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Nanoparticles for gene editing

Nanočástice pro využití v genové terapii

Bakalářská práce

Školitel: Mgr. Klára Grantz Šašková, Ph.D.

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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, 11. 5. 2016

Zuzana Kružíková

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Abstrakt

Časné genové terapie založené na DNA byly testovány pro terapeutické účely, dříve či později se ovšem objevila řada překážek a rizik spojených s jejich využíváním, což zastavilo další klinické testování. Poměrně nedávno byly tyto metody nahrazeny rychle se rozvíjejícím genovým editováním za pomoci programovaných nukleas, které jsou schopny štěpit specifické sekvence DNA a tak vytvořit přesné genomové modifikace. Jako potenciální terapeutika jsou testovány nukleasy s motivem zinkového prstu (ZFN), dále tzv. „transcription activator-like effector" nukleasy (TALEN) či CRISPR/Cas9 systémy. Největším rizikem, kterému je nutné zabránit, jsou chybná štěpení mimo cílové sekvence. Jako nejvhodnější metoda pro aplikaci do buněk se jeví cílené dopravování nukleas ve formě mRNA. Nanočástice různých typů umožňují přenos mRNA a usnadňují tak dopravování nukleas do buněk. Tato bakalářská práce popisuje některé z těchto nanočástic společně s charakterizací programovaných nukleas.

Klíčová slova: genová terapie, nukleasy, editování genů, nanočástice, dopravování mRNA

Abstract

Early DNA-based therapies were tested for therapeutic applications, but they sooner or later revealed multiple hurdles and risks preventing their use in further clinical trials. Recently, they have been replaced by rapidly evolving gene editing using programmed nucleases capable of precise genome modifications by cleaving specific DNA sequences. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 system are currently under investigation as potential therapeutics. However, their off-target effects must be controlled. Targeted delivery of nucleases in a form of mRNA seems as the most promising method. Various types of nanoparticles enable mRNA transfer and could be used to facilitate the nuclease application. Some of these nanoparticles together with characterization of the programmed nucleases are described in this thesis.

Key words: gene therapy, nucleases, gene editing, nanoparticles, mRNA delivery

List of abbreviations

CCR2	C-C motif chemokine receptor 2
CCR5	C-C motif chemokine receptor 5
CFTR	cystic fibrosis transmembrane conductor receptor
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPR/Cas	CRISPR associated proteins
crRNA	CRISPR RNA
DSB	double-strand break
DC	dendritic cell
HDR	homology directed repair
HIV	human immunodeficiency virus
HR	homologous recombination
mRNA	messenger RNA
NHEJ	nonhomologous end joining
NLS	nuclear localization signal
PAM	protospacer adjacent motif
PCL	poly(caprolactone)
PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
pre-crRNA	precursor of crRNA
RCT	rolling circle transcription
RVD	repeat-variable diresidue
SCID	severe combined immunodeficiency disease
sgRNA	single guided RNA
TALE	transcription activator-like effector
TALEN	transcription activator-like effector nuclease
tracrRNA	trans-activating crRNA
ZF	zinc finger
ZFN	zinc finger nuclease
ZFP	zinc finger protein

Contents

1. Introduction

For the last decades, many experiments have been carried out to prove the concept of gene therapy. The initial idea of understanding the genetic basis of the problem seemed promising to solve the real cause of basically any genetic disease (Kay et al., 2000; Cavazzana-Calvo et al., 2000). Several concepts, which appeared to be very efficient in research models, entered clinical trials. However, many of them failed with serious consequences.

The main scheme of gene therapy was initially presented as a delivery and replacement of missing or damaged genes of the genome. In most of the cases, donor DNA templates were tested for therapeutic purposes. Besides, mRNA molecules encoding absent gene products were used for temporal effects. Specific challenges and dangers are associated to both of these methods. It can be either instability of RNA or risks of malignant insertions of DNA sequences. Finding suitable delivery methods also proved very challenging. The risk of potential cellular inflammation or cytotoxicity and immunogenicity of the transfecting agents is enormous.

Numerous knock-out and knock-in genome modifications were generated in the past via insertion or homologous recombination with donor sequences, however, they were too far from their *in vivo* therapeutic applications, since the precision appeared to be quite low. An alternative method for gene editing was discovered recently with programmed nucleases. The relative simplicity of their design and their precision makes them an attractive tool.

Therefore, a novel approach of targeted nuclease delivery, in order to edit genome *in vivo,* seems very promising and might provide a new alternative way for successful development of gene therapy. Nevertheless, ethical aspects about editing of human genes are concerning and gene editing of human germlines is prohibited in most of the countries worldwide.

2. DNA-based therapies

Based on the viral capability to enter the cell or even the cell nucleus, the early methods of gene delivery used viral vectors. The whole genes could be thus transported into the cells. They either temporarily stayed in the cell or they were permanently integrated into the genome. Modified viruses such as retroviruses (Cavazzana-Calvo et al., 2000), their subfamily lentiviruses (Kordower et al., 2000), adenoviruses (Gerdes et al., 2000) or adeno-associated viruses (Kay et al., 2000) were used for the initial gene delivery approaches.

For generating such modified viral vectors, all viral genes enabling replication of their genome must be removed and replaced by therapeutic DNA sequences. Only the genes required for viral assembly or for integration into the host genome are not modified (Thomas et al., 2003).

The adenoviruses naturally transfer their genetic information into the cell nucleus. Therefore, they served as a first suitable model for delivering therapeutic genes. Nevertheless, their toxicity became quite an issue as a patient died after being treated with adenoviral gene therapy in a clinical trial in 1999. He was treated for deficiency of liver enzyme ornithine transcarbamylase, causing the accumulation of high ammonia concentrations in the blood and brain, which leads to encephalopathy and coma. He received a high dose of vector ($3{,}8 \times 10^{14}$ particles) which activated cytokine cascade, led to disseminated intravascular coagulation, acute respiratory distress and finally multiorgan failure (Thomas et al., 2003). To avoid side-effects, such as the immune response or cellular inflammation, low concentrations of viral vectors with strong promoters must have been used in further cases (Gerdes et al., 2000).

Based on the adenoviral model, adeno-associated viruses were further developed for their potential use as delivery vectors. They are small and simple which significantly reduces the risk of toxic reactions. They were used, for example, to deliver factor IX for haemophilia patients. (Kay et al., 2000).

As another example, a possible gene therapy of Parkinson's disease was examined on primates. Glial cell line-derived neurotrophic factor was delivered in lentiviral vectors into the striatum and *substantia nigra* of rhesus monkeys and it successfully prevented the neuron degradation (Kordower et al., 2000).

Another study suggested a potential treatment for severe combined immunodeficiency disease (SCID). SCID patients carry a mutation for γc cytokine receptor causing a block of

T and natural killer (NK) lymphocytes differentiation. In a clinical trial, $CD34^+$ cells were separated from the patient's bone marrow and infected with a retroviral vectors encoding γc receptor. Cells with γc transgenes were then infused into the patients. This resulted in the effective correction of the SCID phenotype and it seemed as a promising SCID therapy (Cavazzana-Calvo et al., 2000). However, three years later it was found that one of the patients treated by γc transgene $CD34^+$ cells developed lymphocytosis and leukaemia as a consequence of the integration of the retroviral vector carrying the γc into the LMO-2 locus. The integration caused malignant expression of LMO-2 gene as it occurs in lymphoblastic leukaemia (Hacein-Bey-Abina et al., 2003). This case pointed at the danger of insertional mutagenesis connected with the usage of retroviruses for gene transfer.

As illustrated above, substantial research work and several relatively successful trials with knock-in genes for replacing the missing or damaged genes in humans had been performed. In addition, experiments for gene knock-outs were also a significant part of the research.

Recombinant adeno-associated viruses were used to generate specific gene knock-outs in human somatic cells. They targeted, for instance, genes of colon cancer cells *in vitro*. The hypothesis assumed that these vectors generate the homologous recombination (HR) which leads to gene disruption (Kohli et al., 2004). However, after several studies, it was shown that therapeutic transgenes do not trigger HR and do not cause biallelic gene knock-outs and thus do not change the mutated loci and cannot be used to treat genetic disorders (Urnov et al., 2005). The need of alternative techniques was still urgent.

3. RNA-based therapies

The theory of gene therapy via nucleic acids encoding specific protein products included both DNA and RNA. At first, most of the studies focused on DNA delivery, mainly because the mRNA is less stable in the cell which makes it harder to use it for therapeutic purposes. Nonetheless, using mRNA has several advantages.

First, DNA must be transported into the nucleus to be transcribed and then translated into a functional protein, whereas, mRNA is immediately translated after entering the cell cytoplasm which eliminates the problem of crossing the nuclear membrane. In addition, mRNA does not integrate into the genome so there is no risk of insertional mutagenesis or unwanted multiple translations. After some time, mRNA is naturally degraded in the cell.

Transferred mRNA must be complete in order to be functional, which means that beside the therapeutic sequences, it also requires 5' cap and 3' poly(A)tail as well as the start and stop codons and untranslated terminal regions (UTR). Preparation of mRNA occurs via *in vitro* transcription and the mRNA is transfected into the cells afterwards (Sahin et al., 2014).

Injected mRNA enters the cells via endocytosis. The cell has a protective mechanism and it recognizes the RNA in endosomes as possible infectious viral RNA. This causes a Toll-like receptor 7 signalling which can result in interferon-mediated inflammatory reaction (Diebold et al., 2004).

Nonetheless, in a study using mRNA encoding luciferase as reporter gene, it was proven that mRNA injected directly into a mouse muscle *in vivo* undergoes translation. Luciferase expression in the cells was detectable until 60 hours after injection (Wolff et al., 1990). With this knowledge, possible mRNA-based therapies became more real and significant subject of further research. An mRNA was used mainly to substitute missing protein products or as a source of antigens for infection or cancer treatment.

The first clinical trial with mRNA started in 2001. The therapy targeted metastatic prostate cancer. Autologous dendritic cells (DCs) were transfected *ex vivo* with mRNA encoding prostate-specific antigen. Transfected DCs were then infused into patients which led to translation of mRNA and successful stimulation of T-cell-mediated antitumor immune response (Heiser et al., 2002).

During e*x vivo* transfection by electroporation or lipofection, mRNA is kept safe from extracellular ribonucleases. Delivering mRNA into cells *in vivo* is far more challenging, but such treatment would be faster and could be applied in more patients.

A preclinical animal model revealed that intranodal application of naked mRNA *in vivo* successfully triggered T-cell antitumor response due to DCs uptake of the naked mRNA and presentation of encoded antigens (Kreiter et al., 2010).

However, another study about delivery of mRNA into dendritic cells indicated the opposite results. It was shown that mRNA encapsulated in nanoparticles was delivered with high efficiency, whereas the naked mRNA did not transfect any cells *in vitro* (Phua et al., 2013). Some cell types can actually spontaneously take up naked mRNA, but the uptake rate in most cell types is just about 0,01 % of injected mRNA molecules (Sahin et al., 2014).

This indicates that the mRNA in nanoparticle format could facilitate the cell entry and protect the mRNA from degradation.

A new approach appeared with the development of precise nucleases. Encoded by mRNA, they have already entered the preclinical trials. Advances in research and the ways of delivery of such agents are analysed further.

4. Nucleases

Gene delivery into the cells did not prove any long-term therapeutic effect using temporary mRNA factors and it showed a danger of random integrations of virus-delivered genes into the genome. On the other hand, site-specific nucleases were found and designed to target mutated genes and repair the original cause of genetic or other disorders with high specificity. After several failures of gene therapy in clinical trials, the focus of research moved towards the development of precise nucleases (Gaj et al., 2013).

Experiments with programmed nucleases showed that it is possible to design them to cleave desired sequences, or even substitute genes using donor vectors together with nucleases. They enable to silence, insert or delete a gene of interest from genomic DNA *in vitro*. The goal is to prove the safety and possibility of doing so also *in vivo* and to use gene editing as a possible treatment for human diseases.

The most commonly used nucleases are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) with CRISPR associated proteins (CRISPR/Cas9). They all cause double-strand breaks (DSB) of the DNA. The cell has its own mechanism to repair DSBs, which is either nonhomologous end joining (NHEJ), or homology directed repair (HDR) (Santiago et al., 2008). In case of HDR, the sister-chromatid is used as a template for homologous recombination. This usually occurs in yeasts, whereas the mammalian cells use NHEJ more often, because the chromatin is typically highly condensed and two sister-chromatids are separated, making the search for homology very difficult in the cell. Nevertheless, during the NHEJ, exonucleases are active and produce deletions, hence, it induces errors in the repaired DNA sequence (Sonoda et al., 2006).

In order to use genome editing as a therapy for human diseases, many potential risks must be handled. Numerous experiments were performed to find the most suitable nuclease with precise genome modifications, high specificity and without off-targets and cytotoxicity. The known nucleases with the history of their development and current progress are described and compared below.

4.1. Zinc fingers

Zinc finger (ZF) is a DNA-binding motif and it is widely used by the eukaryotic cells. For example, most of the transcription factors binding DNA have the Cys_2His_2 zinc finger structure (Hossain et al., 2015). ZFs were originally found in *Xenopus laevis* in their transcription factor IIIA, which contains domains binding zinc ion (Miller et al., 1985).

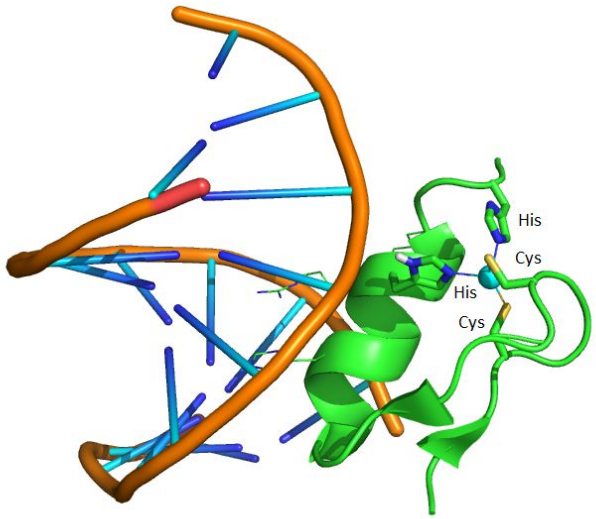

Fig. 1 **Zinc finger bound to DNA.** α-helix and two β-sheets of the zinc finger are linked by a zinc ion (blue sphere) coordinated by two cysteine and two histidine residues. The picture was created by Pymol using structure of the PDB code 5egb (Patel et al., 2016).

ZF consists of an α-helix and two β-sheets linked by a zinc ion, which is usually coordinated by two cysteine and two histidine residues (the Cys_2His_2 motif) (Fig. 1). Each finger binds 3 bp of DNA through the α-helix which is inserted into the major groove of DNA (Fig. 1). The Cys_2His_2 zinc finger proteins bind specific sequences of the DNA which can be used for targeting designed sequence for gene editing. It is possible to form ZF to bind almost

any triplet and to combine those fingers to bind any desired sequence. However, adjacent fingers interact with each other and thus, they influence their binding specificity (Isalan et al., 1997).

4.2. Zinc finger nucleases

Chandrasegaran et al. were the first who used the ability of ZFs to bind specific sequences for gene editing. They made a chimeric protein by fusion of ZFs with a catalytic domain of restriction endonuclease FokI type II, which makes a DSB in DNA (Fig. 2). After this fusion, they got the first programmed endonuclease (Kim et al., 1996). They also had to dimerize the protein, since the FokI works as a dimer in order to make a DSB and eventually the gene knockout (Kim et al., 1996). DSB caused by ZFN is typically repaired by NHEJ (Santiago et al., 2008).

ZFN can be theoretically designed to bind any sequence. Libraries of various ZF domains were constructed and can be used as modules for further assembly of ZFN (Segal et al., 1999). However, the construction and selection of certain ZFN is very time- and material consuming.

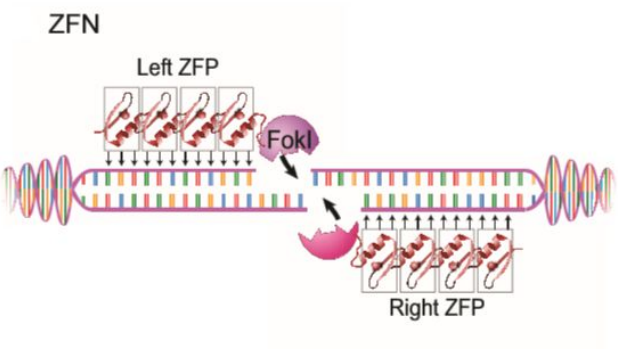

Fig. 2 **Zinc finger nuclease.** ZFN binding the DNA and causing a DSB (Adapted from Peng et al., 2014).

ZFNs were used to make various mutants in many model organisms. For human cells, for example the IL2Rγ gene, carrying the X-linked severe combined immune deficiency mutation (SCID), was modified by designed ZFNs. Two ZFNs containing four ZF motifs each were used to bind DNA surrounding the SCID mutation locus. Human cells were then incubated with ZFNs and with a donor plasmid encoding a fragment of IL2Rγ locus carrying a silent point

mutation. After 4-day incubation, the genome was mutated by ZFNs in 20 % of the cells. Moreover, both alleles of the gene were modified in 7 % of the cells (Urnov et al., 2005).

Furthermore, ZFNs were designed to target *CCR5 (human chemokine receptor 5)* gene in the primary human $CD4^+$ T lymphocytes. ZFNs targeted a sequence upstream of the natural *CCR5*Δ32 mutation which results in a HIV-1 resistance (Dean et al., 1996). After the incubation with ZFNs, 23 % of alleles were mutated, 7 % of the cells showed biallelic mutations. However, 4 % of the related *CCR2* gene was mutated as well showing thus quite high off-target effect (Perez et al., 2008).

ZFN gene editing revealed some of their disadvantages, like the time-consuming preparation and their numerous off-target activities. To increase the specificity, ZFN should target a unique site which differs from the most closely related sequences within genome by at least three mismatches. Furthermore, ZFN should be designed without excess DNA-binding affinity and they should be used at the lowest concentration possible to decrease the tolerance for off-target effects. Despite the fact that this could lower the chance of off-targets, their prevalence makes the usage of ZFNs as a therapeutic tool still unacceptable (Pattanayak et al., 2011).

4.3. Transcription activator-like effectors (TALEs)

After ZFNs, another protein with the ability of binding DNA was discovered - TALE. TALEs were originally found in Gram-negative bacteria *Xanthomonas*. These bacteria were studied as pathogens of crop plants causing significant agriculture damage. They inject the effector proteins into the plant cells via type III secretion system. TALEs contain a nuclear localization signal, a central domain of tandem repeats for binding DNA and transcription activating domain. Once in the plant cell, they are translocated into the nucleus and they activate transcription. The specific activity of TALE is determined by their amino acid sequence and number of repeats (Boch et al., 2009). Each repeat unit contains two variable amino acid residue called repeat-variable diresidue (RVD). One repeat unit binds one specific DNA base (Moscou and Bogdanove, 2009) according to specific recognition code (Fig. 3). A T base is conserved and precedes the natural recognition sequence at 5'end and it seems to be important also for designing the engineered TALEs (Boch et al., 2009).

4.4. TALE nucleases

Using ZFN for gene editing highlighted their propensity for non-specific off-targets. Since TALEs seemed to be more specific in DNA binding, an idea of creating TALE nucleases (TALENs) soon appeared. Analogically to ZFN, TALEs were linked to the cleavage domain of FokI endonuclease. Since the FokI functions as a dimer for DSB, the whole protein was dimerized (Fig. 3). In the study of Miller and co-workers, only TALE truncation variants were used in the TALEN construction for efficient gene modification (Miller et al., 2011). Other groups used the whole TALEN protein, nonetheless there can be a lower protein stability of longer protein variants (Mussolino et al., 2011) and it soon proved advantageous to use the truncated variant.

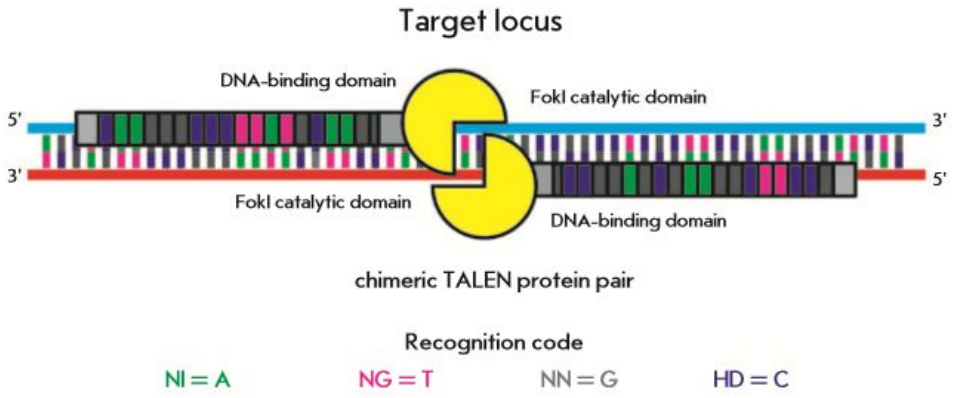

Fig. 3 **Structure of TALEN protein.** TALEN dimer causing a DSB of DNA with the recognition code of amino acid residues binding nucleotides. FokI depicted in yellow (Adapted from Nemudryi et al., 2014).

In contrast to ZFN, one TALEN RDV binds only one DNA base and adjacent RDVs do not influence each other. Therefore, TALEN monomers can be combined to create a protein binding basically any DNA sequence. Several methods for TALENs preparation have been described. For example, the Golden gate cloning method was used to generate TALENs (Cermak et al., 2011). TALENs were also constructed by fast ligation-based automatable solid-phase high-throughput (FLASH) and targeted to various human genes (Reyon et al., 2012). Another strategy was ligation-independent cloning technique (Schmid-Burgk et al., 2013).

TALENs and ZFNs were both used to target the human CCR5 gene, the co-receptor for HIV entering the T cell. Whereas ZFNs showed numerous off-targets at homologous CCR2 locus, TALENs were more precise and significantly less cytotoxic. Mutation frequencies for ZFNs were 14 % at CCR5 and 11 % at CCR2 locus. TALENs mutated 17 % of CCR5 loci and only 1 % of CCR2. Nevertheless, not an insignificant number of off-targets remains, which is still a major risk for potential gene therapy *in vivo* (Mussolino et al., 2011).

A major disadvantage of TALENs represents the big size of the genes encoding the nucleases, which makes it hard for a suitable delivery system. The size of TALEN cDNA is approximately 3 kb, whereas the size of ZFN cDNA is only 1 kb (Gupta and Musunuru, 2014).

4.5. Clustered regularly interspaced short palindromic repeats

Since 2013, the interest of the gene editing research was shifted from ZFNs and TALENs to the newly (re)discovered CRISPR/Cas9 system. Even though the sequence of CRISPR was originally found much earlier, the function of these repeats remained unknown for many years. In 1987, a group of Ishino was studying *Iap* gene in *Escherichia coli*. Analysing the structure of the gene, they found a sequence containing five directed homologous repeats of 29 nucleotides interspaced by 32 unique nucleotides called spacers (Ishino et al., 1987). Similar repeats of 21 to 37 nucleotides were later found in many diverse bacteria and *Archaea* species. To unify the nomenclature, they got their name CRISPRs.

It was discovered that prokaryotes containing CRISPRs, contain also CRISPR-associated (Cas) genes. Cas genes code proteins with helicase and nucleases activity and they are always located next to the CRISPR locus. However, the CRISPR loci were found at different locations of the genome in each microorganism, which indicated that they could exist as mobile elements and be transferred among bacteria (Jansen et al., 2002).

There are more subtypes of CRISPR repeats, but they are always identical for a particular Cas protein. Cas proteins can bind, regulate or differently interact with their DNA or expressed RNA repeats. Therefore, a hypothesis of existence of various CRISPR/Cas systems was formulated (Haft et al., 2005).

4.6. CRISPR/Cas system

CRISPR/Cas system was proven to work as a bacterial natural defence mechanism against viral infections. It was shown that spacer sequences in CRISPR loci are homologous to various bacteriophage or plasmid DNA sequences. The experiment with *Streptococcus thermophilus* revealed that after viral infection of bacterium, new spacer is derived from phage DNA and incorporated into the CRISPR locus. The immunity during the next phage infection is provided via a mechanism based on RNA interference (via crRNA – transcribed CRISPR repeats with complementary spacers) and via Cas proteins functioning as nucleases and causing DSB of the phage DNA (Barrangou et al., 2007).

Spacers in the CRISPR sequences are complementary to the phage sequences called protospacers. Moreover, the site-specific cleavage of protospacers is also determined by protospacer adjacent motif (PAM), consisting of 20 nucleotides and adjacent 5'-NGG sequence, located in the phage DNA (Jinek et al., 2012) (Fig. 4).

There are three types of CRISPR/Cas systems and they differ in the crRNA transcription and maturation mechanism. The most important is type II, which is used for genome editing. Type II systems includes several different Cas proteins and they all transcribe the CRISPR DNA into two components: precursor of crRNA (pre-crRNA) and trans-activating crRNA (tracrRNA). TracrRNA is complementary to direct repeats in pre-crRNA and it helps crRNA maturation by activating host RNase III and Cas proteins (Deltcheva et al., 2011).

As written above, there are more types of Cas proteins with various functions. However, only the Cas9 protein of CRISPR/Cas system type II, originally derived from *Streptococcus pyogenes*, showed both RNA interfering and nucleases activity. Cas9 protein sequence contains a McrA/HNH-nuclease and a RuvC/RNaseH-like nuclease motif. Each of these two nucleases cleaves one strand of dsDNA (Sapranauskas et al., 2011). Cas9 protein can thus enable the crRNA maturation and also target DNA cleavage. Therefore, Cas9 protein is the one used for genome editing via CRISPR/Cas system.

Normally, the tracrRNA interacts with crRNA and they form a duplex which is involved in the DNA cleavage via Cas9 protein. These two molecules can be fused into chimeric single-guided RNA (sgRNA) with crRNA at the 5' end and tracrRNA forming a hairpin structure at the 3' end (Fig. 4). The sgRNA cooperates with Cas9 protein and it can be easily designed to target any DNA sequence by changing only the sequence of complementary crRNA spacer (Jinek et al., 2012).

Overall, for the successful DNA cleavage and gene editing in the cell, necessary components of CRISPR/Cas9 system are: non-coding RNAs - tracrRNA (trans-activating antisense RNA providing the cleavage and thus maturation of crRNAs) and pre-crRNA as two molecules in prokaryotes, whereas for eukaryotes they are often fused into one molecule sgRNA, RNase III for maturation of crRNA (in case of prokaryotes, eukaryotic cell uses its own RNases), and the Cas9 protein (Nemudryi et al., 2014).

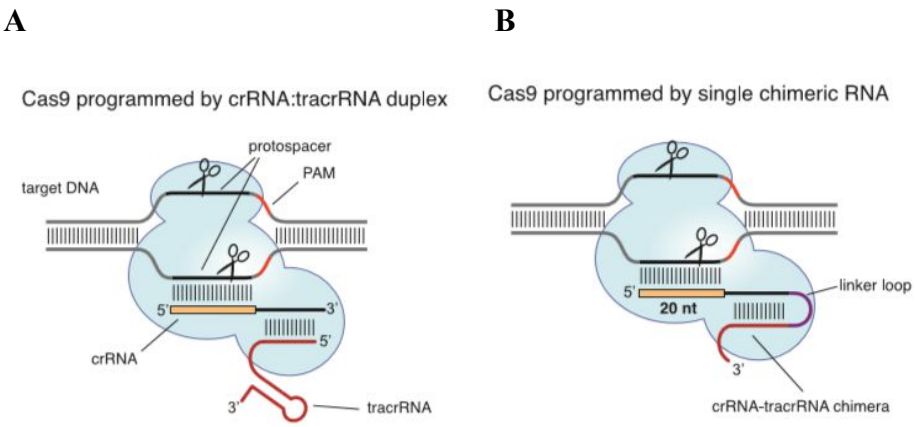

Fig. 4 **Cas9 protein cleaving dsDNA. A:** Cas9 guided via two RNA molecules: targeting crRNA, which forms duplex with activating tracrRNA. **B:** Cas9 guided by single chimeric RNA molecule consisting of tracrRNA and crRNA fused together (Adapted from Jinek et al., 2012).

The difference in DNA editing by CRISPR/Cas9 system and TALENs and ZFNs is that Cas9 protein produces blunt ends while the FokI produces 5' overhangs (Jinek et al., 2012). Nevertheless, it still makes DSB of DNA which is naturally repaired in mammalian cell (Cho et al., 2013).

An advantage of CRISPR/Cas systems is their easier design in comparison to ZFNs and TALENs. Only the targeting crRNA sequence must be changed, but there is no need of changing the protein component (Cho et al., 2013). Furthermore, only the CRISR/Cas system can encode multiplexed gene disruption. Cas9 can also be transformed into nickase, generating only single strand breaks of the dsDNA, and facilitate the homology-directed repair (Cong et al., 2013).

The high efficiency of CRISPR/Cas9 was proven in a study with mouse embryonic stem cells, in which CRISPR/Cas9 targeted two genes simultaneously. Biallelic mutations in both targets were generated in 80 % of the cells (Wang et al., 2013).

CRISPR/Cas9 were also used for the functional repair of cystic fibrosis transmembrane conductor receptor (CFTR). Intestinal stem cells were isolated from cystic fibrosis patients homozygous in Δ508 mutation of CFTR gene. Isolated cells were cultured to make organoids and then transfected with engineered CRISPR/Cas9 and donor vector encoding wild-type CFTR to repair the mutation. According to the reports suggesting that sgRNAs can tolerate mismatches in 20bp protospacer target sequence, 29 potential off-target sites were identified. However, no off-target mutations were found, proving the high specificity and efficiency of this system (Schwank et al., 2013).

The size of the Cas9 gene (4,1 kbp) is smaller than the dimerized TALEN gene (approximately 6,1 kbp) which makes it highly advantageous for gene delivery (Cho et al., 2013).

The discovery of CRISPR/Cas9 system is one of the most important successes of the molecular biology of the last decades. At the time of CRISPR/Cas discovery, more research groups were studying the CRISPR/Cas9 for utilisation in gene editing. Three articles were published in the short period of time (Jinek et al., 2012; Gasiunas et al., 2012; Mali et al., 2013).There are some disagreements about the patent rights which currently belong to Zhang (Mali et al., 2013), conversely, the authors of the two other articles (Gasiunas et al., 2012; Jinek et al., 2012) are more favoured to be awarded with the Nobel Prize. Since 2013, there have been a lot of further research works trying to improve CRISPRs and use them for genome editing *in vivo*. However, ethical concerns about editing of the human DNA do not allow the clinical application yet.

5. Delivery methods

In order to edit the cellular genome, programmed nuclease must be first delivered into the target cell and subsequently into its nucleus. It can be delivered as a functional protein – ZFNs alone, for example, can naturally penetrate through the cellular membrane due to positively charged zinc finger domains (Gaj et al., 2012). Moreover, a nuclear localization signal (NLS) can be incorporated into the ZFNs (at the N-terminus) and enable the protein

to cross the cell membrane and get to the nucleus with higher efficiency. Tandem NLS repeats usually lead to significantly improved cell-permeability and they also lower off-targets rate (Liu et al., 2015). On the other hand, most proteins are not able to cross the phospholipid bilayer without additional modifications. There is also risk of their degradation by proteases in direct protein transport and, secondly, even engineered nucleases in a form of protein would have significant immunogenicity. One should also consider that in case of CRISPR/Cas9 system, cotransport of protein and sgRNA would be necessary.

A specific nuclease, in the form of a functional protein, could be also delivered into the target cell via various virus-like particles. Cai and co-workers used modified ZFNs and TALENs fused with lentiviral Gag precursors. After their delivery, they induced efficient gene disruption of the targeted sequences with minimal off-target effect. Additionally, the authors also incorporated new genes into the viral particles to replace those originally removed (Cai et al., 2014).

The major advantage of nuclease delivery in the form of protein lies in the fact that they function during a relatively short period of time, thus minimizing the possibility of undesirable off-target effects. On the other hand, the process and form of delivery, especially via virus-like particles, brings about multiple problems related to the packaging of relatively huge protein molecules and the immunogenicity of the vector itself.

5.1. Delivery of mRNA encoding programmed nucleases

The majority of approaches use DNA and lately also mRNA encoding nucleases for their cell delivery. DNA, in contrast to mRNA and proteins, can integrate into the host genome, which is related to a risk of multiple translations of the nucleases and an increase of off-target events (Wurtele et al., 2003). There are multiple other disadvantages connected with DNA encoding nuclease delivery already summarized in Chapter 2.

Quite oppositely, nucleases delivered in the form of mRNA represent a promising tool for gene editing. As mentioned in Chapter 3, the mRNA in nanoparticle format is transported into the cells with high efficiency, in contrast to the mRNA in naked format (Phua et al., 2013). Moreover, encapsulating the mRNA into the nanoparticle provides better stability and

protection against nucleases and adds the possibility of targeting. A brief review of currently available nanoparticles suitable for mRNA delivery is provided further.

Briefly, the nanoparticles entering the cells should be positively charged, with an approximate diameter of 100 to 200 nm. The goal is to obtain nanoparticles with no cytotoxicity and no immunogenicity. They should also protect its mRNA cargo against nuclease degradation.

5.1.1. Viral vectors

TALEN genes were initially transferred via adenoviral or lentiviral vectors in the form of DNA (Holkers et al., 2013). It was shown that adenoviral vectors are able to deliver undamaged functional TALENs into the cultured human cells. Packaging into the lentiviral vectors, conversely, led to recombination and rearrangements within the multiple TALE repeat sequences (Holkers et al., 2013), and thus lentiviral vectors are most probably unsuitable for TALEN-DNA delivery. However, lentiviral vectors were used to deliver mRNA encoding TALENs (Mock et al., 2014). These vectors contained a genetically inactivated reverse transcriptase which enabled delivery of intact mRNA encoding TALENs. Therefore, modified lentiviral vectors can be considered as a tool for therapeutic application (Mock et al., 2014).

5.1.2. Lipid nanoparticles

Lipid nanoparticles (Fig. 5) have been used for nucleic acid delivery for decades. They are generally used as transfection reagents for DNA or RNA into the cultured cell lines. One of those transfection reagents is lipofectin, which is used for mRNA delivery. Lipofectin is a liposome with an incorporated synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride. Lipofectin can transfect a wide variety of cell types, however, it should be mentioned that positively charged lipids are toxic. They remain a respectable research tool for *in vitro* delivery, but on the other hand, they are not suitable for *in vivo* utilisation unless modified or mixed with other molecules (Malone et al., 1989).

Such modifications represent, for example, the lipid-enveloped pH-responsive polymer nanoparticles. Coated by a phospholipid bilayer, the cytotoxicity of the cationic core is significantly reduced. A pH-responsive poly(β-amino-ester) (PBAE) core was used to enable the endosomal escape of the nanoparticle by promoting endosome disruption. mRNA in this

system is absorbed onto the surface of the nanoparticle. While transfected into dendritic cells *in vitro*, the transfection and translation efficiency of the mRNA encoding fluorescent protein was approximately 30 %. *In vivo*, mice were intranasally injected with the same nanoparticles carrying mRNA. The successful expression of reporter genes was observed 6 hours after the injection (Su et al., 2011). This and other experiments (Islam et al., 2015) suggest that lipid or "liposome-based" nanoparticles could be successfully used for mRNA delivery. The advantage of such system is significantly reduced immunogenicity compared to virus-like particles and numerous possibilities for incorporating of specific targeting moieties.

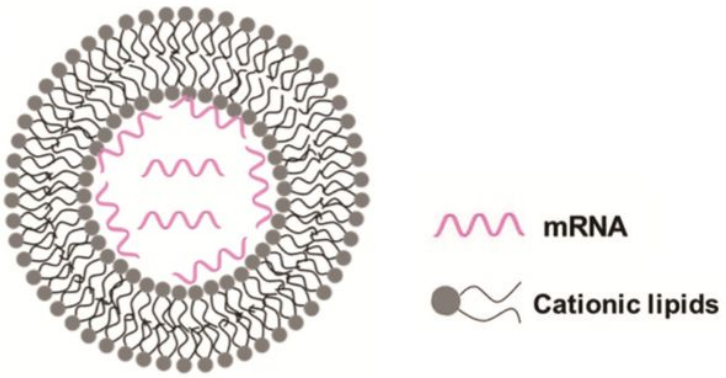

Fig. 5. **Lipid nanoparticle for mRNA delivery.** (Adapted from Islam et al., 2015).

5.1.3. Polymeric nanoparticles

Another type of nanoparticles suitable for mRNA delivery could be formed by a mixture of poly(ethylene imine) (PEI) and poly(ethylene glycol) (PEG) (Fig. 6). PEI had been widely used for plasmid DNA delivery (again as a transfection reagent) since it can promote endosomal escape via proton sponge effect. However, cationic PEI is quite toxic for the cell. Blended with PEG, the cytotoxicity of PEI is significantly reduced and the transfection efficiency is enhanced. Debus and co-workers formulated PEI-PEG copolymers with different nitrogen to phosphate ratios (N/P ratios) containing mRNA and they obtained stable nanoparticles with a diameter of approximately 200 nm, which enabled their successful cell delivery (Debus et al., 2010).

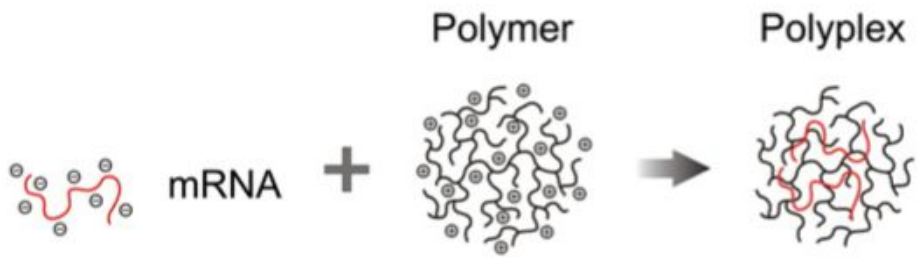

Fig. 6. **Structure of polyplex.** Cationic polymer with mRNA forming a polyplex nanoparticle. (Adapted from Debus et al., 2010).

Furthermore, poly(ε-caprolactone) (PCL) nanoparticles with mRNA-protamine hydrophilic core were generated as a new promising mRNA delivery method representing another possibility for mRNA therapeutic strategies. Cationic protamine binds mRNA and stabilizes the complex since mRNA with PCL shows negative zeta potential. The protamine also offers controlled pH-dependent release of nanoparticle cargo (mRNA). The nanoparticle is stable at pH 7.4, whereas it releases the mRNA at pH 5.0, which allows the endosomal escape. Moreover, PCL provides high colloidal stability, facile transport via endocytosis and low cytotoxicity of the nanoparticle (Palama et al., 2015).

5.1.4. Messenger RNA nanoparticles

Another alternative for mRNA delivery was recently presented as self-assembled mRNA-nanoparticles (Kim et al., 2015). In that study, plasmid DNA was transcribed by rolling circle transcription (RCT) into long RNA strands encoding green fluorescent protein. The template plasmid DNA used for RCT contained the T7 polymerase promoter region, the eukaryotic ribosomal binding sequence and the coding sequence enclosed by start and stop codons. After approximately 20 hours, the mRNA strands from RCT entangled and self-assembled (Fig. 7) into positively-charged mRNA-nanoparticles of spherical shape with diameter of 100 to 200 nanometres.

The main advantage of such mRNA-nanoparticles is no risk of cytotoxicity as they are made only from mRNA strands. Moreover, the study showed that mRNA packed

in mRNA-nanoparticles is protected from nucleases, and that after being introduced into the cell, mRNA-nanoparticles slowly undergo translation (Kim et al., 2015). Thus, mRNA-nanoparticles can be considered as a possible method for clinical therapy.

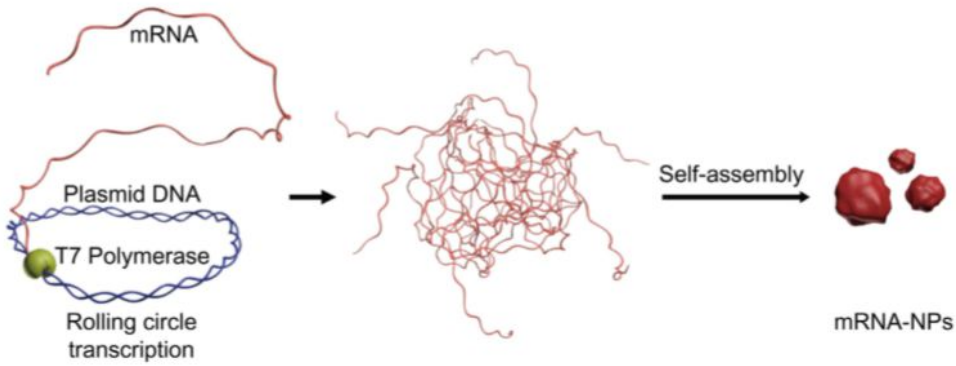

Fig. 7. **Synthesis of mRNA-nanoparticles.** Rolling circle transcription generating mRNA strands which are further self-assembled into nanoparticles (Reprinted from Kim et al., 2015).

5.1.5. Gold nanoparticles

Gold nanoparticles have been studied mainly for DNA and siRNA delivery. Nevertheless, they were proven to deliver mRNA as well (Yeom et al., 2013). The nanoparticles are formed by a gold core with conjugated DNA oligonucleotides, which are used to anchor mRNA (Fig. 8).

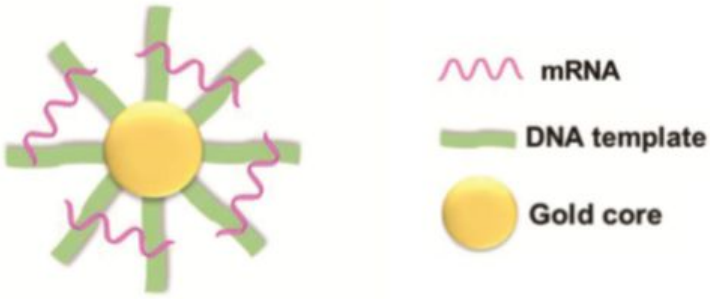

Fig. 8. **Gold nanoparticle-DNA oligonucleotide conjugates.** Gold core with conjugated DNA strands holding mRNA strands for mRNA transfection (Adapted from Islam et al., 2015).

Such gold nanoparticles were used to transport the *BAX* mRNA into human cells *in vitro* and into xenograft tumors generated in mice. The *BAX* protein is a pro-apoptotic factor. Encoded by mRNA, it was successfully translated into a functional protein in the cells and inhibited the tumor growth. However, more information about the delivery mechanism, cytotoxicity and clinical viability is needed (Yeom et al., 2013).

6. Conclusion

The recently discovered system of CRISPR/Cas9 programmed nucleases seems as the most suitable one for genome editing. Together with TALENs, they present the most widely used nucleases in *in vitro* gene editing. Although many improvements of these nucleases were made to increase their precision, their off-target effects are still considered dangerous for *in vivo* studies and further research is needed to obtain full knowledge about their function, in order to eliminate all hazards.

Nucleases in the form of proteins or encoding DNA or mRNA have been already delivered into the cells in many preclinical studies. Delivery of a large nuclease protein is very challenging. DNA delivery must contain transport into the nucleus and it is associated with risks of multiple translation of the protein and multiple deletions within the genome increasing thus the probability of off-targets. Hence, mRNA encoding nucleases might represent an appropriate choice.

The relative instability of mRNA can be partially solved when delivered in nanoparticle. Packed in the nanoparticle, mRNA is protected against nucleases, which presents one advantage over the naked format delivery. The major advantage of the nanoparticle format is its potential of specific targeting. There are diverse types of nanoparticles enabling mRNA delivery. The main requirement is no cytotoxicity and immunogenicity. The function of nanoparticles, besides the prevention of mRNA degradation and the possibility of precise targeting, is also activating the cellular uptake and promoting the endosomal escape of the mRNA cargo.

As already mentioned, the possibility of targeting of the nanoparticles into specific cell types by their surface modification, is their important aspect. Specific targeting moiety (antibody, small ligand,…), which is recognized by cellular receptor, can be incorporated in the nanoparticle. Such nanoparticles containing programmed nucleases and targeting moieties could significantly lower the possible off-targets of the nuclease by delivering them only into the “damaged” cells. However, such approaches still remain in the proof-of-the-concept state.

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