionates as a biosensor combining the specific gene-targeting abilities of CRISPR-Cas9 with sensitive detection properties of gFET, to achieve fast, selective and facile detection of a sequence of interested within intact genome DNA. The thee-terminal gFET, makes use of functionalized graphene, dCAS9 CRISPR complex, to channel between the drain electrodes and the source. It scans the whole genomic sample by unzipping the double helix at PAM (Mekler et al., 2017). In the current research, scientist been able to show Duchenne muscular dystrophy associated gene sequences in genome samples. In only a quarter of an hour, with a sensitivity of 1,7 fM, without making use of additional amplification, a significant output of signal was observed in contrast to the low output of the lacking target sequence (Strecker et al., 2019). CRISPR-Chips further expand the broad application of the CRISPR technology with the ability to on-chip electrical detection of nucleic acids. In the future, it could enhance the efficiency and ease in diagnostics and clinical application. In addition to diagnostics purposes of CRISPR, this technique is able to actively avert diseases if the genomic sequence is known.

4.2 Scientific research in human diseases

CRISPR-Cas is not only a subject of research but also a reliable tool to conduct research. By introduction of knockout and knock-in organism models can gain specific genetical traits that for example mimic human diseases. To obtain these designer animal models has become relatively effortless. A researcher can order online which gene he wants to alter in what kind of organism, then receive the CRISPR requirement to obtain these trades over mail. This makes CRISPR-Cas accessible and leads to practical applications. In diseases like ALS and Duchenne muscular dystrophy, an improved muscular function in animal models is introduced by CRISPR therapy, suggesting a possible treatment for humans (Gaj et al., 2017). CRISPR is also a viable tool in the research of cancer treatment. For example, KRAS is a mutated oncogene and one of the most frequently present in cancers. Introducing a spCas9 system with a sgRNA targeting the mutant allele resulted in an inhibition of the mutant KRAS gene expression, leading to a hold of the cancer cell proliferation. This finding shows a potential CRISPR-based treatment of cancer with driver gene mutations (Gao et al., 2020).
Other treatments surpassed animals-studies and entered clinical trial. A Treatment against cancer uses CRISPR-Cas9 (and TALENs) to enhance T-cell response in immunotherapy (Levi et al., 2017). The first clinical trial of injection CRISPR-Cas9 altered T cells in human patients with aggressive lung cancer occurred by Chinese oncologist at Sichuan University at the West China Hospital (Yang et al., 2019).
Not only somatic cells are altered, CRISPR is also used in human germline cells and embryos. Scientists have shown in recent research that point-mutation in HBB and G6PD can be corrected in human zygotes (Tang et al., 2017).
Dr Jiankui He of the Southern Chinese University of Science and Technology has claimed to create the world's first "HIV-immune" babies using CRISPR-Cas, which shocked the scientific community (Wang et al., 2018).

Twin girls were born to an HIV-negative mother and an HIV-positive father, through in vitro fertilisation (IVF) treatment in combination with CRISPR-Cas9 genome editing. The CRISPR treatment was targeted on the CCR5 genes of the zygote. The chemokine receptor CCR5 is linked with the attachment process of HIV. The treatment results phenotype identical to the naturally occurring CCR5/ Δ32 mutation and permanently guarantees in an absence of CCR5-expression on HIV target-cells, resulting in a cellular immunization from infection due to the virus is unable to enter the cell (Matos et al., 2019). Editing the genome of early embryos or germ cells are still in the stage of basic research, its validity and safety need to be fully evaluated. Considering an incomplete scientific validation and unpredictable safety risk, Jiankui He and his colleagues have not adhered to scientific integrity and ethical norms. Regardless of the ethical consideration of the HIV-immune babies, alteration of the CCR5 gene by CRISPR is a promising technique for the treatment for HIV.

4.3 CRISPR and infectious diseases

Infectious diseases caused by viruses, bacteria, and parasites still account for a quarter of deaths worldwide and the leading cause of death in low income countries (WHO., 2020). In addition, infectious diseases have a persistent potential for destabilizing pandemics. CRISPR-Cas9 is identified as an adaptive immune system for bacteria against invading viruses and foreign plasmid DNA. the system can be reprogrammed to fight bacterial pathogens with their own defence. CRISPR can immunize bacteria against the spread of multidrug-resistant plasmids or target the drug resistance genes directly (Doerflinger et al., 2017).
CRISPR could be an effective against parasitic Malaria disease as a sterile Insect technique (SIT) agent. The insect pest control via SIT has been available for several decades. SIT is commonly done by irradiation whereby the males are sterilized and mass released. These sterile males mate with wildtype females and will not generate offspring, creating a decrease in population for the next generation (Zhu et al., 2000). Repeating this process will eventually reduce a population to a manageable size. Irradiations is very costly and time-consuming work. Sterilising insect pests with CRISPR is more efficient and in multiple organisms inducible. It could eradicate multiple mosquito-transmitted diseases in the next decade (Häcker et al., 2018).
Another interesting way of controlling mosquito-transmitted diseases by CRISPR is the gene driver technology. An individual with a single loss-of-function allele normally will have a wildtype phenotype appearance, while an organism with a homozygous copy of the allele will show a mutant phenotype. With the method (referred to as) mutagenic chain reaction (MRC), integrating the CRISPR-Cas9 editing system for generating autocatalytic mutation to introduce a homozygous loss of function mutations (Agustian et al., 2014). There are four requirements to introduce an autocatalytic insertional mutant: A Cas9 gene, a gRNA targeted to a sequence of interest, payload gene, and homology arms flanking the Cas9/gRRA sequence matching the two sequences immediately adjected on both sides of the target cleave site. Following these four components: a plasmid containing the Cas9 should cut the genomic target at the site of interest determined by gRNA and insert a copy of the Cas/gRNA sequence into the locus via HDR. The inserted Cas/gRNA will repeat the same procedure to the opposing allele (Figure 4) (Champer et al., 2016). The parental organism with the gene driver will always pass the gene to its offspring. In the offspring, this gene will trigger an MRC and transform the heterozygote altered allele into a homozygous genotype. Gene drivers have the potential to distribute beneficial genes in a wild population or to reduce harmful species.

In addition to human disease prevention by pest control, agriculture could benefit by SIT and gene drivers by targeting organism that threaten crops.

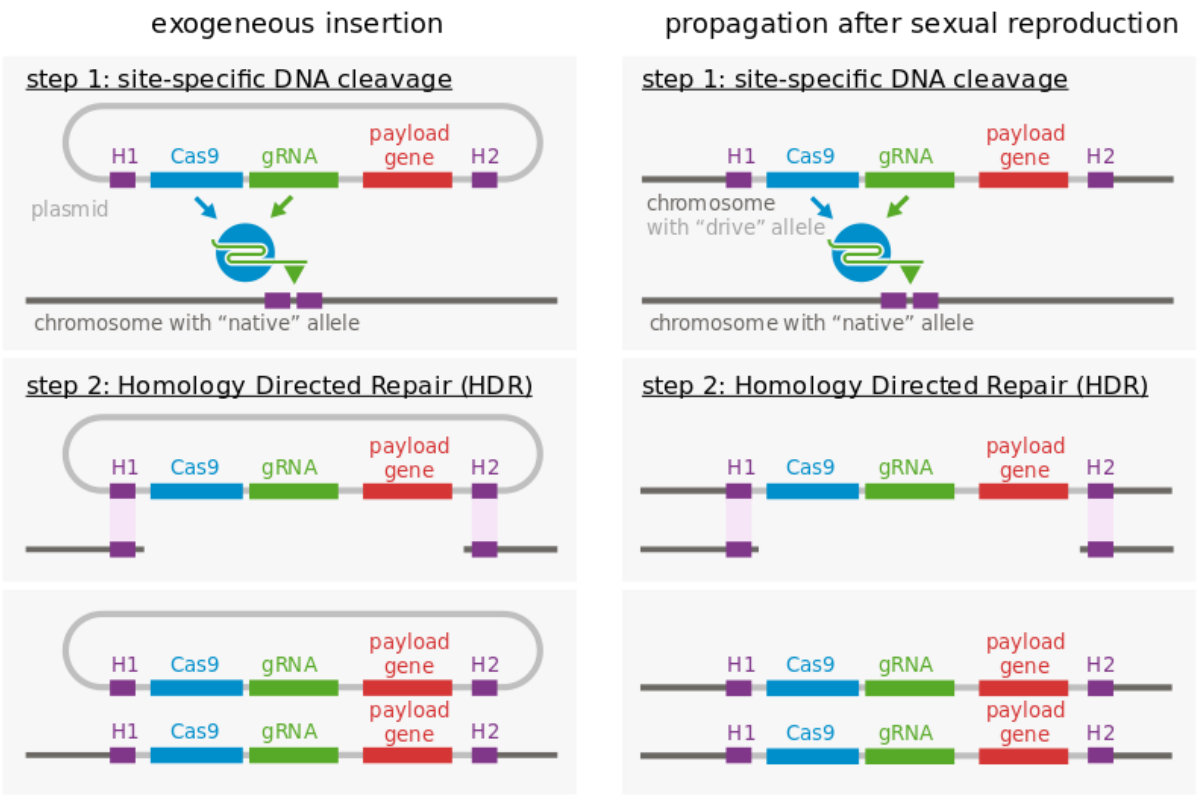

Figure 4| Molucular mechanism of gene drivers. Exogeneous insertion Step 1 Insertion of gene driver by a plasmid. Plasmid contains: A Cas9 gene, a gRNA targeted to a sequence of interest, payload gene, and homology arms. Step 2 insertion copy of the Cas/gRNA sequence into locus via HDR. Propagation after sexual reproduction Insertion of Cas/gRNA into native allele via HDR **(Unckless et al., 2017).**

4.4 Agriculture

In the agricultural field, many CRISPR-based techniques are already established. CRISPR-Cas based techniques cover almost all areas of genome engineering applications in plant cells. It can enhance nutritional value, improve genetic traits, DNA/RNA viruses can be dealt with by either direct interference with the viral genome or the editing/targeting host genes, resistance against bacteria such as Citrus canker, pest resistance, certain fungi (such as powdery mildew and rice blast) (Hameed et al., 2019).

More than 300.000 plant species are on our planet, under 200 of these species are used commercially. Furthermore, only three species deliver most of the energy for animal and human consumption: Corn, rice and wheat (Massawe & Mayes, 2016). These 200 species have genetical treats in common, so-called domestication genes. Domestication genes are causing a favourable plant architecture, for example, abiotic stress tolerance, larger fruits and simultaneous flowering (Østerberg et al., 2017).

By targeting these specific genes with CRISPR it is possible to increase the domestication of uncultured species. One example of this technique is done on an ancestral tomato relative called Solanum pimpinelifolium that has an astounding higher degree of stress tolerance than today's domesticated tomatoes (Zsögön et al., 2018). CRISPR-Cas 9 targeted six domestication genes with the result of significant multiplication of fruit number and nutritional value within a single generation. Domestication of new species and increase the defences of existing crops can lead to an increase in food production in harsh environments and lower waste and the use of pesticides.

5 Discussion

5.1 Overview

With selective breeding, humans made their first alteration in organisms' genome. In the last two centuries the field of genetics developed and artificial alteration for genes were adopted. Artificial genome editing often makes use of DSB with NEJH repair mechanism, either induced by chemicals (or other external factors) or by nucleases. Recent techniques like ZFN, TALEN and CRISPR-Cas greatly increase the plasticity, efficiency, precision and effectiveness. They induce a specific cut at a sequence of choice, where they can remove or alter the genome. CRISPR-Cas being the most flexible and efficient recent technique.

The discovery of CRISPR started with findings of repeating sequences in distance microbial relatives. These repeats are of great importance to the adaptive immunity of microbes. The genetical information of pathogens are stored in spacers. In microbes, CRISPR-Cas proteins are constantly scanning the genome of the host in search for a match, followed by a deletion of these harmful sequences. It is possible to reprogram CRISPR with a custom-designed spacer sequence to target a DNA site of choice.

CRISPR-Cas has many applications the different functional domains can introduce NHEJ for knockouts, knock-in, epigenome modifiers, base editors and transcriptional regulators, other functional domains can interact with RNA rather than DNA. Newly found techniques can edit the genome without the use of a DSB. CRISPR can be introduced in mammalian cells by making use of AAV. The continues search for different Cas protein will lead to more application in the future.

Numerous techniques have already applications in the field. The CRISPR-Chip acts as a biosensor combining the specific gene-targeting abilities of CRISPR-Cas9 with sensitive detection properties of gFET, to achieve fast, selective and facile detection of a sequence of interested within intact genome DNA. CRISPR-Cas is not only a viable tool for diagnostics, but for research as a whole. CRISPR-Cas can make tailor-made animal models that optimize the drug/treatment research. The first claimed human germline alteration happened in China. Twin babies were immunized against HIV by mimicking the natural occurring CCR5/ Δ32 mutation. Other infectious diseases can be antagonist by targeting bacteria directly or by means of SIT and gene drivers. Gene drivers have the potential to alter the genome of a population or even the complete species. In addition, the agricultural sector benefits from pest targeted such techniques. Agriculture can also benefit from CRISPR by targeting bacteria or making plants more resilient and nutritious. Therefore, CRISPR can be of help in for the world's hunger problem, reducing pests and antibiotics, and other environmental problems.

5.2 Ethical consideration regarding CRISPR

5.2.1 Risk-benefit ratio

An important factor in ethical decisions involves assessing potential risk-benefit ratios, with the goal to maximize the benefit while minimizing the risk. In addition, the range of possible outcomes and consequences need to be considered as well. Firstly, there are concerns about the technical limitations and capability of CRISPR. These include the possibility of incomplete editing, inaccurate on- or off-target editing, and limited on-target editing efficiency (Peng et al., 2016). Perhaps in the future, these concerns become absolute, due to the incredible pace of development, as of this moment these worries are relevant. Secondly, what about the future of the modified organism. Whether the alterations are indefinitely. When these organisms reproduce will their offspring have the same traits, affecting them in unexpected ways. Finally, biological systems are very complex, the relation between genetic information and the outcome of the phenotype is not completely understood. One alteration in a gene in the germline or somatic cell can have many unforeseen side effects. Most biological traits come to expression by complex regulatory actions of numerous genes. As long as the biological outcomes of gene expression and modifications are uncertain, it is difficult to assess potential risk-benefit ratio. This forms a problem on its own and makes efficient ethical decision making harder.

Leading to the need of more well-regulated and monitored research in the foundation of CRISPR before it can be fully implemented in organisms. The possibility also exists with continuous research that a novel genome editing agent will be discovered or invented with a far better risk-benefit ratio, making CRISPR obsolete.

5.2.2 CRISPR and society

Other important ethical questions arise by enhancement of livestock, crops, human features and gene drivers. Livestock and Crops enhancement by CRISPR could be of great importance for humanity. In many cases, the general opinion opposes GMO's. As a result, producers could have reservations about applicating CRISPR. A better understating of the advantages and risks of GMO's to the general public lead to an increase in utilization. CRISPR can improve nutrient content in food. It has the potential to fortify foods efficiently for a great deal of the human population suffering from a lack of basic nutrients. In addition, a concern arises by this beneficial feature will such foods be available and accessible for those who need it. Other problems could occur by adjusting the genetical genome of livestock and crops. The goal is to increase fitness, making them stronger and more resilient to specific pathologies. If these genetically altered livestock or crops infiltrate the wild, they might be more successful than wildtypes. This could lead to disturbance in the ecosystem. Leading to the extinction of other animals and other ecological disasters.

Gene drive technology has unprecedented potential. This remarkable effective technology could have a great impact on public health, agriculture, research and species conservation. However, this technology has many expected and unexpected risk and consequences that need to be fully considered. The nature of a gene drive is to affect eventually every individual in a species. Hence, it is advisable to intergrade an off switch: control, inhibit and eliminate the gene driver.

The most pressing ethical concern is about the enhancing of human features such as vision, muscle mass, height, or cognitive function like memory or learning. This question becomes particularly difficult to answer when the distinction between medical necessity and improvements to meet the standard is unclear. In time genes could be edit that lead to the reduction of bad cholesterol. This hypothetical scenario would be beneficial for the individual and society eventually but is difficult to classify as an enhancement or medical need. At this moment it is not allowed to engage in any kind of genetical enhancement. However, in the future genetic modification of a person who might transfer edited genome to future generations could be allowed following specific conditions and strict regulation: treatment for serious genetic diseases (with no alternative treatment), for basic research, to treat other serious diseases (The National Academies of Sciences Engineering and Medicine, 2017). Yet another concern arises regarding the enhancement of human features when editing in the human germline. The understanding of human embryo status is incomplete and therefore it very hard to state the precise moment an embryo achieves 'personhood'. As of this moment, the status of embryo itself prohibit all type of modification (Zhang et al., 2020).

5.2.3 Accessibility of novel CRISPR technology

Ethical concerns arise about the distribution of CRISPR across society. Almost all new biomedical development needs to be profitable for their stakeholders. Therefore, the products will be very costly as long as the patents are valid. Making novel CRISPR costly therapies only available for a select group of people across society. Many CRISPR researches are partially funded by the government, and thus taxpayers. To deny potentially lifesaving features of the technology to the people who funded most of the research is unethical. Furthermore, if such technologies would give wealthy individual a greater advantage above people who can't afford it, the gap between rich and poor could become even bigger. This problem is not unique to CRISPR technology. However, unaddressed continuation of extravagant high pricing could cause psychological, physical and economic harms. Encouraging the formation of anti-price gouging laws, to dam some of these concerns.

5.3 Prospects of CRISPR

5.3.1 Germline editing

When regulations become clearer and reliability and safety of CRISPR increases the prospects are numerous. The CRISPR-Chip or other novel diagnostical techniques could scan germline of potential parents or infants very rapid, in search of any genetical abnormality or heritable disease. When an abnormality is found, selective genetic alterations can be made. Eventually when enough people cure their abnormalities a group like immunity will occur, leading to an insufficient abundance of genetical trademarks for the appearance of certain inheritable diseases. In addition, the human genome will be enriched by an introduction of a vaccine-like DNA sequence. This sequence contains for example: 1) Common sequences of pathologies and a CRISPR Cas property functioning as an addition to the immune system, mimicking bacterial immune system; 2) epigenome modifiers to upregulate healthy beneficial biological processes and downregulate negative processes; 3) machinery to repair common acquired mutations or autoimmune diseases.
The human germline or zygote can be altered to enhance human features. Increasing strength, intelligence, cognitive functions, metabolism, desirable aesthetics, endurance, resistance or vitality. Evolution could be made absolute. Genetical desirable changes with a better survival rate can be done in a lab in a fraction of the time evolution needs. Potential parents could, in theory, compose a baby with features of their desire. However, such services should be very strictly regulated by governments. With increasing improvements in safety and efficiency, the possibility of such services become more realistic followed by supply and demand. Making this hypothetical scenario to be considered by future generations.

5.3.2 Applications in plants, animals and humans

CRISPR has the ability to cause important changes in sustainability and nutrition. In search of novel properties, it could be of interest to investigate the genetic diversity in uncultured and wild species. With CRISPR-Cas domestication in a fraction of time by targeting domestication genes. In addition, favourable properties could be isolated in one specie and integrated in, for example, a faster-growing or easier to manage specie. Features of plants used for chemicals, industry or medicine can be upregulated to make extraction more efficient. The same concept could be used in animals or bacteria for similar principles. Animal pest threatening crops or human health can be dealt with the use of gene drivers. Infectious diseases such as malaria could be eradicated by targeting the vector. Organisms can be altered to closely mimic human features for animal models in research. In addition, animals with increased humane features could be utilized for xenotransplantation to minimalize rejection. Possibly, CRISPR could replace specific recognition genes in donor organs with genetical information of the receiver to lower the change of rejection. Further, altering recognition genes could be of use in autoimmune diseases.
CRISPR based cancer therapies have a promising future, already some therapies entered clinical trials. Therapies could target many aspects of the cancer lifecycle: development, proliferation, differentiation and invasion. By epigenomic interference, up or down-regulation can be modulated for tumour suppressor genes and oncogenes respectively. This interference can lead to a reduction of tumour growth. Other epigenomic treatments can upregulate the immune system to increase the autonomic defences mechanisms against cancer. Cancerous cells could be attacked directly by CRISPR therapies to crippling the genome of these cells, resulting in activation of the apoptotic pathway.

5.3.3 Healthy ageing

As organisms age biomolecular processes change leading to senescence of cells. Many age-related dysfunctional processes find their origin in the DNA; genomic instability, epigenetic alteration, stem cell exhaustion and telomere attrition. Future CRISPR techniques may have valuable features to slow or even revert these processes. CRISPR could be employed to regulate and stimulate juvenile biological processes. Embryonic gene activity could be simulated to promote organ renewal or regeneration of other tissue. The life expectancy of people is increasing, correlating with a growth in the number of people affected by neurodegenerative diseases. CRISPR may play a role in the treatment or prevention of these diseases. A few hypothetical examples; 1) Modification of genes related or responsible for Alzheimer's; 2) Activating genes responsible for the production of dopamine in the brain by Parkinson: 3) Cleave out excessive repetitive repeats by Huntington disease; 4) Introduction of genetical information for novel enzymes that have the ability to cleave protein aggregations in the brain. CRISPR has the ability to grand people a higher life expectancy and an extension in years of good health.

6 Conclusion

CRISPR Cas is discovered in bacteria, where it functionates as a microbial immune system. CRISPR is divided into repeats and spacers, within the spacer genomic information of plasmid conjugation or bacteriophages are stored. The genome of bacteria is actively scanned by CRISPR proteins, when a match between the spacer and the genome is found Cas proteins cleave out this section and neutralizes the pathogen. By modifying the spacer sequence in a sequence of choice, CIRPSR Cas9 can be reprogrammed to make adjustments at any desirable location in the genome. The use of sgRNA made CRIPSR Cas9 available for in vivo usage. Building the foundation for CRISPR as custom gene editor.

Depending on the functional domains combined with RNP components CRISPR can modify the genome in diverse fashions: introduction of knockouts, knock-ins, BE, epigenome modifiers, and transcriptional regulators. Based on these principles CRISPR finds applications in plants, animals and humans. Plants can be reinforced to resist hash environments and increase nutrition. Pest threatening plants and human health can be targeted by gene drivers. With CRISPR Cas edited animal can be used as reliable animal models in human disease research. CRISPR based therapies have been applied in cancer patient and human germline. CRIPSR has a promising future. The increase of effectiveness and efficiency will lead to a broader application of techniques. The human germline could be altered to eradicate hereditary diseases. Favourable traits can be inserted to enhance human features, both cognitive and physical. In addition, the epigenome can be regulated to promote beneficial biological processes. By selective targeting biological processes responsible for senescence aging could be slowed or even reversed. However, for many techniques are applied to both humans and other organisms more research is necessary to confirm the quality and consequences of CRISPR. Regulation surrounding CRIPSR should be very strictly monitored by governments or on global scale to prevent misuse. The CRISPR technology may give humans the ability to set evolution to their hand, we have to act responsible.

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