Future prospects of the applications of CRISPR/Cas9 in cancer research and therapy

The potential of CRISPR technology in cancer research and therapy

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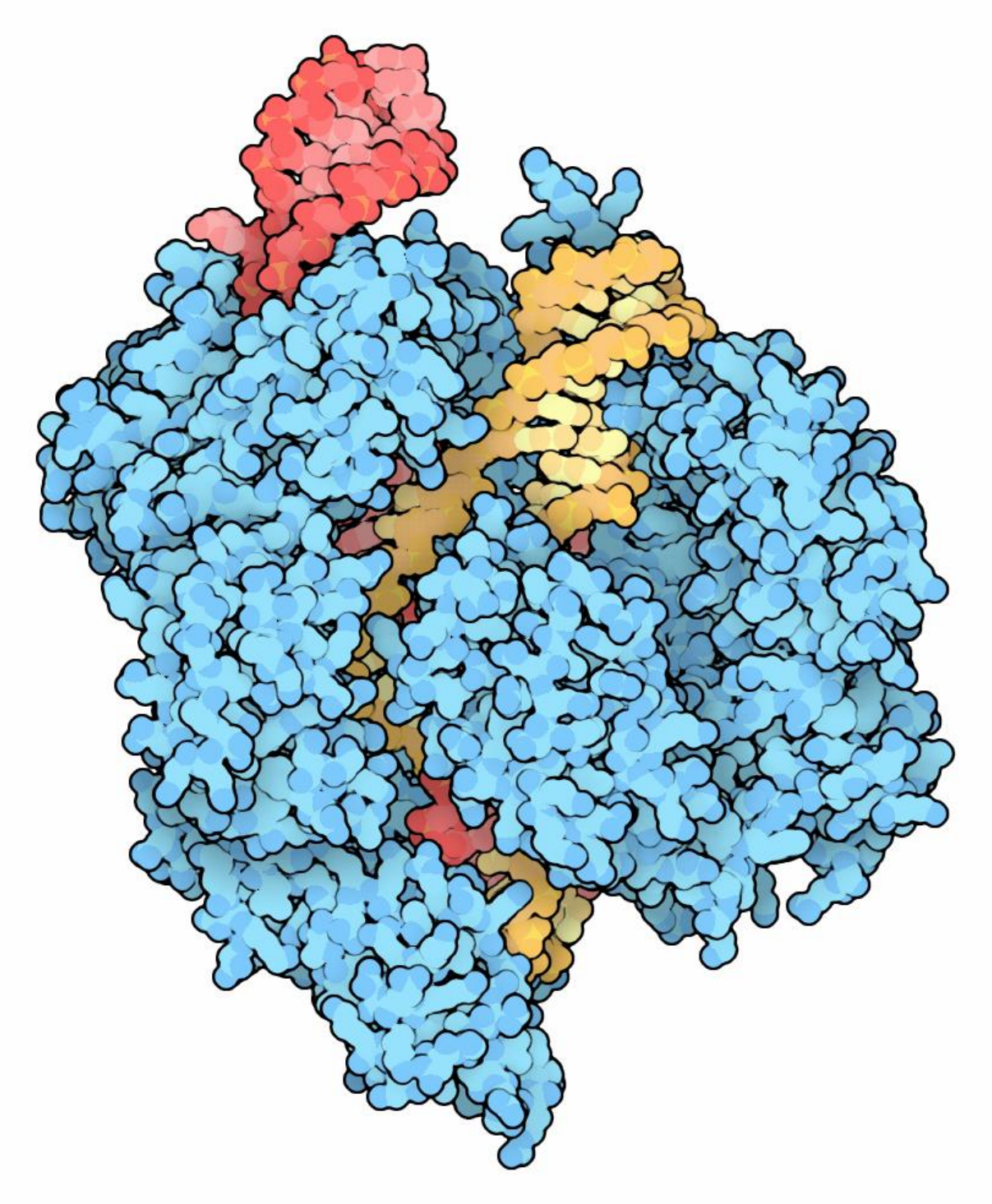

Figure 1 – Schematic Type II CRISPR/Cas with bound viral DNA. Cas9 protein bound to CRISPR RNA (red) and target viral DNA (yellow). (Goodsell, D., 2015). From PDB-101, 'Protein of the month: 181' http://pdb101.rcsb.org/motm/181

"CRISPR has completely transformed the ways biologists study biology. It has given basic scientists a new and more powerful way of asking questions like, 'What genes are involved in cancer becoming metastatic?' and opened new avenues for drug development."

Sam Sternberg, PhD student at the pioneering Doudna Lab

INTRODUCTION

Globally, cancer is the second leading cause of death after heart diseases among humans. It is and will remain a major economic and social burden on both society and individuals. Currently, scientists globally are conducting extensive research toward novel cancer therapies and improving current therapies. Cancer also referred to as malignant neoplasms, is a polygenic disease, characterized by the accumulation of multiple (epi)genetic alterations in the cancer cell genome, which drives cancer pathogenesis, development and organ deterioration (Yi & Li, 2016). These typical (epi)genetic alterations of genes are commonly found in oncogenes (e.g. ErbB), tumour suppressor genes (e.g. p53, PTEN), epigenetic factors and their control loci(e.g. DNMT1), chemoresistance genes (e.g. MDR1), and others (Hanahan & Weinberg, 2011). Current therapies include the surgical removal of cancerous tumours, eradication through radiation therapy and intensive, usually multiple, chemotherapy intravenous infusions. Unfortunately, due to the high tendency of relapse and the primary or acquired chemo- or radiation resistance these therapies often results in poor prognosis and consequences. Therefore, more effective novel therapies, show a lower amount of relapses and which are less radical to the host's body are in high demand (Reis, 2014). More recently, targeting and/or reversing the malignant (epi)genetic changes that cause cancer through genomic editing tools directly, have shown to be a more promising therapeutic method in the fight against cancer.

Since all cancer types are underpinned by genomic mutations, genomic editing represents a prominent tool disease prevention as well as disease treatment. The ability to make precise and targeted changes to live genome *in vivo*, *ex vivo* or *in vitro* by correcting one or more sections of the cancer cell genome, yields an interesting approach for various fields of research. Thus, genomic editing is a promising therapy for cancer, as well as for a wide range of conditions such as haematological diseases, AIDS, diabetes, cardiovascular problems and neurodegenerative diseases (Reis, 2014). Elucidating its relevance, over 2000 gene therapy clinical trials have been conducted worldwide, and several products resulting from gene therapy have already been approved by authorities worldwide (Xiao-Jie, Hui-Ying, Zun-Ping *et al.*, 2015). Studies conducted over the past decade aimed at manipulating genetic phenotypes employed forced homologous recombination and RNA interference (RNAi) for the wanted genetic alterations. More recently, other approaches with different programmable nucleases were introduced: Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Unfortunately, homologous recombination and RNAi were proved inefficient, and ZFNs and TALENs were expensive and slow to reproduce. Therefore, the disadvantages to past technology indicated that the state of genomic editing was limited by the instruments used.

Because of this, research groups around the world investigated new and more potential genomic editing toolboxes. In 2012, a publication by the Doudna lab proposed the currently used CRISPR/Cas9 gene editing system for animal and cellular models, which, when introduced into the model makes site-specific changes to genomic sections (Doudna & Charpentier, 2014). This proposal was followed by the involvement of Doudna in the first reported publication concerning the insertion of manipulated DNA with CRISPR/Cas9, a breakthrough for genomic editing. Only much later, these studies were extended to experimentally demonstrate that CRISPR and its CRISPR-associated proteins (Cas) are linked to the adaptive immunity targeting foreign DNA (Zhan, T., Rindtorff, Betge *et al.*, 2019).

The appearance of an RNA-guided genome editing tool named 'Clustered Regularly Interspaced Short Palindromic Repeats/Cas9' (CRISPR/Cas9), a programmable nuclease, showed potential in the genome-editing field. Because of its relative affordability, efficiency, scalability, precision as well as programmability compared to other genetic editing tools, CRISPR/Cas9 technology is praised as the biggest biotechnological discovery of the century

according to Martinez-Lage *et al.*, as it has opened a door to new possibilities and chances for precise genome editing (Martinez-Lage, Puig-Serra, Menendez *et al.*, 2018). Compared to the current genomic editing arsenal, the tool shows unprecedented potential to study and target diseases with a profound genetic foundation, such as cancer.

In this thesis, I will investigate my research question: What will the recent developments concerning the CRISPR/Cas9 complex effectuate in cancer research and therapy? To be able to do this thoroughly, I will explain the origin and functioning of the CRISPR/Cas9 complex, followed by the current application of CRISPR/Cas9 in cancer research and therapy. Afterwards, I will discuss the potential risks and pitfalls for the complex. Within this thesis, I will also delve how the CRISPR/Cas9 technology poses itself as preventative or curative medicine for cancer. Finally, I will provide a conclusion for my literary research and provide personal insight into the future of CRISPR/Cas9, including the highly relevant ethical aspect of the subject.

CRISPR/CAS: BACKGROUND AND MILESTONES

In 1980, Ishino et al. Published a paper in *'The Journal of Bacteriology', in* which they described the genetic sequence of the CRISPR/Cas protein complex of E. *coli.* Not much later the group of Ishino found the genetic foundation of the CRISPR system in other organisms such as other bacteria and archaea. However, the absolute functioning of the system was not clarified until much later by a different group.

CRISPR/Cas is a prokaryotic component, which functions primarily as an automatic and stand-alone system. Specifically, CRISPR/Cas9 identifies and cleaves DNA and RNA as a way to defend against genetic injections of viruses and other mobile genetic elements (Rath, Amlinger, Rath *et al.*, 2015). The CRISPR locus is a collection of short repeated sequences separated by integrated spacers with distinctive sequences. After the acquisition of new spacers by the CRISPR/Cas9 complex, viral DNA and RNA are recognized, cleaved and removed through the same complex. This activity of CRISPR requires the activation of a subset of CRISPR-associated (Cas) genes. The Cas proteins transcribed from this locus are essential to the response for the protrusion of foreign genetic material inside the cell, subsequently binding with the spacers as 'recognition elements' to search and destroy foreign genomes.

In 2005 several groups described similarities of the CRISPR sequences in the native genome of *Streptococcus* strains to phage DNA (Bolotin, Quinquis, Sorokin *et al.*, 2005). This raised the hypothesis that the concerning sequences were related to the adaptive immune system. However, due to the complexity of the adaptive immune system, the demonstration of such in prokaryotes was a surprise. In 2007 a research group led by Barrangou demonstrated that the bacterium *Streptococcus Thermophilus* could acquire resistance against a bacteriophage through the integration of a genomic fragment of an infectious virus into its CRISPR locus (Barrangou, 2013; Karvelis, Gasiunas, Miksys *et al.*, 2013). Therefore, CRISPR/Cas9 was hypothesized to act as a native immune system in specific unicellular organisms.

Following its initial proof of concept in 2013, the complex was used to manipulate various genes in multiple cell lines and organisms, including bacteria, plants, zebrafish, *C. elegans*, *X. tropicalis*, *S. Cerevisiae* and *D. melanogaster*. The CRISPR/Cas9 genome editing technique showed unprecedented clinical potential compared to the older RNAi, ZFNs and TALENs in precision, specificity and reproducibility. Not only to induce specific genomic alterations and to discover novel drug targets, but also the ability to anatomize chemical-genetic interactions. This period of rapid discoveries concerning genome editing therapies climaxed in 2013 when CRISPR/Cas9 protein *Streptococcus pyogenes* (SpCas9) was used for RNA guided DNA cleavage in mammalian cells *in vivo* for the first time. This showed that CRISPR/Cas9 was not limited by *in vitro* experiments, and could

be applied to living tissues. This was proven by the research of a Chinese group in 2016, led by the oncologist Lu You of the Sichuan University in Chengdu. The group used native immune cells from the recipient's blood and disabled the PD-1 gene using CRISPR/Cas9, which is known to regulate immune responses and often abused by carcinogenic cells to proliferate uncontrollably (Cyranoski, 2016). The editing through CRISPR/Cas9 was performed *ex vivo*, and the experiments turned out successful, becoming one of the earlier genetic immunotherapies in oncology through CRISP/Cas9. The success of this research exemplified the potential of the CRISPR/Cas9 complex but also gave rise to ethical concerns towards this powerful genomic tool.

In 2018 He Jiankui, a Biophysicist professor from the Southern University of Science and Technology in Shengzen, shocked the world when he announced at the World Summit of Human Gene Editing that he and his team had disregarded worldwide regulations on research and medical ethics through the alteration of human embryonic genes. The team had edited the C-C chemokine receptor type 5 (CCR5) through CRISPR/Cas9 and implanted the embryos in two women. One of them gave birth to a twin, of which the researchers proclaimed they were to be immune to the renounced HIV infection. Even though the evidence and the accuracy of the results of this experiment remain to be disclosed, the manuscript, experimental design and data presented at the summit revealed ethical misconduct. (Wang & Yang, 2019). This research was globally presumed unethical and unauthorized research and was loaded with political critique while blemishing the name of genomic research. At the same time, the group took the scientific world by storm on a previously undiscussed topic: the emergence of CRISPR technology as a result of the alteration of a variety of genomes is no longer a dystopian, abstract and hypothetical subject. The applications of CRISPR are exceeding expectations. Venturing beyond research and biomedical therapies, it gives rise to new and innovates fundamental ethical concerns.

Throughout recent history, the CRISPR technology appears as a potential genomic editing toolbox, used widespread from research to medical application. However, this same history makes evident what risks the usage of the CRISPR technology envelops. With the rising need for genomic editing which is simple, cost-efficient and easy to amplify for large-scale operations tool, the usage of CRISPR has an undeniable role on the forefront of research. At the same time, the application of the CRISPR technology comes with clear ethical issues. Recent objections towards the implementation of CRISPR/Cas9 in genome editing include "an enormous threat", "categorical wrong" and "playing God" (Locke, 2020) enveloping the disunity on the subject. When applied correctly, however, it has the supposed potential to provide mankind with a tool of unprecedented possibilities. As Brokowski stated with regards to what is currently valued as the most versatile genomic engineering tool created in the history of molecular biology: "Moral decision making should evolve as the science of genomic engineering advances and hold that it would be reasonable for national and supranational legislatures to consider evidence-based regulation of certain CRISPR applications for the betterment of human health and progress." (Brokowski, 2018)

CRISPR/CAS9: IDENTIFICATION AND FUNCTION

Considering the diversity and complexity of architecture within the various CRISPR/Cas subgroups, consistent classification and definition have proven to be difficult. According to the group of Rath, the diversity of Cas proteins, the presence of multiple CRISPR loci and frequent horizontal transfers of CRISPR/Cas systems makes the specification of subtypes within the CRISPR system a complex task (Rath *et al.*, 2015). Through global efforts, however, three major subgroups within the CRISPR/Cas system are differentiated. Each subgroup contains group-specific sets of Cas proteins essential to correct and particular functioning (Makarova, Wolf, Alkhnbashi *et al.*, 2015). Each of these group-specific sets of Cas proteins resembles CRISPR cascades of genomic editing, represented throughout various organisms.

A more recent classification introduced 3 additional subtypes (II-A, II-B and II-C) because of the identification of additional active Cas proteins. However, the niche function of these subtypes remains to be proven. Therefore the latter subtypes will be excluded from this thesis (Makarova *et al.*, 2015). Due to the relative ease in application, and targeted derivation from *E. coli*, the Type II (II-A) CRISPR/Cas9 system has been the most studied in comparison to the other systems since its first finding (Bolotin *et al.*, 2005; Martinez-Lage *et al.*, 2018). Therefore the focus of this thesis will be the type II CRISPR system and its possibilities.

The CRISPR/Cas genome exists of Cas genes, associated with different cellular interactions. Previous studies dictate that the proteins transcribed are most commonly interactive with nucleic acids, such as nucleases and helicases or RNA-binding proteins. As shown in Figure 2, the Cas protein system is classified into four distinct groups which are referred to as modules. Each module represents another set of Cas-proteins involved in different stages and functioning of the immunizing CRISPR/Cas activity. Furthermore, Cas proteins are sorted among classes, based on their functionality in the CRISPR/Cas system. Most commonly, Cas proteins work in concert to enact targeting and cleaving processes (Barrangou, 2013). One exemption is the ancillary module which is comprised of accessory proteins to the overall CRISPR complexes. They are not included in the core complex of CRISPR, though have other applications. For example, Cas4 plays a role in programmed cell death and Csn2 plays a role in the prevention of native genome damage, though has no active role in CRISPR. (Makarova *et al.*, 2015) Cas1 and Cas2 are prominent subtypes throughout various CRISPR types and represented in the majority of CRISPR/Cas systems (Grissa, Vergnaud, & Pourcel, 2007). This protein couple forms a quasi-autonomous complex that is required for the inclusion of viral DNA in the CRISPR/Cas locus within the hosts' genome. The endonuclease activity of the Integrase Cas1 protein combined with the nonenzymatic interferase activity of the Cas2 protein forms a

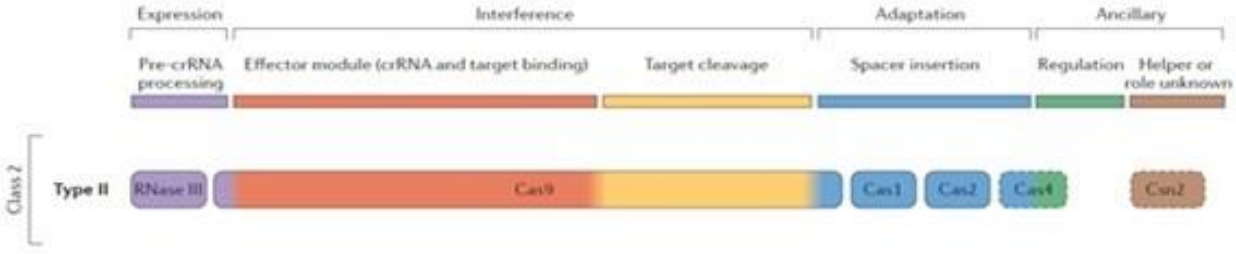

Figure 2 – Functional classification of Cas proteins in Type II CRISPR/Cas9. Protein names follow the current nomenclature and classification of Makarova et al, 2011. Designation of Classes, Types, and inherent Cas proteins involved in each module of activity. Important is the broad multi focus proteins found in Class 2. The function and activity of the Cas proteins in each Type are listed (at top) and split between four modules of influence. (Makarova et al., 2015).

complex which envelops the spacer acquisition and integration, representing the 'expression module' in the CRISPR/Cas systems (Makarova *et al.*, 2015).

The type II CRISPR/Cas9 complex is distinguishable from types I and III through signature Cas9 protein activity. Similar to other systems, the type II system also encodes for Cas1, Cas2 and occasionally Csn2 and Cas4 (Rath *et al.*, 2015), even though the type II complex is lowest in total genes associated (Grissa *et al.*, 2007). Unlike types I and II however, the multi-domain Cas9 protein involves the full extent of the effect of the CRISPR/Cas9 complex. This allows the condensation of the multi-focus function of the immune system into one protein. Both the function as an RNA-guided endonuclease with tracrRNA-crRNA duplex directed target sequence recognition and the function as the lead molecule directing protein-mediated DNA cleavage are centralized within the same CRISPR/Cas9 molecule (Barrangou, 2013). According to the research group of Makarova "the relatively large Cas9 protein, of about 950–1,400 amino acids, is required for all three of the functional steps of CRISPR-based immunity (adaptation, expression and interference) in type II systems and thus concentrates much of the CRISPR–Cas system's function in a single protein."

The enacting complex of CRISPR/Cas9 is defined through the nuclease cleavage-active domains referred to as RuvC-like Nuclease and HNH Nuclease Domain. Both of these domains are required for DNA cleavage (Barrangou, 2013). Furthermore, type II system-based interfering of DNA requires the presence of Protospacer Adjacent Motif (PAM) sequences. These PAM sequences are 2-6 base pair DNA sequences immediately following the target DNA sequence (Zhang, Adikaram, Pandey *et al.*, 2016). PAM sequences are called 'non-self-activation' mechanisms prevent the CRISPR/Cas systems from targeting its CRISPR loci, and thus are only identified within the viral genome. (Makarova et al., 2015). An important side note is that different Cas types recognize different PAM sequences, therefore CRISPR systems throughout various organisms enact different activities according to specific strands of harmful viral DNA.

The CRISPR/Cas genome exists of short segmented palindromic repeats, interspaced with 'spacer DNA', each individually uniquely combining into what is referred to the 'CRISPR' segment (See figure 3). These spacer DNA segments are parts of DNA matching to previously registered viral RNA, incorporated into the CRISPR/Cas locus through Cas1 and Cas2 proteins. During the immunizing response of the CRISPR/Cas9 protein, these spacers are transcribed to crisprRNA (crRNA) which are complementary to the target sequence. The Type II CRISPR/Cas9 system requires complete crRNA-protospacer complementarity in the seed region. This seed region is the locus of interest by the CRISPR/Cas9 complex. The seed region is referred to as a 'protospacer' and thus is inversely complementary to the spacer in the CRISPR loci (Makarova, Haft, Barrangou *et al.*, 2011). This pre-crRNA is processed into the crRNA through RNAse III inside multiple Cas9 proteins aligned (Deltcheva, Chylinski, Sharma *et al.*, 2011). Furthermore, within the CRISPR/Cas9 complex, trans-activating CRISPR RNA (TracrRNA) hybridizes with and further matures crRNA into an RNA duplex (Makarova *et al.*, 2015). This results in tracrRNA-crRNA-Cas9 complex formation. It is important to note that the TracrRNA is also transcribed from CRISPR/Cas locus. This crRNA-tracrRNA duplex is referred to as guide RNA (gRNA) (Jinek et al., 2012). This

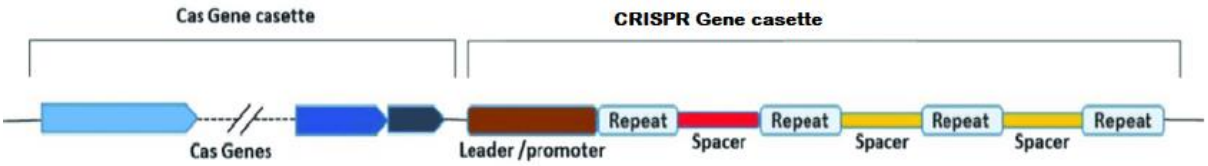

Figure 3 – Schematics of a general CRISPR locus. The figure shows the general structure of the Cas and CRISPR gene cassettes (Seahyoung *et al.* 2015).

duplex allows replacement by a synthetic fused chimeric single gRNA (sgRNA), which enables the use of CRISPR/Cas9 in genome engineering (Martinez-Lage et al., 2018). These fused strands act as a guiding sequence, directing Cas9 towards the invasive DNA. Association of the crRNA-tracrRNA duplex with Cas9 effectuates a conformational change in the nuclease, which allows the duplex to bind a PAM site. Afterwards, the viral strand is identified through binding with the crRNA sequence. Both recognition of a PAM site and complementarity of the crRNA is required for cleavage. The Cas9 complex binds and consequently cleaves the identified strand and the complementary strand through the nuclease proteins NHN and RuvC1-Like respectively (Khadempar, Familghadakchi, Motlagh *et al.*, 2019). Thus, the cleavage of the viral strand results in a double-strand break (DSB) rendering the strand ineffective. For a functional overview of the functionality of CRISPR/Cas9, I refer to figure 4.

To summarize the function of CRISPR/Cas9 Type II described above, the type II cascade requires three main components: endonuclease Cas9, crRNA, and tracrRNA. The cascade of CRISPR/Cas9 is most commonly divided into three stages: (1) Acquisition; (2) maturation, and (3) interference (Makarova *et al.*, 2015). Acquisition (1) is initiated through the presence of viral DNA, leading to the recognition through the Cas1-Cas2 complex and resulting in the inclusion of new spacers within the CRISPR locus (Sterling, 2017). During the maturation (2) stage, characterized by crRNA biogenesis, the CRISPR system recruits the Cas proteins to location and produces and matures the crRNA and tracrRNA. These three components are combined as a complex, facilitated by the enzymatic function of RNAseIII. Finally, the interference (3) stage is started when the Cas9 complex is loaded with the crRNA-tracrRNA duplex. The effector complex targets

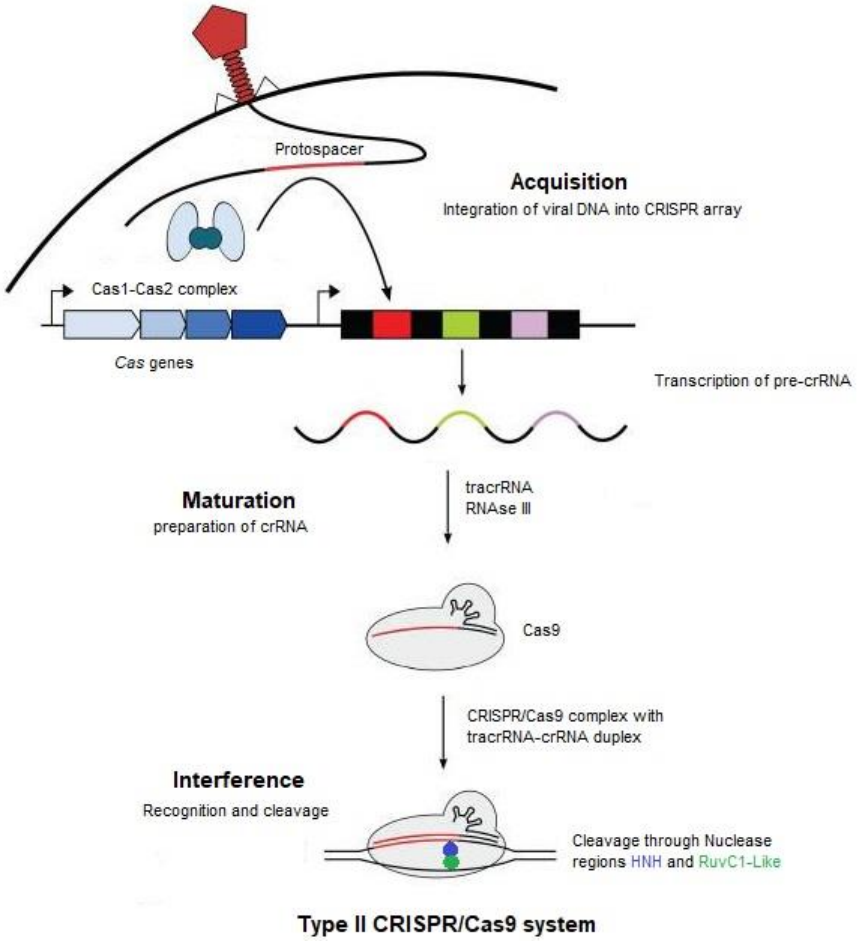

Figure 4 – Functional overview of the function of Type II CRISPR/Cas9 systems. The figure above shows the functionality of type II CRISPR/Cas9 systems. The cascade includes the three main stages of CRISPR immunization: acquisition, maturation and interference. The two nuclease sites have been coloured accordingly (HNH is blue, RuvC1-Like is green). For the purpose of serving this specific thesis, the original picture has been altered (Hille *et al.* 2016).

complementary viral DNA of the gRNA strand along with fitting PAM sequences for target site recognition. This is results in cleavage by Cas9 with the internal nuclease regions.

Cleavage of a strand of native DNA with the nuclease sites of CRISPR/Cas9 results in non-homologous end joining (NHEJ) or homology-directed repair (HDR). However, NHEJ is error-prone and often causes mutations such as point mutations, deletions and frameshifts. This leads to an altered genetic product, potentially eliminating function, though this technique is favoured for most genetic knockout experiments and applications. HDR is most frequently the preferred DNA restoration pathway and a more precise genomic editing tool. However, HDR is naturally prevalent in only a select number of stages of the mitotic cycle. During HDR a part of DNA shows partial sequence homology to the target site. This artificially inserted or naturally occurring available part of DNA is then used for homologous repair. This DNA segment allows the integration of the desirable DNA sequence wanted at the target site (Hille & Charpentier, 2016). The wanted type of DNA recovery can forcefully be shifted towards the preferred repair pathway. For example, various CRISPR/Cas types such as Dead Cas9 (dCas9) and scaffold-incorporating single guide RNA (sgRNA) (Xue, Ji, Gao *et al.*, 2016) trigger different repair pathways compared to the Wildtype CRISPR/Cas9. An important note is that these processes occur through a different mechanism, or do not occur at all, in viral DNA or RNA strands injected by viruses. NHEJ will result in joining of DNA ends through induction of natural DNA repair mechanisms. A functional overview of cleavage through CRISPR/Cas9 is shown in figure 5.

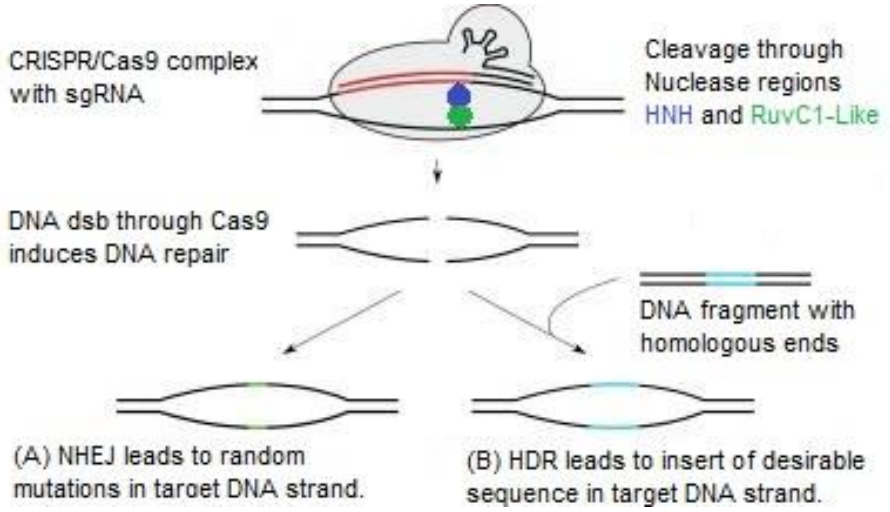

Figure 5 – Functional overview of cleavage through CRISPR/Cas9. The figure shows the cleavage of the two target strand through the nuclease proteins on Cas9. By cleavage two possible DNA repair pathways are optional: (A) NHEJ and (B) HDR. For the purpose of serving this specific thesis, the original picture has been altered (Hille *et al.* 2016).

CRISPR/CAS9: APPLICATION IN CANCER THERAPY AND RESEARCH

Malignant neoplastic diseases (cancer) are a collection of (epi)genetic disorders, resulting from cancer-driving genetic mutations. These oncogenic mutations are characterized by the activation of oncogenes and/or the deactivation of tumour suppressor genes. They cause uncontrolled cell cycle progressions, aberrations in DNA repair mechanisms and inactivation of the body's natural apoptotic mechanisms, which, in turn, are symptomized through uncontrolled cell growth and the procurement of metastatic properties (Sarkar, Horn, Moulton *et al.*, 2013). Untreated progression of cancer often results in death. Luckily, due to the common genetic nature of both cancer and CRISPR technology, CRISPR/Cas9 poses as a possible toolkit for cancer therapy and research. The wide range of applications of CRISPR/Cas9 in this field includes screening, prevention and anti-cancer therapy implying the curative and preventive possibilities which accompany artificial modifications in the genome. However, the active application of the protein by oncologists as 'treatments' is still an issue. Especially in cancer, where genetical errors are the foundation of the disease profile, genomic editing requires high editing efficiency, dynamic targeting and adequate delivery systems (Xiao-Jie *et al.*, 2015). This defines cancer as a challenging disease in potential genomic editing therapies, relying on dynamic efforts and high personalization.

Due to the polygenicity and heterogeneity of cancer, the statutory aberrations in cancer are genetic mutations, epigenetic alterations and chromosomal translocations. Unfortunately, the high inconsistency of cancer-origin profiles throughout different individuals creates a broad spectrum of variability in pathology. The previously discussed somatic oncogenic mutations are attained during an individual's lifetime as a result of malfunctions in mitosis or meiosis, exposure to carcinogenic substances, exposure to radiation, among other less prevalent causes. However, cancer also has a heritable factor. Estimates state that 5-10% of the cancer cases are explained through inherited genetic mutations (Ranchod, 2018). Some of these heritable mutations are BRCA1/2 in both heritable breast cancer (HBC) and heritable ovarian cancer syndrome (HBOC) (Romagnolo, Romagnolo, & Selmin, 2015), RB1 in familial retinoblastoma (RB) (Chakraborty & Rahman, 2012) and MLH1, MSH2/6 and PMS2 in Lynch syndrome (LS) (Ngeow & Eng, 2016). However, in most known hereditary malignant syndromes the elevation of risk for malignant neoplasms is due to a singular genetic defect, also referred to as a monogenic hereditary disease (Rahner & Steinke, 2008).

Whereas polygenic cancer types with obtained oncogenic mutations are hard to exterminate through CRISPR-technology, monogenetic hereditary cancer types provide a better target for genomic engineering tools such as CRISPR/Cas9. The biggest hurdle of polygenic cancer for the treatment with CRISPR/Cas9 is the systemic applications to multiple genetic sites. However, interception of malignant mutations in germline cells could even provide a solution for familiar cancer with a long-term hereditary genetic timeline. Therefore, genomic editing tools such as a CRISPR/Cas9 pose an interesting tool to possibly prevent or resolve cancer. In the remaining part of this chapter, this thesis will describe a selection of applications of CRISPR technology within cancer research, prevention and resolution which after theoretical research I deemed most valuable and promising for future application.

The identification of carcinogenic genes, drug resistance genes and possible anticancer drug targets is an important development in possible cancer therapy and prevention. The CRISPR/Cas9 complex can be applied to cancer screening by drug target validation, in resistance mutation experiment and in exploring new cancerous mutation sites (Yi & Li, 2016). The potency of the CRISPR/Cas9 applications in cancer screens has become evident in various researches. A CRISPR/Cas9-mediated knockout screen showed the Dck-gene as a primary contributor in resistance to specific chemotherapy in acute

myeloid leukaemia cell lines (Yi & Li, 2016). Furthermore, a genome-wide library screen conducted by Shalem *et al*. in melanoma cells resulted in the identification of four new candidate genes for cancer therapy (Shalem, Sanjana, Hartenian *et al.*, 2014). CRISPR/Cas9 is a tool for quick and systematic identification of carcinogenic genes, drug resistance genes and anti-cancer drug targets, which are extremely beneficial in drug development and introduction of new therapies. CRISPR/Cas9 is especially valuable compared to other screening methods because of the easy generation of sgRNA, the omnipotent function of CRISPR/Cas9 in respect to various genes and the simplicity over other knockout tools. However, to achieve higher efficiency more sgRNA strands need to be added to genomic libraries, and new more consistent application and delivery methods need to be developed.

Given that cancer is primarily the result of the accumulation of mutations in oncogenes and tumour suppressor genes, mutated oncogenic and tumour-suppressor genes could be considered as attractive therapeutic targets (White & Khalili, 2016). They might play an important role in the correction of the cancer-driving genes or targeting knockout genes necessary for cancer survival resulting in reduced pathogenesis of cancer. Theoretically spoken, applying CRISPR/Cas9 to manipulate a mutated gene could be applied to any kind of malignant neoplastic monogenic disease originating from an (epi)genetic alteration (Xiao-Jie *et al.*, 2015). Using CRISPR/Cas9, researchers can effectively create knock-in and knock-out mutations (Nagata, Takahashi, Matsuba *et al.*, 2018). This occurs most potently in mice embryos (Wu, Zhang, Peng *et al.*, 2019). Possible therapeutic targets for cancer malignancies include P21, E-cadherin, hBax (Yi & Li, 2016), PD-1/PD-L1 (Dermani, Samadi, Rahmani *et al.*, 2019), PLK1 (Liu, Sun, & Wang, 2017), Survivin (Soleimanpour & Babaei, 2015) and Tyrosin Kinases (Krause & Van Etten, 2005). Moreover, dCas9 can be recruited to target specific DNA sites, and used to activate (express) or repress (silence) either malignant oncogenic targets (Sander & Joung, 2014; Zabaleta, Barberia, Martin-Higueras *et al.*, 2018). Again, theoretically, CRISPR/Cas9 could be applied to any kind of malignant neoplastic genetic alteration, however, due to current editing inefficiencies, off-target effects and lacking delivery methods practical implementation remains troublesome. Most of these disadvantages will be addressed in 'The CRISPR/Cas9 complex: Potential pitfalls and risks'.

Furthermore, epigenetic and transcriptional modifications play an essential role in carcinogenesis (Sarkar *et al.*, 2013). Targeting epigenetic regulators through CRISPR/Cas9 as a potential epigenetic inhibitor shows potential though is often limited by a lack of specificity (Yi & Li, 2016). Recurrent evidence indicates that the activation of CRISPR/Cas9 in combination with RNA scaffolds can be used alongside effector proteins to target epigenetic regions of the OCT4 promoters and induce site-specific epigenetic and transcriptional modifications. The OCT4 gene is known to play an important role in therapy resistance and tumorigenesis (Fogarty, McCarthy A, Snijders KE *et al.*, 2017; Zalatan, Lee, Almeida *et al.*, 2015). Furthermore, dCas9 can site-specifically bind to epigenetic DNA elements and domains to suppress their transcriptional activities through a process called CRISPRi or fusion with the epigenetic modifiers. Unfortunately, unlike monogenic cancer types that can be resolved at a low gene correction efficiency (0,25%), epigenomic manipulation for cancer therapy requires high editing efficiency due to the unimpaired malignancy of unedited cancer cells. Because unedited cells remain malignant, these cells are provided with a selective advantage over-edited cells effectively rendering the therapy ineffective after a certain timeframe. Even though, epigenetic modifications are recurrent throughout cancer, indicating that CRISPR/Cas9 mediated strategies can be implemented in anti-cancer therapies. Epigenomic modifications can bypass DNA-repair pathways, deferring the usage of NHEJ or HDR. This proves that epigenetic modulation might prove less challenging than CRISPR/Cas9 induced genetic modification and insertion.

Oncolytic viruses (OVs) are another emerging application of CRISPR/Cas9 mediated cancer therapy. OVs are anticancer agents, which explicitly interact with cancerous cells *in vivo* to attack and lyse the target cells through virus-mediated cytotoxicity (Martinez-Lage *et al.*, 2018). Through

CRISPR/Cas9, inactivated virus strains are engineered and transformed with the deletion of viral genes such as ICP34.5 (neurovirulence), ICP6 (ribonucleotide reductase), P53 genes and genes from the RB pathway (Martinez-Lage *et al.*, 2018). After the deletion of these specific genes, the virus is lethal to cancerous cells, while it maintains prevented replication and promotes safety in wild-type cells. These OVs consists of multiple strains of viruses including Herpes Simplex Type I, Adenoviruses and Vaccinia Virus (Yi & Li, 2016) One of the examples of OVs as an application in therapy, is the knockout the *E1A* gene from oncolytic adenoviruses (Martinez-Lage *et al.*, 2018). The *E1A* gene encodes for a protein that binds pRB. This activates the release of transcription factors (most importantly E2F) and consequently stop cell cycle checkpoints. E2F specifically allows for the generation of new virions, lysis of the infected cell and thus the further spread of the virus. Since cancerous cells usually have a mutation in the Rb pathway, this allows for the generated selection of cancer cells while maintaining healthy cells integrity (Annunziato, de Ruiter, Henneman *et al.*, 2019; Chen, Tsai, & Leone, 2009).

Cancer immunotherapy is a growing vital component of cancer therapy. Immunotherapy is the engineering and multiplication of native immune cells *in vitro* to develop various anticancer properties and to be reintroduced, to specifically recognize and exterminate cancerous cells. This therapy, also referred to as adoptive cell therapy, has shown significant effectiveness in the treatment of multiple cancer types including lymphomas, leukaemia and sarcomas (Yi & Li, 2016). Engineered T-cells through CRISPR/Cas9 such as the chimeric antigen receptor T (CAR-T) cell emerged as promising therapeutic strategies in oncology. Currently, two noteworthy variations of adoptive cell therapy undergoing research involve the knockout of programmed cell death protein-1 (PD-1) and production of next-generation CAR-T cells engineered to possess tumour targeting receptors.

The immune checkpoint regulator programmed PD-1 protein receptor is located at the cell surface of T-cells and in normal physiological circumstances responds to PD-L1/2 ligands. In healthy somatic cell lines, the PD-1 protein interaction prevents cellular damage in response to chronic infection and inflammation (McGowan, Lin, Ma *et al.*, 2020). It is known that cancer abuses PD-1 mutations through upregulation of the surface protein, effectively evading the host immune system. Furthermore, research in renal carcinomas provides that a high PD-L1 expression in the tumour microenvironment increases tumour aggression and risk of death by 4.5 fold (Thompson, Gillett, Cheville *et al.*, 2004) Lu You *et* al injected a patient with aggressive lung cancer with PD-1 deficient T-cells edited through CRISPR/Cas9 (Xia, He, Wang *et al.*, 2019). Through CRISPR/Cas9-mediated PD-1 gene knockout, T-cells can be reintroduced into their hosts where the cells will activate the previously mutated immune response and improve efficiency for further immunotherapy against cancer. Figure 6 shows a systemic overview of PD-1 Knockout mediated therapy.

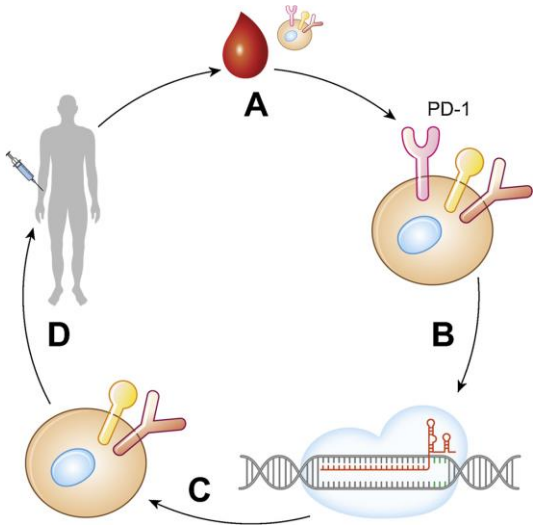

Figure 6 – General overview of PD-1 knockout mediated therapy. Peripheral blood lymphocytes are collected from the patient with a solid tumor (A) and CRISPR/Cas9 mediated knockout of the immune checkpoint gene PD1 is performed in T-cells (B–C). The PD1-knockout T-cells are expanded *ex vivo* and then transfused back to the patient (D), where they are supposed to induce immunological response against tumor cells (Zhan *et al. 2019).*

Furthermore, the production of next-generation CAR-T cells holds great promise. CAR-T cell therapy relies on the activation of the host's immune system to attack and eradicate cancer cells. T-cells are genetically modified *In vitro* through CRISPR/Cas9 where genes are inserted and CAR proteins are transduced and expressed on the cell-surface, reprogramming t-cells to attack malignant neoplasms. These CARs include both an intracellular signalling domain and an extracellular binding domain which activate T-cells and recognize antigens expressed on malignant cells respectively (Benmebarek, Karches, Cadilha *et al.*, 2019). Preclinical studies indicate that targeted gene delivery of a CAR to the T-cell locus using CRISPR/Cas9 produced CAR-T cells with enhanced tumour rejection activity compared to regularly produced CAR-T cells using genetic vectors (Zhan, T. *et al.*, 2019). Even though adoptive cell therapies have shown promise in clinical trials on various forms of cancer, patients have been reported to die during clinic trial phases most like due to cytokine release syndrome and neurotoxic difficulties in mild or severe forms (McGowan *et al.*, 2020). This means that while adoptive cell therapies show great promise in cancer therapy and research, the application in clinical trials is still impossible to manage in regards to the mild to severe consequences.

THE CRISPR/CAS9 COMPLEX: POTENTIAL PITFALLS AND RISKS

CRISPR/Cas proved itself as a potent genomic editing tool, facilitating simple, cost-efficient and high-throughput epigenetic editing. However, earlier research on CRISPR/Cas9 made apparent that the application of this technology had more complications than initially foreseen. Peng *et al*. described in their review of 2016 a large cohort of studies on genomic editing through CRISPR/Cas9. This group elucidated the pitfalls of CRISPR/Cas9 mediated genome editing: off-target mutations, substandard targeting efficiency, the balance of incidence between HDR and NHEJ and complicated delivery. Additionally, fundamental aspects of the CRISPR complex remain unclear as yet, including the catalytic mechanism, mechanisms of target site identification and PAM-dependence (Peng, Lin, & Li, 2016). Quoting Peng *et al*. on why the focus of current research on CRISPR/Cas9 should shift towards the improvement of recurring problems: "Aiming to improve these potential pitfalls of the highly promising CRISPR/Cas system will aid in improving efficiency, specificity, and generating highly specific CRISPR tools" (Peng *et al.*, 2016).

Firstly, a disadvantage to the CRISPR/Cas system is off-target cleavage. Cleavage is enacted in sequences integral to the complementary-strand of the crRNA sequence in the Cas protein, with the only prerequisite being a proximate PAM sequence. Multiple studies propose that potential off-target sites can differ from one to six nucleotides in the complementary seed region. Therefore a typical protospacer region will have up to thousands of potential off-target sites (Sander & Joung, 2014). Presently, it is still unidentified why some sites have a higher affection to be cleaved. Furthermore, what still needs to be clarified is how genomic and epigenomic characteristics affect Cas-DNA interactions. Previous research implies a possible influence of GC nucleotide repeats, DNA-methylation and chromatin structure on the Cas-DNA interaction (Sander & Joung, 2014). Nevertheless, off-target cleavages depend on factors like the Cas9 concentration, Cas9 structure, cell type and cellular state (Eid & Mahfouz, 2016). Multiple approaches have been proposed to reduce off-target cleavage. For example different Cas types such as nCas9 with nickases and dCas9 fused to FokI (fCas9; contains a different nuclease protein than Cas9) (Xiao-Jie *et al.*, 2015) or the usage of truncated gRNA (tru-gRNA) (Khadempar *et al.*, 2019). tru-gRNA strands can carry 17 or 18 nucleotides (whereas normal gRNA carries approximately 20) of complementary strand and function as efficiently as normal length

gRNA according to studies (Khadempar *et al.*, 2019; Rodriguez-Rodriguez, Ramirez-Solis, Garza-Elizondo *et al.*, 2019). Through physically decreasing the number of nucleotides involved, and combing the complex with specificity adding proteins, Khadempar *et al.* propose that the functional selectivity of the CRISPR/Cas9 complex will increase. What also should be noted, is that multiple studies report elevated off-target mutations and effects in oncogenic cells where the genetic mutations are already the underlying cause (Xia *et al.*, 2019). This underlines the urgency of solutions to this specific disadvantage.

Secondly, the imbalance of incidence between HDR and HDEJ is a disadvantage to the CRISPR complex. Editing through Cas9 always results in NHEJ or HDR. Treatments using NHEJ are moderately efficient and occur at all cell-cycle stages. However, NHEJ gives rise to uncontrolled genomic gene disruptions (Xiao-Jie *et al.*, 2015). In cancer, NHEJ applies to some knockout therapies, generating knockout mutations at the desired location rendering cancer-causing gene inactive (Yi & Li, 2016). Many treatments prefer HDR because the template strand is supplied and inserted into the DNA strand to replace the undesirable sequence (Khadempar *et al.*, 2019). HDR relies mostly on the availability of a repair template during the timing of cleavage in the cell cycle and the nature of the donor strand (Xiao-Jie *et al.*, 2015). Noteworthy is that non-dividing or post-mitotic cells are practically in submissive to gene editing through HDR. Much progress has been made by shifting NHEJ to HDR in cells by using different Cas subtypes (Yi & Li, 2016), releasing small molecule inhibitors to suppress NHEJ pathways (Yi & Li, 2016), conducted time delivery with different delivery vectors (Xiao-Jie *et al.*, 2015), Cas9 fusion with various proteins (Jayavaradhan, Pillis, Goodman *et al.*, 2019) and covalent tethering of CRISPR/Cas9 to a DNA repair template (Aird, Lovendahl, St. Martin *et al.*, 2018). It should be emphasized that undesired mutations as a result of CRISPR/Cas9 treatment could complicate further treatment. Rapid division of cancer could lead to mosaicism and therefore untreatable mutations, accentuating the urgency of research on the exploitation of HDR and NHEJ for therapeutic applications in cancer treatment.

Lastly, the efficient delivery of the Cas9 complex accompanied by the required sgRNA strand is a complicated matter. This matter is especially urgent in cancer where the required delivery is to primary tumour cells as well as secondary metastatic sites. The efficient, safe and most commonly used vector for CRISPR/Cas9 transduction is Adeno-associated virus (AAV) because of its low immunological response and cytotoxicity, high amount of serotypes and high efficacy in transducing a variety of cell types (Sander & Joung, 2014). Furthermore, the AAV proved successful usage in mouse models as well as in more recent clinic trials (Martinez-Lage *et al.*, 2018). However, in the delivery of large Cas9 proteins, commonly used viral vectors are not applicable due to their limited limit genetic cargo space. This forces researchers to resort to separate viral vectors for the sgRNA and Cas9 delivery (Xiao-Jie *et al.*, 2015). This implies that not only transportation is the complication, but the timing and localization of the reuniting of the vectors are also essential to the case as well. Furthermore, consecutive expression of Cas9 and AAVs caused a humoral immune response, with minor cellular damage (Zhan, Tianzuo, Rindtorff, Betge *et al.*, 2019). This lead to the research of other methods of transportation for CRISPR/Cas9 such as electroporation mediated transfer, cell penetration through peptides and nanoparticles (Yi & Li, 2016). Gold-based nanoparticles Cas9-sgRNA complexes plus donor DNA were delivered in mice, and significantly induce HDR (Martinez-Lage *et al.*, 2018). Efficient delivery of Cas9 to the target site is a challenge for future application, and currently limits the usage of CRISPR/Cas9 in cancer research and therapy. Therefore, extensive research to potent delivery methods is needed, and future successes of the CRISPR/Cas complex application will highly depend on this issue.

Conclusively, the possible relevance and potential applications of CRISPR/Cas in cancer research and therapy will develop and advance with the number of research conducted. The most urgent and obvious issues rising from the CRISPR/Cas9 complex are off-target mutations, substandard targeting efficiency, the balance of incidence between HDR and NHEJ and complicated delivery.

However, fully understanding the complications involved in such a possibly beneficial tool is arguably even more important than understanding its functionality. The current, relatively infant state of CRISPR technology naturally is still vastly determinative to its possible direct application for clinical trials. Due to the discussed potential drawbacks, we will have to tread carefully with the transition to CRISPR as a curative medicine for cancer. Finding workarounds or solutions for these potential pitfalls of the highly promising CRISPR/Cas9 system will aid in improving efficiency, specificity, and generating highly specific CRISPR tools (Peng *et al.*, 2016).

CONCLUSION AND DISCUSSION

Unicellular organisms have deployed CRISPR for billions of years as an immune system against viral intrusions. It might be regarded as surprising, therefore, that only as late as 2012 pioneer Jennifer Doudna revealed the scientific capabilities and implication of CRISPR/Cas9 on life sciences. Technological advances in genetic editing (RNAi, ZFNs and TALENs) have provided researchers with a helpful tool in research with genetic knock-outs and knock-ins. However, through usage, it became evident that these tools were inefficient and expensive to reproduce.

CRISPR technology, however, completely overhauled and transformed the way researchers work. This specific technology turned out to provide life sciences with a highly demanded tool which exceeds current gene-editing technology in affordability, efficiency, scalability, precision as well as programmability. As a result, CRISPR has seen a quick adoption in research programmes (Locke, 2020). Moreover, as a potential genomic editing toolbox with usage widespread from research to medical application, CRISPR has the potential to revolutionize the way we think about synthetic biology, genetics and genetic interaction, genomic screening and diseases with a genetic foundation.

This thesis specifically studied the possibilities of CRISPR/Cas9 in the search for innovation in research and new therapies in cancer, and what previous developments would effectuate for the cancer field. Despite the fact that the past decades have yielded a more thorough understanding of the genetic origin of cancers, the disease is still characterized by a high mortality and high degree of therapy-resistant clinical cases. This obviously represents the demand for more efficient therapeutic options. With the increasingly growing understanding of molecular aberrations related to cancer onset, progression and metastasis, researchers longed for a therapy which is simple yet versatile. Accordingly, this thesis explored and discussed the application of CRISPR/Cas9 in cancer-influence gene screening, genetic intervention, epigenetic modifications, oncolytic viruses and immunotherapy. Oncolytic viruses are emerging as relevant tools in anti-cancer, though their full functioning, as well as their therapeutic potential remains, to be further examined. However, especially anti-cancer immunotherapy is at the forefront of this branch because of the abundant possibilities of CRISPR/Cas9 in mediated immunotherapy. Conclusively, because of the genetic foundation of both CRISPR/Cas9 and cancer, CRISPR emerges as a valid option. It promises to broaden the spectrum of potential cancer strategies, providing a useful tool in the solution, prevention and research of cancer. The recent developments in CRISPR/Cas9 will effectuate systemic change in the view on cancer research and therapy, however it will affect every field with a genetic connection. CRISPR/Cas9 is bound to represent a vast branch of next-generation therapeutics in the battle against cancer.

Even though CRISPR/Cas9 overturned current therapies, details of the potential pitfalls and risks naturally need to be addressed to further gain benefit from this technology, as well as to improve its specificity and efficiency. As stated in the body of this thesis, fully understanding the complications and implications accompanying such a relevant tool is arguably at least, but possibly even more important to understand the functionality. Implicative studies concerning CRISPR/Cas9 circumvent the problems on the possibility for off-target mutagenesis, the imbalance in HDR and NHEJ providing unwanted DNA consolidation and the challenge of efficiently

delivering in target tissues or cells, among others. However, the same inquiries propose various solutions and future possibilities. The current state of the CRISPR technology still resides in infancy according to its application for clinical trials.

Before this complex is translatable to clinical application, future improvements will need to be made to pave the road for the therapeutic use of CRISPR in the future. This implies that rigorous validation of the desired effect in animal models and cell tissues is required to meet the requirements of therapeutic application. Therefore, conducting more and further specified research on CRISPR/Cas9 and its context is needed. While the potential has already been demonstrated in research, CRISPR/Cas-mediated editing still has a long way ahead to fully realize the implementation of CRISPR/Cas as the foundation for therapeutic strategies to treat neoplastic malignancies in human patients.

Following the extensive background research concerning every aspect of CRISPR/Cas, I feel scientifically obliged to provide a recommendation for future of CRISPR/Cas9 research regarding the application of CRISPR/Cas in cancer research and therapy. For cancer especially, a few serious concerns arose during this theoretical research.

Firstly, the stage of intervention for the interception of malignant neoplasms is an important factor to take into account. Where hereditary types of cancer in germlines can be intervened after the screening, somatic types of cancer require a more systemic approach. Herein a systemic approach means an approach where every target cell is affected. Determination of the stage of intervention requires in-depth knowledge and understanding of different cancer therapies.

Secondly, the choice of sgRNA target sites needs to be considered. Designing a sgRNA strand requires the identification of the target region while there is a broad spectrum of variability in cancer pathology due to the inconsistency in (epi)genomic mutation profiles. The identification of personal mutation genetic profiles requires an intimate understanding of (epi)genetic interactions and will provide obstacles and hurdles to overcome for CRISPR/Cas9 based therapy specificity.

Lastly, the delivery of CRISPR/Cas9 components to target and metastatic sites should be investigated more thoroughly. Efficient delivery has been an essential problem in general genomic editing tools. However, especially in cancer where metastatic sites potentially can be found in the entire body, efficient delivery to the target location(s) is of even higher importance. Therefore, developing functional delivery tools with high efficacy like nanoparticles (Mangeot, Risson, Fusil *et al.*, 2019) might even be more important before we think about the implementation of CRISPR/Cas9 in cancer therapy.

As a future research prospect for cancer with high yield would, according to me, be the research of systemic interaction of CRISPR/Cas9 on a multiplication of genes. I propose this can ben achieved through the addition of different Cas proteins, or through scaffolding proteins.

The CRISPR/Cas9 machinery is said to transform the current state of genomic medicine and biotechnology. CRISPR technology has been proclaimed by many as having “unprecedented potential to revolutionize innovation in basic science” (Brokowski, 2018) and potentially being “the biggest biotechnological discovery of the century” (Martinez-Lage *et al.*, 2018). Interestingly, as Locke described: “Unless you were around to witness the development of immunology by Louis Pasteur in the 1870s, it is hard to imagine biotechnology that has generated more acclamation than CRISPR.” (Locke, 2020) referring to the unrestrained opinion of the press on the matter, which is reluctant and effusive to the usage of general genomic editing tools, and especially in regards to CRISPR/Cas9. Especially after Jiankui and his fellow researchers brought their shocking research to light, the mainstream attention was notably negative and political concerns arose surrounding the dissident subject.

The emergence of CRISPR technology and genomic editing is no longer a dystopian, abstract and hypothetical subject. Genomic editing with CRISPR/Cas9 has reached a stage where fiction is becoming reality. Mankind is regarded to be on the doorstep of scientific breakthroughs in eugenics.

Interestingly, in a review by Dijke *et al.* within a cohort of 180 articles, 13 articles were identified to express concerns about bioscientists "playing God" referring to germline editing (van Dijke, Bosch, Bredenoord *et al.*, 2018). Aside from the question of what "playing God" means, the implication of this question is beyond the limits of 'a greater deity'. While theology is not within the rationality of most researchers, this issue cannot remain unaddressed or be ignored. Locke refuted this notion by a compelling argumentation in '*The Promise of CRISPR for Human Germline Editing and the Perils of "Playing God*"'. Where one of his arguments was: "It is when we use such (read: genome editing) power in the absence of the moral attributes of God that we tend to draw this particular interpretation of the criticism 'playing God'." Locke implied that, if the biological community can show and prove that we, the scientific community, can responsibly carry out germline editing, the full extent of the effect of CRISPR/Cas and its accompanying ethical concerns can be publicly debated. Additionally, there are multiple modern-day examples of intervening in life sciences which would be characterized as "Playing God" including but not limited by imposing forced natural selection on cattle and crops, *In Vitro* fertilization and transgenderization.

Furthermore, one might argue that while being a quintessential part of human nature, natural selection and the evolutionary process are slow, contain suboptimal designs and represent survivability over optimality. As Harris cleverly stated "What human reproduction does not do very well is improve (read: the human genome)" thereby expressing positive stimulation for the usage of CRISPR/Cas where it could be implemented to add to the future of mankind.

In the near future, multiple arguments and critical monikers on genomic editing and eugenics will rise, and the scientific community ultimately has to welcome this. CRISPR/Cas technology is driving research forward in a pace that would not have been possible just half a decade ago. And even at this time, the full potential of CRISPR/Cas for cancer research has yet to unfold. Currently, CRISPR/Cas9 seems to have no limitations in regards to applications and researchers idealize a possible application within all disciplines of life science and beyond. What we do know, however, is that CRISPR/Cas has the potential to revolutionize the next generation personalized treatments, improve the quality of human life and is able to rewrite our entire vision on genetics.

Momentarily, CRISPR/Cas9 has yet to be applied as a treatment in clinical studies, and we need to be extremely aware of the profound risks and issues that accompany the application of CRISPR/Cas9 in research and therapy. Though the potential of this tool cannot go to waste. "Allowing its potential benefits to be unnecessarily stalled would be like Louis Pasteur allowing the theory of spontaneous generation to be foisted on him by his critics, instead of refuting it as he did." (Locke, 2020). CRISPR/Cas9 is one of the highest impact discoveries of our time and it is our scientific duty to never cease to address the public critique on the matter to clarify the future importance of this technology. CRISPR/Cas9 has the capabilities to contribute to a promising future of mankind and could allow our species to flourish through times, though we as mankind need to be morally and scientifically motivated enough to use this powerful tool for good since its only limit is our own imagination.

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