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# Programmable nucleases in human therapy

# Využití programovatelných nukleáz v lidské terapii

Bachelor´s thesis

Supervisor: Mgr. Petr Kašpárek, Ph.D.

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**Poděkování:**

Mé poděkování patří Mgr. Petru Kašpárkovi, Ph.D. za odborné vedení, trpělivost a ochotu, kterou mi v průběhu zpracování bakalářské práce věnoval. Dále bych ráda poděkovala Alžbětě Horákové za pomoc při gramatické kontrole anglického jazyka.

**Prohlášení:**

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 5. 1. 2019 Marta Šlaufová

## Abstract:

Most genome disorders cause severe symptoms and are usually incurable. Recent, rapid development of programmable nucleases (PNs) brought new possibilities for the treatment of many diseases, such as genetic disorders, infectious diseases or cancer. PNs are enzymes, which enable site specific DNA cleavage that can lead to targeted modification of desired genomic loci. They are composed of separable non-specific cleavage domain and DNA-binding domain. The DNA binding domain is in the form of modular DNA-binding proteins or complementarity-based pairing of the oligonucleotide. The non-specific cleavage domain mediates DSB stimulation, which is necessary for further genome editing. Development of zinc finger nucleases (ZFNs) followed by transcription activator-like effector nucleases (TALENs) enabled the first therapeutic approaches based on targeted manipulation of human genome. The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas technology brought further simplification to the method and broadened the availability of PN-based toolkits. This thesis will provide a summary of the recent developments, application of PNs in the therapy of human patients and potential obstacles preventing their implementation in clinics.



## Keywords:

programmable nucleases, human therapy, gene therapy, ZFN, TALEN, CRISPR-Cas, genome disorders, clinical trial

## Abstrakt:

Většina genomických mutací vede k závažným symptomům, které obvykle nejsou vyléčitelné. Intenzivní vývoj technik spojených s programovatelnými nukleázami (PN) v posledních letech otevřel nové možnosti při léčbě mnoha nemocí, jako jsou například geneticky podmíněné choroby, infekční nemoci, nebo rakovina. PN jsou enzymy, které umožňují místně specifické štěpení DNA, jež může vést k cílené modifikaci daného genomického lokusu. Skládají se z domény nespecificky štěpící DNA a z DNA-vazebné domény, která má buď formu modulárních proteinů vázajících se na DNA, nebo formu oligonukleotidů, které se na základě "Watson-Crickovského" párování váží na odpovídající sekvenci DNA. Doména nespecificky štěpící DNA zajišťuje vznik dvouvláknových zlomů, které jsou nezbytné pro následující úpravy genomu. Vývoj zinc finger nukleáz (ZFN) a poté i TALE nukleáz (transcription activator-like effector nucleases, TALEN) umožnil první terapeutické postupy založené na cílené úpravě lidského genomu. Technologie CRISPR-Cas (clustered regularly interspaced short palindromic repeats) přinesla další výrazné zjednodušení a rozšíření metod využívajících PN. Tato práce si klade za cíl poskytnout přehled o současném stavu, vývoji a překážkách v oblasti využití PN při léčbě lidských pacientů.



## Klíčová slova:

programovatelné nukleázy, lidská terapie, genová terapie, ZFN, TALEN, CRISPR-Cas, geneticky podmíněné nemoci, klinické zkoušky

# Table of Contents

# Introduction

This thesis aims to discuss recent approaches for treating selected genetic disorders using programmable nucleases for gene therapy. I chose this theme because of its importance and perspective for the future of human therapy.

Sequencing was one of the milestones which enabled progress in the field of genome engineering (Lander 2011). Since the development of sequencing techniques for human genome, sequencing data have fuelled the development of research, clinical medicine and targeted therapeutics. A single mutated gene can cause serious health problems, because of the broad interconnectedness of all human cell processes. Up until now, there have been reported mutations in more than 4,000 of the approximately 25,000 annotated genes of human genome that are related to pathological phenotypes ('OMIM Gene Map Statistics' n.d.). Many genetic diseases, however do not yet respond to any recent treatments, such as dietary therapy, drug therapy or gene product replacement therapy. That is why many attempts for the development of new technologies to treat genetic diseases at the site of the primary defect, meaning the genetic level, have started to arise (Friedmann and Roblin 1972).

Gene-based therapeutics are from a broad perspective defined as an introduction of nucleic acids into cells, where they alter gene expression and prevent, suspend or reverse pathological progression. The genetic therapeutic technologies can be divided into gene therapy and RNA interference (RNAi). RNAi mediates targeted repression of mRNAs and has been used as a cure for cancer, age related macular degeneration and transthyretin (TTR)-amyloidosis. RNAi have poor specificity and sometimes decrease the effectiveness of treatment (David Benjamin Turitz Cox, Platt, and Zhang 2015). However, RNAi approach would not be further discussed in this thesis. Gene therapy can be realized by gene addition, gene correction, gene disruption or by a combination of these approaches (M. A. Kay 2011). The therapeutic DNA integrates into the host genome or persists as an episomal vector, depending on the type of delivery system. The broad range of delivery systems and techniques are summarized and described in comprehensive reviews (Du et al. 2018; Naldini 2015).

First gene therapy clinical trials have been published in the 1990s (Rosenberg et al. 1990), almost 20 years after the proposal to replace defective DNA of those who suffer from genetic defects for wild type (wt) DNA (Friedmann and Roblin 1972). These therapeutic approaches were based on random DNA integration. As a consequence, the off-target integration often occurred and led to undesired side effects. These complications

limited the spectrum of curable genome disorders. They were abandoned due to safety issues, as some of the treated patients developed leukaemia (Cavazzana-Calvo 2000). The main reason for frequent failures was the absence of technologies that enabled targeted gene manipulation.

Homologous recombination (HR) represents a more advanced way how to reach strictly targeted integration of donor DNA. DNA was firstly edited via HR in the late 1970s, but the absolute frequency of successful HR was quite low (Hinnen, Hicks, and Fink 1978; Capecchi 1989). Following experiments showed that DNA double-strand break (DSB) can significantly increase the frequency of HR (Latt 1981).

Some of the first attempts used meganucleases, such as the homing endonuclease I-*Sce*I that is able to recognize a 18-bp long target sequence, but it was nearly impossible to direct them to any chosen sequence, due to its complicated structure (Choulika et al. 1995). This fuelled research to find a more simply structured endonuclease with the possibility of redirection. Chimeric molecules composed of DNA binding domain and non-specific cleavage domain represent more interesting and versatile tools. The first concepts were based on the zinc-finger (ZF) binding domains and type IIS restriction enzyme *Fok*I nuclease activity (Y. G. Kim, Cha, and Chandrasegaran 1996). *Fok*I is one of the restriction enzymes that has physically separable binding and cleavage activity. It was proved that substitution of the recognition domain leads to the redirection of the cleavage (Y. G. Kim and Chandrasegaran 1994). Zinc-finger nucleases (ZFNs) are the first approaches of programmable nucleases (PNs) to be introduced. Another of the PN tools, transcription activator-like effector nucleases (TALENs) were discovered a couple years later (Christian et al. 2010).

It was the clustered regularly interspaced palindromic repeats (CRISPR) - CRISPR-associated-9 (Cas9) system that elevated targeted genome editing to a higher level, because of the simplicity of the technology (Jinek et al. 2012).

The development of PNs initiated the development of novel treatment strategies for gene therapy of a variety of disorders that require site-specific gene modification. In 2005, Urnov and his colleagues were the first to use targeted manipulation of DNA by ZFNs in human cells (Urnov et al. 2005). Only three years later, in 2008, groups of scientists begun reporting various approaches for human genome disorders treatment using ZFNs and later even other PN platforms (Perez et al. 2008; Ding et al. 2014; Z. Hu et al. 2015; Dever et al. 2016).

In the first chapter of this thesis, I aim to describe the principles of the three PN platforms and to compare their pros and cons relevant to gene therapy. In the second chapter, I give examples of the mainly used treatment strategies for human genome disorders of a variety of origins and introduce selected, ongoing clinical trials. The final, third chapter is about controversial, ethical issues of gene editing mediated by PNs in humans, especially in the human germline.

# 1. Principle of Programmable Nucleases

## 1.1 Zinc-Finger Nucleases

Programmable nucleases described in this thesis are composed of separable DNA-binding domain and non-specific cleavage domain. These two domains are connected by a peptide linker. Zinc-finger nucleases were the first widely used type of PNs of such composition to be presented.

The ZFN's DNA binding domain is the zinc-finger (ZF) motif, that was originally discovered in *Xenopus* and it is the most common DNA binding motif in all of metazoan (Miller, McLachlan, and Klug 1985). The DNA binding domain of these nucleases is located at the N-terminal and it is composed of a set of $Cys_2His_2$ ZFs. The crystal structure of the ZFs bounded to DNA indicates that each finger cooperates with one atom of zinc and contacts three base pairs (bp) of DNA in a major groove (Pavletich and Pabo 1991). The DNA binding domain usually contains three or four ZFs that bind 9-bp or 12-bp DNA target sites. Up to six fingered ZFNs were also tested (Urnov et al. 2010). The study of Kim *et al.* in 2009 showed that four-finger ZFN binds the DNA target more stably and specifically than the three-finger ZFN (H. J. Kim et al. 2009).

The nonspecific cleavage domain at the C-terminal is usually the restriction enzyme *Fok*I. The *Fok*I nuclease mediates the DSB only as a catalytic dimer, thus the two ZFNs must be designed. The first binds the sequence at the leading strand of the DNA and the second binds the opposite sequence at the lagging strand. The sequence between these two target half-sites is called a spacer, which is usually 5 - 7 bp long and this is where the cleavage by the *Fok*I dimer takes place (J. Smith et al. 2000; Bibikova et al. 2001).(Fig. 1.)

The key residues in the dimer interface have to be exchanged in order to prevent the homodimerization of *Fok*I nucleases and thus also prevent off-target activity of the ZFN (Szczepek et al. 2007). The heterodimerization of *Fok*I also brings a further increase in ZFN specificity and a decrease in off-target activity, because the target sequence in DNA is twice as long (Urnov et al. 2010).

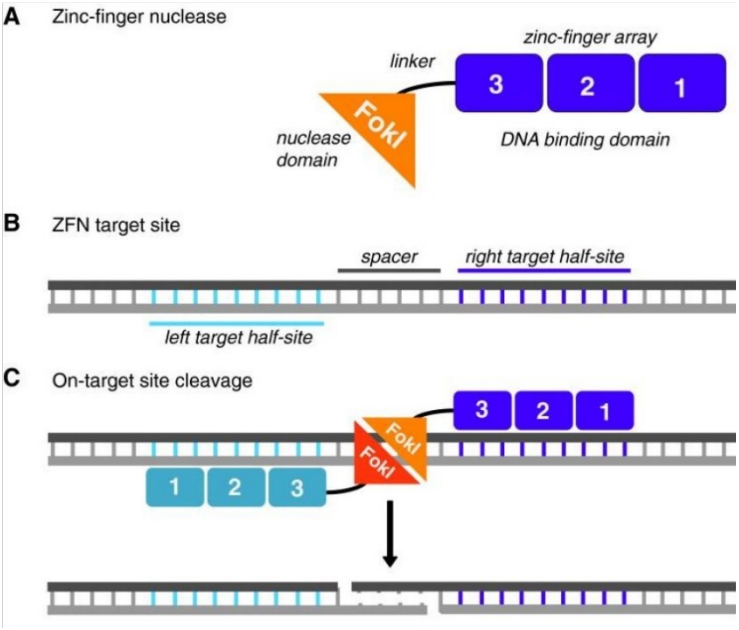


Fig. 1. **(A-C)** Zinc-finger nuclease-mediated DNA cleavage.
**(A)** The 3-finger ZFN architecture
**(B)** Constitution of a target site for two ZFNs.
**(C)** After dimerization of the two FokI domains, the nuclease cuts the DNA within the spacer sequence, leaving a 5′-overhang. Adapted from (Rahman et al. 2011).

The most common approach for ZFNs' design is a "modular assembly" method, which identifies each finger for each component triplet of the target sequence and then links them together as a multi-finger peptide (Segal et al. 2003). The specificity of these ZFN is still partly unpredictable and the process of ZFN production is still quite expensive and laborious (Chandrasegaran and Carroll 2016), despite the effort of several academic laboratories that formed the Zinc Finger Consortium, which put together and provided free specific ZFN protocols, plasmids and software for ZFN design (available at: http://www.zincfingers.org/) (Sander et al. 2010). Production complications prevent their widespread use.

## 1.2 Transcription Activator-like Effectors

Transcription activator-like effectors (TALEs), another programmable DNA-binding system, was found in a plant pathogen genus *Xanthomonas.* It works as a protein tool for the manipulation with a gene expression of a host plant (S. Kay and Bonas 2009). The protein structure of TALE DNA binding domain is modular. It is usually composed of 15 – 20 modules and each module consist of 34 amino acids. These are largely invariant except of the two variable residues at a position 12 and 13, called the repeat variable diresidue (RVD). A DNA binding specificity of TALEs is highly predictable, because there is simple code, where one RVD of a specific constitution binds one nucleotide in the target DNA (Moscou and Bogdanove 2009). This fact suggests that a simple protocol for the production of the versatile binding domain of TALENs could be developed. Boch *et al.* in 2009

experimentally identified the recognition preferences of TALEs: HD = C; NG = A; NS = A, C, G or T; NN = A or G; and IG = T (Boch et al. 2009).

In the case of TALENs the cleavage domain is usually the same as in ZFNs (Christian et al. 2010) meaning, that the restriction enzyme *Fok*I must dimerize for DSB stimulation, therefore two TALENs must be designed. In this case, the spacer between two target half-sites is a bit longer, about 10 - 15 bp (Mussolino et al. 2011). (Fig. 2.)

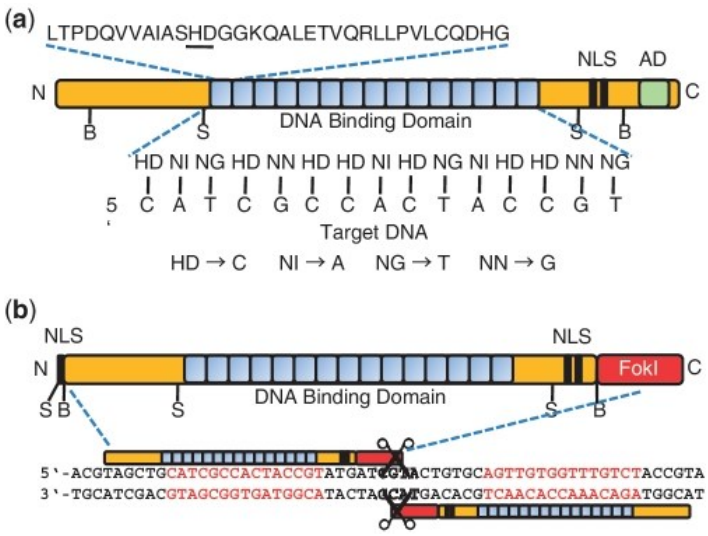


Fig. 2. (**a**) Structure of a TALE: An invariant repeat sequence with underlined RVD. The composition of RVDs determines the target sequence. The nuclear localisation signal *NLS* directs the TALE into the nucleus of the host cell. The transcriptional activation domain *AD* cause an activation of a host gene transcription. BamHI *B* and SphI *S* serve as target places for a restriction enzyme that are used for constructing TALENs. (**b**) Structure of a TALEN: Two TALENs are needed for a FokI dimerization and DNA cleavage. Adapted from (Cermak et al. 2011).

Cermak *et al.* deposited all their plasmids for expressing TALENs and TALEs in a free clone repository AddGene (available at: www.addgene.org) ('Addgene: Homepage' n.d.) and also created a freely accessible software for TALEN site selection and design (available at: https://tale-nt.cac.cornell.edu/) (Cermak et al. 2011; Doyle et al. 2012). The more simple design and protocols for modular assembly have enabled this PN tool to expand.

## 1.3 CRISPR-Cas System

The most recent technology, clustered regularly interspaced short palindromic repeats with CRISPR-associated protein, was discovered as an adaptive defence system in a bacteria and archaea (Mojica et al. 2000). This system is a multistep process: 1) Non-self-viral nucleic acids, called protospacers, are incorporated as spacers into a CRISPR array of a host genome.; 2) CRISPR array is transcribed as a pre-crRNA which is than cleaved into small CRISPR RNAs (crRNAs) (Brouns et al. 2008).; 3) An individual crRNA in conjunction with a Cas protein binds to a foreign nucleic acid by the recognition of a

protospacer sequence and a protospacer adjacent motif (PAM). This state initiates the cleavage of a viral, foreign nucleic acid (Deveau et al. 2008).

Depending on different microorganisms, there can be many modifications to this defence system. Target genome engineering usually uses the type II system, in which the Cas9 protein plays the main role (Jinek et al. 2012). However, Cas9 requires a 3' RNA-RNA hybridization structure for correct binding and cleavage activity, so there is an additional RNA in this system, called the trans-activating crRNA (tracrRNA). This tracrRNA binds to the crRNA due to its complementarity and together they create a guiding complex, guide RNA (gRNA) (Deltcheva et al. 2011). A chimeric tracrRNA:crRNA molecule, the so-called single-guide RNA (sgRNA), was established for scientific purposes (Fig. 3.). The Cas9 protein maintains the DSB of DNA sequence 3 bp upstream of the PAM and creates blunt ends. The optimal PAM sequence recognized by the most commonly used Cas9 protein derived from a *Streptococcus pyogenes* is 5'-NGG-3' (Jinek et al. 2012; Gasiunas et al. 2012).

Another potential of the Cas9 protein is its ability to bring together all the major classes of biopolymers (RNAs, DNAs and proteins) without the cleaving activity simply by the utilization of a nuclease-null version of the Cas9 protein. This way we can repress or activate the transcription, elucidate the role of the unknown factors, engineer a chromatin modification or remodel the genome architecture (Mali, Esvelt, and Church 2013). In this thesis, I will focus on the cleaving activity of this tool in human therapy and not the approaches mentioned above.

One can simplify the sgRNAs design for their project by using a broad range of online tools such as the one developed by Maximilian Haeussler (available at: http://crispor.tefor.net/) which introduces a scoring system that chooses sgRNA sequences with minimum off-target sites (Haeussler et al. 2016).

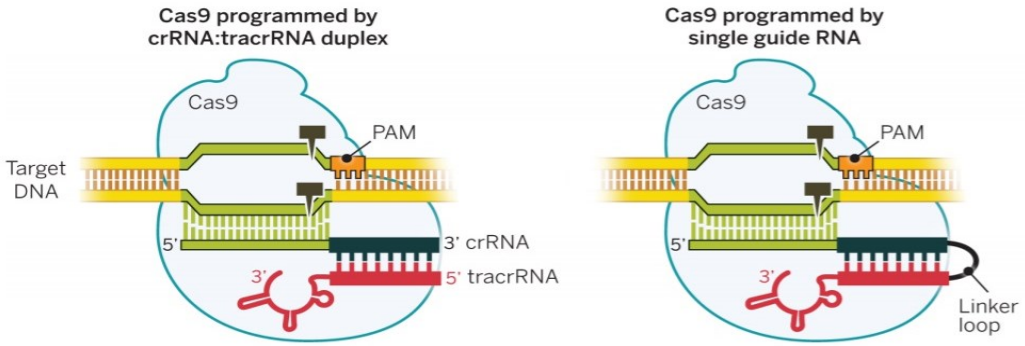


Fig. 3. Arrangement of a CRISPR-Cas9 components directed by a gRNA (left) / sgRNA (right). Adapted from (Doudna and Charpentier 2014).

## 1.4 Comparison of PN Tools

Up until now, I have described the three main platforms that are used in genome engineering. To compare these systems, I would like to focus on a few issues that are relevant for human therapy.

### 1.4.1 Size

The size is an important property of each PN tool, because of the limited cargo size of delivery systems. In general, the smaller the PN tool is, the more preferred is for therapy.

There are two ZFNs and two TALENs required in contrast to the CRISPR-Cas system which is able to cleave both strands as a monomer. It is also good to keep in mind the mRNA length of each system. Although the mRNA for two ZFN proteins is about 2.2 kb (for the 18-bp target), for two TALEN proteins it is more than 5.0 kb (for the 36-bp target). This is because the TALE motifs and ZFs are the same size, whereas one TALE module recognizes 1 bp and one ZF recognizes 3 - 4 bp of the target sequence (Boissel et al. 2014). A single commonly used Cas9 protein from the *Streptococcus pyogenes* is also quite large, it is composed of 1368 amino acids (in case of mRNA about 4.1 kb). Fortunately, employing smaller Cas9 orthologs, smaller than 1100 amino acids, in the case of the *Staphylococcus aureus*, also seems to be effective (Mali, Esvelt, and Church 2013; Ran et al. 2015).

### 1.4.2 Tolerance of Epigenetic Changes of DNA

Epigenetic modifications, such as methylation, acetylation and phosphorylation are naturally represent in many locuses of the genome. The tolerance rate of PNs to epigenetic changes is closely related to versatility of their use.

It is not a problem for the sgRNA of the CRISPR-Cas system to bind to the DNA with epigenetic changes, whereas the protein-based binding domains of ZFNs and TALENs are not able to bind to it. Epigenetic changes of DNA occur frequently during carcinogenesis, thus the CRISPR-Cas is the system of choice in these cases (Khan et al. 2016).

### 1.4.3 Production and Design

Production of ZFNs is very complicated, due to the complicated process of designing ZF sets for new target sequences. Many ZF sets have to be prepared and tested before utilization and usually only a few of all attempts work well. Theoretically, sixty four different modules are requisite to bind all possible triplets. Though, the sequence of TALENs is clearly given and only four different subunits, one for each nucleotide, are

required, the process is still a bit laborious and time consuming. On the contrary, the CRISPR-Cas system is composed of a single, constant Cas9 protein that is present in many bacteria and archaea. In this case, only the sequence of the sgRNA needs to be changed to redirect the cleavage, thus the CRISPR-Cas system is the most economical. Another advantage of a single, constant protein requirement is that in the case that several targets need to be addressed there is the possibility of simultaneous delivery of multiple sgRNAs (Cradick et al. 2013; Chandrasegaran and Carroll 2016; Deng et al. 2012).

### 1.4.4 Resulting Products

HDR vs NHEJ balance in the process of DSB reparation may be influenced by the shape of resulting DNA ends (Zetsche et al. 2015). There are differences in the form of the DNA ends after a DSB. ZFNs and TALENs generate overhanging ends, which are prone to modifications and occurring of indels. Quite the reverse, the CRISPR-Cas9 system generates blunt (non-overhanging) ends, which are more stable (Bibikova et al. 2001; Nerys-Junior et al. 2018). It does not apply to all Cas proteins. For example the Cpf1 protein is an alternative to the Cas9 protein and it mediates a staggered double stranded cut with 5' 4 or 5 nt long overhang and requires only a 42 nt long crRNA without a tracrRNA (Zetsche et al. 2015).

### 1.4.5 Specificity and Off-target Activity

Dimerization of ZFNs and TALENs creates higher target specificity. The sgRNA in the CRISPR-Cas system typically targets a 20-bp sequence compared to TALENs, which usually bind 30 - 40 bp long target (Cradick et al. 2013).

The length of the target corresponds to the level of specificity, which is important for the safety use of the tool.

Off-target activity of PNs is a big issue, it can generate multiple oncogenic mutations and cause severe complications and thus this is the main concern for bringing PNs into human therapy. Off-target activity is closely related to the specificity of the PN. Usually, the longer recognition sequence is, the more specific the PN. It is necessary to mention, however, that even highly specific endonucleases, such as I-*Sce*I, that recognize the 18-bp sequence not present in the entire human genome, have off-target DNA cleavage activity when presented to a human cells (Petek, Russell, and Miller 2010).

All three platforms have a low ability to avoid of off-target sequences. In the case of CRISPRs, the system tolerates even a few mismatches in a target sequence, this is probably because of its original purpose, which is to recognize viral genomes that evolve rapidly and can contain many mismatches (Chandrasegaran and Carroll 2016). Another

reason for this can be naturally occurring differences in the PAM sequence that can be, albeit less efficiently, recognized by a Cas9 protein also as 5'-NAG-3' or 5'-NGA-3' (Hsu et al. 2013).

This is why many scientists are trying to find the way how to increase the specificity of each platform and reduce off-target activity. Examples are given in tab. 1.

| | |
|---|---|
| a selection of a target sequence which is unique in the entire genome (Szczepek et al. 2007) | All PNs |
| a modification of the *Fok*I cleavage domain that allows the formation of heterodimers instead of homodimers (Szczepek et al. 2007) | ZFNs and TALENs |
| an addition of two extra guanine nucleotides at the 5' terminus of the sgRNA (D. Kim et al. 2015) | CRISPR-Cas |
| a truncation of the ds part of a sgRNA (tru-sgRNA) from 20-nt to 17-nt ensures the enhanced sensitivity of mismatches, caused by reduced binding energy a mutation of one of the Cas9 active sites converts it to a nicking enzyme that cleaves only one strand of the DNA target sequence, thus it is necessary to have two sgRNA directed at two different target sequences in close proximity and two mutated Cas9 proteins (Ran et al. 2013) | CRISPR-Cas |
| a transient exposure to PNs by using, for example a ribonucleoprotein delivery instead of stressful plasmid transfection (S. Kim et al. 2014). | All PNs |

Tab. 1.

Off-target activities must be monitored carefully and supressed as much as possible. Identifying potential off-targets and assessing their magnitude is quite challenging and many laboratories came up with strategies for capturing the cleavage sites more broadly, the majority of them are reviewed by Koo *et al.* (Fig. 4.).

The application of PN tools and the selection of reagents, therefore depend on a risk vs. benefit analysis comparing parameters like efficacy, specificity as well as many others. These parameters are clearly stated in tab. 2.

In a next chapter of this thesis, I would like to introduce some selected strategies that use PN platforms for the treatment of significant human diseases.

| | ZFNs | TALENs | CRISPR-Cas9 |
|---|---|---|---|
| Target sequence recognition | Zinc fingers protein, protein-DNA interactions | Repeat variable diresidues (RVDs) repeats, protein-DNA interactions | sgRNA, RNA-DNA interactions |
| Endonuclease | FokI | FokI | Cas9 and its variants |
| Endonuclease construction | 3–4 Zinc fingers domains | 8–31 RVD repeats | sgRNA synthesis or cloning |
| DNA sequence recognition size | (9 or 12 bp) × 2 | (8–31 bp) × 2 | 17–20 bp + NGG × 1 |
| Cytotoxicity | High | Low | Low |
| Targeting efficiency | Low | Moderate | High |
| Off-targeting effect | Moderate | Low | Variable |
| Cost of experiment | High | High | Low |
| Easy of experiment | Difficult and time consuming | Moderate and time consuming | Easy and rapid |

Tab. 2. The comparison of a properties of the three main PN platforms. Adapted from (Yi and Li 2016).

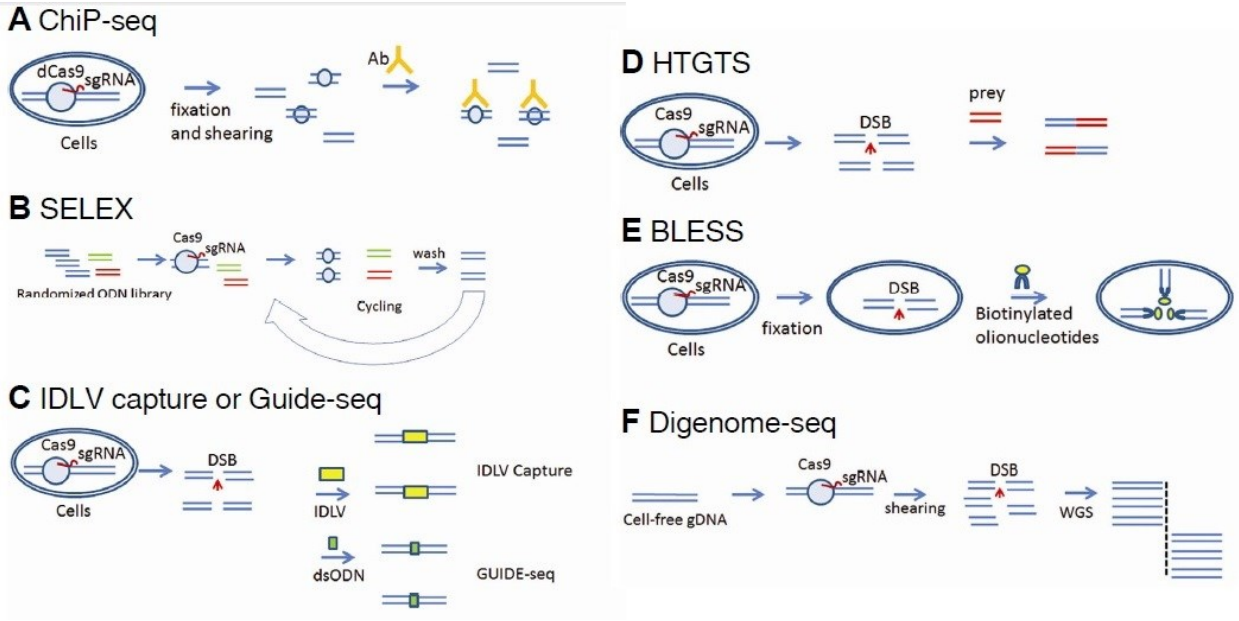


Fig. 4. **(A-F)** Schematics of methods for measuring an off-target activity **(A)** Chromatin immunoprecipitation coupled with deep sequencing using an anti-Cas9 antibodies *Ab* and catalytically-dead Cas9 *dCas9* (*ChIP-Seq*). **(B)** Systemic evolution of ligands by exponential amplification (*SELEX*) using a randomized oligodeoxynucleotide *ODN* library. **(C)** Integrase-deficient lentivirus (*IDLV*) capture or genome-wide, unbiased identification of DSBs enabled by sequencing (*GUIDE-seq*). **(D)** High-throughput genomic translocation sequencing (HTGTS). **(E)** Breaks labelling, enrichments on streptavidin and next-generation sequencing (BLESS). **(F)** In vitro nuclease-digested genome sequencing using the whole-genome sequencing method WGS (Digenome-seq). Adapted from (Koo, Lee, and Kim 2015)

## 2. PNs in Human Therapy

Some of genome disorders will affect most people at some point in their lives. Even rarely occurring disorders, such as monogenic diseases, affect about 6% of all people (Aymé et al. 2011). Diagnosis and treatment of genome disorders is still insufficient. Till recently the care was primarily palliative, focusing on disease management and aftermaths treating without addressing the underlying genetic defects. A loss of function of some gene is a quite simple process, whereas repair of the function is quite challenging. (Prakash, Moore, and Yáñez-Muñoz 2016)

PNs can be used for targeted gene manipulation by creating short insertion/deletion mutations (indels), large chromosomal deletions, chromosomal inversions, chromosomal translocations, non-functional gene corrections or expression cassette integrations. These manipulations are achieved by two main repair pathways. The induced DSB can be repaired by the predominant non-homologous end joining (NHEJ) or by the highly accurate homology-directed repair (HDR) in the presence of donor DNA. Naturally, the NHEJ occurs mostly during the G1 phase of the cell cycle and the HDR is active mostly during the S and G2 phases, when homologous chromatids are available. The HDR uses the homologous donor sequence as a repair template, whereas during the NHEJ the two DNA ends are simply re-ligated and can prior undergo few nucleotides addition or removal (Fig. 5.). (Rahman et al. 2011; Haas, Dettmer, and Cathomen 2017)

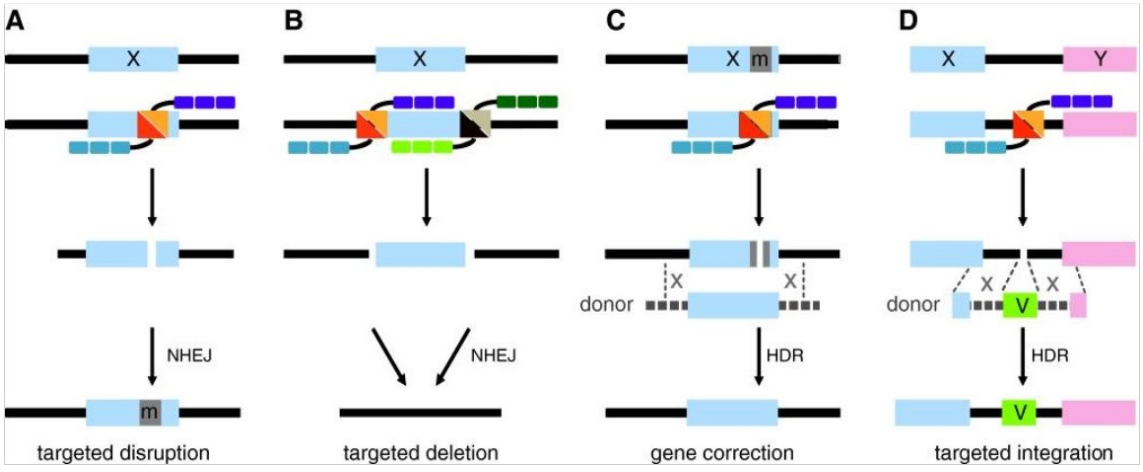


Fig. 5. **(A-D)** Targeted DSB mediated by ZFN. **(A)** Targeted disruption: DSB is repaired by a NHEJ pathway resulting in indel mutations *m* at the cleavage site **(B)** Targeted deletion: two simultaneous DSBs are repaired by NHEJ resulting in a deletion of the targeted locus. **(C)** Gene correction: DSB is repaired by HDR and the co-introduced wt donor DNA fragment resulting in a correction of the mutation *m*. **(D)** Targeted integration: DSB is repaired by HDR and the co-introduced donor DNA fragment with inserted cassette *V*. Adapted from (Rahman et al. 2011).

Some of genome disorders allow treatment that cause gene disruption. In these cases, we can exploit NHEJ. Some genome disorders, however, have to be solved through HDR. Current gene-editing approaches using HDR are limited by efficacy of modification, safety concerns related to it and delivery of gene-editing tools to target cell types. That is why, most of recent strategies edit the patient cells *in vitro*, reproduce successfully-edited cells and afterwards provide them to patient.

Germ-line editing is another possibility how to treat such diseases and even to eradicate such diseases causing genes forever. It is mediated by targeting of a genome in germ cell or embryo instead of already developed somatic cells that can't contribute to gamete formation. It causes changes that are theoretically present in all cells of treated organism which could be very hazardous (Ormond et al. 2017).

In this chapter I would like to introduce selected genetic diseases that are exemplary candidates for targeted gene therapy using ZFNs, TALENs or CRISPR-Cas9 system.

## 2.1 HIV/AIDS

An acquired immune deficiency syndrome (AIDS), caused by an infection of the human immunodeficiency virus type 1 (HIV-1), has threatened society since the late 1970s (Centers for Disease Control (CDC) 1982). The C-C chemokine receptor type 5 (CCR5) is expressed on the surface of our CD4+ T-cells and serves as one of the major co-receptors for the HIV-1 cell entry. To prove that fact, it was found that some of the Northern Europe populations carry the genetic mutation CCR5Δ32 that causes the non-functional CCR5 production and thus also causes protection from infection of CCR5-tropic HIV-1 (Oh et al. 2008). Homozygosity for CCR5Δ32 doesn't however, mean complete resistance to the HIV-1, non-CCR5-tropic variants, such as a CXCR4-tropic viruses are able to use C-X-C chemokine receptor type 4 (CXCR4) as a co-receptor for cell entry (Westby et al. 2006).

Progress in HIV therapy accelerated very quickly, because of the high incidence of this disease. To date, the most effective treatment technology seems to be the highly active antiretroviral therapy (HAART) which, prolongs the life of HIV-positive patients and slows down the progression of the disease. Unfortunately, it requires lifelong medication that can lead to cumulative toxicities and an escape of viral resistance mutants (Rossi, June, and Kohn 2007). And so, replacing HAART by once-in-a-lifetime treatment would be a great asset for both, patients and the health care system. Allogeneic human stem-cell (HSC) transplantation is a possibility, nevertheless a lack of finding HLA-matched donors, who are screened for homozygosity in CCR5Δ32, is a problem (Hütter et al. 2009).

The publication of a successful bone marrow transplant from the naturally CCR5Δ32 homozygous, HLA-matched, unrelated donor into the HIV positive patient with acute myeloid leukaemia proved the concept's efficiency. The donor cells repopulated the peripheral blood, the viral replication was terminated and the patient reversed into a persistent aviremic state for more than 27 months without requiring additional antiretroviral therapy (Hütter et al. 2009).

The ZFN discovery broke the "HLA-match" limitation, because it allowed a *de novo* production of HIV-1-resistant genotype and the utilization of an autologous CD34+ HSC transplant for each patient. This strategy was firstly used by Perez *et al.* in 2008 when the CD4+ T cells of healthy donors were transduced using adenoviral ZFN expression vector. The ZFN pair was engineered to target the DNA sequence encoding the first transmembrane domain TM1 of CCR5 on a chromosome 3. The created DSB was then repaired by an NHEJ pathway and indel mutations were introduced. This led to the disruption of the open-reading frame and non-functional CCR5 transmembrane protein. Statistically, 12 clones expanded from 52 treated CD4+ T-cells containing CCR5 disruption and only 4 of these 12 mutant clones were homozygotes. They also observed appreciable off-target activity at the C-C chemokine receptor type 2 (CCR2) locus, which is the closest relative of CCR5 in human genome (Perez et al. 2008). A concomitant loss of CCR2 and CCR5 shouldn't be problematic, but the fusion epitope can lead to the elimination of the engineered T-cells by the host immune system (M. W. Smith 1997).

Another step made by Holt and his colleagues was to knock out the CCR5 locus using nucleofection of ZFN expression plasmids into the isolated HSCs and co-transplant them with the HIV-infected peripheral blood cells into immunodeficient mice. Whereas these mice underwent selection for CCR5-negative cells and had significantly lower levels of HIV-1, the control mice, who received untreated HSC with the HIV-infected peripheral blood cells, displayed profound CD4+ T cell loss. Some of Holt's results are shown in (Holt et al. 2010).

There are a few ongoing clinical studies who use ZFN platforms for the treatment of HIV infected patients (NCT03617198, NCT00842634, NCT01044654, NCT02500849, NCT02225665 and NCT01543152). All these clinical studies have more or less a similar purpose, to observe the safety and the antiviral activity of the infusion of the ZFN-modified autologous CCR5 -/- CD4+ T cells in HIV positive patients. The majority of the mentioned clinical studies are in phase 1, which means they examine the safety of the drug and focus on the adverse effects, drug degradation and drug excretion. Healthy volunteers are usually

used for phase 1 of clinical studies. Two of the mentioned studies are already in phase 2, gathering preliminary data on whether a drug works on individuals with a certain condition (HIV positivity in this case) ('Glossary of Common Site Terms - ClinicalTrials.Gov' n.d.).

Other PN tools are also used for CCR5 gene disruption. Liu *et al.* in 2017 designed the lentivectors with combined sgRNAs targeting both, the CXCR4 and the CCR5 simultaneously. The sgRNAs-Cas9 lentivectors could induce a mutation of both genes in the murine CD4+ T cells without any nonspecific editing and without obvious cytotoxic effects, which suggests that the loss of CXCR4 may be immune tolerant (Liu et al. 2017).

There is an ongoing clinical study using such CRISPR-Cas9 modification of CCR5 gene in CD34+ T cells (NCT03164135).

An additional finding about CCR5 locus is, that individuals, who are naturally homozygous for CCR5Δ32, are healthy and show no visible phenotype differences, thus we can presume that CCR5 is probably a "safe harbour" locus for the insertion of other therapeutic genes (Camenisch, Brilliant, and Segal 2008). Genomic safe harbours are locations, where some transgenes can integrate without disrupting other gene activity or stimulating cancer. An integration near or within the cancer-related genes poses a big concern and the avoidance of such genes is the main priority (Sadelain, Papapetrou, and Bushman 2012).

Another approach, which has been developed, uses the highly specific targets within the conserved HIV-1 LTR U3 region. Its disruption, the RNA-guided Cas9-mediated genome cleavage inactivates the viral gene expression and replication in the latently infected T cells. Concurrently, the pre-existence of the sgRNA-Cas9 system in cells leads to the elimination of the new HIV-1 virions before they integrate into the host genome and also leads to the immunization of the cells against HIV-1 infection (W. Hu et al. 2014).

## 2.2 Cancer

Cancer exists in multiple complex forms and for this reasons it is difficult to find a versatile tool for its treatment. The current treatment strategies are surgery, radiotherapy and chemotherapy. Alterations of cancer-related genes, such as activated oncogenes, inactivated tumour suppressors, mutations in genes that confer chemo-resistance or repair mechanisms, provide an opportunity for an PN approach (Yi and Li 2016). Recently, ZFNs and TALENs were used to treat cancer, but their limitation in targeting epigenetic changes

was an obstacle. For this reason, the CRISPR-Cas9 is in many cases the system of choice (Khan et al. 2016).

Here are some examples of the CRISPR-Cas9-mediated cancer-related gene modifications reviewed by Yi and Li in 2016 (Yi and Li 2016): Effectively and specifically inhibited bladder cancer cell proliferation and induced cancer cell apoptosis caused by activation of tumour suppressors, such as the p21, E-cadherin and hBax.; Reversing drug resistance of cancer cells by knocking out the multidrug resistance gene 1 (MDR1) by targeting its exon 5 in the osteosarcoma cell lines.; Proposal to correct or destroy the mutated gene for the epidermal growth factor receptor (EGFR) in the lung cancer.; An induced apoptosis of the Burkitt lymphoma cells due to a deletion of the induced myeloid leukaemia cell differentiation protein (MCL-1).

There is another approach for the treatment of various cancers, it is based on PN-mediated editing of immune system cells instead of cancer cells. One of these cancer immunotherapy strategies is the disruption of the programmed death protein 1 (PD-1). PD-1 is a receptor presented on the activated T cells surface. In case it binds to the ligand PD-L1, the T cell's immune response is inhibited. PD-L1 is not expressed only in individual's tissue to mediate peripheral tolerance, but it is also expressed on some tumour cells to inhibit the antitumor immune response (Keir et al. 2008). Su *et al.* demonstrated the enhanced immune response of the CRISPR-Cas9 mediated PD-1 KO T cells in 2016. They targeted the exon 2 of the PD-1 gene in primary human T cells using co-transfection of sgRNA-Cas9 expression plasmid via electroporation, which provided intended transient down-regulation of the PD-1 (Su et al. 2016).

There are many dose-escalation studies of ex-vivo mediated PD-1 knockout-T cells in patients with various cancer types (NCT03081715, NCT02863913, NCT02867345, NCT02867332 and NCT02793856).

Another cancer immunotherapy strategy, is based on manipulation with high-avidity T-cell receptor (TCR) α and β chain genes. These genes are isolated from the rare tumour-specific lymphocytes and transferred into the polyclonal T cells to create chimeric antigen receptor (CAR) T cells. The transduced cells, however, already contain endogenous TCR α and β chains, so they express four different TCR chains, which compete for the TCR formation. These dual-specific T cells have lower avidity for targets and it can also can lead to mispairing of exogenous and endogenous α and β chains causing harmful unpredictable specificities (Kessels et al. 2001). Provasi *et al.* introduced the ZFN-based strategy for disruption of endogenous α and β chain genes to overcome this adverse pairing.

They combined the ZFN-driven disruption of endogenous α and β with lentiviral delivery of Wilms Tumor Antigen-specific TCR. The ZFN-edited T cells infused in mice showed enhanced tumour killing ability and sharply reduced alloreactivity. (Provasi et al. 2012)

There are two ongoing phase 1 clinical studies, who evaluate the feasibility and safety of the CRISPR-Cas9 mediated PD-1 KO T cells and endogenous TCR KO CAR T cells in patients with multiple solid tumours (NCT03545815 and NCT03747965).

## 2.3 HPV Infection

Cervical cancer, caused by the malignant transformation abilities of a high-risk human papillomavirus (HR-HPV), is commonly diagnosed in the women patients who are persistently infected with this virus (Walboomers et al. 1999). The malignant transformation is dependent on the sustained expression of the viral E6 and E7 oncogenes which makes it an ideal target for gene therapy. Host cell-cycle progression is promoted because of the E6 product, which degrades the tumour suppressor p53, and the E7 product, which interacts with the tumour suppressor RB1, releasing the transcription factor E2F (Moody and Laimins 2010).

There are various non-invasive cervical cancer treatment studies describing the targeted cleavage of the E7 or the E6 oncogene in HPV-positive cervical cancer cell lines using ZFNs (Ding et al. 2014), TALENs (Z. Hu et al. 2015) or CRISPR-Cas9 systems (Z. Hu et al. 2014). A down-regulation of expression of these oncogenes leads to the restoration of the tumour suppressor genes RB1 and p53, an apoptosis and growth inhibition of the PN-treated HPV-positive cervical cancer cells.

There are some clinical studies in progress which evaluate the safety and efficacy of human cervical intraepithelial neoplasia treatment using mentioned non-invasive approaches (NCT02800369, NCT03057912 and NCT03226470).

## 2.4 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a genetic disease that leads to muscle degeneration, loss of mobility and premature mortality in male patients. It is caused by various deletions leading to the disruption of the dystrophin-gene reading frame. Fortunately, a milder form of this disease exists, Becker muscular dystrophy (BMD). BMD is caused by in-frame deletions of the dystrophin gene. In contrast to the non-functional dystrophin protein in DMD, BMD results in the expression of truncated, but partially functional dystrophin protein. (England et al. 1990)

In 2016, Tabebordbar *et al.,* and soon after them Nelson *et al.,* used the CRISPR-Cas9 system to convert the DMD form of the dystrophin gene to the BMD form. They used a mouse model of DMD, which has a nonsense mutation in exon 23, and treated it with the adeno-associated virus (AAV) vector. They directed the sgRNAs to target the introns 22 and 23 in order to excise the exon 23. The purpose was to produce internally truncated, but functional, dystrophin protein, typical for BMD. They delivered components of the CRISPR-Cas9 system to the terminally differentiated skeletal muscle fibers and cardiomyocytes. The muscles were, after few weeks, harvested and analysed for the presence of exon 23. This method led to the recovery of dystrophin expression, muscles morphology recovery and reduction of fibrosis. (Tabebordbar et al. 2016; Nelson et al. 2016)

## 2.5 Haemophilia B

Most genetic diseases affect organs that do not enable *ex vivo* cell manipulation, one of such diseases is haemophilia B. The disorder is caused by congenital deficiency of blood coagulation factor IX (F.IX), which is encoded by the F9 gene and produced by the liver. The severe form of this disease is characterized by the circulation of F.IX below the level of 50 ng/ml, whereas the mild form at a level of at least 250 ng/ml. (Scriver 1995).

In contrast to the editing strategies mentioned above, the following strategy uses HDR pathway of DSB repair instead of NHEJ, it co-delivers the wt donor of the F9 gene fragment. In 2011, Li *at al.* created ZFNs that targeted the first intron of the F9 gene and co-delivered them with the wt cDNA fragment of exon 2 - 8 to encompass all the mutated variants of the F9 gene. (Fig. 6.). They injected the hepatotropic AAV vector expressing F9 ZFNs from a liver-specific enhancer into the tail vein of the human-haemophilia B mouse model to evaluate the targeted delivery of such a vector into the liver. Their results showed that the *in vivo* AAV delivery of the donor template and the ZFNs induced gene targeting caused the correction of the F9 gene and increased the circulating F.IX levels. (Li et al. 2011).

An ongoing clinical trial, (NCT02695160), examines therapeutics for ZFN-mediated genome editing of the F9 gene. The corrective copy of the F9 transgene is placed under the control of highly expressed endogenous albumin locus of patient hepatocytes. It is expected that this correction would provide permanent, lifetime, liver-specific expression of the F.IX.

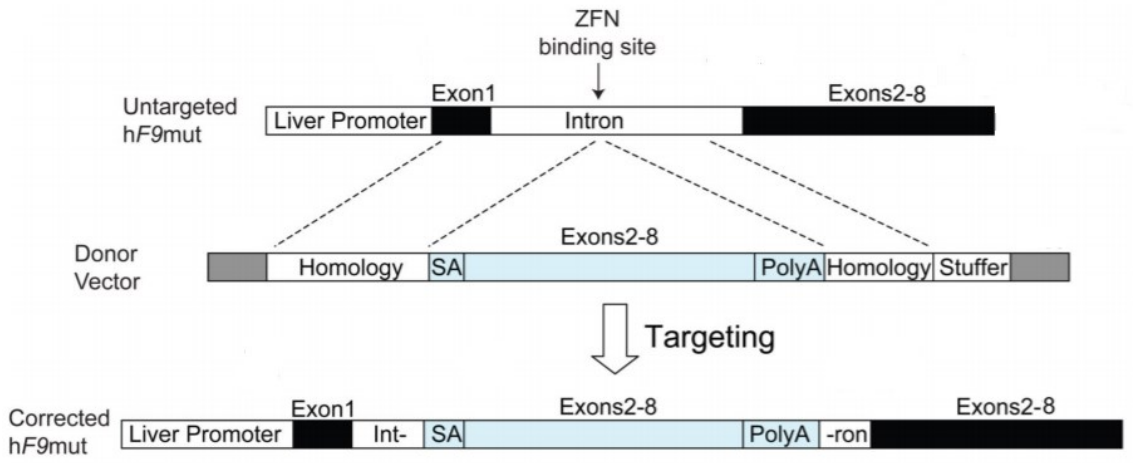


Fig. 6. Design of the F9 gene correction. ZFNs cause the DSB who is marked by arrow and promotes integration of the donor wild-type F9 exons 2–8 into the hF9mut intron 1. The left homology arm spans from the beginning of exon 1 to the ZFN target site. The right homology arm spans intronic sequence 3' of the ZFN target site. Adapted from (Li et al. 2011).

## 2.6 Tyrosinemia

Tyrosinemia type I, a metabolic liver disorder, is often drug resist and requires a liver transplant. This hereditary disease is caused by recessive mutation of the Fah gene, which encodes the last enzyme, the fumarylacetoacetate hydrolase, in the tyrosine catabolic pathway. The lack of this enzyme leads to the accumulation of the cytotoxic homogentisate and its oxidative products (Scriver 1995).

In 2016, Pankowicz *et al.* introduced principle, metabolic pathway reprogramming, which is based on the findings from Endo *at al.* (Endo et al. 1997). This strategy uses the power of the CRISPR-Cas9 for the genetic deletion of the Hpd, another key gene of the tyrosine catabolic pathway. Deletion of the Hpd gene disables accumulation of the homogentisate and therefore supports growth, repopulation of the affected liver and lethal phenotype rescue (Fig. 7.). Pankowicz *et al.* designed the CRISPR-Cas9 platform to target introns next to the exon 3 and 4 of the Hpd gene. Using the mouse model, they successfully converted the (Fah -/- ; Hpd +/+) hepatocytes to the (Fah -/- ; Hpd -/-) hepatocytes by the hydrodynamic injection of the pX330 vector into the tail vein (Pankowicz et al. 2016).

It is necessary to say that both the above mentioned strategies, haemophilia B as well as tyrosinemia I, use vector hydrodynamic injection into the tail vein. Unfortunately, this approach is not considered to be clinically practicable on humans (Guan et al. 2016).

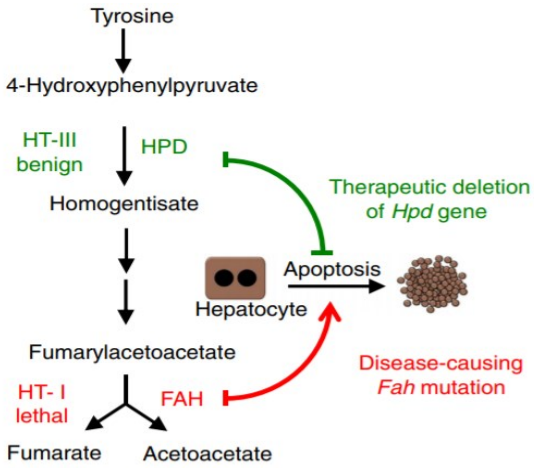


Fig. 7. Metabolic pathway reprogramming, the therapeutic conversion of Hereditary tyrosinemia type I *HT-I* to Hereditary tyrosinemia type III *HT-III* by the Hpd gene deletion. Adapted from (Pankowicz et al. 2016).

## 2.7 Sickle Cell Disease and β-Thalassemia

The treatment of genetic disorders, such as sickle cell disease and β-thalassemia, uses an attractive approach for the correction of β-globin, utilising the occurrence of haemoglobin switching. (M. D. Hoban et al. 2015; Megan D Hoban et al. 2016; Dever et al. 2016) (NCT03728322). The transcriptional factor BCL11A silences the γ-globin, the fetal globin gene, during development, and regulates fetal haemoglobin (HbF) switching (Sankaran et al. 2008). Approaches focused on BCL11A gene disruption were developed, because it was discovered, that adult individuals with elevated HbF levels have milder symptoms of β-haemoglobinopathies (Uda et al. 2008). Bjurström *et al.* discuss the utilization of all three PN platforms for BCL11A gene disruption in human primary CD34+ cells as well as in immune-deficient mice (Bjurström et al. 2016).

Restoration of the functional erythrocytes, which is mediated by the reactivation of the γ-globin gene through NHEJ pathway, is a much simpler procedure than the HDR-mediated correction of the β-globin gene. For this reason the majority of ongoing clinical trials evaluate the safety and efficacy of the autologous CD34+ human stem and progenitor cells (HSPCs) with PN-mediated BCL11A gene disruption. All of them are already in phase 2 of a clinical study (NCT03655678, NCT03745287, NCT03432364 and NCT03653247).

All mentioned clinical studies are available at https://clinicaltrials.gov ('Home - ClinicalTrials.Gov' n.d.).

# 3. Question of Ethics

I have mentioned some strategies that use programmable nucleases for the treatment of hereditary disorders. All of which are focused on editing genetic information in somatic cells which is not able to be passed on from individuals to their offspring. The stated strategies intend to treat patients with life threatening symptomatology. Even this very straightforward scenario raises serious worries about unintended consequences. There are still limits to our knowledge of gene-environment interactions and the disease/treatment pathways that can differ in each patient (Baltimore et al. 2015).

Numerous discussions have been raised since germ-line modification was facilitated by PN platforms. A controversial question for those involved in these discussions is, whether or not gene editing of human embryos for therapeutic reasons should be considered or categorically ruled out (Bosley et al. 2015). Ormondo *et al.* divides the ethical concerns of germ-line genome editing into two categories, those arising from its potential failure and those arising from its success (Ormond et al. 2017). Often repeated concerns are for example: the fear of possibility altering essential characteristics, insufficient specificity of the PNs, the permanency and heredity of the targeted changes, the lack of knowledge about the consequences of such alterations, the inability to obtain informed consent of yet unborn patients or the fate of the children being produced by such technology. Moreover, nobody can decide if it is appropriate to exchange disease-causing genetic mutation to a sequence more typical among "healthy" people. Though there are some exceptions, most of the prominent scientists claim that human germ-line modification should only be undertaken for prevention, diagnostic or therapeutic purposes (Chandrasegaran and Carroll 2016; Baltimore et al. 2015; Bosley et al. 2015; Ormond et al. 2017). The summary of recommendations and attitudes of major scientific institutions related to human germ-line gene editing is seen in (tab. 3.).

An article published in 2015, by a group of Chinese scientists, has introduced the use of CRISPR-Cas9 in human tri-pronuclear zygotes and has provoked international outcry. The paper, initially submitted to journals like *Nature* and *Science,* was eventually rejected, because of the involved ethical considerations (Krishan, Kanchan, and Singh 2016; Liang et al. 2015).

Equally, the very recent Chinese born twins, who have secretly had the CCR5 gene disruption edited by the CRISPR-Cas9 tool, have provoked shock and outrage among scientists around the world ('Statement on Claim of First Gene-Edited Babies by Chinese

Researcher' 2018; 'Statement from the Organizing Committee on Reported Human Embryo Genome Editing' n.d.).

| Arguments | Organizations | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | The Hinxton Group | NAS, NAM, CAS, and UK Royal Society International Summit | NAS and NAM Committee on Human Gene Editing | ASGCT and JSGT | ISSCR | Baltimore et al. | EGE | Lanphier et al. | ACMG | NIH | HFEA |
| Basic research should be conducted | x | x | x | x | x | x | | | | | x |
| Preclinical research should be conducted | | x | x | | | | | | | | |
| There should be a partial or full moratorium on research | | | | | | | x | x | | x | |
| Diverse stakeholders should be involved in decision making | x | x | x | x | x | x | x | x | x | | |
| Clinical use should not proceed currently | x | x | x | x | x | x | x | x | x | | |
| Clinical use should proceed *only* if safety and efficacy issues are resolved | x | x | x | x | x | x | x | | x | | |
| Clinical use should proceed *only* if society has agreed on bounds | x | x | x | x | x | | x | | x | | |
| Clinical use should proceed *only* if appropriate oversight is in place | x | x | x | | | | x | | | | |
| Clinical use should proceed *only* if justice and equity concerns are addressed | x | | | x | | | x | | | | |
| Clinical use should proceed *only* if it is transparent | | | x | | | x | | | | | |
| Clinical use should be discouraged worldwide | | | | | | x | | | | | |
| Any public policies regulating this | x | | | | | | | | | | |

Tab. 3. The summary of recommendations and attitudes of major scientific institutions related to a human germline gene editing. *NAS* - US National Academy of Sciences; *NAM* - US National Academy of Medicine; *CAS* - Chinese Academy of Sciences; *ASGCT* - American Society for Gene and Cell Therapy; *JSGT* - Japan Society of Gene Therapy; *ISSCR* - International Society for Stem Cell Research; *EGE* - European Group on Ethics in Science and New Technologies; *ACMG* - American College of Medical Genetics; *NIH* - National Institutes of Health; *HFEA* - UK Human Fertilization and Embryology Authority. Adapted from (Ormond et al. 2017).

## Summary and Prospects for the Future

Programmable nucleases are a very useful tool that have fuelled the genome engineering revolution further by introducing the possibility of targeted editing. In my thesis, I have focused on strategies that are used for the treatment of life-threatening genetic disorders of various origins, having discussed examples of the most widely used approaches.

One of them being, the disruption of the gene that enables the disease to develop, such as the CCR5 gene, which mediates the HIV entry into the T-cells. The same principle is used for treating haemoglobinopathies, where the disruption of the BCL11A gene leads to the restoration of fetal haemoglobin, which replaces the function of non-functional adult haemoglobin.

Another important example is the disruption of the pathogen genome, which is suitable for infectious diseases, such as HPV or HIV infection.

The modification of polyclonal T-cells is useful for treating cancer, where the mutations can be highly variable. Enabled by the disruption of the endogenous high-avidity T-cell receptor (TCR) α and β chain genes and addition of the exogenous TCR α and β chain genes that are isolated from the rare tumour-specific lymphocytes.

There are other modifications that are able to compensate the function of the affected protein. In case of treating Duchenne muscular dystrophy, the non-functional dystrophin protein is partially knocked out for the purpose of creating truncated, but partially functional, dystrophin protein typical for Becker muscular dystrophy. Tyrosinemia is treated with the targeted conversion of the (Fah -/- ; Hpd +/+) hepatocytes to the (Fah -/- ; Hpd -/-) hepatocytes, which doesn't cure the non-functional tyrosine catabolic pathway, but it prevents the toxic product accumulation.

Lastely, the most challenging of the strategies mentioned, which is used in the rest of the cases, where we cannot use other easier strategies, is the correction of a mutated gene. This strategy is demanding because of the necessity of donore DNA presence and low efficiency of involved HR. It is used for example for haemophilia B, where we replace the majority of the mutated gene.

Up until now, all the above discussed approaches are still in testing and improvement phases. The treating strategies that are based on gene disruption and achieved by NHEJ are much closer to common clinical treatment. I dare say that, for example, the disruption of the CCR5 gene in T-cells of HIV positive individuals could soon be a

commonly available treatment strategy. On the contrary, gene correction is still very challenging and primarily its safety needs to be improved.

In regards to the individual characterisation of each PN platform, one has to decide which of the PN tools is suitable for the specific use. It depends on their properties, such as size, specificity, ease of production, cost *etc*. So far, ZFNs and TALENs are closer to clinical application then the CRISPR-Cas9 system, due to the length of time they have been in the testing process. On the other hand, the majority of scientists are using the CRISPR-Cas9 system for the development of new therapeutic strategies. It is the system of choice because of its versatility, simplicity and availability even to small laboratories.

In order to implement the use of programmable nucleases into clinics is critical to further understand the physiology of the cells and tissues. In my opinion, we still don't know enough about all the cellular pathways and potential consequences. As with any other treatment strategy, the risks can be tolerated only when the reward of success is higher than the loss caused by the disease. Unfortunately, in many cases it is not yet possible to make this decision, firstly we have to improve the assessment methods.

Avoidance of potential off-target activity, which is still quite frequent and is the obstacle to introducing these technologies in clinics, is another field that should be upgraded. Some of the developed strategies for off-target activity inhibition are mentioned in tab. 1.

Similarly, the delivery systems of PN-tools need to be further evaluated and optimized because the delivery into human tissues still causes problems. For this reason, many studies attempt to create similar conditions, such as human murine models or monkeys. The testing of such delivery systems, as well as off-target activity, can be newly done on human organoids, the self-organized 3D tissue cultures that are derived from HSCs. The use of organoids was extensively reviewed by Clevers *et al.* (Clevers 2016).

This thesis does not discuss other possible therapeutic activities of programmable tools, such as RNA base editing technology using the type VI CRISPR-associated RNA-guided RNase Cas13. This tool controls cellular processes at the transcript level, which represents safer strategy then the DSB induction by PNs (David B. T. Cox et al. 2017). Similar programmable tools have potential for further research and therapy.

Utilization of PNs in the treatment of human patients still raises numerous discussions and social concerns. These discussions have intesified since germ-line modification was facilitated by PN platforms. The arisal of strict rules is necessary for the clinical utilization of PNs.

It is a matter of time before the use of programmable nucleases, especially the CRISPR-Cas system, is implemented in daily clinical use or in personalized and precision medicine. It will depend on our ability to eliminate the above mentioned obstacles and improve the safety assays.

# Abbreviations

**AAV** - adeno-associated virus
**AIDS** - acquired immune deficiency syndrome
**BMD** - Becker muscular dystrophy
**bp** - base pair
**CAR** - chimeric antigen receptor
**Cas** - CRISPR-associated protein
**CCR2** - C-C chemokine receptor type 2
**CCR5** - C-C chemokine receptor type 5
**CRISPR** - clustered regularly interspaced short palindromic repeats
**crRNA** - CRISPR RNA
**CXCR4** - C-X-C chemokine receptor type 4
**DMD** - Duchenne muscular dystrophy
**DSB** - double-strand break
**EGFR** - epidermal growth factor receptor
**F.IX** - blood coagulation factor IX
**HAART** - highly active antiretroviral therapy
**HbF** - fetal haemoglobin
**HIV** - human immunodeficiency virus
**HDR** - homology-directed repair
**HR** - homologous recombination
**HR-HPV** - high-risk human papillomavirus
**HSC** - human stem cell
**HSPC** - human stem and progenitor cell
**indel** - insertion/deletion mutation
**KO** - knockout
**LTR** - long terminal repeat
**MCL-1** - myeloid cell leukaemia 1
**MDR1** - multidrug resistance gene 1
**NHEJ** - non-homologous end joining
**PAM** - protospacer adjacent motif
**PD-1** - programmed death protein 1
**PN** - programmable nuclease
**RNAi** - RNA interference
**RVD** - repeat variable diresidue
**sgRNA** - single-guide RNA
**TALE** - transcription activator-like effector
**TALEN** - transcription activator-like effector nuclease
**TCR** - T-cell receptor
**tracrRNA** - trans-activating crRNA
**tru-sgRNA** - truncated sgRNA
**U3** - unique sequence at 3’ end of the LTR region
**wt** - wild type
**ZF** - zinc finger
**ZFN** - zinc-finger nuclease

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