Crispr/cas9 applications, limits and drawbacks

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Abbreviations

Alt-NHEJ	Alternative Non-homologous End Joining (also called MMEJ)
CIBN	Cryptochrome Interacting Basic Helix Loop Helix 1
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRY2	Light sensitive Cryptochrome 2
dCas9	Dead Cas9
DSB	Double Stranded Break
FISH	Fluorescent in-situ Hybridization
FokI	Non-specific DNA cleavage domain
FRET	Fourier Energy Transfer
GMO	Genetically Modified Organism
HDR	Homology-Directed Recombination
HIV	Human Immunodeficiency Virus
HNH	(Nuclease Domain)
KRAB	Kruppel-Associated Box
MMEJ	Microhomology-Mediated End Joining (also called Alt-NHEJ)
NHEJ	Non-homologous End Joining
NLS	Nuclear Localization Signal
OPEN	Oligomerized Pool Engineering
PAM	Protospacer Adjacent Motif
REC domain	Recognition Domain
RUvC	(Nuclease Domain)
I-SceI	(Endodeoxyribonuclease protein)
ssODN	Single-stranded Oligodeoxynucleotides
TALE	Transcription Activator-like Effector
V16 / V64	Transcription Activation Domain
ZFN	Zinc Finger Nuclease

Summary

Discovery of the CRISPR/Cas9 system is a promising breakthrough in genome editing, because it can make specific gene alterations more quickly and efficiently than previews techniques (Christopher A. Lino, Jason C. Harper, 2018; Selle & Barrangou, 2015). The CRISPR/Cas system can introduce double stranded breaks (DSB) at specific sites (Ceasar, Rajan, Prykhozhij, Berman, & Ignacimuthu, 2016). These DSBs can be repaired by the non-homologous end joining pathway (NHEJ) and homologous directed repair pathway (HDR). NHEJ will result in random inserts or deletions (Bialk, Rivera-Torres, Strouse, & Kmiec, 2015), whereas HDR repairs DSBs by use of a homologous DNA sequence. HDR can be used for specific genome editing, such as knock-ins or knock-outs. The CRISPR/Cas9 system consists of three components, the Cas9 protein, the guide RNA (sgRNA), and a single stranded oligodeoxynucliotides (ssODN) which provides the homologous sequence carrying the mutation. CRISPR/Cas9 has been used extensively for many applications to great success (Maruyama et al., 2015; Tang & Liu, 2018; Yi & Li, 2016). In this essay, applications, limitations and the potential to overcome these limitations are discussed. CRISPR/Cas9 does have some limitations as genome editing tool. For example, several mismatches are allowed in the guide RNA as well in the PAM site, increasing the change of off-target DSBs. CRISPR/Cas9 has great potential to correct gene alterations in humans, however careful consideration of limitations is required and several hurdles need to be overcome for usage in therapeutic studies (Oude Blenke, Evers, Mastrobattista, & van der Oost, 2016).

Preface

This essay is written in context of my master program Molecular Biology and Biotechnology. After incentive from my supervisor Prof. Haastert I searched for an essay topic, and sticking a little to my comfort zone, I picked Crisp/Cas systems. DNA related research always interests me, especially regulatory and repair systems or DNA editing tools.

''Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.''

Sydney Brenner

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Introduction

When a pathogen enters the human body by evading physical barriers and the innate immune system, the adaptive immune system will fight the pathogen (McDermott, Klein, & author, 2018). The antigen is presented to specific parts of the immune system, and in turn, antibodies attack the invading pathogen. These pathogens will be remembered by the use of B or T memory cells, and subsequent attacks can be fended off quickly. Bacteria and archaea are single cell organisms, and must therefore protect themselves within the cell itself. Bacteria can do this, in contrast to eukaryotic cells, by expanding their genome (Hsu, Lander, & Zhang, 2014; F. Jiang & Doudna, 2015). Higher evolved eukaryotes often have many redundant genes, which are extraneous and redundant repeated genetic parts derived from exogenetic elements. Redundant and extraneous genes are normally lost for strategic reasons in a bacterial genome over time. Beneficial material for prokaryotes however, originating from plasmids, viruses and transposons are taken up in the genome (Horvath & Barrangou, n.d.; Karginov & Hannon, 2010; Sampson, Saroj, Llewellyn, Tzeng, & Weiss, 2013). This coordinated dance of losing redundant genes and acquiring beneficial material, makes bacteria and archaea seemingly easy targets for viruses and plasmids. However, clustered regularly interspaced short palindromic repeats (CRISPR) and Cas proteins together, protect the bacterial genome for invading genetic material (Ostria-Hernández, Sánchez-Vallejo, Ibarra, & Castro-Escarpulli, 2015; Touchon & Rocha, 2010). Understanding of this system has shed light on new possibilities that this system offers in the field of genetic modification.

Cas proteins are endonucleases and are able to introduce double stranded breaks in foreign target DNA/RNA. The target of Cas proteins are dictated by the CRISPR sequence, which are small RNA strands that bind the Cas protein. CRISPR structures were discovered in 1987 in *Escherichia coli* when several fractions of DNA were observed spaced next to each other with a repeating sequence (Matsutani, Ohtsubo, Maeda, & Ohtsubo, 1987; Rath, Amlinger, Rath, & Lundgren, 2015). The name CRISPR was first used in 2002, when CRISPR loci were extensively studied to identify closely related bacterial species. CRISPR/Cas systems can be found in 40% of all bacteria and in ~90% of all archaea (Brodt, Lurie-Weinberger, & Gophna, 2011; Mei, Wang, Chen, Sun, & Ju, 2016). Foreign DNA from plasmids or viruses is recognized by the cell, and acquisition of a part of harmful DNA offers adaptive protection after incorporation into the CRISPR loci. Moreover, this system has been recognized as a new genome editing tool with great potential.

CRISPR/Cas works as an adaptive immune system for bacteria, and provides Cas proteins with a target to cut (Bhaya, Davison, & Barrangou, 2011; Richter, Randau, & Plagens, 2013). Three different stadia can be described; adaptation, were the foreign DNA is identified and taken up in the genome as a spacer in the CRISPR repeat cassettes. Secondly, expression of the crRNA to guide the proteins to their target sequence which is the last step of the CRISPR Cas system, interference(Cady, Bondy-Denomy, Heussler, Davidson, & O'Toole, 2012). Three types of CRISPR Cas systems have been identified in bacteria and archaea, of which type II is arguably the most interesting when it comes to harnessing this system for genome editing, this will be discussed further in chapter 1.

Several important discoveries lie between the first discoveries of the CRISPR spacers and using it as a genome editing tool, which are discussed in this essay. CRISPR Cas has become rapidly a very important tool to edit genes, introduce knockouts and small mutations (Christopher A. Lino, Jason C. Harper, 2018). Moreover, CRISPR systems have been used for genome editing in both bacteria and eukaryotes, which means that it can be used in almost all organisms (Ceasar et al., 2016). The most

powerful aspects of using CRISPR Cas, is the efficiency and the speed at which the editing design can be done compared to other genome editing techniques (Adli, 2018). However this does not mean that this system is perfect or does not have drawbacks. This essay will shed light on the applications of CRISPR Cas as a genome editing tool in context of limitations and its potential to overcome these limitations. The first chapter will explain the Crisp Cas system in further detail, and the way it is modified in order to use CRISPR Cas for specific site mutations. Than other genome editing techniques before CRISPR/Cas are discussed in chapter two. Chapter three and four, give examples of CRISPR Cas research that illustrate the usable cell types / organisms and applications of the technique respectively. As CRISPR Cas has become rapidly so popular, the main question addressed in this assay, is what are the major issues of the CRISPR Cas technique, and if it is safe to use in medicine, for example gene therapy.

Applications and limitations of the CRISPR/Cas system

Chapter 1 CRISPR Cas system

In 1991 Barrangou et. al., discovered that *streptococcus thermophiles* can become resisted to a bacteriophage by adding a fragment of its DNA into the CRISPR locus (Rath et al., 2015). Moreover, CRISPR/Cas systems can be transferred to other bacteria and provide protection against invasive nucleic acids. The CRISPR locus can be found in chromosomal or plasmid DNA, and consists of repeats and spacers, placed alternatively (see figure 1) (F. Jiang & Doudna, 2015). An array of short palindromic sequences are separated by non-repetitive spacers (clustered regularly interspaced short palindromic repeats), giving CRISPR its name (see figure 1). Spacer and repeat length are well conserved within a specific CRISPR locus, however the length can differ between loci or genomes. The spacer length is between 26 and 72 bp, whereas the repeat sequence is between 21 and 48 bp. The total number of spacers may differ greatly, from a few to several hundreds within one CRISPR loci. Spacers can therefore take up a significant portion of the genome (Gasiunas, Sinkunas, & Siksnys, 2014). A highly conserved leader sequence is located at the start of the CRISPR loci, which is rich in A-T base pairs. Newly acquired spacer providing immunity are placed at the leader sequence. The order of acquired spacers can therefore be observed starting from the leader sequence. Targeting of foreign DNA differs greatly between CRISPR/Cas systems, whereas acquisition of exogenous DNA is almost universal for all systems. Adaption involves a specific set of Cas

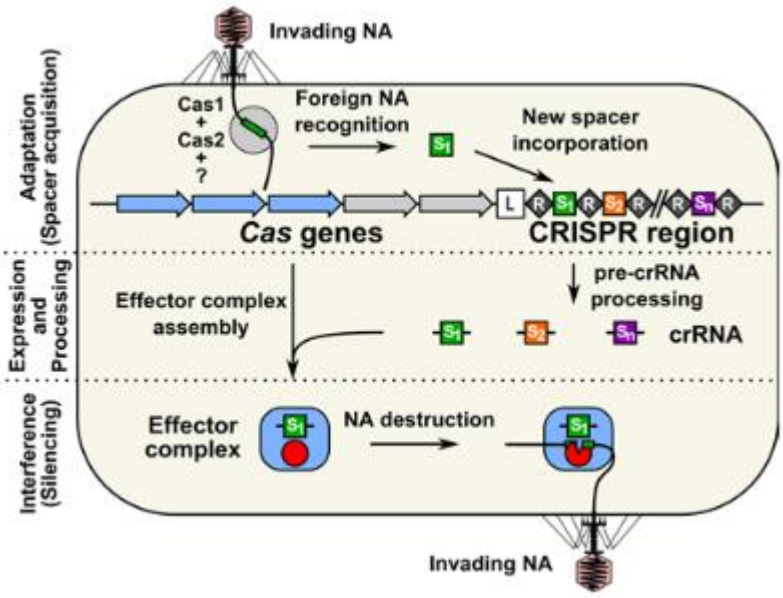

Figure 1 Schematic overview of CRISPR acquisition, expression and interference. Cas proteins are involved in acquisition of harmful DNA sequences and interference. Unique DNA sequences (spacers) are placed in-between repeats (black diamonds) and expressed to guide effector complexes to invading DNA.

proteins, Cas2 and Cas3, assisted by related proteins which differ for each class (Makarova et al., 2011). These enzymes sample several stretches of bases called protospacers, and insert these in the CRISPR loci subsequently.

Before the CRISPR array can be used by the Cas proteins to target foreign DNA, so called expressed pre-crRNA is cleaved by ribonucleases and other Cas proteins (Barrangou, 2015). crRNA will than form CRISPR ribonucleoproteins (crRNP) complexes, which are able to target harmful DNA. Preventing self-targeting is paramount for prokaryotes, because bacterial genomes relay on homology-directed recombination to repair DSB(Farboud et al., 2018). Without a template to repair the induced DSB, the DSB will result in the death or silencing of the prokaryotic cell. crRNA can obviously self-target the CRISPR loci from which it originates, and the CRISPR system can have one of two systems to prevent this. The first system, which is present in most CRISPR/Cas systems, requires a protospacer adjacent motif (PAM), to be able to target the DNA. A PAM site, typically 3 base pairs long, first has to be recognized, and the neighbouring sequence is analysed by the crDNA to initiate subsequent cleavage. The second system, present in CRISPR/Cas systems which lack PAM recognition, requires mismatches adjacent the target sequence. If the flanking parts of the crRNA match the target site, indicating CRISPR loci, the target sequence will not be cleaved. However, self-targeting still occurs more than once out of 500 events according to Gasiunas et. al. (Gasiunas et al., 2014). In only a few cases can this induced DSB be overcome, yet death is the outcome in most cases of self targeting.

Three groups of CRISPR/Cas systems

CRISPR/Cas systems differ between species, and three seperate types are so far established by researches. Recently a fourth group was proposed, however discussion over the specifics of this type still exist. Moreover, several genomes still do not fit in any established type and are therefore unclassified CRISPR-Cas systems (Selle & Barrangou, 2015). Each established CRIPSR/Cas system type consists of several subtypes with a total of (Makarova et al., 2011). System types are established computationally by comparing genomes of many bacterial and archaea species with each other. The three well-established CRISPR types are discussed in this essay in order to explain the system type selection for genome editing.

The type I system consists of six subtypes (I-A – I-F), and are characterized by the Cas3 protein, which is present is all type I CRISPR/Cas systems. Mature crRNA binds with a cascade protein, a large protein, which in turn undergoes conformational change upon correct base pair binding. The conformational change in the cascade protein recruits Cas3 to the target site which facilitates target DNA degradation. Cas3 proteins contain helicase and DNase domains in order to degrade the target sequence. The Cas3 protein must bind with the cascade protein for DNA cleavage, making the type I CRISPR system a multi-protein complex. Type II CRISPR/Cas systems encode the Cas9 gene, which is indicative protein of type II, in addition to the Cas1/Cas2 genes. In some cases of type II systems a fourth protein (CSN2 or Cas4) is present. Type II has been divided into three subgroups (II-A – II-C). Cas9 is a large protein, contains at least two nuclease domains, and is able to induce DSBs as a single protein. This type of system is different from other systems by requiring only one protein and the use of two RNA strands. Normal crRNA, as discussed above and trans-activating crRNA (tracrRNA). TracrRNA is partially complementary to the crRNA and forms pre-CRISPR RNA complex, which is cleaved by RNase III to a mature complex subsequently (see figure 1). TracerRNA and crRNA together, bound to Cas9, are necessary for target binding and degradation by binding to a PAM site

and interrogating the flanking 5' side with crRNA homology. The last identified CRISPR/Cas system group, type III, has Cas10 as unique protein identifier, of which the function is yet unknown. This system can be divided into two subtypes, III-A targets DNA and crRNA forms a complex with the Csm protein. Subtype III-B includes the CMR protein and can cleave RNA, which is the only type which can do so. Both subtypes require multiple proteins for actual cleavage, of which many are still unknown.

Identification of Cas9 as DNA editing tool

The different systems described above are divided into 3 types and 11 subtypes, based upon the presence of specific Cas genes (Gasiunas et al., 2014). Type II CRISPR/Cas systems require only one protein for inducing specific DSBs, whereas the other types involve multiple proteins. This makes Cas9 the ideal type as a genome editing tool. Moreover, by combining the crRNA and tracrRNA into one single-guided RNA (sgRNA), only two components are needed for CRISPR/Cas targeting (Cui, Xu, Cheng, Liao, & Peng, 2018). The crRNA sequence contains a guide part of 20 nucleotides and a 12 nt repeat region (Mei et al., 2016).The tracrRNA has a 12 nt repeat long region anti-homologous to the repeat region on the crRNA and three stem loops, a tetreloop and a linker between stem loop 1 and 2, which are vital for proper function of Cas9 (see figure 2). The Cas9 protein has two lobes which fulfil two functions, recognition of the target sequence, and the nuclease lobe which induces the DSB. The recognition lope, characterized by a long a-helix, consists of an REC1 domain and REC2 domain, which have affinity for the repeat anti repeat duplex and are Cas9 specific. The nuclease lobe consists of three distinct domains, the RUvC, HNH, and PAM interacting domain. RuvC and HNH are both named after known nuclease domains from other structures. RuvC originates from a protein which resolves holiday junctions in Escherichia coli, whereas the HNH domain was found in phage T4 endonuclease VIII, and is responsible for recognition and cleavage of DNA. The Cas9-sgRNA complex recognizes a target sequence, and the PAM site on the non-complementary strand. After binding, the ternary complex is formed which initiates DNA cleavage of the complementary and non-non-complementary strand (DSB).

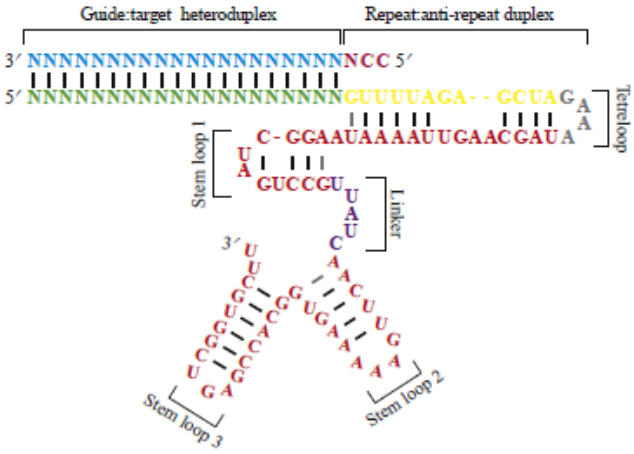

Figure 2 crRNA bound to tracrRNA. RNA heterodimer of crRNA (top) and tracrRNA (below). This complex binds to Cas proteins and promotes a target sequence for cleavage. Mei et al. 2016.

Harnessing CRISPR/Cas for specific DNA modification

Editing of the genome after DSB induction relies on two DNA-repair pathways, non-homologous end joining (NHEJ) and homology directed repair (HDR)(Farboud et al., 2018; Mei et al., 2016). A third repair pathway, which will occur when the NHEJ pathway is inactivated, is alternative end joining (Alt-NHEJ). The Alt-NHEJ pathway is not studied in great detail and is often hard to distinguish from NHEJ because the outcome of repair is often very similar. The NHEJ pathway repairs DSBs without a homologous sequence, which means that loss of nucleotides will not be corrected for. Inappropriate NHEJ DNA repair can lead to small insertions, deletions or larger mistakes such as translocations or telomere fusion (Maruyama et al., 2015). HDR relies on a homologues template which will guide the repair process, resulting in perfect repair by replicating the template. The most common form of HDR is homologous recombination. In this process, the DNA from the 5' side of a DSB is removed and

strand invasion occurs from the 3' side of the DSM onto a homologous sequence (see figure 3). DSBs that are repaired with the HDR pathway are therefore unlikely to include insertions or deletions due to "reading" of a homologous sequence.

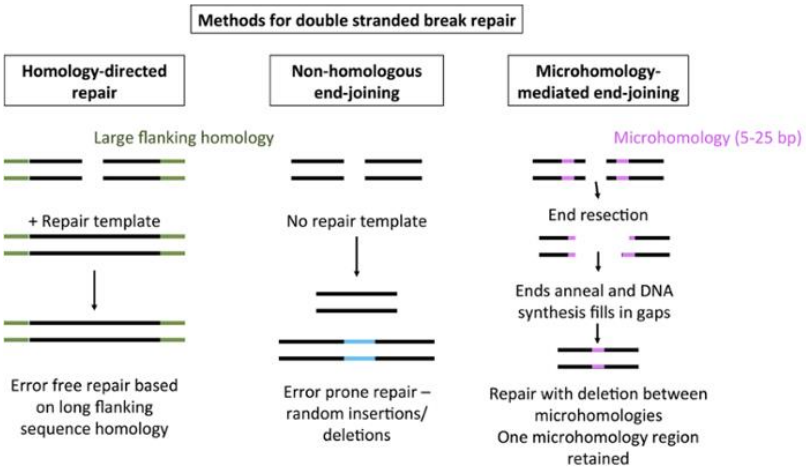

Figure 3 Possible DNA repair pathways after DSB. The efficiency and/or possibility of each pathway depends on the cell type and state. Mei et al. 2016.

Introduction of a homologous DNA strand carrying the gene mutation is required when specific genome editing is desired, in addition to the Cas9/sgRNA hybrid (Ceasar et al., 2016). When the induced DSB is repaired with the introduced DNA strand carrying a mutation, the mutation will also be copied into the repaired DNA site. Whether the NHEJ or HDR pathway is used depends on the host cell. Prokaryotic cells have limited or completely lack NHEJ, and are depend on HDR to repair DSBs. Introduction of specific mutations is therefore more straightforward in prokaryotic cells (Kennedy & Cullen, 2015). Eukaryotic cells however, have a very low rate of homologous recombination and normally repair DSB using the NHEJ pathway (Maruyama et al., 2015).

Chapter 2 Genome editing alternatives

Since the discovery of DNA and understanding of the importance, scientists have tried to alter sequences to observe the phenotype (Arimondo et al., n.d.; Segal & Meckler, 2013). Altering DNA is extremely important in not only molecular biology, but also in medicine and the industry. Since the 80s several techniques have been developed in order to alter DNA, by PCR in vitro and by the discovery of homologous recombination in vivo (Segal & Meckler, 2013). This chapter will shed light on early genome editing techniques as well as modern alternatives to CRISPR/Cas, their possibilities and limitations.

Mega nucleases

In early studies, specific gene alteration by homologous recombination gave unprecedented insights into the functioning of genes in organisms (Mei et al., 2016). However, the efficiency of integration of exogenous DNA was very low (10^{-3} – 10^{-9} cells). Additionally, off target integration is possible, and studies found that uptake of exogenous DNA is very depended on cell state and type (Maruyama et al., 2015). Researches sought to overcome these limitations and discovered that the rate homologous recombination could be increased by introducing a DSB at the target site. Restriction enzymes are of course not specific enough to target specific sites due to their small recognition sites. Endodeoxyribonucleases, or meganucleases, are proteins which can recognize a large nucleotide sequence (12 – 40 bp). I-SceI was the first discovered endodeoxyribonuclease protein able to introduce DSBs in 1990 (Haft, Selengut, Mongodin, & Nelson, 2005). I-SeI recognises an 18 bp long sequence, and would therefore, statistically, only cut once in the human genome. Despite the fact that there are hundreds of different meganucleases, the chance of finding one for a specific sequence can be difficult. To expand this library, existing meganucleases can be modified by

introducing amino acid sequence variations, thereby changing the recognition site. Alternatively, protein domains of different meganucleases can be fused together, creating a chimeric protein. Engineering a new mega nuclease with the goal of creating a specific DSB in a target sequence however, is very labour intensive. And as mentioned before, eukaryotic cells DSBs are mainly repaired through NHEJ and therefore randomly inserts or deletions may occur at the target site.

ZFNs

The next major breakthrough in genome editing came with the discovery of zinc finger proteins (ZFN). ZFNs work in a similar manner as meganucleases, but have several advantages (Gaj, Gersbach, & Barbas Iii, n.d.; Segal & Meckler, 2013). ZFNs get their name from zinc ions which regulate small protein motifs that, similar to tRNAs, are able to recognize a 3 bp sequence. The zinc ion in this protein motifs stabilizes the fold between two Cystine and Histine molecules. The DNA binding loop by these ligands resembles fingers, hence the name ZFNs. These individual ZFNs can be combined to match a specific sequence, moreover the combined recognition fingers are then able to use for genome editing. The second component of the ZFN protein is a nuclease linked to the DNA binding domain which can induce DSBs. FokI was carefully chosen for two specific reasons, firstly, native FokI already has two distinct domains for DNA sequence recognition and cleavage. The recognition domain of FokI was therefore replaced with the ZFN protein. Secondly, FokI operates as a homodimer and two FokI proteins are required for DNA cleavage. This means that two zinc finger modules are needed which target tow sites close to each other to achieve homodimerization. Requiring two ZFN modules for full cleavage greatly reducing the off target effect. Targeting a 3-bp DNA sequence, zinc fingers can be put in tandem of 6 to 7 proteins, which will target a DNA site of 18 – 21 bp long. Zinc fingers of all 64 combinations available, however adjacent recognition may occur, which can result in wrong recognition (Christopher A. Lino, Jason C. Harper, 2018). Researches have tried to increase the number of zinc fingers to reduce off targeting, thereby expanding the target DNA recognition sequence, but with limited success. Alternatively, oligomerized Pool Engineering (OPEN) is a software package which takes into account the context-dependent interactions between neighbouring fingers when designing a zinc finger protein (Xiao et al., 2013). More similar software packages exist, however the effectiveness of designed ZFN proteins have to be tested experimentally.

TALEs

ZFN engineering was followed by Transcription Activator-like Effector Nucleases, which in contrast to ZFN do not recognize 3-bp sequences, but singe nucleotides (Gaj et al., n.d.; Xiao et al., 2013). TALEs are naturally occurring proteins originating from the bacteria *Genus Xantomonas*, and composed of 33 – 35 amino acid repeat domains that each recognize one nucleotide. Like zinc-fingers, TALE proteins are linked together to recognize a specific DNA sequence. FokI is also used as DNA cleavage protein in this technique, and therefore two TALE constructs that target close to each other on a target DNA site are required for DSB. FokI mutants have been developed over time with increased specificity and rate of cleavage. Although TALE genome editing is more flexible than ZFN, cloning of TALE proteins able to recognize a target site is challenging due to the extensive identical repeats. Several cloning strategies have been developed to overcome this hurdle, such as Golden Gate and high-throughput solid-phase assembly (Segal & Meckler, 2013). These techniques allow researchers to insert multiple DNA fragments into one target. One limitation of the TALE technique is the binding site of TALE, which must start with a thymine base. TALE has been used in model organisms such as mice, zebrafish, rat, but also human embryonic stem cells.

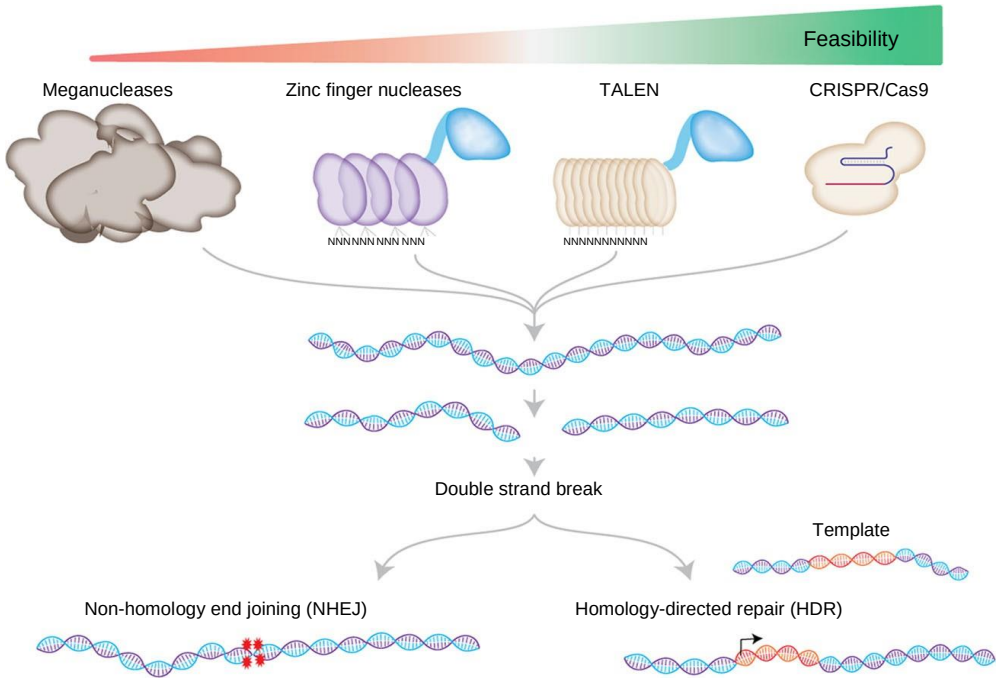

Figure 4 Overview of genome editing techniques. Schematic overview of techniques and feasibility.

Chapter 3 Possibilities and applications of CRISPR/Cas9

CRISPR cas9 follows TALE as editing technique and is the most recent breakthrough in genome editing. CRISPR/Cas9 does not require the construct a protein which targets a specific DNA site to induce a DSB. Only a sgRNA is needed to direct Cas9 to a specific site to induce DSBs. Additionally, a homologous sequence to the target site is required if specific genome editing is desired (Cui et al., 2018). As mentioned before, the 20 nucleotide long recognition sequence is responsible for specific targeting of the Cas9 protein. After a DSB, the target site can be repaired by HDR in the presence of a homologous DNA sequence, for specific genome editing. Single-stranded oligodeoxynucleotides (ssODN) are introduced to the cell, which are ~90 nucleotides long and guides HDR for specific editing. Eukaryotic cells have a low rate of HDR however, and mainly repair DSB through NEHJ, which can result in deletions, inserts or inversions.

Since the discovery of Cas9 as a genome editing tool, many modifications have been made to Cas9 to improve functioning or introduce new functions(Hsu et al., 2014). Firstly, the crRNA and tracrRNA are fused to make one sgRNA, making it a 2 component system and ideal easy genome editing. Moreover, Cas9 was human codon optimized and with the addition of a nuclear localization signal (NLS) ready for use in human cells. Yet more modifications have been made since then to make the CRISPR/Cas9 system more versatile. This chapter will discuss modifications to Cas9 and applications, that use the system beyond introducing knock-ins and knock-outs etc.

Developing mice models

CRISPR/Cas9 was first used in traditional models, such as genetically modified mouse models (Choi & Lee, 2016; Li et al., 2015). Before CRISPR/Cas9, genome editing was done with trans genesis or gene targeting in embryonic stem cells. These earlier editing techniques took up large parts of studies because genome editing was very time consuming and labour intensive. CRISPR/Cas9 can in a one-step approach mutate genes in mice to null, conditional or introduce precise mutations. Moreover, reporter, or tagged alleles can easily be obtained with CRIPSR/Cas9. For example,

researches generated mouse models for lung adenocarcinoma, were KRAS, p53 and LKb1 genes were mutated by introducing the sgRNA virally and through reagents (Shi et al., 2016). Efficiency of this technique also means less mice are requied, because modification of several genes can be done in one step.

Therapeutic delivery of CRISPR/Cas

The list of genetic diseases is extensive and many disorders have been directly linked to abnormalities in specific genes (Platt et al., 2014; Yin et al., 2014). These abnormalities can range between single base pair substitutions and large deletions. CRISPR/Cas9 can be delivered in organisms to correct genetic defects, which has been done in mice, but also in humans (Cavazzana, Six, Lagresle-Peyrou, André-Schmutz, & Hacein-Bey-Abina, 2016; Oude Blenke et al., 2016; Yi & Li, 2016; Yin et al., 2014). Study or treatment of genetic disorders have been done for example on Barth syndrome, Duchenne muscular dystrophy, haemophilia and cystic fibrosis. Yin et al. showed in 2014 successful delivery of Cas9 to liver cells using hydrodynamic delivery. 1 in 250 liver cells were corrected in the Fah gene, which is associated with hereditary tyrosinemia. The efficiency rate of correction is not high, however expansion of the modified cells resulted in phenotype rescue. CRISPR/Cas9 has also been used to target human immunodeficiency virus (HIV), giving the cell protection by targeting coding and non-coding sequences during virus integration (Armstrong-James et al., 2017; Mei et al., 2016). These studies show that CRISPR/Cas9 could potentially be used therapeutically, providing protection against viruses.

CRISPR/Cas9 as analogue recording device

Several biological essays exist to investigate the current state of a cell, such as sequesing for the genome, norther blot for RNA and western blot for proteins (Sheth, Yim, Wu, & Wang, 2017; Tang & Liu, 2018). Essays that can give information about the history of a cell are far less developed, despite the fact that this can attribute greatly to studies involving cell stress, such as cancer or other diseases. To address this, researches have made an analogue recording system in both prokaryotic cells and eukaryotic cells with the goal to ‘save’ cellular event in DNA (Tang & Liu, 2018). The study proposes two systems in bacterial cells and one in mammalian cells. The first system, CAMERA1, consists of a writer plasmid (W1) and two recorder plasmids (R1, R2). R1 and R2 are the same except for only a few base pairs. W1 expresses Cas9:sgRNA which targets only R2 due to the few base pairs differences. The ratio of the recorder plasmids R1 and R2 will remain the same over time if Cas9:sgRNA targeting R2 is not expressed. If W1 is activated by an inducer, W1 will introduce a DSB in R2, resulting in the loss of R2, and by extend, change the R1/R2 ratio. Depending on the inducer system, the specific ratio change is a direct readout of amount or duration of exposure. Events from within the cell are recorded in the DNA and stored as ratio changes, depending on the inducer for the writer plasmid. Presence of a metabolic factor can be recorded, event order can be deduced, or exposure to light or a viral presence can be stored within the CAMERA system. This illustrates the amount of diverse applications for which CRISPR/Cas9 can be used (Sheth et al., 2017).

Modifications of Cas9 in biology

RuvC and the HNH domain located at the nucleus lobe (chapter 1) are responsible for introducing a break in the complementary and anti complementary strand. These domains can be mutated to become catalytically inactive, whereby the Cas9 protein is still able to recognize a target site, but is unable to cleave the DNA(Adli, 2018). Instead the Cas9 protein will remain bound at the site, which will inhibit transcription due to physical hindrance. Dead Cas9 (dCas9) will not modify the DNA but

remain bound, which offers great possibilities in biological essays. Repressor factors can be fused with the dCas9 protein, such as the Kruppel-associated box (KRAB), and inhibit gene expression. Activators can also be fused with dCas9, which has been done with VP16 and VP64, which activates specific gene expression. V16 and VP64 are examples of transactivation domain transcription factors, and are a scaffold for expression regulators.

Fluorescent imaging of genes using dCas9

Fusing proteins with a specific function to dCas9 has been done with multiple goals and holds great potential in biology (Komor, Kim, Packer, Zuris, & Liu, 2016). Fusion of a fluorescent protein to dCas9 can give insights in chromosome localization behaviour and gene regulation (Polstein & Gersbach, 2015). Until recently, ZNF and TALE proteins have been used for this purpose, but advances in dCas9 have significantly improved efficiency and scope of genome targeting with fluorescent in situ hybridization (FISH)(Rath et al., 2015). Conceivably, background fluorescence is a significant problem due to the unbound dCas9/fluorescent proteins which also will produce signal in the cell. Up to now, only repeating genomic regions have been able to been studied using dCas9/FISH, which give a good fluorescent signal. Approximately 26 – 36 individual sgRNAs are required for live cell fluorescent imaging of a non-repeating genomic site(Eid, Alshareef, & Mahfouz, n.d.; Rath et al., 2015). Alternatively, it also possible to use a protein which acts as a scaffold for fluorescent molecules. Studies have been done with multiple (>16) binding sites, and due to the expansion of fluorescent imaging techniques, even more possibilities exist. For example, Fourier resonance energy transfer (FRET), which relies on the proximity of another fluorescent molecule for a shift in wavelength.

Changing gene proximity by protein dimerization

Protein dimers fused to dCas9 offer still more possibilities in manipulation of chromatin positioning. The effects of positions for specific genetic loci can be studied in great detail(Adli, 2018; Arimondo et al., n.d.). For example, by introducing artificial chromatin loops and fusing these proximate to endogenous parts of the genome their effect can be studied. Two dimerizable protein domains from a plant-based signalling pathway (ABI1 and PYL1), have been used for forced chromatin loop formation between an enhancer and promoter region, which are normally distant from each other (Adli, 2018). In this research, differences in expression at the β-locus were observed between K562 hematopoietic and HEK293T cells. This system can prove vital for several research fields such as synthetic chromatin biology.

Activation and inactivation of expression

Expression and subsequent activity of CRISPR/Cas9 can be brought under the control of a promoter, however another way of temporary activation can be achieved through light-activated Cas9 (Polstein & Gersbach, 2015). If dCas9 is fused with CIBN (cryptochrome-interacting-basic-helix-loop-helix 1), lose light-sensitive cryptochrome 2 (CRY2) can be recruited to the CIBN molecule under light stimulation. In the presence of blue light CRY2 undergoes conformational change and can heterodimerize with CIBN. An activator or repressor protein fused with CRY2 can than ensure gene expression or inactivation. This system is activated by light stimulation, and can therefore not only provide temporarily genome editing, moreover spatial editing can be achieved.

Chapter 4 Future perspectives of CRISPR/Cas9

Mutational modifications of CRISPR/Cas9 have given the technique many more additional functions and possibilities beyond genome editing. In this chapter, limitations and perspectives for CRISPR/Cas9 are discussed (Arazoe, Kondo, & Nishida, n.d.; Selle & Barrangou, 2015).

Requirements and limitations in the CRISPR/Cas9 system

Several factors are imperative in genome editing, most importantly, delivery, efficiency and off target effects (Maruyama et al., 2015; Oude Blenke et al., 2016). 3 components are needed for genome editing, Cas9, sgRNA and ssODN. This makes delivery is quite straightforward compared to meganucleases, ZFN and TALE, because the production and design of a sgRNA and ssODN is greatly less labour intensive.

The target DNA sequence is recognized by the 20 nucleotide long sequence on the sgRNA, however three mismatches are tolerated between the recognition sequence and target site. Some studies have shown that off target DSBs occur with substantial frequency (Eid et al., n.d.; Maruyama et al., 2015). Interestingly, truncating the spacer to 17 – 19 nucleotides increases the specificity due to intolerance of mismatches. However, while off target events occur less often, on-target efficiency is decreased. Longer recognition sites (>20) do not increase the specificity and also decrease the on-target efficiency. Several software packages exist which can predict the on-target efficiency based upon experimental data. Examples of such software packages are E-CRISP, CHOPCHOP and PROTOSPACER (Cui et al., 2018). As mentioned in the previous chapter, increasing the off target efficiency can also be achieved by two nickase Cas9 proteins target close to each other.

Rather than inactivating both endonuclease domains, RuvC and HNH, mutational inactivation of one domain will result in a nickase, because one strand will remain intact (Eid et al., n.d.; Rees et al., 2017). Cas9 mutant D10A shows RuvC inactivation, whereas an H874A abolishes HNH domain activity. By combining two individual sgRNA, which target close together, a staggered DSB can be induced which can improve the efficiency and specificity of the CRISPR/Cas9 system greatly. Two separate cutting sites decreases the off target effect, moreover, it can shift the host preferred DNA repair pathway.

Optimal PAM site recognition requires a 5'-NGG-3', however recognition also takes place with a low frequency on 5'-NGA-3' and 5'-NAG-3' PAM sites(Maruyama et al., 2015). This makes the PAM an important factor to consider when choosing a target site for Cas9. Additionally, several Cas9 variants exist which show affinity for PAM sites other than NGG. For example, a VQR variant shows a high affinity for 5'-NGAN-3', and a VRER Cas9 variant recognized a 5'-NGCG-3' PAM site robustly. These mutants can further be developed to decrease off-targeting of Cas9, moreover it is possible to use specific DNA sequences as PAM sites which occur repeatedly.

Ethics of CRISPR/Cas9 usage in biology

With the power of easy genome editing comes the responsibility of using the technique wisely. The tools themselves do not pose a threat, but extreme careful consideration is needed for use in organisms regarding biosafety and ethically (Maruyama et al., 2015; Smalley, 2018). Several aspects of the CRISPR/Cas9 system are still poorly understood and its usage could potentially change an entire species. Consequently, this could have a significant impact on the ecological balance (Mei et al., 2016; Pope, 2017). Gene editing has been done in human embryos, which raised questions about the usage of CRISPR/Cas9. Several countries have banned CRISPR/Cas9 for studies in human

embryos, and genetic editing of embryos has become controversial among scientists. A gene therapy study in 2002, which involved 20 children suffering from immunodeficiency (SCID X-1), was initially very successful with 17 children cured (Cavazzana et al., 2016). However, 5 children developed T-cell leukaemia, because the SCID X-1 correcting gene was inserted near tumor-promoting regions, which caused activation of those genes.

Discussion and conclusion

The field of genome editing has been in existence long before the discovery of CRISPR/Cas9, however the field has been rapidly evolving due to the discovery. The ease by which DNA can be edited has improved significantly, requiring only a small RNA sequence to target an individual DNA site. The common drawbacks inherit to genome editing, such as off target effect and efficiency still exist and this makes CRISPR/Cas9 by no means a perfect tool (Ran et al., 2013). However, specificity can be increased by use of Cas9 nickases and other Cas9 mutants, moreover, base editors can be fused to dCas9. Understanding of sgRNA binding and mismatch tolerance is still lacking, despite the existence of many computational sgRNA design tools. Furthermore, CRISPR/Cas has become undoubtedly an essential tool in research, but has still yet to prove itself as a valid treatment in medicine.

CRISPR/Cas9 can attribute greatly to the field of gene therapy, over 3000 genes to date have been associated with disease-causing mutations (Cavazzana et al., 2016; Christopher A. Lino, Jason C. Harper, 2018; Yi & Li, 2016). CRISPR/Cas9 has the potential to target these problems at the source, by either introducing DSB for inactivation or re-activating genes by providing additional homologous template DNA (Kennedy & Cullen, 2015). Several key problems need to be solved before CRISPR/Cas9 can be applied to medicine treatments. These factors include prevention of random integration of introduced DNA, cargo delivery vehicle of the system and control over the transcription levels. The availability of many Cas9 mutants, for example dCas9, Cas9 nickase and light induced Cas9 offers great potential to overcome these particular hurdles.

The real power of the CRISPR/Cas9 tool is not only the easy design and multifunctional domains of Cas9. The fact that sgRNA is a separate factor guiding the Cas9 protein offers many possibilities for applications. Repression and activation can be achieved for multiple targets, and both the Cas9 gene and sgRNA can be brought under a promoter. This offers many possibilities when using different promoters for CRISPR/Cas9 components, which can be induced by external or internal stimuli. As shown in chapter 4, cell event history, duration, and event order can be deduced by recording this information in DNA.

CRISPR/Cas9 can be used in prokaryotic cells, eukaryotic cells, archaea and plants (W. Jiang, Bikard, Cox, Zhang, & Marraffini, 2013; Mei et al., 2016; Woo Cho, Kim, Min Kim, & Kim, 2013). Differences can be observed in efficiency due to inherited variations in host DNA repair pathways. HDR is the most or only pathway used in prokaryotes, whereas DSB in eukaryotes are mainly repaired by NHEJ. Only a few CRISPR/cas9 applications are developed for archaea, which are naturally difficult to study with traditional genome manipulation techniques (Bhaya et al., 2011; Brodt et al., 2011). Genome editing in plants holds enormous potential in improving food crops. CRISPR/Cas9 editing was first done in *Arabidopsis* in 2013 (Ceasar et al., 2016). While CRISPR/Cas9 might be very important in the future for strengthening food output, GMO regulation has prevented large scale cultivation of these plants, especially in Europe and Asia. CRISPR/Cas9 has made great strides in human studies towards

correcting disease-causing genetic mutations (Mei et al., 2016). Although these studies are very promising, some studies have been met with tragedies, as mentioned in chapter 4. It is important to point out that some terrible outcomes in studies are due to the therapeutic delivery method, which is a critical factor in gene therapy (Oude Blenke et al., 2016).

No doubt exists about the importance of the discovery of CRISPR/Cas9 as genome editing tool. The system will continue to help researches answer biological questions, and countless other applications can be found and explored. Several key shortcomings have still to be addressed to prevent off-target events and improve efficiency of genome editing. Cas9 mutants have been developed to expand the field of possibilities, which also include nuclease activity and overall specificity. In the future, more applications and theraputical strategies can be expected from CRISPR/Cas9. Limitations of CRISPR/Cas9 as discussed in this essay can be overcome over time, which makes it one of the most promising tools in biology at this moment.

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