UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

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Dissertação para obtenção do Grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação:

Professora Doutora Leonor Cancela - orientadora

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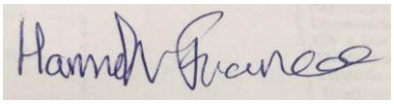
Declaração de autoria de trabalho:

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Universidade do Algarve, 2018



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RESUMO

O sistema CRISPR (do inglês: clustered regularly interspaced short palindromic repeats) faz parte de um processo de imunidade adquirida existente nas bactérias, sendo constituído por sequencias de bases de ácido desoxirribonucleico (ADN) curtas e repetitivas que fornecem uma imunidade adquirida contra vírus e plasmídeos tendo como alvo o ácido nucleico dos invasores de uma forma específica. Os loci CRISPR consistem num arranjo de sequências repetitivas curtas de aproximadamente 30-40 pares de bases e parcialmente palindrómico, intervaladas por sequências de 'espaçador' igualmente curtas de origem viral ou de plasmídeo. Os espaçadores fornecem um registo genético de uma infeção anterior, o que permite ao hospedeiro prevenir futuras invasões do mesmo agente infecioso.

A Cas9 é uma endonuclease de ADN, multifuncional, com aproximadamente 1,368 aminoácidos. Esta endonuclease cliva ADN de cadeia dupla, 3 pares de bases a montante do Motivo Adjacente ao Protoespaçador (do inglês: Protospacer adjacent motif, PAM) através de dois domínios distintos de nucleases. Tem um domínio de nuclease tipo HNH que cliva a cadeia de DNA complementar à sequência guia de ácido ribonucleico (ARN), também conhecida como a cadeia alvo, e um domínio de nuclease tipo RuvC responsável por clivar a cadeia de DNA oposta à cadeia complementar. Adicionalmente, a Cas9 também participa na maturação do ácido ribonucleico CRISPR (do inglês: CRISPR ribonucleic acid, crRNA) e da aquisição do espaçador.

A edição de genoma é um tipo de engenharia genética na qual ADN é inserido, deletado ou substituído no genoma de organismos celulares através de nucleases programáveis e altamente específicas como a CRISPR/Cas9. O sistema de CRISPR/Cas9 depende de ARNs pequenos como o crRNA, crRNA de ativação em trans (do inglês: trans-activating crRNA, tracrRNA) e ARN guia (do inglês: single-guide RNA, sgRNA) para clivagem de sequências específicas de ADN. A Cas9 necessita apenas de uma sequência de 20 nucleótidos no sgRNA que se emparelha com os pares de bases do ADN alvo e a presença de um PAM adjacente à região de complementaridade.

As quebras de cadeias duplas induzidas por nucleases podem ser reparadas por ligação de extremidades não homólogas (do inglês: Non-Homologous End-Joining, NHEJ) e por reparação

direcionada por homologia (do inglês: Homology Directed Repair, HDR). As modificações baseadas em NHEJ são propensas a erros e incluem pequenas inserções ou deleções (indels). Por outro lado, as modificações baseadas em HDR usam um modelo de ADN nativo ou desenhado para substituir o alelo alvo por uma sequência desenhada pelo processo de recombinação. Existem outras vias de reparação de ADN que também podem produzir edições de genoma. Na ausência de um modelo de reparação, as quebras de cadeias duplas são religadas através do processo NHEJ, onde ocorrem mutações indel. No entanto, pode ser fornecido um modelo de reparação para promover o caminho HDR, que permite alta fidelidade e edição precisa.

Uma vez que o CRISPR acompanhado pela Cas9 pode ser programado para editar qualquer local de interesse no genoma, torna-se possível a correção de erros e mutações que o genoma possa ter sofrido, e ainda ativar ou desativar genes em células e organismos, de forma eficaz, rápida e económica. Desta forma, o processo de CRISPR/Cas9 tem sido aplicado em diversas áreas de interesse como medicina, biotecnologia, biologia e agricultura para controlar por exemplo a transcrição, modificar epigenomas, realizar rastreios genómicos, manipular circuitos biológicos facilitando a geração de materiais sintéticos, corrigir mutações genéticas e controlar a expressão de genes inteiros.

Especificamente, este mecanismo tem sido intensamente explorado para aplicação em patologias humanas como a investigação dos mecanismos responsáveis de diversas doenças, rastreio para descoberta de novos fármacos e desenvolvimento de novas terapias, diagnóstico rápido, edição *in vitro* e *in vivo* e correção de transmissões hereditárias. Atualmente, o CRISPR/Cas9 já tem sido aplicado em doenças sem atual cura clínica de origem viral como o vírus da imunodeficiência humana (VIH), vírus Epstein-Barr (VEB), vírus da hepatite B, papilomavírus humano (HPV), vírus de John Cunningham (VJC) e o vírus herpes simplex (VHS). Igualmente já tem sido utilizado em doenças genéticas sem cura como a talassemia β, fibrose cística, distrofia muscular de duchenne e doença de Huntington. A crescente resistência aos antibióticos também despoletou interesse na utilização do CRISPR/Cas9 nesta área. Por fim, esta tecnologia tem sido utilizada no estudo da área da oncologia visto que esta é uma doença devastadora de origem genética que provoca uma em cada seis mortes mundialmente e, infelizmente, o arsenal

terapêutico existente para o cancro tem limitações na sua capacidade de cura e muitos efeitos adversos relatados incluindo toxicidade.

Apesar do CRISPR/Cas9 apresentar resultados positivos e promissores em diversas patologias, este sistema tem algumas limitações tais como mutações fora de alvo, problemas com o sistema de entrega, dependência do PAM e questões éticas que devem ser ultrapassadas de modo a que seja possível progredir para estudos clínicos posteriormente.

Seguramente, o futuro desta nova tecnologia aparenta ser promissor e digno da atenção de toda a comunidade científica, visto que poderá possivelmente constituir a resposta ao tratamento muitas patologias humanas que neste momento são consideradas incuráveis.

No primeiro capítulo desta revisão bibliográfica, é apresentada uma breve introdução da história do CRISPR desde a primeira vez que foi descrito, passando pela descoberta da sua função em bactérias, até ao presente em que este método de edição genética é adaptado para uso laboratorial. Posteriormente no mesmo capítulo introdutório, são descritas as estruturas do CRISPR e da Cas9 e o seu respetivo mecanismo de ação. Por fim, ainda é descrita a transição da sua aplicação na edição do genoma a nível laboratorial que inclui também a comparação do CRISPR/Cas9 com os métodos de edição genética anteriores e a discussão sobre o desenvolvimento dos diversos métodos de entrega deste sistema ao local de ação pretendido.

No segundo capítulo são abordadas as aplicações gerais do sistema de edição genética do CRISPR/Cas9 em áreas como a medicina, biotecnologia, biologia e agricultura e, mais especificamente em algumas patologias humanas de origem viral, genética, bacteriana e ainda um subcapítulo dedicado somente às doenças oncológicas. É feita uma breve descrição das patologias abordadas, descrevendo a sua origem, o mecanismo responsável pelo desencadear da doença, os seus sintomas, assim como o seu tratamento e as limitações associadas e por fim a utilização do sistema CRISPR/Cas9 no desenvolvimento de novas terapias.

No terceiro capítulo são discutidas as limitações desta nova tecnologia incluindo as mutações fora de alvo, a sua dependência do PAM, as fragilidades dos métodos de entrega do CRISPR/Cas9 e as questões éticas levantadas pelo uso deste método de edição genética.

Por fim, no quarto e último capítulo são abordadas as considerações finais deste tema e as perspetivas futuras do CRISPR/Cas9.

Palavras-chave: CRISPR, Cas9, edição do genoma, patologias humanas

ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR) is an adaptive immune system of bacteria, of prokaryotic deoxyribonucleic acid (DNA) that contain short, repetitive base sequences which mediate acquired immunity by targeting viral DNA and plasmids in a sequence-specific manner. Paired with Cas9, a large multi-domain and multifunctional DNA endonuclease, this system can be programmed to edit nearly any genomic location of interest making it conceivable to correct genome errors while also permitting to relatively easily, quickly and at a reduced cost to turn genes on or off in cells and organisms.

The versatile CRISPR/Cas9 system has been getting increased applications in diverse areas such as medicine, biotechnology and biology. Recently, the possibility of using CRISPR/Cas9 in several human diseases has been thoroughly investigated as it may reveal to be a valuable addition to their therapeutic arsenal, displaying promising results. However, this system also has its limitations such as off-target mutations, delivery vehicle problems, PAM dependence and raising ethical concerns that must be surpassed in order to consider further its clinical applications.

The future of this new technology certainly seems bright and deserves a great attention of the scientific community as it may prove to provide the means to cure many high burden diseases currently affecting humanity.

Chapter one presents an introduction to CRISPR/Cas9, unfolding its history, describing its structure and associated mechanism and finally explaining the transition to its use in genome editing. In **chapter two**, the general applications of CRISPR/Cas9 are described as well as its specific applications for a defined set of selected diseases affecting mankind that can be targeted by this genome editing technique. The current existing limitations are discussed in **chapter three** and finally conclusions and future prospects for CRISPR/Cas9 are presented in a final section, **chapter four**.

Keywords: CRISPR, Cas9, Genome editing, human pathologies

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ABBREVIATIONS

AAV	Adeno-associated virus
AIDS	Acquired immunodeficiency syndrome
BART	BamHI-A rightward transcript
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Cas	CRISPR-associated system
CAR	Chimeric antigen receptor
cccDNAs	Covalently closed circular DNAs
CNS	Central nervous system
CPPs	Cell penetrating peptides
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNAs	CRISPR ribonucleic acid
dCas9	Deactivated variants of Cas9
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
dsDNA	Double-strand DNA
dsRNA	Double-strand Ribonucleic acid
EBV	Epstein-Barr virus
EHM	Engineered heart muscle
ELISA	Enzyme-linked immunosorbent assay

EU	European Union
E. col	*Escherichia coli*
gRNAs	Guide RNAs
HBB	Human haemoglobin beta
HBV	Hepatitis B virus
HBsAg	Hepatitis B Antigen
HD	Huntington's Disease
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HDR	Homology-directed repair
HEs	Homing Endonucleases
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSPC	Hematopoietic stem and progenitor cells
HTT	Huntingtin gene
iPSCs	Induced pluripotent stem cells
JCV	John Cunningham Virus
miRNAs	microRNAs
mRNA	Messenger Ribonucleic acid
NHEJ	Non-homologous end-joining
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction

PD-1	Programmed cell death protein-1
PML	Progressive multifocal encephalopathy
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
saCas9	Staphylococcus aureus Cas9
sgRNA	Single guide RNA
SRSRs	Short regularly spaced repeats
ssDNA	Single-stranded DNA
STI	Sexually transmitted infection
S. pyogenes	*Streptococcus pyogenes*
S. thermophilus	*Streptococcus thermophilus*
T-ag	T-antigen
TALENs	Transcription Activator-Like Effector Nucleases
TCR	T cell receptor
tracrRNA	trans-activating CRISPR RNA
USA	Unites States of America
WHO	World Health Organization
ZFNs	Zinc Finger Nuclease

1. INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR) is an adaptive immune system, used by bacteria, through which prokaryotic deoxyribonucleic acid (DNA) that contain short, repetitive base sequences mediate acquired immunity by targeting viral DNA and plasmids in a sequence-specific manner[1].

CRISPR/Cas9 is the basis of a genome editing technology that allows permanent modification of genes within organisms making it conceivable to correct genome errors while also permitting, relatively easily, quickly and at a reduced cost, to turn genes on or off in cells and organisms. The CRISPR/Cas9 system has been used for several laboratory applications including functional genomic screens, live imaging of the cellular genome and rapid generation of both cellular and animal models. The potential use of CRISPR/cas9 to edit human genomes would offer us the potential to cure any genetic disorder, and it is a promising field that is advancing at a noteworthy pace[2,3].

1.1 History

The first description of CRISPR was in 1987 from Yoshizumi Ishino who accidently cloned part of a CRISPR together with the *iap* gene, from a genome of *Escherichia coli* [4].

Later in 1993 Francisco Mojica at the University of Alicante, Spain, pioneered the studies of CRISPR while he was working on *Haloferax mediterranei*, an archaeal microbe with extreme salt tolerance[5]. Meanwhile, it was discovered that the salt concentration of the growth medium seemed to influence how restriction enzymes were cutting the microbial genome, and Mojica decided to describe the altered fragments[5]. In a DNA fragment examined, he found a structure with several copies of a palindromic repeated sequence of 30 bases, separated by spacers of approximately 36 bases that did not look like any family of repeats previously known. Mojica initially named it short regularly spaced repeats (SRSRs), but later it was renamed as CRISPR[5].

In 2000 Mojica classified interspaced repeat sequences as an exclusive family of repeat elements that usually occur in clusters present in 90% of archaea and over 40% of sequenced bacteria[6]. Two years later, Mojica and Jansen created the acronym CRISPR to unify the description of microbial genomic loci comprising an interspaced repeat array. At the same time Jansen observed several clusters of signature CRISPR-associated system (cas) genes typically adjacent to the repeat elements, but its function was still unknown[7].

The first clue regarding the biological function of CRISPR was unearthed in 2005, when three distinct research groups nearly concurrently saw that the seemingly random sequences separating identical CRISPR repeats showed homology to invasive DNA sequences, such as viruses and plasmids[8–10]. One of the research groups that observed this was Mojica's group. They removed each spacer and inserted it into to the Basic Local Alignment Search Tool (BLAST)[11] program to search for correspondences with any other known DNA sequence. Subsequently, they positively identified, in a CRISPR locus from an *E. coli* strain, one of the spacers which matched the sequence of a P1 phage that infected several *E. coli* strains. However, the strain carrying the spacer was known to be resistant to P1 infection. This made it clear for Mojica that this loci must encode an adaptive immune system that protected against specific infections [10]. Simultaneously, Bolotin and colleagues observed that the spacers, all have a similar sequence[8] at one end which would later end up to be called the protospacer adjacent motif (PAM)[12].

After suggesting CRISPR as an immune system in prokaryotes, the scientific community moved on to the demonstration of its biological function. In 2007, Barrangou and his team at Danisco (food ingredient company) had available the various pieces required to prove the theory that CRISPR is an adaptive immune system. They possessed in silico datasets containing the genomes of bacteria encoding diverse CRISPR–Cas systems and the genomes of bacteriophages able to infect them, the biological material, including bacterial isolates that carried active CRISPR–Cas systems and lytic phages that could readily infect these hosts and finally the information from the existing literature [13].

During attempts to develop molecular methods for strain differentiation of *Streptococcus thermophilus*, Barrangou discovered the existence of CRISPR loci. Initially, his team used CRISPR sequences to compare numerous *S. thermophilus* strains and observed the conservation of some

spacers across groups of strains. Curiously, strains clustered in groups for which the CRISPR genotypes associated with phage resistance phenotypes. Also, they noticed that occasionally strains that had been isolated at different moments in time did carry additional repeat–spacer sequences, suggesting phage-induced and time-dependent variability of CRISPR sequences[13]. Through the comparison of data between sequences of CRISPR loci of industrial strains and phage genome sequences, the link between the CRISPR genotype and the phage resistance phenotype became noticeable[13]. Subsequently, Barrangou and his team started a series of experiments that confirmed that CRISPR systems are indeed an adaptive immune system. Using a *S. thermophilus* strain, that was well-characterized for being phage-sensitive, and two bacteriophages, they performed genetic selections to isolate bacteria that was resistant to bacteriophages. Instead of developing resistance mutations, the resistant strains had acquired DNA sequences of bacteriophage origin at their CRISPR loci. Furthermore, the insertion/deletion of multiple spacers correlated with increased/decreased resistance, respectively. They also demonstrated that Cas genes control spacer acquisition and corresponding phage defense[13].

The *S. thermophilus* model[1] was the first CRISPR–Cas system in which adaptation[13], biogenesis of CRISPR ribonucleic acids (crRNAs)[14,15] and interference[16] were characterized in native conditions. Recently, these steps were also characterized in *Pseudomonas aeruginosa*[17–19].

During the following year (2008), a series of studies[20,21] revealing the mechanisms of CRISPR defence system were published and helped to establish the mechanism as well as function of CRISPR loci in adaptive immunity. Van der Oost and his team, through studying the type I CRISPR locus of *Escherichia coli*, was able to demonstrate that CRISPR arrays are transcribed and further processed into shorter crRNAs that contain distinct spacers with the function to guide Cas effector proteins such as Cascade[20]. In the same year, CRISPR-mediated defence from *Staphylococcus epidermidis* was demonstrated to block plasmid conjugation, proving that the target of Cas enzyme activity is DNA rather than RNA[21].

Having recognised CRISPR as a DNA-encoded, RNA-mediated and DNA-targeting immune system, a series of following studies investigated the biochemical and genetic processes supporting the mechanism of action[22] . One critical discovery occurred also in 2008, consisting

on the existence and preservation of a CRISPR topic flanking the targeted phage sequences[12,23,24], which was renamed the protospacer adjacent motif (PAM)[12].

Shortly after, in 2010, Moineau and colleagues used genetic studies in *Streptococcus thermophilus* to demonstrate that Cas9 (formerly called Cas5, Csn1, or Csx12) is an endonuclease that precisely creates double-stranded breaks in target DNA at specific positions, 3 nucleotides upstream from the 3‘ edge of the protospacer sequence[16]. They also confirmed that Cas9 is the only protein required for cleavage in the CRISPR-Cas9 system [16].

Subsequently in 2011, Emmanuelle Charpentier and colleagues performed small RNA sequencing on *Streptococcus pyogenes (S. pyogenes)* which has a CRISPR type II system and revealed that, in these types of systems, an auxiliary noncoding RNA, the trans-activating crRNA (tracrRNA) hybridizes with crRNA to create a dual RNA guide for Cas9[25]. Still in 2011, Siksnys and colleagues set out to see whether the CRISPR system from *S. thermophiles* (Type II system) could be reconstituted, in a fully functional form, in an evolutionary distant bacteria, *E. coli*. So, they cloned the CRISPR-Cas locus from *S. thermophiles* and expressed it in *E. coli,* thus positively confirming that it can provide plasmid resistance[26].

In 2012 two huge milestones were reached, almost concurrently by two distinct groups, Emmanuelle Charpentier/Jennifer Doudna[27] and Virginijus Siksnys[28]. Siksnys and his team proved that the Cas9–crRNA complex of the *Streptococcus thermophilus* CRISPR/Cas system introduces *in vitro* a double strand break in DNA that held a sequence that complemented the crRNA. They also proved that the DNA cleavage is performed by Cas9, using separate active sites (RuvC and HNH) to produce site specific nicks on opposite DNA strands[28] and that the Cas9–crRNA complex acts as an RNA-guided endonuclease which uses RNA for the recognition of its target and protein (Cas9) mediated DNA cleavage. They also noted that the crRNA could be trimmed down to a 20-nt (nucleotide) stretch, which is enough for efficient cleavage. Most remarkably, they demonstrated the possibility of reprogramming Cas9 by changing the sequence of the crRNA which consequently allows to target any desired site[28].

Furthermore, Emmanuelle Charpentier in collaboration with Jennifer Doudna reported similar findings as Siknys, but they also reported that the crRNA and the tracrRNA could be fused

together to engineer a single guide RNA (sgRNA). This sgRNA has two vital features including the 20-nt sequence at the 5' end of the sgRNA complementary to the DNA target site, and the double-stranded structure at the 3' side of the guide sequence that binds to Cas9, thus creating a system that by changing the guide sequence of the sgRNA can be used to engineer CRISPR-Cas9 to target any desired DNA sequence that is adjacent to a PAM[27].

In light of these studies, it became increasingly clear that there are several and diverse CRISPR–Cas systems in nature that share features (DNA-encoded, RNA-mediated, nucleic acid targeting), but also carry out peculiarities (various guide RNA types and structures, as well as cleavage outcomes), especially with regard to the type of nucleic acid targeted as some CRISPR–Cas systems can actually target RNA[29,30].

Within months of the publishing of Emmanuelle Charpentier and Jennifer Doudna's paper, a number of laboratories showed almost simultaneously that CRISPR–Cas9 molecular machineries can be repurposed to generate double-stranded breaks in DNA and lead to genome editing of mammalian cells using DNA repair systems[31–33], giving rise to what has been termed the 'CRISPR craze'[34] and the transformation of genome editing[35,36]. Disruptive CRISPR-based technologies have since taken over, based on a number of Cas-based molecular technologies (Cas9, Cpf1, deactivated variants of Cas9) that enable genome editing, transcriptional control[37–39] and epigenetic alterations[40]. The many advantages and features of Cas proteins (programmability, transferability, affordability, specificity and efficiency, amongst many) have empowered geneticists throughout the world and the phylogenetic spectrum to change at will the genome and transcriptome of all genetically characterized species tested to date[39].

Current studies of CRISPR–Cas systems investigate the various aspects of the mechanism of action, with emphasis on novel immunity acquisition genetics and machinery[41–44], as well as explaining the molecular and structural basis of interference by Cas nucleases[45], and its evolutionary implications, particularly with regards to host/virus interactions[19] and the impact of CRISPR immunity and targeting countermeasures[17,46].

1.2 Genome Editing

Genome editing is a category of genetic manipulation that involves DNA being deleted, inserted or replaced in the genome of cellular organisms through engineered, programmable and highly specific nucleases such as meganucleases, Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9, which can induce site-specific double-stand breaks (DSB) at the genomic locus to be modified[47].

Meganucleases or homing endonucleases (HEs) are modified forms of restriction enzymes that occur in nature that are characterized by having long DNA recognition sequences (12 to 40 base pairs, (bp)). However, the downfall of meganucleases is the difficulty of manipulation due to the fact that these enzymes have their cleavage activity and DNA recognition tangled in only one domain[48,49].

TALENs and ZFNs are engineered restriction enzymes that are obtained by fusing a nonspecific DNA cleavage domain from the FokI endonuclease to a modified DNA binding domain. The repeat domains of TALEs and Zinc fingers that have tailored specificities can be combined into collections that bind to extended DNA sequences[50].

ZFNs are one of extensively used engineered nucleases that comprise a nuclease domain of the FokI restriction endonuclease and a Cys2–His2 DNA-binding domain[50]. However, it has been challenging to robustly produce engineered zinc finger arrays since it is required to consider how the distinct finger domains have context-dependent effects between them in an array[51].

TALENs originated from a protein present in the bacteria *Xanthomonas* that is responsible for various diseases in plants. The DNA-binding domain of these restriction enzymes contain a conserved and repeated sequence of 33 to 35 amino acids, that individually recognize a specific nucleotide[50]. TALENs specificity to target DNA sequences can be modified by rearranging repeated amino acid recognition motifs. It appears that TALE repeat domains have less context-dependent effects and can thus be produced to recognize almost any desired DNA sequence[52], through the application of an easy code on a one-to-one basis between the distinct repeats and the four potential DNA nucleotides[53].

More recently, CRISPR/Cas9 system offers another method for genome editing instead of the previously described meganucleases, ZFNs and TALENs. Contrary to the mechanisms of DNA cleavage of ZFNs and TALENs, the CRISPR/Cas9 system exploits small RNAs (crRNA, tracrRNA and sgRNA) for sequence-specific DNA cleavage *in vitro*[47]. The requirements of Cas9 to search for a DNA target are humble, demanding only a sequence of 20 nucleotides (nt) on the sgRNA that is able to base pair with the target DNA while simultaneously needing, adjacently to the complimentary region, the presence of a DNA protospacer adjacent motif (PAM).

In Mammalian cells, nuclease-induced DSBs can be repaired by two pathways that function in almost every cellular organism. These are denominated homology-directed repair (HDR) and non-homologous end-joining (NHEJ) (*Figure 1*). The error-prone NHEJ-based modifications include small insertions or deletions (indels) that can interfere with the binding sites of factors that act in a trans-acting manner in enhancers or promoters and disturb the translational reading frame of a coding region. Thus, the NHEJ mechanism is mostly useful for gene inactivation and knock out. On the other hand, HDR-based modifications use a native (or engineered) DNA template to replace the targeted allele with a designed sequence by recombination. Additional DNA repair pathways such as single-strand annealing, alternative end joining, microhomology-mediated joining, mismatch and base- and nucleotide-excision repair can also produce genome edits[54,55].

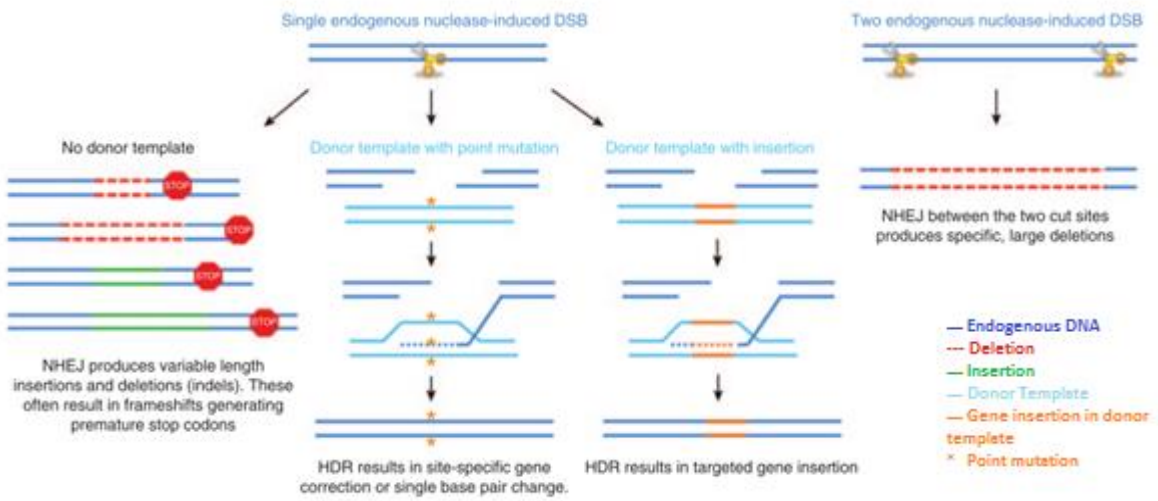

Figure 1- Mechanisms of double strand break repair*. Nuclease-induced DSBs can be repaired by two pathways that function in almost every cellular organism, these are denominated homology-directed repair (HDR) and nonhomologous end joining (NHEJ). Repair facilitated by the NHEJ pathway is imprecise and can result in variable length indel mutations at the DSB location which, ultimately, can possibly lead to the formation of premature stop codons due to frameshifts. Repair facilitated by the HDR pathway is more precise and can result in the introduction of targeted insertions or point mutations from either ssDNA or dsDNA donor templates. NHEJ produces specific large deletions of DNA segments between two cut sites. Adapted from Maeder et al. 2016 (ref 65)*

As mentioned previously, nuclease-induced DSBs can be repaired by either NHEJ or HDR. In the absence of a repair template, DSBs are re-ligated through the NHEJ process, where indel mutations occur[2]. However, the HDR pathway can be favoured by using a repair template, which permits to edit precisely and with high fidelity[56]. Targeted DNA alterations require the use of traditional double-stranded plasmid-based donor DNA repair templates containing homology arms flanking the target site[57] or, for small changes in a specific locus, the use of single-stranded DNA oligonucleotides (ssODNs)[58].

The SURVEYOR nuclease assay[59] or sequencing can be used to detect any indel mutations in cells that got co-transfected with sgRNAs to perform a genomic inversion or deletion[2]. HDR facilitated by both repair template deliveries can be checked through DNA amplification mediated by a polymerase chain reaction (PCR), followed by either a restriction-fragment length polymorphism (RFLP) analysis or by sequencing the altered site[2]. Both Sanger or deep sequencing can be used to detect the induced alterations in the genome[2].

Cas9 offers several potential advantages over ZFNs and TALENs. Cas9 is effortlessly customized as it can be easily retargeted to new DNA sequences by merely altering the 20-nt

guide sequence[2]. On the other hand, retargeting of TALEN is a more exhausting effort as it entails making a couple of novel TALEN genes and even though several protocols for producing TALENs exist[52,60–62], it is more time consuming to obtain two of these enzymes. Since it is recognised that *S. pyogenes* Cas9 (SpCas9) performs a blunt cut between bases at the 17th and 18th position in the sequence of interest[27], it is possible to convert it into a DNA nicking enzyme by altering catalytic residues in the HNH or the RuvC nuclease domain of the enzyme[27,63]. Contrary to Cas9, TALENs have a non-specific DNA cleavage in the linker of 12–24 bp between the couple of TALEN monomer-binding sites[64]. Finally, while both SpCas9 and TALENs are known to mediate genome editing proficiently in a diversity of cell types and organisms[65], Cas9 appears to be more efficient since it allows co-delivery of several sgRNAs into the target cells. This fact represents an advantage since this endonuclease can be used to edit numerous genomic loci concurrently.

1.3 Structure and Mechanisms of CRISPR/Cas9

Most archaea and numerous bacteria have acquired immunity against plasmid transmission and infections caused by viruses that affect these prokaryotes called bacteriophages. This immunity is achieved by a highly developed adaptive immune system that is guided by RNA sequences which are encoded in CRISPR loci and the associated Cas genes (*Figure 2A*). CRISPR loci contain short, repetitive and partially palindromic base sequences of around 30 to 40 bp that are separated by similarly small and unique spacer sequences (*Figure 2*) obtained upon infection from plasmids or bacteriophages. Therefore, spacer sequences are responsible for yielding a genetic record of previous infections which in turn allows the prokaryote to avoid future infections from identical invaders *(Figure 2B)* [41,66].

Streptococcus pyogenes Cas9 is a large (1,368-amino-acid) multi-domain and multifunctional DNA endonuclease. It cuts double-strand DNA (dsDNA) 3 bp upstream of the PAM through its two different nuclease domains. The first one is an HNH-like nuclease domain that cleaves the DNA strand complementary to the guide RNA sequence, also known as the target strand, and an RuvC-like nuclease domain responsible for cleaving the DNA strand opposite to

the complementary strand, the nontarget strand[27,28,67]. Additionally, Cas9 also participates in crRNA maturation and spacer acquisition[42].

The immunity provided by the CRISPR array can be divided into two phases, as shown in *Figure 2*. Initially during the immunization phase (*Figure 2A*), a Cas complex identifies sequences from plasmids or viral genome and integrates a newly acquired repeat-spacer section at the leader end of the CRISPR array, resulting in the immunization of the prokaryote. Subsequently, if another infection attempt of the same invader occurs, the immune response is activated (*Figure 2B*). During this stage of defence, the transcription of CRISPR sequence is set in motion resulting in a long pre-crRNA transcript that is processed into smaller and mature crRNAs that contains one spacer sequence at the 5' end while the 3' end contains a piece of CRISPR repeat sequence (*Figure 2B*). Afterwards, upon an infection of a familiar plasmid or bacteriophage, the mature crRNAs are used to guide the Cas endonucleases into recognizing and inactivating the foreign genetic material by cleaving plasmid or viral DNA (*Figure 2B*)[13].

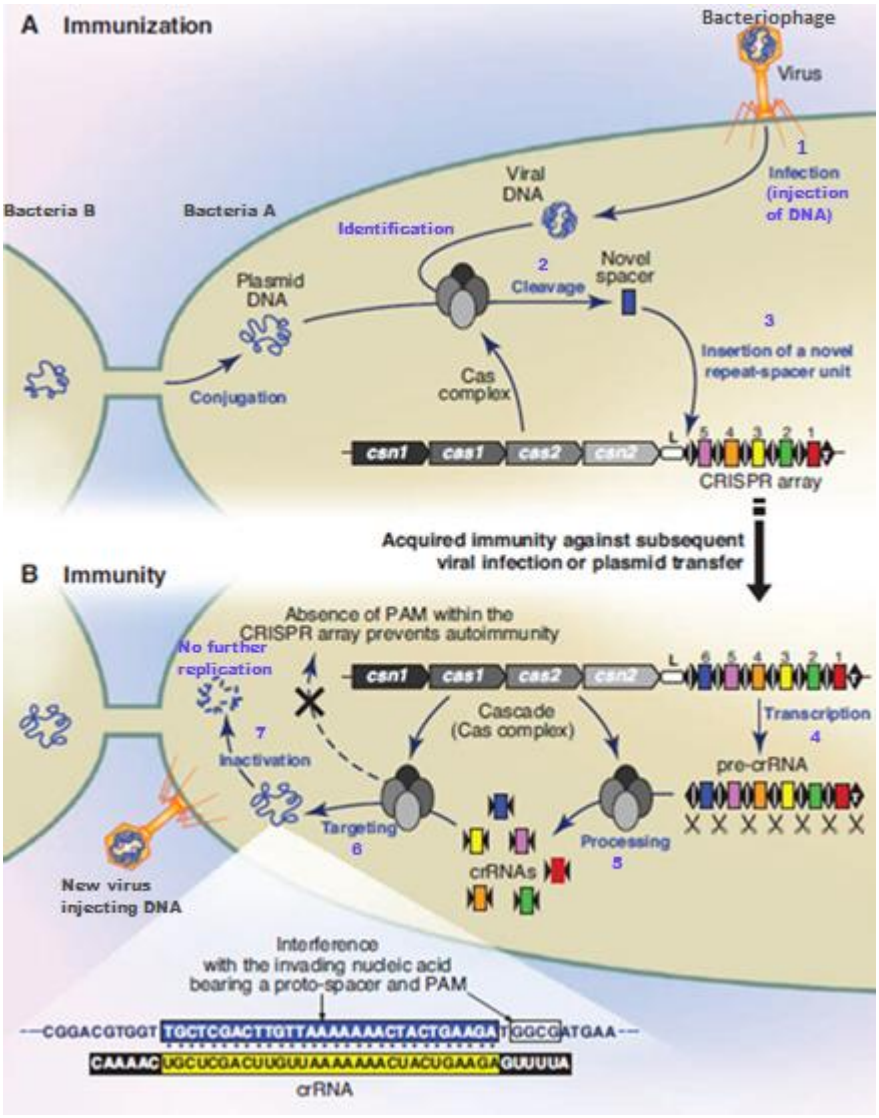

Figure 2-Crispr/Cas mechanism of action. A) *Immunization phase: Upon insertion of foreign DNA into the affected prokaryote, the genetic material is recognised by the Cas complex and, subsequently, cleaved and integrated as a newly acquired spacer into the leader end of the CRISPR array;* ***B)*** *Immunity phase: Transcription of the CRISPR array occurs and pre-crRNA is obtained which is further processed into short and mature crRNA. These mature crRNAs are then used as guides for Cas RNA-guided nucleases that typically form a complex to interfere with the invading DNA. The Cas complex then identifies and cleaves the corresponding target sequence of the foreign nucleic acid, thus inactivating it. The spacers are represented as coloured rectangles, the repeat sequences as black rhombus and the CRISPR leader sequence as a white box labelled L. Adapted from Horvath et al. 2010 (ref 1)*

1.3.1 Mechanisms of different types of Crispr-Cas

The cas1 and cas2 genes are present in all CRISPR/Cas systems and are known to be essential to the first phase of immunity provided by this defence mechanism. However, three distinct system types exist that can be distinguished based on the presence of accessory cas genes[68]. Despite the fact that the three types of CRISPR/Cas systems use the same method of DNA cleavage through crRNA-guided nucleases, they diverge in targeting requirements and the generation of crRNAs[68].

The immunity provided by the type I CRISPR/Cas system is carried out by the Cas3 nuclease in combination with the Cascade complex (*Figure 3A*)[20]. After the pre-crRNA is produced by transcribing the CRISPR sequence, a Cascade subunit denominated Cas6e with endoribonuclease activity is responsible for the cleavage into small and mature crRNAs that stay linked to the complex and are, subsequently, employed by Cascade to find the target protospacer sequence[20]. Situated right upstream of the target sequence is a small sequence motif that is recognized by an additional subunit known as Cas8[69].

For the immunity mediated by the type I CRISPR/Cas system to occur, it is compulsory to recognize a PAM sequence. For this reason, an autoimmune response is avoided with the absence of a PAM sequence in the repeat sequences since crRNA is thus unable to target the spacers of the CRISPR sequence. When a matching sequence is flanked by a PAM, the Cascade complex is able to bind to the target DNA which results in an R-loop between the dsDNA and the crRNA. Finally, the target recognition by Cascade recruits and engages Cas3 that is responsible for introducing single-stranded DNA (ssDNA) breaks into the target plasmid or viral DNA[70], consequently inactivating them[71].

The immunity by the type II CRISPR/Cas system is achieved by using only a single cas gene called cas9 in combination with a crRNA guide for target recognition (*Figure 3B*). In contrast with the other types, the type II CRISPR/Cas system requires an additional short RNA known as the trans-activating crRNA (tracrRNA). This RNA is partially complementary to crRNA as it has an area that shares complementarity with the repeat sequences of CRISPR and additionally it establishes

a secondary structure facilitating its aggregation with the enzyme Cas9. The pre-crRNA is further processed into short crRNA guides by the cleavage mediated by ribonuclease (RNase) III that cleaves the double-strand RNA (dsRNA) formed between the pre-crRNA and the tracrRNA[25].

Similarly, immunity mediated by the type II CRISPR/Cas system can only occur in the presence of a PAM sequence. This sequence is recognised by Cas9 through a PAM-binding domain but, differently from type I, in this immunity the PAM is located directly downstream of the sequence that is targeted. Cas9 has two nuclease domains known as HNH and RuvC that are responsible for the specific dsDNA breaks in both the DNA strands of the foreign DNA that is facilitated by the type II CRISPR/Cas system[71]. The target recognition is initiated by Cas9 transiently binding to PAM sequences of the foreign DNA, enabling the denaturation of the two DNA strands that are directly upstream of the PAM sequence. Targeted cleavage occurs after an R-loop forms due to a productive interaction of the denatured DNA with the crRNA guide in the aforementioned target area[71].

In the immunity provided by the type III CRISPR/Cas system, a repeat-specific endoribonuclease that does not belong to a complex, called Cas6, is responsible for cleaving the pre-crRNA (*Figure 3C*). A sequence at the 5' end of the spacer denominated crRNA tag contains 8 nucleotides remaining from the repeat sequence due to the CRISPR/Cas type processing [72]. Afterwards, the short crRNAs produced through the cleavage mediated by Cas6 are relocated, by an unidentified mechanism, to a Cas10–Csm complex in case of a type III-A system or to Cas10–Cmr complex in case of a type III-B system[73]. These complexes are responsible for trimming the 3' end of the crRNAs at intervals of 6 nucleotides, resulting in a mature crRNAs[74]. While the other system types depended solely on recognizing DNA sequences, the immunity provided by the type III CRISPR/Cas system additionally necessitates the transcription of the target sequence and complementarity to the transcript and the target non-template DNA strand by the crRNA for cleavage to occur[75]. Therefore, type III Crispr/Cas is able to target both the foreign DNA and its transcript, leading to the crRNA-guided cleavage by the Cas10 complex[76]. The cleavage of the non-template strand occurs in the palm domain of Cas10, while the cleavage of the RNA transcripts for the type III-A is mediated by the backbone subunit Csm3 and for the type III-B system by the backbone subunit Cmr4[76].

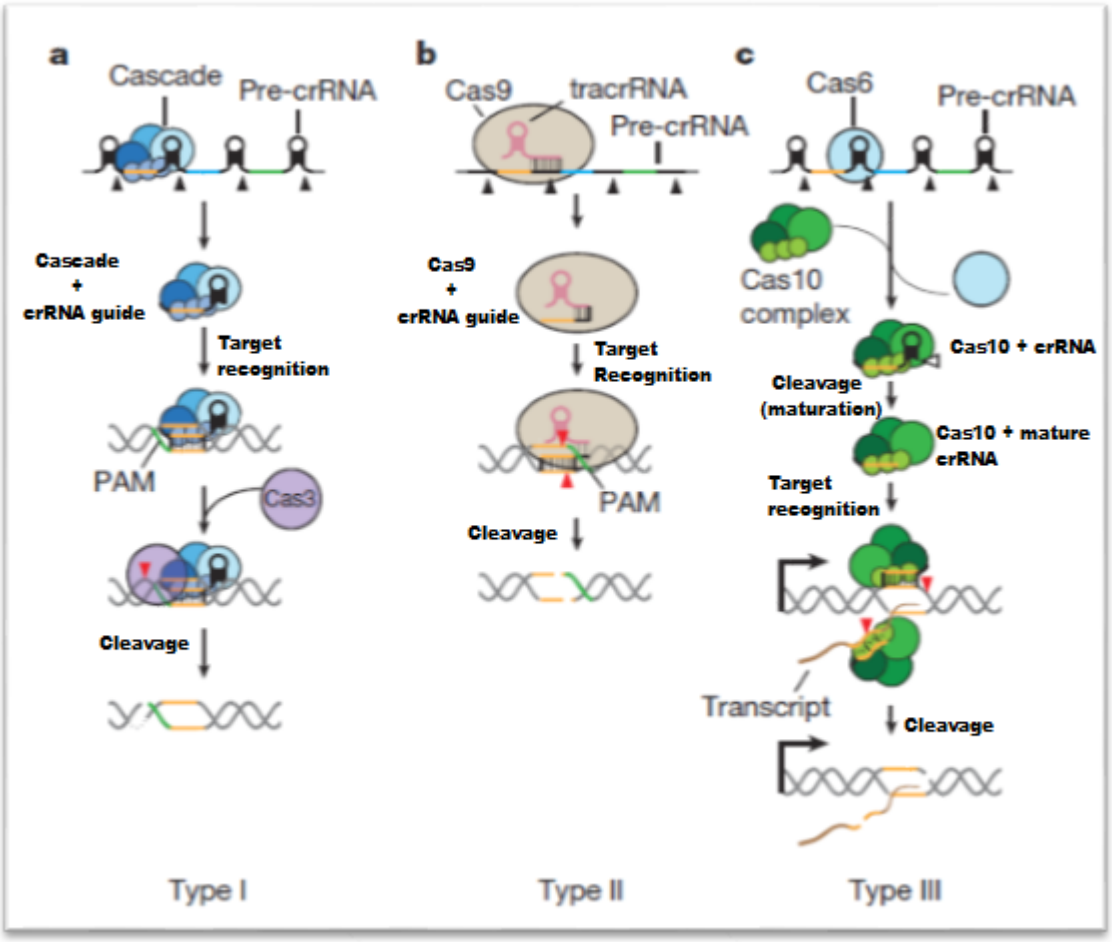

Figure 3- Mechanisms of different Crispr/Cas types. A) Type I systems possess a Cas protein complex (Cascade) that is responsible for cleaving each repeat at the base of the stem–loop structure in the pre-crRNA, which results in the formation of short crRNA guides. Subsequently, the Cascade in combination with crRNA examines the target DNA to find a protospacer that is flanked by a PAM sequence. Finally, the crRNA anneals to the target strand forming an R-loop and, afterwards. the Cas3 nuclease is recruited and activated into cleaving the target sequence downstream of the PAM, resulting in the destruction of the opposite strand; **B)** Type II system encode a small tracrRNA that is aggregated to Cas9 and is partially complementary to repeat sequences of CRISPR. RNase III is responsible for cleaving the repeat/tracrRNA to obtain crRNA guides for the Cas9 nuclease. This enzyme after recognizing a target sequence, mediates the cleavage of both strands of the protospacer/crRNA R-loop; **C)** Type III systems encode a repeat-specific endoribonuclease that does not belong to a complex called Cas6, which is responsible for cleaving the pre-crRNA into crRNA that is subsequently relocated into the Cas10 complex where it is cleaved to produce a mature crRNA. The complex additionally necessitates transcription of the target sequence and complementarity to the transcript and the target DNA non-template strand by the crRNA for cleavage of both sequences to take place. Adapted from Marrafini *et al. 2015 (ref 22)*

The type III CRISPR/Cas system depends on the disparity in base pairing of the crRNA and the sequences that are adjacent to the protospacer to avoid targeting the CRISPR array[72]. Therefore, no autoimmunity is caused by this system because the DNA targeting is prevented when the crRNA tag is totally complementary to the DNA repeats within the CRISPR locus.

Contrarily, DNA targeting is permitted in case of incomplete complementarity between the sequences flanking the protospacer and the crRNA tag[72].

1.4 Delivery Methods of CRISPR/Cas9

The sgRNAs and Cas9 can be delivered by different methods depending on the application including DNA constructs, mRNA constructs or Cas9 ribonucleoproteins[77]. While both crRNA and tracrRNA can be delivered separately, the combination into a single chimeric gRNA simplifies both design and delivery. The gRNA can either be expressed from a plasmid inside the cell or generated *via in vitro* transcription[77]. One way of delivery is through PCR amplicons containing an expression cassette[2]. PCR-based sgRNA delivery attaches the custom sgRNA sequence onto the reverse PCR primer used to amplify a U6 promoter template and the resulting amplicon could be co-transfected with a Cas9 expression plasmid. Since this method removes the need for plasmid-based cloning and sequence verification, it is suitable for testing or co-transfecting numerous sgRNAs for generating large knockout libraries or other scale-sensitive applications[2].

Early efforts in mammalian cells focused on encoding Cas9 in the form of DNA plasmids[63,78]. In this method, a single plasmid can be used to encode both the chimeric gRNA and the Cas9 under separate promoters. Typically, the Cas9 is encoded as a fusion to a nuclear localization signal that mediates intranuclear transduction upon expression, thus allowing access to the genetic material of the cell[63,78,79]. The delivery of the plasmid DNA into the cells is attained either *via* standard chemical transfection or electroporation methods[77].

A more complex delivery method is packaging the Cas9 DNA in a single-stranded form within a non-integrating virus such as adeno-associated virus (AAV)[80]. However, the maximum packaging capacity for AAV is approximately 4.5 kb[81], which makes combination of both the Cas9 and cognate gRNA into a single capsid challenging. As such, smaller Cas9 variants[82] and several systems to create split Cas9 enzymes[83–85], allowing division between two AAV vectors, have been developed but both suffer from reduced efficacy[82–85]. Another path for AAV-based delivery lies in the use of significantly smaller Cas9 orthologs from other species as reported by Ran *et al.*[86].

Cas9 isolated from *S. aureus* can edit mammalian genomes with similar efficiencies to *S. Pyogenes* Cas9 while being more than 1 kb shorter when encoded in DNA form, allowing packaging within a single AAV capsid[86].

Another way of reducing the size of Cas9 delivered to cells is to generate RNA transcripts of the gene. Cas9 mRNA delivery has been extensively adopted for the ex vivo modification of mammalian embryos by microinjection[87–89], and more recently in human somatic cell lines[90], or primary cells[91] via electroporation. RNA constructs can also be delivered in viral form, usually in the form of integrase defective lentiviral particles. The lentivirus capsid has a substantially larger capacity for nucleic acid (approximately 8 kb)[92] compared to AAV, and so it can be engineered to express both Cas9 and up to four gRNAs simultaneously[93,94].

A more recent line of investigation has been to deliver ribonucleoproteins (RNPs), which are composed of Cas9 protein precomplexed with gRNA[90,95,96]. Delivering Cas9 in protein form leads to fewer observed off-target mutations than delivery *via* plasmid DNA[90,95,96]. Non-standard techniques for the delivery of Cas9 RNPs are also being explored. These include lipid-based transfection reagents[97], nanoparticle carriers of Cas9[98] and non-lipid carriers that mimic vesicle-like structures[99].

The range of delivery options for Cas9 RNPs is further expanded upon modification of the Cas9 protein termini to allow for covalent functionalization such as conjugation to cell penetrating peptides (CPPs)[100,101].

2. APPLICATIONS

Engineered nucleases have quickly become a frequently used method by researchers for targeted genome editing, especially because of the recent arrival of CRISPR with the combination of the highly customizable Cas9[102].

Genome editing with these engineered nucleases has been used in a quick, easy and efficient manner to modify endogenous genes in a diverse set of biomedical relevant cell types and in organisms that have previously been difficult to modify genetically [32]. It has quickly overcome TALENs[103] and ZFNs[104] where editing was excessively complex and arduous.

In the research arena, versatile CRISPR-enabled genome editing has been used in various fields, such as medicine[65], biotechnology[105] and biology[106] with diverse applications such as: controlling transcription, modifying epigenomes, conducting genome-wide screens, imaging chromosomes, manipulating biological circuits facilitating the generation of synthetic materials, correcting gene mutations, control the expression of entire genes offering longer term expression alteration compared to other methods such as RNAi[102,107]. CRISPR systems are already being used to improve genetic disorders in animals and are likely to be employed soon to treat human diseases of the eye and blood[108]. Beyond biomedical applications, this system is already being used to expedite crop[109,110] and livestock breeding[111], develop new antimicrobials[112] and control insects that carry diseases with gene drives[113]. The CRISPR technology is already providing a new class of genetic models that are more suited for diverse applications such as rapid diagnostics, fundamental disease research, drug screening and therapy development, *in vivo* editing and correction of inherited conditions[114].

Even though the genome-wide specificities of the CRISPR/Cas9 technology remain to be fully understood, the ability of these systems to execute targeted alterations of genome sequences and gene expressions with high efficiency will unquestionably alter biological research and encourage the development of new molecular therapeutics for diseases affecting mankind.[114]

The applications of the CRISPR/Cas9 system in human pathologies will be further explored in the following chapters.

2.1 Antiviral Therapy

Antiviral therapy through designer nuclease CRISPR/Cas9 has become a promising new tool against human infecting viruses. This method has been used successfully to target viral genes or host genes that encode vital receptors to inhibit infection and replication of viruses[115].

2.1.1 HIV

The human immunodeficiency virus (HIV) is a member of the genus *Lentivirus*, part of the family *Retroviridae*[116] that causes a viral infection and progresses to an acquired immunodeficiency syndrome (AIDS). AIDS is responsible for a progressive deterioration of the human immune system, allowing lethal opportunistic infections and cancers to flourish[117]. Transmission of HIV occurs by blood transfer, pre-ejaculate, semen, vaginal fluids, and from mother to child during pregnancy, delivery or breastfeeding[118].

HIV infects essentially the human immune system cells including the vital helper T cells (CD4+ T cells) (Figure 4), dendritic cells and macrophages. The infection, consequently, leads to low levels of helper T cells through numerous mechanisms, including apoptosis of uninfected bystander cells, pyroptosis of abortively infected T cells, direct viral killing of infected cells, and by CD8 cytotoxic lymphocytes killing the infected cells. Cell-mediated immunity can be lost if helper T cell numbers drop below a set level, which leads to increasing susceptibility to opportunistic infections[119].

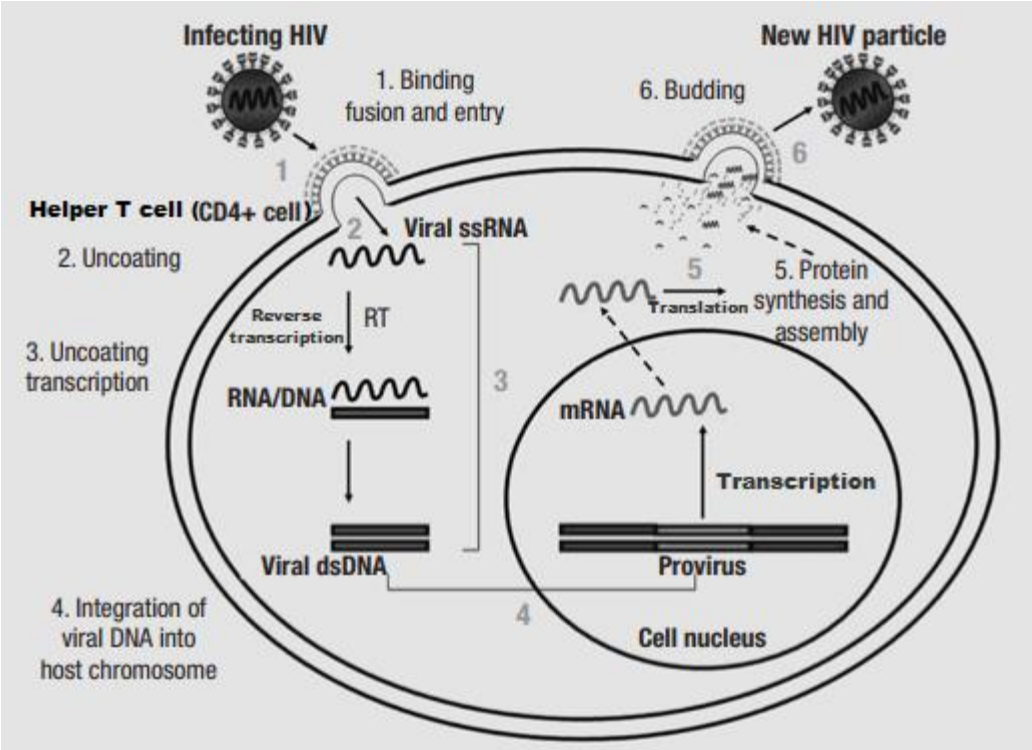

Figure 4- HIV replication cycle*. The main steps in HIV replication are sequentially numbered from 1 to 6. (1) Virus binds to CD4 and the appropriate coreceptor resulting in fusion of the viral envelope and the cellular membrane, leading to the viral nucleocapsid being released into the cytoplasm. (2) The viral RNA is uncoated. (3) subsequently, reverse transcription mediated by the Reverse Transcriptase (RT) occurs. (4) The obtained viral dsDNA then migrates into nucleus of the affected CD4+ cell and is posteriorly integrated into the cellular DNA by the enzyme Integrase. (5) Afterwards, transcription of the proviral DNA mediated by the cellular RNA polymerase II occurs producing mRNAs. Then, translation of these RNA molecules is facilitated by the cellular polyribosomes. (6) Genomic RNA and proteins of viral origin are transported to the cellular membrane, where assembly occurs. Immature virions are released. The viral protease is then responsible for processing the polypeptide precursors to generate mature viral particles. After migrating to the cell's plasma membrane, the virus particles suffer a budding process, resulting in the release of the new HIV particle. Adapted from Fanales-Belasio et al. 2010 (ref 122)*

Therapeutic interventions mainly target two key retroviral enzymes: reverse transcriptase and protease. In combination, these antiviral drugs have greatly diminished both mortality and morbidity in HIV infected patients. However, when administered over prolonged periods, these drugs induce considerable toxicity, and their effectiveness is undermined by the emergence of drug-resistant strains of HIV[120].

Although current anti-HIV therapies can inhibit HIV-1 replication, the viruses that have integrated within the host genome in a latent state can still potentially reactivate at any time.

The CRISPR/Cas9 system has proved to be useful for eliminating latent HIV-1 by targeting its genomic DNA. Several groups have reported that they successfully disrupted the expression of HIV-1 provirus in infected cells utilizing the CRISPR/Cas9 system[121–124]. More recently a team successfully excised the HIV-1 provirus in three different animal models using an all-in-one adeno-associated virus vector to deliver multiplex sgRNAs and Staphylococcus aureus Cas9 (saCas9)[108]. The published results demonstrate the plausibility of engineering Cas9/gRNA to precise and efficiently obtain a prophylactic and therapeutic method against AIDS.

2.1.2 Epstein-Barr Virus

Epstein-Barr virus (EBV) or human herpesvirus 4, belongs to the herpes virus family and is one of the most common human viruses[125]. The virus usually spreads through bodily fluids, mostly saliva[125]. EBV can cause several health issues such as infectious mononucleosis, some forms of cancer and can also affect the nervous and autoimmune systems[125].

Most infections occur during infancy and early childhood and present either no symptoms or nonspecific symptoms. However, in adolescents and young adults, EBV infection commonly results in infectious mononucleosis with symptoms including fever, sore throat, lymphadenopathy and splenomegaly. Additionally, other symptoms and signs may be present such as headache, fatigue, rash and hepatomegaly[125].

EBV infects B cells of the immune system and epithelial cells. After controlling the initial lytic infection, EBV persists latently in B cells for the rest of the affected person's life. In some cases, the virus may reactivate in asymptomatic form and people with weakened immune systems can even develop symptoms. If EBV reactivates it can infect others[125].

Currently there is no treatment for Epstein-Barr Virus, however some measures can be taken to help relieve the symptoms including fluid intake, resting and using medication for pain and fever[125].

In 2014, Wang *et al.*[126] reported to have successfully applied CRISPR/Cas9 as an antiviral treatment in human cells by specifically targeting the genomes of latent Epstein-Barr virus. There

was a halt in proliferation and an associated reduction in viral load in cells from a Burkitt's lymphoma with latent EBV after being exposed to the CRISPR/Cas9 system that targeted the viral genome.

In 2015 Yuen *et al.*[127] used two gRNAs to introduce a 558bp deletion in the promoter region of BamHI-A rightward transcript (BART) which encodes viral microRNAs (miRNAs) on the EBV genome. Numerous latently infected human epithelial cell lines such as nasopharyngeal carcinoma C666-1[128] cells were successfully edited. They observed efficient editing of the EBV genome by the CRISPR/cas9 system as the whole pBART region was eliminated which resulted in the loss of miR-BART expression and activity. Their results represented the first genetic evidence that the major promoter for the expression of BART is pBART. Finally, after cells expressing Cas9 and gRNAs were selected with puromycin, a recombinant virus with the intended deletion was obtained and deep sequencing revealed no off-target cleavage[127].

Van Diemen and his team[129] showed that the CRISPR/Cas9 system is able to directly edit the genome of latent EBV in EBV-positive tumor cells and that targeting vital areas of the virus dsDNA efficiently reduces the content of viral genome in latently infected cells. They designed gRNAs aiming for the viral EBV nuclear antigen 1 (EBNA1) and numerous parts of the origin of replication (OriP) of the Epstein-Barr virus that are involved in its replication and episome maintenance[130,131].

Their team used Burkitt's lymphoma Akata-Bx1 cells as model system. These cells carry a recombinant EBV expressing green fluorescence protein (eGFP) under control of the cytomegalovirus (CMV) promoter[132]. Therefore, the expression of the eGFP is a signal of EBV presence in these cells[129]. After the transduction of the cells with their corresponding gRNAs, a loss of eGFP expression was observed. A near full loss of eGFP from most of the cells was observed after introducing a combination of these active gRNAs. Finally, they showed that the most efficient method was targeting EBNA1 with two different gRNAs, which induced over 95% loss of EBV genomes[129].

The results obtained by Van Diemen's team demonstrate that viral genome content in latently EBV infected cells can by efficiently reduced by the CRISPR/Cas9 system when it is

targeted at vital areas of the EBV dsDNA and that this reduction could culminate on a termination of the tumorigenic, cell cycle-promoting roles carried out by EBV and the loss of anti-inflammatory functions and counter-apoptotic features of gene products encoded by the virus. Therefore, this approach might be a new therapeutic strategy to fight malignancies associated with the Epstein-Barr virus[129].

In early 2017, Ma and colleagues[133] used the CRISPR/Cas9 system to perform genome-wide loss-of-function screens to identify host dependency factors that are critical for the EBV infected lymphoblastoid and Burkitt lymphoma B cell growth and survival. They managed to identify multiple non-redundant mechanisms by which EBV prevents apoptotic responses to oncogene stress in transformed B cells and identified key EBV-induced synthetic lethal targets for therapeutic intervention.

Yuen *et al.*[134] reported suppression of EBV in latently infected nasopharyngeal carcinoma cells when using the CRISPR/Cas9 system. They studied the possibility of the CRISPR/Cas9 system to induce a reduction of EBV levels by targeting the genome of the virus in infected nasopharyngeal carcinoma cells. They engineered several gRNAs aimed towards different areas of the viral genome and transfected them into C666-1 cells[128]. The team observed a reduction by half of the viral DNA levels in C666-1 cells and even though this effect lasted for weeks[134]. Lastly, they observed that the survival of C666-1 cells did not change. However, these cells became sensitized to the chemotherapeutic effect of 5-fluorouracil and cisplatin[134]. The authors consider that this work provides the proof-of-concept for suppressing EBV DNA load with CRISPR/Cas9 and that it may lead to a potential new strategy to sensitize EBV infected nasopharyngeal carcinoma cells to chemotherapy which in turn might reduce the necessary dose of these drugs, thereby alleviating the side effects. However, the team has concerns about possible off-target effects and the adequate delivery vehicle. All these issues need to be addressed before CRISPR/Cas9 technology can enter the next phase of medical applications[134].

2.1.3 Hepatitis B virus

Hepatitis B is a viral infection affecting the liver caused by the Hepatitis B virus (HBV)[135]. The virus is transmitted from person to person through contact with the infected blood, semen or other infected body fluids[136]. Normally, it is an acute infection with a short duration, but it can also become a lasting, chronic infection. The risk of developing a chronic infection is associated with the age of the infected person. Most (90%) infected infants become chronically infected, while only a small fraction of infected adults (2% to 6%) become chronically ill[135]. It is best to prevent Hepatitis B infection by getting vaccinated as the chronic illness can lead to severe health problems, including hepatocellular carcinoma or cirrhosis[136].

It may take approximately 90 days for symptoms to appear. The initial infection may present no symptoms in some people, while others may develop a rapid onset of sickness. The symptoms commonly last some weeks and this initial stage of the disease rarely results in death of the infected person[135]. The presence of symptoms differs depending on the age. Most children under age 5 and newly infected immunosuppressed adults are asymptomatic, whereas 30% to 50% of persons over 5 years old have initial symptoms[136]. The symptoms are mainly non-specific and can include: Fatigue, fever, nausea, loss of appetite, vomiting, abdominal pain, joint pain, jaundice, dark urine and clay-coloured bowel movements[135].

Since 1982, there has been an effective vaccine against hepatitis B. The vaccine is 95% effective in preventing early viral infection and the progress into chronic illness and liver cancer[136]. There is no specific treatment for acute hepatitis B and the only therapeutic option available is to treat the patient's symptoms relieving their discomfort. For example, lost fluids from diarrhea and vomiting are replenished to guarantee appropriate nutritional balance[136]. On the other hand, chronic hepatitis B infection can be treated with antiviral medication. The treatment can slow down the development of cirrhosis, decrease occurrence of liver cancer and ultimately improve long-term survival of those affected by the virus. However, the abovementioned antiviral therapy only suppresses the viral replication and therefore does not provide a cure for hepatitis B[136].

Several *in vitro* and *in vivo* studies demonstrated that using the CRISPR/Cas9 system to target the HBV cccDNA is successful and efficiently inhibits HBV replication[137–142]. Seeger *et al.*[137] showed that their team could inhibit HBV infections up to eightfold by testing several HBV-specific gRNAs. They demonstrated that Cas9 could certainly cleave the virus-derived cccDNA and that the resulting cleaved cccDNA is swiftly repaired, possibly by the NHEJ pathway. Even though the authors of this study did not show the experimental feasibility, they consider that this method can be applied to future investigations that study the role of host genes in the viral life cycle by inactivating relevant cellular genes.

Lin *et al.*[138] designed eight sgRNAs that target the P1 and XCp genes on HBV. In this study, they found a significant reduction in the production of core and HBsAg proteins in Huh-7 hepatocytes derived from cellular carcinoma cells. In a mouse model, it was shown that this method could cleave the intrahepatic plasmid that contained the viral genome and enable its clearance, which resulted in a decrease of serum surface antigen levels[138].

Furthermore, Zhen *et al.*[139] used CRISPR-Cas9 to target the Hepatitis B antigen (HBsAg) on HBV *in vitro* culture and within *in vivo* systems that were confirmed by quantitative enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR). The total amount of HBsAg secreted into the cell culture and in the mouse serum was reduced after editing the gene with CRISPR-Cas9. In the same study, they also found that no HBsAg-positive cells persisted in the liver tissue of CRISPR-Cas9 treated mice, and this was confirmed by immunohistochemistry[139].

CRISPR/Cas9 system has also been used by Dong and colleagues[140] to target the HBV cccDNA, which is highly stable, and a prime target for the inhibition of HBV infection. This work showed a reduction in the generation of the virus in Huh7 cells and in HepG2.2.15 HBV-replication cells[140]. Similarly, Kennedy *et al.*[141] used Cas9 and HBV-specific sgRNAs. The total viral DNA load was reduced by up to 1000-fold and cccDNA was reduced by up to 10-fold, and outstandingly remaining viral DNA was mutated[141].

These studies can be further expanded and explored with the hope of developing a novel therapeutic strategy, not only against chronic HBV infection, but also against the wider family of other hepatitis viruses in the future.

2.1.4 Human papillomavirus

Human papillomavirus (HPV) is a DNA virus belonging to the papillomavirus family and can cause infection in humans affecting the skin and the moist membranes lining of the body (cervix, anus, mouth and throat)[143,144].

HPV is the most common sexually transmitted infection (STI) worldwide and there are more than 170 types of HPV, of these at least 13 are known to cause cancer[143,144]. The virus is primarily transmitted by sexual interaction and, generally, people are infected with it soon after the beginning of sexual activity[144]. Most HPV infections do not cause symptoms but some HPV types (primarily types 6 and 11) can cause warts[143]. A small proportion of infections with some types of HPV can persist and progress to various types of cancer such as: cervical cancer (approximately 70% of cervical cancer cases can be attributable to HPV infection), cancers of the anus, vulva, vagina, and penis[144].

Moreover, persistent infection with specific types of HPV, most frequently types 16 and 18, have the highest risk for development of genital cancers[143]. Symptoms of cervical cancer normally only appear after it has gotten to a more advanced stage and may include non-specific symptoms: loss of appetite and weight loss; fatigue; leg, back or pelvic pain; a single swollen leg; vaginal odorous discharge or discomfort; and irregular, abnormal or intermenstrual vaginal bleeding after sexual intercourse[143].

There are currently 3 vaccines that protect against some cancer causing HPV types: Cervarix® protects against HPV types 16 and 18[145], Gardasil® protects against HPV types 6, 11, 16, 18[146] and Gardasil 9® which in addition to protecting against infection by strains covered by the previous generation of Gardasil, also protects against five other HPV strains (31, 33, 45, 52, 58) that are the cause for one fifth of cervical cancers[147].

There is medical interest in the high-risk HPV E6 and E7 proteins because of their associated tumor suppressor p53 and retinoblastoma (Rb) interference that can result in the formation of tumors. The E6 protein promotes cell proliferation through stimulating the degradation of the tumor suppressor p53 protein by the formation of a trimeric complex containing E6, p53 and the cellular ubiquitination enzyme E6-AP. E6 degradation interferes with the biological functions of p53 disturbing the control of the cell cycle progression and resulting in increased tumor cell growth[148].

E7 binds to a region of the Rb protein that is essential for its tumor suppressor function. One of the major biochemical functions of Rb as a tumor suppressor is to bind E2F-family of transcription factors and repressing the expression of replication enzyme genes[149]. E7 disrupts the interaction between Rb and E2F, resulting in the release of E2F factors in their transcriptionally active forms, thus stimulating replication and cell division[150].

Kennedy *et al.*[151] have successfully used CRISPR-Cas9 machinery to disable the viral genes E6 and E7 in both HPV18 and HPV16, using HeLa and SiHa cervical carcinoma cell lines. They reported that the expression of Cas9 RNA-guided endonuclease, together with sgRNAs specific for E6 or E7 cleave the HPV genome, resulting in gene inactivating indel mutations. This induces p53 and Rb expression that are able to stall the cell cycle and initiate apoptosis.

Recently, Liu *et al*.[152] reported that a CRISPR-Cas9 system using a dual guide RNA vector successfully targeted and deactivated the E7 gene in HPV types 6 and 11 in keratinocytes. The results showed that silencing of E7 led to inhibition of cell proliferation and induction of apoptosis in E7-transformed keratinocytes. The data showed potential for the development of an adjuvant therapy for genital warts.

2.1.5 John Cunningham Virus

Lytic infection of oligodendrocytes by John Cunningham Virus (JCV) in the central nervous system (CNS) is the cause of the fatal demyelinating disease, progressive multifocal encephalopathy (PML)[153]. This disease mostly develops in people who have underlying

immunosuppressive conditions, such as AIDS[154,155], Hodgkin's lymphoma[156], lymphoproliferative diseases[157], and in those undergoing antineoplastic therapy[158,159]. Similarly, this disease seems to be steadily increasing amid patients with autoimmune diseases, such as Crohn's disease[160] and multiple sclerosis[161–163], who are treated with antibody therapies.

PML is a sub-cortical white matter disease of the brain and exhibits signs and symptoms suggestive of the involvement of multiple regions of the brain[164–166]. While demyelination can develop in any location in the white matter, it can also occur in other regions of the CNS[164–166]. The most common symptom of PML is visual deficit, accounting for 35 to 45% of the cases. Mental deficits, another devastating sign of PML, and motor weakness is also observed in a considerable number of patients accounting for a third and 25 to 33% of all cases, respectively[164]. The disease can progress quickly, causing death within four to six months, although clinical signs and symptoms may persist stable for a longer period[164,165,167].

Currently, there is no effective treatment for PML[168] even though several therapeutic approaches such as heparin sulfate, cytarabine and interferons have been used to treat this illness[153]. Therefore, the development of novel effective therapies for PML is urgently needed. In this regard, two studies involving the use of the CRISPR/cas9 system as a therapeutic strategy targeting the JCV have been reported[169,170].

T-antigen (T-ag) is a viral early protein that is essential for directing viral reactivation and lytic infection, which is encoded in the small circular double stranded DNA of the JCV genome[171]. Wolebo *et al.*[169] employed *in vitro*, the CRISPR/cas9 system to introduce mutations in the viral genome and, by inactivating the gene encoding T-ag, they inhibited viral replication. This method could potentially be used to eliminate both actively replicating virus in patients with PML and asymptomatic persistent virus in persons without the disease but that are prone to develop it.

In the work reported by Wolebo and colleagues[169], it is probable that before the initiation of DNA replication, the viral genome was already targeted since gRNAs and the CRISPR/Cas9 system were already inside the cells before introducing the aforementioned viral genome. To further assess this, Chou *et al*.[170] studied the possibility of effectively restricting viral replication with the CRISPR/Cas9 system in cells that were already acutely infected prior to the introduction

of CRISPR/Cas9. It was observed that the replication of the JCV was greatly impaired when the host cell unsuccessfully repaired the DNA cleavage by CRISPR/Cas9 in susceptible regions of the JCV genome. In addition to Wolebo's team findings[169], these results confirm that CRISPR/Cas9 in conjunction with the appropriate gRNAs targeting the JCV genome can indeed hinder viral replication and thus the infection when introduced into cells that were already acutely infected prior to this insertion[170].

The results described above suggest that the CRISPR/Cas9 system could be of valuable interest in the quest of finding an effective therapy against the deadly PML. However, both teams admit that development and optimisation of an efficient delivery vehicle is needed before further clinical consideration[169,170].

2.1.6 Herpes simplex virus

Infection with herpes simplex virus currently results in a lifetime infection and can be due to either herpes simplex virus type 1 (HSV-1) or herpes simplex virus type 2 (HSV-2). Generally, HSV-1 is associated with orolabial disease, whereas HSV-2 is almost entirely associated with genital disease[172]. However, genital herpes caused by HSV-1 has been reported[173–176]. Globally, an estimated 3.7 billion people under the age of 50 (67%) and 417 million people aged 15-49 (11%) have HSV-1 or HSV-2 infections, respectively[177].

Although both genital and oral herpes infections are usually asymptomatic, some symptoms can still occur. In oral HSV-1 infections symptoms include painful blisters or open sores in or around the mouth and before the presence of these ulcers there might be a sensation of burning, itching or tingling in the affected area. Similarly, anal or genital ulcers or blisters can occur in people with genital herpes. Newly acquired genital herpes infections can present additional symptoms such as swollen lymph nodes, fever and body aches. When the cause of infection is HSV-2, preceding the appearance of genital ulcers there might be feelings of localized pain or mild tingling[172,177]. Possible complications may occur including severe disease, neonatal

herpes and psychosocial impact. Additionally, HSV-2 and HIV influence each other by improving the transmission rate and severity of disease[172,177].

Currently, there is a limited amount of options for treating the symptomatology of a herpes infection and all the existing antiviral agents that are approved have the viral DNA polymerase as a common target during the lytic phase of infection[178]. However, herpesvirus also possess the ability to evade the human immune system by establishing a latent state in which the virus expresses limited gene products[179]. Furthermore, viral DNA polymerases do not actively replicate the herpesvirus genome during this state which ultimately leads to ineffective antiviral therapies that target this enzyme[178].

Van Diemen *et al.*[129] designed an *in vitro* study to determine if the CRISPR/Cas9 system can be reprogrammed to effectively hinder an infection by herpes simplex during both productive and quiescent stages of its life cycle. With this approach, they intended to achieve successful editing of the HSV-1 genome, which consequently, would lead to reduced virus replication by directing gRNAs to essential viral genes. However, the research team observed that when they used only one gRNA targeting the herpesvirus genome, a selection of mutants resistant to the CRISPR/Cas9 editing arose when there was a continued proliferation of infected cells. The team then envisioned that they could more successfully hinder viral replication by concurrently targeting several vital viral genes and consequently avoid the formation of mutants. They tested this idea by using two gRNAs to target the HSV-1 genome and they successfully achieved a halt in the replication of the virus in human cells.

Van Diemen and colleagues[129] also assessed whether the CRISPR/Cas9 system could target the latent state of HSV-1 in infected cells but, in contrary to the results obtained in the productive stage, CRISPR/Cas9 seemed to be ineffective at targeting latent HSV-1 genomes but the viral replication could again be successfully stopped upon virus reactivation. The authors consider that CRISPR/Cas9 system may be inefficient in accessing the tightly repressed state[180] of the HSV-1 quiescent genome. However, further *in vivo* and *in vitro* studies are necessary to clarify if the CRISPR/Cas9 system can target latent state HSV-1.

The study presented above indicates the potential of the CRISPR/Cas9 system as an attractive new strategy to combat pathogenic human herpesvirus by directly hindering viral replication and thus eliminating this pathogen from infected cells and, upon optimization, could also be used to target latent HSV-1.

2.2 Genetic disorders

A genetic disorder is a condition caused by an abnormality or mutation in an individual's DNA sequence. Mutations can be caused by an error in DNA replication, environmental factors or can also be hereditary[181].

Genetic disorders can be categorized in three different groups, monogenic disorders, chromosome disorders and multifactorial disorders[181]. Monogenic disorders are caused by a mutation in a single gene and can be dominant or recessive[181]. Autosomal dominant disorder means that only one mutated copy of the gene is necessary to develop the disease, the chance of a child inheriting this type of disorder is of 50%[181]. Autosomal recessive disorder requires the presence of two copies of the mutated gene for a person to be affected by the disease, the chance of inheriting this type of disorder is of 25%[181]. If the mutated allele is in the X chromosome, a disorder can be recessive in women but dominant in man and the inheritance can be different if the child is male or female. Chromosome disorders result from alterations in the number or structure of the genes that are in the chromosomes[181]. Multifactorial disorders are caused by a combination of multiple gene mutations associated to environmental and lifestyle factors[181].

Recently, CRISPR/Cas9 has been used as a novel editing tool for the development of new treatments of genetic disorders, which will be further discussed below.

2.2.1 β-thalassemia

β-thalassemia is a genetic disorder that affects the human haemoglobin beta (HBB) gene that presents over 200 distinct point mutations and occasionally some deletions[182]. These point mutations frequently happen in an intron, leading to abnormal splicing. β-thalassemia is characterized by reduced, abnormal or absent synthesis of β-globin chains[182]. Patients homozygous (β-thalassemia major) have the most severe form of this disease, causing ineffective erythropoiesis and hepatosplenomegaly, leading to severe anaemia and typically need recurrent iron chelation and transfusions[183].

Currently, the only available cure for this disease is transplantation of hematopoietic stem cells when there are available donors with matching histocompatibility[183]. Until now, a patient with HBE1-β-thalassemia, who had a normal HBB gene delivered through the use of a lentiviral delivery vehicle into his hematopoietic stem and progenitor cells (HSPC) has not needed additional blood transfusions to treat his condition[184]. Nevertheless, gene therapy that involves viral vectors has serious limitations and possible harms as these vectors can integrate randomly into numerous locations of the genome of the treated patient, as already observed in other genetic disorders[185,186].

In a recent study[183], the combination of CRISPR/Cas9 to cleave the HBB gene and the piggyBac transposon (a mobile genetic element that efficiently transposes between vectors and chromosomes) to select for homologous recombination events, efficiently corrected two different β-thalassemia mutations and converted homozygous β-thalassemia to the heterozygous states in induced pluripotent stem cells (iPSCs) from β-thalassemia patients[183]. The research team did not observe off-target effects in the treated iPSCs, the karyotypes were normal and the cells preserved full pluripotency. They also observed that iPSCs that had been successfully edited showed an improved HBB expression following differentiation into erythroblasts, when compared to their parental cell line. These corrected iPSCs display normal function and could potentially provide a source of cells for transplantation in patients, offering a new strategy to cure this disease[183].

CRISPR Therapeutics, a company founded by Rodger Novak, Emmanuelle Charpentier and Shaun Foy in November 2013, is developing a treatment of both Sickle cell disease and β-thalassemia called CTX001[187]. CTX001 is a therapy that is performed *ex vivo*, where the patient is the source of the autologous cells that are collected. The CRISPR/Cas9 system is then used on the harvested cells to perform a unique genetic change with the goal of obtaining a boost in fetal haemoglobin present in the donated blood cells of the patient. Subsequently, the previously harvested cells are reintroduced into the patient's circulation with the hope that they differentiate into fetal haemoglobin rich erythrocytes, circumventing the deficit of haemoglobin that these illnesses originate[187]. CTX001 is anticipated to enter clinical trials in 2018 in Europe for β-thalassemia[187].

2.2.2 Cystic Fibrosis

Cystic fibrosis (CF) is a genetic disease that affects the mucus glands and is generally a progressive, chronic and life-threatening illness. It mainly affects young people's digestive and respiratory systems, while it can also possibly involve the reproductive system and sweat glands[188]. Patients that suffer from this genetic disease normally lose high levels of salt through sweat resulting in an unbalanced sum of minerals in the blood that can originate irregular heart rhythms. Additionally, the affected patients have uncommonly thick mucus that can accumulate in lungs and intestines. These disease traits are responsible for inadequate growth, malnourishment, breathing difficulties, recurrent infections of the respiratory system, and ultimately can lead to permanent lung damage[188]. In developed countries, CF patients have an expected lifespan of 42 to 50 years, where respiratory complications account for approximately 80% of deaths[189].

This genetic disorder is caused by mutations in the pair of copies of the cystic fibrosis transmembrane conductance regulator (CFTR) gene that produces the CFTR protein and is hereditary in an autosomal recessive way[189]. People that have one copy of the gene that is operational are considered carriers of the CF gene but display normal function of the mucus

glands. More than 1500 CFTR mutations have been identified, but only the functional importance of a small number is known. The absence of phenylalanine at position 508 (Phe508del, also known as F508del), accounts for about two-thirds of mutated alleles in northern European and North American populations[189].

Cystic fibrosis has no cure at the moment but management of its symptoms and reduction or even prevention of future complications is possible[189]. Treatment for associated problems may include: antibiotics, digestive enzymes, airway clearance techniques, inhaled hypertonic saline and salbutamol, vitamin supplementation, bronchodilators, steroid medication and ultimately lung transplantation[189].

CRISPR/Cas9 genome editing was successfully employed in a culture of intestinal stem cells of people afflicted by CF and the CFTR locus was efficiently corrected by homologous recombination[190]. The corrected gene was expressed and fully functional, CRISPR/Cas9-mediated repair of the CFTR locus restored cAMP-induced intestinal stem cell organoid swelling, lost in CFTR mutant organoids of cystic fibrosis patients[190]. This study demonstrated the possibility of gene correction mainly in adult stem cells derived from patients with a monogenic hereditary defect, and combined with a previous study, in which *in vitro* expanded organoids were transplanted into the colons of mice successfully[191], provides a potential strategy for future gene therapy in cystic fibrosis patients[190].

2.2.3 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a genetic disorder that is known for its progressive muscle deterioration and associated weakness. The disorder is X-linked recessive and is caused by a mutation in the gene for the protein dystrophin (DMD gene), which is important to maintain the muscle fibres membrane. This protein is located primarily in skeletal muscles and in cardiac muscle[192].

People affected with DMD show an elevated level of the enzyme creatine kinase in their blood. Currently, there is no cure for muscular dystrophy[192]. However, some symptoms can

be attenuated using surgery to correct common issues related to DMD such as spinal curvature caused by scoliosis or deformities of the foot and physical therapy braces, while those affected with deteriorated muscles responsible for breathing might benefit from assisted ventilation. Muscle deterioration can be slowed down by using steroids, while immunosuppressants can be used to postpone injury to muscle cells that are dying and, finally, anticonvulsant medication has the ability to control some activity of the muscles and seizures[192].

CRISPR/Cas9 has been successfully applied by Li *et al*.[193] to restore the dystrophin protein in DMD patient derived iPSCs. The team performed three correctional methods, exon skipping, frameshifting, and exon knock-in, and found the latter to be the most effective approach. Additionally, they differentiated the CRISPR/Cas9 edited iPSCs into skeletal muscle cells and confirmed the expression of dystrophin protein. Similarly, several independent groups[194–197] used adeno-associated virus to deliver CRISPR/Cas9 system into the DMD mouse model to remove the mutated exon 23. This experiment included local and systemic delivery resulting in deletion of exon 23 and consequently restoring the expression of the dystrophin gene in myofibers and cardiomyocytes. More recently Long *et al.*[198] myoedited iPSCs from multiple patients with DMD genes that harbour point mutations, large deletions or duplications and their team was able to reinstate the expression of the dystrophin protein with relative success in derivative cardiomyocytes. Myoediting of mutations responsible for the disease in three-dimensional engineered heart muscle (EHM), resulted in the reinstatement of the protein expression and the matching mechanical force of contraction. It was enough to amend only a subsection of cardiomyocytes from the mutant EHM phenotype in order to improve it to normal control levels. These reports attest to the capacity of CRISPR/Cas9 mechanism as a future therapy for DMD patients.

2.2.4 Huntington's Disease

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disorder caused by mutations in the huntingtin (HTT) gene[199]. The mutations involve the expansion of the CAG repeats in the first exon of the HTT gene that provides the genetic

information to produce the huntingtin protein. Generally, in healthy people the HTT gene has 10 to 35 repetitions of the CAG segment[199]. However, an increase of these CAG segment repetitions that ranges from 36 to over 120 times is observed in people affected by Huntington's disease. It has been observed that patients who have 36 to 39 CAG repeats might possibly show symptoms and signs of the disease, while those that possess more of these repeats most certainly acquire the illness[200]. The expansion of the CAG segment results in an abnormal long huntingtin protein. This lengthened HTT protein is divided into toxic small fragments that have the ability to remain jointly and gather in neurons, resulting in a disturbance of the standard functions of these specialized cells[199].

The dysfunction and death of neurons cause the symptoms of Huntington disease such as loss of cognitive skills, depression, involuntary movements, and alterations in personality as well as difficulty in thinking. Symptoms generally start between age 30 to 45, and life span is around 15 to 20 years after the first signs appear. There is no cure for Huntington's disease, in later stages of the disease full time care is necessary and treatments can ease some of the symptoms[199].

Dabrowska *et al*.[201] proved that the CAG repeat can be precisely excised from the HTT gene using the Cas9 nickase strategy. The team used as a model fibroblasts of a HD patient with various numbers of CAG repeats. Excision of the repeat disabled the HTT gene and inhibited huntingtin synthesis. Since Cas9 nickases are specific and reliable this approach may lead to the future cure of Huntington's disease.

2.3 Antibacterial therapy

Antibacterial agents obstruct the proliferation of bacteria and by interrupting vital bacterial cellular functions they can quickly kill them. Most bacteria are typically vulnerable to solely a restricted variety of antibacterial agents and possess an intrinsic ability to withstand the therapeutic effect of others, also known as resistance. Additionally, bacteria can obtain resistance to one or more antibiotics that would usually have an effective therapeutic effect. Acquired resistance can arise by mutations in genes often associated with the mechanism of

action of the compound and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer. Unfortunately, some types of bacteria are able to acquire resistance to multiple drugs which, in extreme cases, can ultimately result in bacteria that show resistance to most or all of the agents that would commonly be used to fight them[202].

Whenever an antibiotic is used to treat an infection, there is possibility that the agent will select, in the population of bacteria infecting the organism, for bacteria that demonstrate to have acquired a resistance to the antibiotic in question, therefore causing an unsolved infection in supposedly treated patient. A further selection of resistant bacteria may also occur in the patient's commensal flora, resulting in colonisation by resistant bacteria, which possibly will be accountable in the future for a different infection at the same or alternative location in the body. The resulting resistant bacteria will have the opportunity to spread to other people, especially within hospitals leading to growing rates of resistance to antibiotics[203]. The causes of the fast spreading of antibiotic resistance are identified as the over-prescribing of antibiotics, patients not taking antibiotics properly, an unnecessary use in agriculture, poor infection control in clinics and hospitals, a lack of rapid laboratory tests and poor hygiene and sanitation practices[204].

Each year, infections caused by multidrug-resistant bacteria in the European Union (EU) result in extra healthcare costs and productivity losses of at least 1.5 billion euros and is even responsible for 25 000 deaths[205]. The World Health Organization (WHO) has named antibiotic resistance as one of the three most important public health threats of the 21st century[206]. However, there has been a lack of research and development for new antibiotics, in the last decades, as the identification of new active compounds became harder and the attention of pharmaceutical companies shifted to more profitable investments[205–207].

Several studies have demonstrated that CRISPR-Cas systems can offer a promising solution to overcome current antibiotic resistance while even having a possible better selectivity[112,208,209].

Gomaa *et al.* [209] have showed in their article that they could effectively and specifically target bacterial genomes by using CRISPR/Cas systems resulting in a significant elimination of distinct strains and species of bacteria. They utilized as a model the bacteria *Escherichia coli* and

a type I CRISPR/Cas system for the bacterial genome targeting. Their team discovered that the removal of bacteria could be produced by using either imported or native systems and that the transcriptional activity, genomic location and strand of the target sequence didn't influence the effectiveness. Moreover, the crRNAs targeting was precise enough to easily discriminate between even vastly similar strains in mixed or pure cultures. However, an appropriate delivery vehicle was lacking, exposing an obstacle to overcome if control of bacterial populations in diverse ecological niches and scientific fields were to be used.

Later, Citorik *et al.*[208] and Bikard *et al.*[112] reported the use of CRISPR-Cas systems as antimicrobials in which they overcame the delivery vehicle challenge using phagemids, plasmids with phage packaging signals[210]. Citorik's team used *Escherichia coli* as a model, whereas Bikard and colleagues used *Staphylococcus aureus*. The bacteria these teams used are particularly pertinent since they both have described resistances to antibiotics[211], predominantly the multidrug-resistant *Staphylococcus aureus* (MRSA)[212].

The bacterial genome was targeted by the engineered phagemids, resulting in a fast and comprehensive elimination after an increase in the quantity of phagemid employed. Additionally, the packaged phagemids were equally used in both studies to target plasmids that had acquired resistance to antibiotics, resulting in the effective elimination of the plasmids[112,208]. Citorik *et al.*[208] also investigated conjugation as a means of delivery, but the transfer efficiency was too low to substantially reduce cell counts.

Both Citorik's[208] and Bikard's[112] teams used mixed bacterial communities and were able to eradicate solely the targeted strains of bacteria whereas other strains were unaffected. The team led by Citorik was also capable of identifying a change in a single base-pair when looking at two different bacteria strains, thus underlining the target accuracy.

The research of both teams was additionally enriched by performing studies *in vivo*. Citorik *et al*.[208] used larvae of *Galleria mellonella* as an infection model for enterohemorrhagic *E. coli,* while Bikard *et al*.[112] used fluorescence to their advantage. Their team used a co-culture of two strains of *S. aureus* in a model of skin infection in mice in which one strain was fluorescent and the other was not, with the former being the target of the technique employed. They equally

demonstrated that the target strains were substantially harmed by the applied phagemids which was verified by an improved survival of the larvae or a decrease in fluorescent *S. aureus* present on the skin of the animal[112,208].

The studies mentioned above demonstrate the plausibility of using CRISPR-Cas systems as antibacterial agents, particularly in antibiotic resistant strains. While these systems have several advantages over traditional antimicrobials, the authors also recognize that to exploit its capabilities, the development and optimization of delivery strategies is needed[112,208,209].

Regardless of the challenges ahead, CRISPR seems to be an interesting and worthwhile option to explore as a novel antibacterial agent.

2.4 Oncology

Cancer is a group of diseases that is characterized by an abnormal cell growth in any body part, which can grow outside of their normal borders, invade adjacent structures and, consequently, spread to other tissues and organs (metastasizing)[213,214]. Cancer cells are less differentiated than normal cells and do not possess specific functions that mature cell types have and, consequently, continue to divide. In addition, cancer cells are able to bypass checkpoints that regulate cellular growth and might impact normal molecules, cells and blood vessels that are around a tumour and feed it[213]. During the mestastatic process, cancer cells are able to modulate the microenvironment by releasing extracellular signals, by inducing peripheral immune tolerance and promoting tumour angiogenesis [213,215].

According to the World Health Organization[214], cancer is a leading cause of death globally, that accounts for close to 9 million deaths which translates to be the cause of nearly 1 in 6 deaths. Additionally, cancer has an economic impact that is considered to be noteworthy and growing, with an estimated US$ 1.16 trillion of total annual economic cost[216].

Cancer is a genetic disease that starts with the conversion of a normal cell into a tumour cell during a multi-step process[213,214]. The transformation of a benign mass into a tumor mass is

the result of the interaction between external agents and the patient genetic background. The external agents can be classified in physical, chemical or biological carcinogens. Common physical external agents include ultraviolet (e.g. the sun) and ionizing radiation, while asbestos and components of tobacco smoke are ordinary chemical carcinogens. Finally, biological agents include infections from some bacteria, parasites or viruses[213,214]. Some of these were discussed in previous chapters.

The genetic modifications that are responsible for the development of cancer usually target DNA repair genes, proto-oncogenes and tumor suppressor genes. Usually, proto-oncogenes are responsible for regulating cell growth and division but when these genes acquire mutations and become unusually more active, thus promoting more cell growth and division, they convert into oncogenes and contribute for cancer development. This enables cells to grow and survive in conditions that otherwise would not. Similarly, tumor suppressor genes are equally responsible for controlling cell growth and division by repressing cell division and inducing DNA repair or apoptosis. Cells that hold certain alterations in these genes may divide irrepressibly. DNA repair genes are responsible for fixing DNA that got damaged by the agents mentioned earlier and cells that possess mutations in these genes are predisposed to acquire further mutations in other genes[213].

The genetic modifications can occur by distinct mechanisms and at independent levels. Chromosomes can be entirely added or deleted due to misfunctions in mitosis. However, mutations that change the nucleotide sequence of genomic DNA are more common[217,218].

Large-scale mutations include the addition or deletion of a portion of a chromosome. Gene amplification occurs when a cell gets copies of a small chromosomal locus, which normally contains several oncogenes and some adjacent genetic material. Translocation happens when two distinct chromosomal regions become unusually fused. On the other hand, small-scale mutations include point mutations, insertions and deletions that can occur either in the promoter region of a gene and, consequently, affect its expression, or in the coding sequence of the gene and alter the function or stability of its protein. Disruption of a gene can also result from an integration of genomic material from a virus that, consequently, lead to expression of viral oncogenes in the affected cell[217,218].

Current treatments of cancer depend on a correct diagnosis for the adequate and effective treatment since every cancer type requires a specific treatment regimen that includes one or more modalities such as chemotherapy, radiotherapy, surgery, immunotherapy, targeted therapy, hormone therapy and stem cell transplant[214,219]. The primary goal of treatment is usually to cure cancer or to considerably prolong life. Another important factor is improving the cancer patient's quality of life which can be achieved by supportive or palliative care and psychological support[214].

Sadly, the existent therapeutic arsenal for cancer has limitations in its curing ability and many reported side-effects and toxicity[217,220]. In this regard, CRISPR/Cas9 may present a new and exciting opportunity in the battle against this devastating disease. Since cancer arises from the transformation of normal cells into tumor cells through modification of various but specific genes, CRISPR/Cas9 can be used to study these alterations or to restore the normal function of these cells and consequently halt the spread of cancer, ultimately leading to a clinical cure of the disease.

CRISPR/Cas9 uses in cancer research ranges from functional validation of genes involved in the progression of cancer to the modelling of the disease and new therapeutic designs[221]. From research on a small scale[222–224] to high-throughput screens of gene function[225–227], this technique has unravelled new knowledge about the development of cancer and the metastatic process and even guided towards the identification of novel potential therapeutic targets.

CRISPR/Cas9 can be used in screens, as it is a powerful functional genomic tool that aids on the discovery of new targets for cancer therapy. Pooled screening with CRISPR/Cas9 requires a cell population with a diversity of gene knockouts to be generated in a multistep process with the main goal of identifying genotype-specific vulnerabilities[228]. The identified essential genes can turn out to be potential drug targets[228]. When compared to other methods, CRISPR was shown to be more sensitive in detecting essential genes[229], leading to more large-scale CRISPR/Cas9 screens being conducted to systematically discover essential genes across several cancer cell lines[230–232].

A complementary screening approach to identify new drug targets relies on the concept of synthetic lethality which appears when the expression of two or more genes have a combination of insufficiencies that ends up in the death of the affected cell, while an insufficiency in a single gene does not[228,233]. In cancer, this means that previously non-essential genes can become essential due to the loss of a specific tumor suppressor or the activation of an oncogene[233]. Due to the increasing knowledge on the structural alterations of cancer, this method could vastly expand the spectrum of potential drug targets[228]. Several CRISPR/Cas9 screens have been carried out to identify synthetic lethal interactions[234–236] but a future challenge will be to unravel the underlying molecular mechanisms and identify synthetic lethal interactions that can be translated into therapies[228].

CRISPR/Cas9 screening can also be used to study chemico-genetic interactions, which gives insights into how cancer responds to drug treatment. Using a drug as perturbation in a pooled CRISPR screening, allows the identification of gene knockouts that act synergistically with or confer resistance to the agent[228]. Several studies have used this method to identify synergetic or resistant interactions of anti-cancer drugs and genes that modulate cellular response to specific drugs[237–240]. Similarly, this approach has been used to explore the mode of action of poorly characterized anti-neoplastic drugs such as apilimod[241] and in a combinatorial screening in which two predefined sgRNAs are encoded in lentiviral plasmids that allows not only the dissection of genetic interaction of pairwise gene knockouts, but also enables identification of synergistic drug targets[242,243].

Most of the human genome consists of non-protein coding regions that contain many regulatory elements such as enhancers or non-coding RNAs. Since the expression of these non-coding RNAs is known to be deregulated in cancer[244] and the transcription of oncogenes can be controlled by near and distant enhancer elements[245], the comprehensive understanding of non-coding elements will provide deeper insights into cancer biology. In this regard, CRISPR/ Cas9 has been shown to be a strong screening tool for the identification of non-coding elements such as enhancer regions[246,247] and/or other functionalities[248–251].

CRISPR/Cas9 can be used to generate organoid cancer models by first isolating and cultivating stem cells from adult tissues and later stimulating them with tissue specific growth

factors, thus differentiating and forming organoids in a cell culture dish. This opens the possibility to culture both healthy and cancerous tissue which in turn enables the study of tumor development and progression *in vitro*[228]. In this regard, The CRISPR/Cas9 system has been used in several studies[221,228] as a tool to transform the healthy human organoids into their cancerous counterparts by disrupting both tumor suppressor genes and oncogenes which, ultimately, provides the opportunity to unravel the responsible mechanisms of cancer formation[228].

Additionally, CRISPR/Cas9 can be used for *in vivo* screening, where factors that influence the microenvironment are present. *In vivo* can be either achieved by implanting ex vivo edited cancer cells or by delivery of CRISPR/ Cas9 components to mouse tissues, thereby generating tumors[221,228]. The transplantation of *ex vivo* CRISPR edited cells enables screening of sgRNAs with high-throughput which allows identification of key genes in cancer development. This method has already been applied in several cancer models such as lung[226], liver[252] and colon[253] cancer cells. The direct *in vivo* approach of genome editing is a potent way to study the effect of genetic alterations on carcinogenesis in a native tissue microenvironment[228]. Several studies[221,254–259] have demonstrated the applicability of this method where the delivery of sgRNAs and Cas9 targeting oncogenes and tumor suppressor genes resulted in the formation of the desired cancer model yielding new insights into the function of cancer-associated genes.

Gene therapy trials have been previously performed in earlier years. However, they have mainly been unsuccessful for several reasons such as: somatic silencing of gene products, host immune response, viral vectors and mutagenesis[260]. Fortunately, the new technologies of gene editing which includes CRISPR/Cas9 may soon overcome some of the current obstacles and allow permanent modification of somatic cells[228].

The first clinical trial using CRISPR for cancer therapy took place at West China Hospital, Sichuan University in Chengdu in 2016[261,262]. It is a non-randomized, open-label phase I study where the safety of programmed cell death protein-1 (PD-1) knockout engineered T cells ex vivo is evaluated in treating metastatic non-small cell lung cancer that has progressed after all standard treatments[261]. PD-1 is a T-cell receptor responsible for the inhibition of T-cell activation, thus regulating immune tolerance and decreasing autoimmune reactions. However, this also allows cancer to escape an immune response[263]. In this study, patients provide a sample of

peripheral blood lymphocytes and the T-cells are later edited *ex vivo* by the CRISPR/Cas9 system to obtain PD-1 KO. The edited lymphocytes are then selected, expanded and subsequently infused back into the patients[261]. Similarly, four other trials are applying the same concept of PD-1 knockout for the treatment of other cancer types, including bladder, esophageal, prostate and renal cell cancer[262], while another registered phase I/II trial adds PD-1 knockout to EBV-specific autologous T-cells for the treatment of EBV positive cancers[264]. The first clinical trial using CRISPR/Cas9 for cancer therapy outside of China was approved in the Unites States of America (USA) in the University of Pennsylvania[265]. It is a phase 1 trial of autologous T Cells engineered to express NY-ESO-1 TCR and CRISPR gene edited to eliminate endogenous T cell receptor (TCR) and PD-1 to test whether NY-ESO-1 is expressed on certain tumor tissues[266].

Another *ex vivo* method is the generation of chimeric antigen receptor (CAR) T-cells with CRISPR/Cas9. CARs are engineered receptors that are transduced into T-cells and are able to reprogram these cells to target malignant cancer cells. In a preclinical study, they directed a CAR to the T-cell receptor α constant (TRAC) locus using CRISPR/Cas9 produced T-cells with immensely enhanced activity against tumours compared to conventionally produced CAR T-cells using randomly integrating vectors[267]. CRISPR/Cas9 is also being used in two distinct clinical trials to generate CAR T-cells. One of them is a phase I/II study of universal CRISPR/Cas9 gene-editing CAR-T cells targeting CD19 in patients with relapsed or refractory CD19+ leukemia and lymphoma where they will test whether it can evade host-mediated immunity and deliver antileukemic effects without graft-versus-host disease[268]. To overcome the fact that that a subset of patients in this trial relapsed due to the loss of CD19 in tumor cells, another phase I/II study has started. This new trial pretends to evaluate if using this CRISPR/Cas9 gene-editing CAR-T cells targeting CD19 and CD20 or CD22 would recognize and kill the CD19 negative malignant cells when they express CD20 or CD22[269].

The only *in vivo* registered phase 1 clinical trial pretends to use TALEN or CRISPR/Cas9 plasmids targeting HPV16 and HPV18 E6/E7 DNA (previously discussed in chapter 2.1.4). The constructs are delivered with a gel that is locally applied to the HPV infected cervix and the safety and dosing regimen will be determined. Likewise the change of HPV 16 or 18 DNA titers, as well as cervical cytology and histology will be evaluated[270].

CRISPR/Cas9 is proving to be a powerful tool in both studying and providing potential new therapies for cancer. Combined with existing techniques and therapies, it might provide an additional hope for those affected by cancer as it has shown promising results. Improvements in the specificity of Cas9 for targeting specific genomic regions without off-target effects and more efficient delivery methods to target specific tumours or organs with CRISPR/Cas9 constructs will certainly spark the interest of further developing *in vivo* gene editing clinical trials in the future[228].

3. LIMITATIONS

Despite the countless potential applications of the CRISPR/Cas9 system in human pathology, there are some important issues that need to be addressed before its application for clinical use, such as off-target mutations, PAM-dependence and delivery methods of CRISPR/Cas9[271].

Generally, large genomes hold several sequences of DNA that are either vastly homologous or equal to target DNA sequences. CRISPR/Cas9 can cleave both these sequences, which can consequently lead to off-target mutations that can result in the transformation or death of the cell[2,271]. The extent to which a given guide sequence exhibits off-target activity depends on a combination of factors including the abundance of similar sequences in the target genome[2] and the dosage of CRISPR/Cas9, which should be carefully controlled[272,273].

Several efforts to reduce the cellular toxicity of CRISPR/Cas9, by reducing the off-target mutations have been reported[2,63,272,274,275]. Moreover, Cas9 can be converted into nickase to assist in reducing the mutations in undesired sites, without changing the effectiveness of the desired targeted cleavage executed by CRISPR/Cas9[63].

One of the biggest lasting limitations of the CRISPR/Cas9 system is effectively delivering its endonuclease into tissues or cells in order to accomplish the desired therapeutic result. A promising approach to deliver nucleases into cells is by transfecting the nuclease and gRNA expression-cassette bearing plasmid DNA. Regardless of this technique being easy to implement, it has not been extensively employed due to several shortcomings such as cytotoxicity related to the use of DNA and bacterial DNA sequences in plasmid backbones, low transfection efficiency of primary cells and a possibility of plasmid fragments being arbitrarily introduced into the gene[65,276].

It is preferred to use vectors based on adeno-associated virus in somatic gene therapy since this type of vectors are not pathogenic, only induce a residual immune system response and can target non-dividing cells[65,277]. Nevertheless, as previously mentioned in earlier chapters, these vectors have limitations on the amount of DNA that can be cloned into their

backbones, where the coding sequences of Cas9 and sgRNA barely fit. As such, smaller Cas9 variants[82] and several systems to create split Cas9 enzymes[83–85] have been developed but both suffer from reduced efficacy[82–85]. Alternatively, Cas9 can be delivered to cells as RNA transcripts of the gene via microinjection[87–89], electroporation[90,91] or in in the form of integrase defective lentiviral particles[93,94].

The development of non-DNA-dependent alternatives including RNPs paved the way for novel methods for delivery in gene therapy, allowing effectively a lot of genomic modification through microinjections and electroporation[90,95,96], lipid-mediated transfection[97] or other non-standard techniques[98–101]. When comparing delivery methods of previously assembled complexes of protein and RNA with nucleotide delivery methods based on vectors for genome targeting by CRISPR, the former displayed less cell toxicity and enhanced reliability resulting in the elimination of the safety issues related to the insertion of foreign DNA[65].

Despite recent advances in new delivery methods, the development and optimisation of efficient delivery vehicles is needed before further clinical consideration of the CRISPR/Cas9 system.

Hypothetically, CRISPR/Cas9 can be used to target any DNA sequence via engineered customizable gRNA but for this system to be precise there must be a PAM sequence composed of two to five nucleotides that is positioned directly downstream of the target sequence. Furthermore, it is compulsory that the gRNA is complementary to the target sequence[27]. The PAM dependence of CRISPR/Cas9-mediated DNA cleavage constrains the frequencies of targetable sites in genomes but simultaneously increases the specificity of CRISPR/Cas9. Therefore, there should be less observed off-target mutations in CRISPR/Cas9 systems that demand longer PAM sequences[47].

Conceivable genome editing in the human germline has seen ethical apprehension, as this is the transmittable genome to future generations from different origins including embryos, fertilized eggs or gametes. The reasoning behind these concerns stems from the fact that editing germline cells can in one hand, have the expected gene correction and on the other hand induce mutations in undesired locations that will be later transmitted to innumerable

generations. Therefore, an ethical discussion has arisen on whether the benefits compensate the associated hazards of using CRISPR/Cas9 as a method to genetically edit the human germline. Additionally, informed consent has been object of doubt as this is unobtainable by the descendants that acquire unwanted effects of the application of this technique and finally it is ambiguous who is accountable for the possible genetic harm that is transmitted to numerous generations[278].

Lastly, CRISPR/Cas9 could be used for purposes other than therapeutic ones including individual enhancement which raises another ethical concern. This could be attained by editing germ or somatic cells using CRISPR/Cas9 and selecting favourable phenotypic features such as improved intellectual or athletic performance which could ultimately lead to unfairly designed offspring. This in turn may have a profound social impact. What are the requirements to choose who can benefit from editing the genome, who makes that decision, how do we obtain an informed consent from the beneficiary, are some of the pertinent questions that come along with the beauty of manipulating genes using CRISPR/Cas9 technique[278].

4. CONCLUSION

Currently, many diseases affecting mankind still do not have a clinical cure and finding a cure for these pathologies has shown to be quite challenging. In this regard, genome editing is an interesting field of study that has been advancing at a remarkable pace to achieve new therapeutic treatments for many of these pathologies.

Genome editing can be accomplished through engineered, programmable and highly specific nucleases such as meganucleases, Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and more recently CRISPR/Cas9. The latter technique has quickly surpassed all the others, owing to its easy and non-time-consuming customization and its simple and highly efficient mechanism of editing compared to the other methods.

As previously discussed during this work, CRISPR/Cas9 has been the subject of an increasing number of studies in numerous different fields, ranging from medicine to agriculture, with diverse applications such as controlling transcription, modifying epigenomes, conducting genome-wide screens, manipulating biological circuits facilitating the generation of synthetic materials, correcting gene mutations and controlling the expression of entire genes. The possibilities of this newfound technique seem to be endless. Specifically, the use of the CRISPR/cas9 system in therapeutic research of incurable diseases such as some viral diseases, genetic disorders and different types of cancers has refreshed the hope of finding cures to many of the debilitating diseases discussed in this work as it has shown promising results.

However, CRISPR/cas9 also possesses some limitations which hinder the progression of its use in clinical research. One of the limitations concerns the possible off-target mutations that can result in cell death or transformation. Thus, before CRISPR/cas9 can be further used in clinical research, the specificity and possible cellular toxicity needs to be addressed. Another issue of this powerful editing technique seems to lie in the delivery process of the CRISPR/cas9 system into tissues or cells to achieve its therapeutic effect. While adeno-associated virus based vectors are favored for use in somatic gene therapy, they are limited by their packaging limit and, even though ways to circumvent the AAV shortcomings have been investigated, they suffered from

reduced efficacy. Alternatively, RNA transcripts via microinjections, electroporation or in the form of defective lentiviral particles have been tested but presented its own flaws. Finally, RNPs paved the way for novel methods for delivery with improved fidelity and reduced cell toxicity. Nevertheless, despite the recent advances in new delivery methods, the development and optimization of efficient delivery vehicles is needed before further clinical consideration of the CRISPR/Cas9 system. Finally, ethical concerns have been raised regarding the use of CRISPR/cas9 in the human germline, questioning if the benefits indeed outweigh the risks and raising the issue of informed consent and the possibility of using it as an enhancement agent.

The future of CRISPR/Cas9 seems promising with seemingly endless possibilities and applications of this new genome editing technique. Nevertheless, some flaws must be addressed before its further use in clinical research. The specificity, safety and toxicity must be closely controlled and improved so that the benefits outweigh the risks to test and later use it on humans as a novel therapeutic agent. Concluding, it is worthwhile to investigate further the CRISPR/Cas9 system as it may turn out to be the long-awaited cure to many incurable diseases with a great impact on humankind.

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