

## REVIEW ARTICLE

# KEY ACHIEVEMENTS IN GENE THERAPY DEVELOPMENT AND ITS PROMISING PROGRESS WITH GENE EDITING TOOLS (ZFN, TALEN, CRISPR/CAS9)

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**Abstract:** Gene therapy concept is based on introduction of the wild-type allele into a patient's genome in order to reverse a specific mutation. It is designed to treat hereditary diseases as well as the other diseases occurring later in life. Gene therapy was first mentioned in the 1960s and 70s, whereupon a series of studies was carried out, and in 1990 the first successful gene therapy was conducted. Since then about 2 600 clinical trials based on this concept were completed or are in progress. The two biggest issues are introduction of an exogenous DNA to target tissue, and its controlled integration in the genome. Until recently, the exogenous DNA sequences were incorporated randomly in the patient's genome. Even though most of these treatments gave positive results, there was always a possibility of insertional mutagenesis. Controlling the integration place has rapidly progressed with the development of gene editing tools: ZFN, TALEN and CRISPR/Cas9. Although they have been used in only several clinical studies, gene editing tools are a small step away from clinical usage. In this review, we will give historical overview of gene therapy development and describe recent tools that can be used in precision medicine.

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## HISTORICAL REVIEW

### *Fundamental discoveries*

In a paper from 1947, the term "Gene therapy" was first mentioned by Clyde E. Keeler.<sup>1</sup> At the time, treatment of genetic disorders using the methods of traditional medicine resulted only in alleviating the symptoms but without curing the cause of the disease. Keeler noted that causes of genetic diseases in offspring were determined by genetic material in germ line cells of parents. Because of mechanisms of inheritance in human (e.g. crossing over), replacement of defective gene with the functional one can result in correction of defective hereditary characteristics. He concluded that this principle could solve a number of "genetic problems". In 1952 two important discoveries contributed to gene therapy development: Lederberg and Zinder described recombination between two bacteria where genetical material from one bacterium to another was carried by bacteriophage (transduction), and Hershey and Chase conducted experiment which proved that DNA is a carrier molecule of hereditary characteristics.<sup>2, 3</sup> In 1962 the first hereditary gene transfer was described by Elizabeth Hunter Szybalska and Waclaw Szybalski. They used the total genomic DNA with a functional copy of the HPRT1 gene (hypoxanthine phosphoribosyltransferase), to transform *HPRT1*-deficient D98S bone marrow cell line. That was the first success of human cells transformation *in vitro*.<sup>4</sup> Basically, they showed that newly acquired hereditary feature, "incorporated feature", is transferred to the next generation of cells and gene aberration is nullified.

### ***Beginnings of gene therapy***

In 1961, while studying chicken cells infected by Rous sarcoma virus (RSV), Howard Temin discovered that mutation caused by virus is stabilized in host cells and transferred to next cell generation. Given that RSV is an RNA virus, this was evidence that information stored in RNA genome can be incorporated into DNA.<sup>5</sup> It was shown that viruses might serve as a tool for insertion of a new gene in any cell, which was an interesting topic discussed by Edward Tatum shortly after.<sup>6</sup> He suggested that if process of gene transfer between two bacteria by virus can be applied to animal cells, introduction of a functional gene into animal host genome to replace a defective gene of interest can be achieved. For such approach it was required to design a non-pathogenic viral vector. Although there was no mention of “gene therapy”, this paper brought description of its basic principles. In order to successfully edit patient’s genome, it would be necessary to propagate patient’s cells in culture, transfect them with functional gene and re-introduce cells with wild-type gene back to the relevant tissue of the patient. Key point that later allowed conduction of such approach was usage of restriction enzymes, discovery for which Nobel prize was awarded to Arber, Nathans, and Smith in 1978.<sup>7</sup>

The first attempt to treat a disease by introducing a foreign gene into the human genome was made in 1970. Patients were two children with rare hereditary disease, hyperargininemia, caused by deficiency of arginase. In this disorder, because of accumulation of arginine in blood and liquor mental retardation occurs. Selected method of treatment was based on the fact that a rabbit infected by Shope papilloma virus (SPV) produces increased amounts of arginase. Moreover, in 35% of laboratory workers exposed to that virus a decrease in arginine concentration can be seen.<sup>8</sup> SPV was directly injected into the bloodstream of patients. There was no improvement in patients’ condition and it was debated if SPV has an arginase coding gene or if it just stimulates the arginase production in healthy individuals from the endogenous gene.<sup>8</sup> Later was elucidated that the infection caused higher production of host arginase. Therefore, patients who lack functional arginase gene cannot be treated using this approach. Although this “gene therapy” attempt didn’t have harmful effects on patients, it became evident that safer and more elaborate treatment methods should be designed prior to any further clinical application.

In 1970 two independent groups discovered reverse transcriptase<sup>9, 10</sup> - enzyme that transcribes RNA into complementary DNA coded by retroviruses to allow viral genome integration into the host genome can be used *in vitro* in order to transcribe any mRNA in the complementary DNA that can be then ligated into the virus vector and then after entering the cell, integrated into the host genome. This feature has become a key element in the technical implementation of gene therapy.

In the same year, Victor McKusick Almond published comprehensive list of 92 genetic disorders in humans with exact enzymes whose deficiency caused a particular disease.<sup>11</sup> Two years later, Theodore Friedmann and Richard Roblin discussed gene therapy as a desirable technique for treating hereditary diseases.<sup>8</sup> Over 1 500 different genetic diseases were known at that time, with new ones being discovered frequently. The exact mechanisms underlying these diseases were still unknown, but associations of genetic defects with different diseases become more and more evident. Such diseases were commonly treated with adjusted diets e.g., diets with low-phenylalanine alleviate mental retardation in case of phenylketonuria. Second treatment option was usage of drugs which block or reduce the accumulation of potentially harmful metabolites, but at the time they were available for only a few genetic disorders such as cystinuria and Lesch-Nyhan syndrome. Third possible treatment was a direct introduction of a wild-type enzyme intended for enzymatic hereditary diseases. All these possibilities gave scarce results and gene therapy looked like a promising new option.

In the paper from 1972, Friedmann and Roblin also discussed technical barriers, and proposed ethical standards that should be taken into consideration before applying the gene therapy in humans. They pointed out that because many human genes are expressed at a low level, and only in certain cell types, strategy for bringing exogenous DNA specifically to those cells should first be developed. Additionally, they emphasized a higher potential of cells whose genome was edited by *in vitro* treatment to develop malignant properties. Conclusion was that gene therapy should never put individual’s life at risk, which is why it is necessary to first understand biochemical properties of the process intended to be affected, to consider differences in the diseases with a different genetic background, and to only apply a well-characterized and safe vector. For the same reasons, all new methods should be first tested on animals.<sup>8</sup> Based on their recommendations, genome editing was planned to be used in clinic.

### ***Beginnings of clinical applications***

The first clinical implementation of gene therapy was done by Martin Cline. His approach was based on experiments showing effects of foreign genes for dihydrofolate reductase (DHFR) and thymidine kinase (TK) incorporated in mouse bone marrow cells.<sup>12, 13</sup> In 1980, without any approval from the institution, he treated two patients who were diagnosed with  $\beta$ -thalassemia using this approach.  $\beta$ -thalassemia is caused by mutations in the human  $\beta$ -globin gene (*HBB*). Patients’ bone marrow cells were harvested and treated with the plasmid DNA which carried an integrated gene for  $\beta$ -globin. Modified cells were then returned to patients’ bone marrows.<sup>14</sup> In the meantime,

his institution (The University of California, Los Angeles) declined the approval based on the fact that the same treatment has not previously been tested on animal models. Cline's work was declared unethical, and any similar treatment was forbidden.<sup>15</sup> The treatment itself showed no results - introduced gene was not expressed. Nevertheless, Martin Cline is still regarded as the first scientist who used recombinant DNA in clinical treatment.

At the beginning of 1989 National Institutes of Health gave official approval for the entry of a foreign gene into the human body to S. A. Rosenberg<sup>16</sup> with an aim of improving melanoma treatment. In previous studies, it has been shown that treatment of metastatic melanoma with interleukin-2 simultaneously with *in vitro* expanded tumor infiltrating lymphocytes (TILs), in some patients reduced the progression of the disease.<sup>17</sup> Therefore, it was necessary to define a connection between TILs activity and regression of malignant disease. The method for this procedure was based on a marker that can help monitor distribution of TILs in patient's tissues. TILs were isolated from the melanoma patients, modified by Moloney murine leukemia retrovirus with an incorporated marker gene, and injected back into the patients. It was shown that these cells can survive for months in the patient's body, and that retroviral vectors were a safe and simple method for introducing foreign genes into human genome.<sup>16</sup>

In September 1990, Food and Drug Administration (FDA) approved first gene therapy that ended with success. Patients were two girls diagnosed with severe combined immunodeficiency (SCID). SCID is a rare autosomal recessive disease caused by B- and T-lymphocytes developmental failure.<sup>18</sup> It is caused by a mutation in a single gene, but different types of the same disease develop due to mutations in different genes. Selected cases were caused by mutations in gene for the adenosine deaminase (ADA).<sup>19</sup> Patient 1 was Ashanti DeSilva. She was diagnosed with ADA deficiency when she was 26 months old. Treatment was polyethylene glycol-modified adenosine deaminase (PEG-ADA) as a replacement for the missing enzyme. Two years later, at the age of 4, the treatment resulted in the normal count of peripheral blood T cells which responded to mitogens *in vitro*. However, other signs of immunodeficiency were still present and Ashanti's T-cells lacked normal activity. Patient 2 was Cindy Kisik, at the age of 9. When she was three years old, she had first hazardous infection and at the age 5 she developed septic arthritis after which a milder form of SCID was diagnosed. She was, like Ashanti, given PEG-ADA treatment which at first led to increase of peripheral T-cells number, but their number again decreased in the third and fourth year of treatment. Both patients were supposed to receive gene therapy in parallel with application of PEG-ADA.<sup>19</sup> The procedure started with peripheral T-lymphocytes isolation from patients' blood that were afterwards, in cell culture, infected by retroviral vector which

contained the human ADA cDNA. Between 9<sup>th</sup> and 12<sup>th</sup> day, the cell population increased 135-fold and transformed T-cells were reinjected into patients' bodies. Ashanti received 11 transformed cell infusions in 2 years, and Cindy received 12 infusions in approximately 18 months. Ashanti had positive tests for T-cell activity 9 months after the beginning of the trial, and Cindy 17 months after the beginning of the trial. Patients' immune function improved and therapeutic doses of PEG-ADA were reduced. The treatment was completed in 1992. Four years later, normal T-cells count was still measured in both patients and the ADA gene expression in T cells continued. All other symptoms of the disease were also undetected.<sup>19</sup> The patients' immune system grew stronger and it was more functional than it was during treatment with only PEG-ADA. After 23 years, the two cured patients participated at the Immune Deficiency Foundation National Conference as two healthy grown women.

In the next few years after the SCID-ADA gene therapy success, development of gene therapy was in progress for various genetic diseases. However, in 1999 at the age of 18 Jesse Gelsinger died after gene therapy treatment for ornithine transcarbamylase (OTC) deficiency.<sup>20</sup> This metabolic disorder causes difficulty in removing ammonia from the body. Children born with it usually die 72 hours after birth. Jesse had a partial OTC deficiency, which was treated with available drugs and special protein reduced diet. Although gene therapy was not necessary, Institute for Human Gene Therapy at the University of Pennsylvania considered Jesse as a suitable candidate for clinical research that would help in the future treatment of other patients with the same disease. Based on patient's consent, recombinant adenoviruses with a functional OTC gene were injected into his hepatic artery. He had an almost immediate immune response to the vector and died four days after receiving the treatment. At that time, approximately 400 gene therapy clinical trials were approved worldwide and Jesse's death was the first and the only one reported.<sup>20</sup>

Afterwards, most research studies continued, but all preclinical studies had to include detailed and comprehensive aspect for each newly developed approach.

### ***The development of commercially available drugs***

In 2003 Zhaohui Peng and his team from the company Shenthen developed an adenoviral vector Ad-p53 for treatment of head and neck squamous cell carcinoma. The study was approved by the State Food and Drug Administration (SFDA) of China and the drug appeared on Chinese market under the name Genticine. It was the first commercially available adenovirus vector intended for gene therapy treatment.<sup>21</sup> Genticine is based on non-replicative and

non-pathogenic virus with inserted sequence for the human *TP53* gene. This gene is the best-known tumor suppressor gene that controls cell division and DNA repair. It codes for a transcription factor of 53 kDa that binds to various gene promoters, and through several steps, stimulates cell apoptosis. In 50% of cancer patients, p53 is mutated, while in a healthy individual this gene is in an inactive form and it is only activated when there is a triggered stress-response. Before Gendicine become available on the market, it was tested in phase I and phase II/III trials. In the first phase, 12 patients with advanced laryngeal carcinoma were treated. Drug was delivered by intratumoral injections, then tumor tissue was surgically removed, and drug was re-injecting in the prior tumor location. The only side effect was fever. All patients were successfully cured after 10 injections.<sup>22</sup> The second and third phase consisted of 135 patients with squamous cell carcinoma of head and neck. Drug was injected directly to carcinoma tissue in combination with radiotherapy. It was shown that gene-therapy based drug in combination with radiotherapy provided faster and more efficient results than treatment with only radiotherapy.<sup>23</sup> After Gendicine become commercially available in 2004, ethical considerations begun to arise because of the less rigorous procedure in processes for drug approval in China, in contrast to those in the USA.<sup>22</sup> In 2005, Chinese SFDA approved another product: Oncorine (H101). Unlike the previous drug, this one was based on oncolytic therapy with a replicative adenovirus designed for treatment of late-stage nasopharyngeal cancer. Oncolytic viruses are therapeutic viruses with ability to directly infect and destroy specifically cancer cells without damaging non-tumor cells. Considering that mutations in the tumor suppressor gene p53 are some of the most common mutations present in malignant cells, this drug was designed as modified adenovirus which carries mutation in its E2B 55kD gene and causes this virus to preferentially destroy only those cells with mutated p53. This drug is still available only on the Chinese market.<sup>24</sup>

Another p53-based gene therapy treatment was designed for patients with Li-Fraumeni syndrome under the name Advexin. Advexin is based on adenoviral vector that carries functional p53 gene. When delivered to the cell that has abnormal p53 gene it is supposed to produce p53 tumor suppressor while not integrating itself into the host's genome and enabling only transient expression of p53 gene. In this way, Advexin could serve as a replacement protein carrier that will help trigger protective mechanisms in a tumor cells and drive them to death. Gendux Molecular Limited applied to EMA for its approval in 2006. The company provided documents showing promising results in experimental models and based on one patient's experience. In December 2008, Gendux withdrew its application based on, as stated in their letter to EMA, "the company's marketing strategy".<sup>25</sup> In September 2008 FDA issued Refuse to File Letter

for the application of Advexin from Introgen Therapeutics, Inc., in this case intended for the treatment of head and neck cancer.<sup>26</sup>

Few years later, in the western world the first drug for gene therapy was approved under the name Glybera. Glybera is an adeno-associated virus (AVV) vector which instead of virus pathogenic genes has an inserted lipoprotein lipase gene (LPL), intended for treating the lipoprotein lipase deficiency. This deficiency is a very rare hereditary disease which leads to an increase of fat in the blood. In clinical studies including 27 patients, it was shown that this drug leads to a reduction in blood fat concentration. Committee on Human Medicinal Products (CHMP) gave the final recommendation in 2012 and the drug was released to the European market.<sup>27,28</sup>

On the European and USA markets, an increase in the number of approved gene therapy medicines has been recorded since 2016. In June 2016 European Medicines Agency (EMA) approved Strimvelis for the treatment of ADA deficiency. The treatment includes *ex vivo* modification of autologous hematopoietic stem cells with drug composed of a functional ADA gene packed into gamma retroviral vectors.<sup>29</sup> Soon after, in August and October of 2017, the FDA approved two drugs for chimeric antigen receptor T cells (CAR-T) based therapy of blood cancer in the USA: Kymriah<sup>30</sup> and Yescarta.<sup>31</sup> Their usage is based on *in vitro* modification of autologous T cells designed to express chimeric antigen receptor (CAR) to target and kill cancer B cells. The only difference between these two drugs is the type of vector which is used; lentiviral vector as a part of Kymriah drug and  $\gamma$ -retroviral vector as a part of Yescarta.<sup>32</sup>

The last gene therapy drug in the western world was approved in December 2017 by FDA under the name Luxturna. It is intended for treatment of retinal dystrophy which occurs as a consequence of biallelic *RPE65* mutation. The treatment is performed by subretinal injections of adeno-associated virus which carry a functional copy of human *RPE65* gene to retinal cells.<sup>33</sup>

Although gene therapy for various diseases became a reality, precise insertion of the introduced gene copy is still one of the challenges. Recent development of genome editing tools might improve this obstacle.

### Genome editing tools

The idea of a precise, highly specific genetic editing tool that could introduce a targeted change in DNA sequence was encouraged by the fact that double stranded break (DSB) in the genomic DNA could be repaired through the process of non-homologous end joining (NHEJ) or homology-directed repair (HDR). Both pathways could be used in gene editing. NHEJ is the main and faster way of DSB repair because it is based on direct religation of cleaved ends of DNA molecule, but it can yield unpredictable mutations such

as deletions, insertions or substitutions. For these reasons it is mainly used in order to knock-out the activity of a target genomic region.<sup>34</sup> HDR is a DSB repair based on the sister chromatid template, or another homologous sequence present in the cell, such as a DNA sequence introduced for therapeutic purposes.

Based on these two naturally occurring mechanisms of DSB repair, genome editing tools were developed in order to specifically modify target genomic regions. Today, three gene editing tools with different levels of specificity and different approaches for eukaryotic cells are developed: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9).

Zinc-finger nucleases (ZFNs) are the first tool designed for targeted genome engineering back in 1996. It consists of an artificial protein that contains one of the most common DNA binding protein domains in eukaryotes, the zinc-finger protein (ZFP) and FokI endonuclease.<sup>35</sup> ZFP is composed of tandem Cys2-His2 binding domains which recognize 3-bp DNA segment.<sup>36</sup> It is usually designed in a way that it contains 3 to 6 zinc finger-like protein domains in order to specifically bind to a unique genomic sequence.<sup>37</sup> Two ZFN monomers are required for the recognition of the target sequence. Each of them recognizes the adjacent sequences in the antiparallel DNA chains separated by a spacer sequence of length between 5 and 7 bp. Spacer sequence provides a place for dimerization of FokI which results in its activity and double-stranded DNA cleavage.<sup>38</sup> ZFNs are the smallest type of genome editing tools, they contain 30 amino acids to identify one base-pair triplet. Nowadays, commercial ZFNs kits can target on average every 50 bp in a random genome sequence.<sup>39</sup> This allows good coverage for gene knock-out by inducing small indels that might result in a frameshift mutation, but genome is not covered well enough for very precise site-specific modifications.

In 2010 another class of sequence-specific nucleases was created by fusion of the transcription activator-like effectors (TALEs) and FokI endonuclease.<sup>40</sup> TALEs were originally identified as a product of plant pathogen bacteria in genus *Xanthomonas*. Their role is to bind sequences in genome of the infected plant cell and cause changes in plant gene expression with the aim of spreading bacterial infection.<sup>41</sup> TALEs recognize specific DNA sequences with tandem repeats of DNA-binding domains composed of 33-35 amino acids. Amino acids at positions 12 and 13 of each domain, called repeat variable diresidues (RVDs), are recognizing one specific base pair.<sup>37</sup> Such domains can be newly engineered, so specifically designed TALEs can recognize any sequence.<sup>40</sup> As endonuclease FokI is active only in a form of a dimer, it is necessary to design two TALEN complexes (similarly to ZFN complexes). Each one should position its FokI

endonuclease domain at an appropriate distance to the other one so that dimer can be formed and DSB produced.<sup>42</sup> If the two pairs of opposite TALENs (or ZFNs) binding complexes are designed in a way that each pair makes one DSB, specific sequence can be removed from the genome by NHEJ repair mechanism. Another option is that, due to HDR, specific sequence can be replaced by the exogenous DNA through the process of homologous recombination.<sup>42</sup> Compared to ZFNs, TALENs are simpler to use since there is a TALEN library which can target all human genes,<sup>43</sup> and they have lower cytotoxicity and lower off-target effect.<sup>44</sup> General disadvantages of TALENs are their size - it requires 33-35 amino acids to identify one base pair, as well as their repetitive sequence which makes packaging and introducing it to the target cells difficult.<sup>45</sup>

Beginning of discovery of the latest genome editing tool started in 1987. Yoshizumi Ishinomiya's group conducted a research on *E. coli* and reported an unusual repetitive sequence downstream of the *IAP* gene. This sequence consisted of five highly homologous 29 nucleotides long direct repeats separated by non-repetitive 32 nucleotides, so called spacer sequences.<sup>46</sup> Similar repetitive DNA sequences were later found in many bacterial and archival species, but not in eukaryotes and viruses. Therefore, these sequences were recognized as parts of a gene family specific for prokaryotes.<sup>47</sup> Because of their characteristic structure they were named the clustered regularly interspaced short palindromic repeats (CRISPR).<sup>48</sup> Shortly afterwards, CRISPR-associated (Cas) protein-coding genes were identified adjacent to CRISPR loci in bacterial genomes,<sup>48</sup> but function of the CRISPR family sequences was unknown until 2007 when Barrangou and colleagues showed that *Streptococcus thermophilus* can acquire immunity to phages by incorporating fragments of their genomic DNA into its own genome. *Streptococcus pyogenes* has four Cas genes (Cas9, Time1, Time2, and CSN2), genes coding crRNA (CRISPR targeting RNA) and tracrRNA (transactivating crRNA), as well as six different 30-nt long spacer sequences derived from viruses and plasmids, flanked at each side by a 36-nt long repeats.<sup>49</sup> In other prokaryotes, spacer sequence length may vary from 21 to 72 nt with the most common length between 32 to 38 nt.<sup>50</sup> During infection of the *S. pyogenes* adaptation happens - a viral or plasmid DNA fragment integrates into a bacterial CRISPR locus, which involves all four Cas genes products.<sup>51</sup> Then, transcription of the locus leads to the assembly of an active DNA endonuclease complex consisting of three parts: (i) crRNA molecule partly complementary to the target DNA sequence, (ii) tracrRNA involved in the maturation of the crRNA, and (iii) Cas9 endonuclease which recognize 3-nt long protospacer adjacent motif (PAM sequence) juxtaposed to crRNA target DNA sequence. After the crRNA and Cas9 recognize the appropriate fragments of foreign DNA, Cas9 with its two nuclease domains; RuvC-like and HNH-like causes a DSB in foreign DNA and

makes bacteria resistant to attackers.<sup>52</sup>

The potential of bacterial immunity was soon recognized in the Doudna and Charpentier laboratory as a source for developing the third genome editing tool. According to the above-described mechanism, in 2012 they developed CRISPR-Cas9 system with only two main components; Cas9 endonuclease and chimeric guide RNA (gRNA) which replaces crRNA and tracrRNA. By providing a synthetic gRNA, this system can recognize any sequence followed by PAM in eukaryotic genome and thus lead to its double-stranded cleavage and modification.<sup>53</sup> Unfortunately, it has been shown that in human cells, Cas9 can sometimes cleave DNA even when there is a mismatch between DNA and gRNA, or when there is a mismatch in PAM sequence. That is why along with target DSB, additional DSBs might be generated. When they are repaired by NHEJ mechanism, indel mutations emerge in different genomic sites. In order to choose optimal gRNA and to predict nonspecific binding, *in silico* tools were designed.<sup>54</sup> Despite that, the off-target activity still exists. Therefore, to avoid it, two alternative variants of original CRISPR-Cas9 system were developed; (i) Cas9 nickase and (ii) Cas9-FokI nuclease. In both cases, position of DSB is determined by the pair of monomeric variants where each gRNA recognizes a particular adjacent sequence around targeted site, effectively making the target recognition sequence doubled. Cas9 nickase is a modified variant of Cas9 nuclease that cleaves only one strand in dsDNA. Adjacent sequences in antiparallel chains are recognized and nicked by a pair of colocalized Cas9 nickase - gRNA complexes, then two formed nicks result with a DSB. The main advantage of paired nicking strategy is reduced off-target mutations because non-specific nicks are repaired with much higher fidelity than DSBs.<sup>55</sup> Unlike Cas9 nickase, Cas9-FokI nuclease needs dimerization for its activity. Two different gRNAs lead Cas9-FokI monomers at the target location and appropriate distance at which FokI nuclease dimerizes and forms DSB. This Cas9 variant proved to be an improvement since it induced lower frequency of indel mutations.<sup>56</sup> Targeting of the CRISPR-Cas9 complex to any sequence in the genome of a cell only requires a simple design and synthesis of a new guide RNA, as opposed to the labor-intensive re-design of protein-based ZFNs and TALENs. It has already been mentioned that all of these genome editing tools cause off-target effects. Despite off-target effects, CRISPR-Cas9 system proved to be the most effective and the best choice for multiple editing of genome in different cell types and organisms.<sup>57</sup>

### **Clinical use of new tools**

Described genome editing tools can be used in *in vivo* and *ex vivo* gene therapy and there are several ways to introduce them into the target cells. In *ex vivo* gene editing, cells are modified outside of the patient's body

and then reinfused back. Usually, DNA or mRNA coding for ZFN, TALEN, CRISPR/Cas9 with gRNA can be delivered to target cells by electroporation, lentiviral vectors or simply by direct introduction of protein complexes.<sup>58</sup> In contrast to *ex vivo*, the *in vivo* gene editing methods are more complex as they include process of bringing these tools to a specific type of cells in the specific tissue of the body. The most studied vectors so far for *in vivo* gene therapy are adeno-associated virus vectors (AAV). These are viruses that instead of their own pathogenic genes carry a coding sequence for genome editing tools and therapeutic genes. The capacity of AAV is approximately 4.8 kb of DNA, whereas the synthesis of only one TALEN monomer requires cDNA larger than 4 kb, and the synthesis of Cas9 originating from *S. pyogenes* requires 4.2 kb cDNA, while ZFN requires only about 1 kb. Therefore, the new goal is to develop a method in which just one vector would code the entire product needed for a successful gene therapy.<sup>58</sup> Additionally, all genome editing tools, ZFNs, TALENs and CRISPR/Cas9 systems show certain level of off-target action. Therefore, the screening of unwanted mutations and further optimization of these tools should improve gene editing specificity.

ZFN was the first of described tools that was used in the clinical trial. The study was conducted between May 2009 and July 2012 with the goal of curing AIDS by creating an immune system resistant to HIV infection. T-cells co-receptor CCR5 is often the one responsible for the entry of HIV into the cell. Homozygotes with a 32-bp deletion in the CCR5 gene are resistant to HIV infection and in heterozygotes progression of the disease is slower. According to these data, in the clinical study conducted on 12 patients, CD4 T cells were cultured and exposed to a pair of CCR5-specific ZFNs. DSB formed within the CCR5 gene region were repaired by NHEJ repair which led to aberrant truncated gene and non-functional protein presented on the cell surface. Study followed the safety and tolerability criteria for a single dose modified autologous CD4 T-cells. In all patients improvement was reported.<sup>59</sup>

Furthermore, ZFNs are the first genome editing tool that has been used in *in vivo* human genome editing trials. At the 56 Annual Symposium of the Society for the Study of Inborn Errors of Metabolism (SSIEM) held in September 2018, the update from a Phase 1/2 clinical trial intended for curing the mucopolysaccharidosis II (MPSII or Hunter Syndrome) was presented. MPSII occurs as a consequence of mutation in the gene for the enzyme iduronate-2-sulphate (IDS) responsible for breakdown of some complex sugars. SB-913 drug which targets the liver cells was constructed. It is composed of two ZFNs-nucleases and one functional donor *IDS* gene packaged in AAV. Sixteen weeks after the treatment onset, in two out of four patients who received a higher dose of medication, reduction in the amount of urinary glycosaminoglycan associated with this disease was

reported. Although expected, there was no increase in the amount of IDS enzyme in plasma. Currently, two more clinical trials are being carried out by *in vivo* ZFN-mediated gene therapy: treatment for mucopolysaccharidosis I (MPSI) and hemophilia B.<sup>60-63</sup> TALEN was applied in clinical treatment for the first time in June 2015. An 11-month-old infant girl in the late stage of acute lymphoblastic leukemia with a barely functioning immune system and with insufficient T-lymphocytes for personalized therapy, intravenously received TALEN-modified T cells of a healthy donor named UCART19 cells. Donor T-cells were modified with inactivation of two genes: (i) T cell receptor gene (so that patient's immune system cannot develop response to foreign cells), and (ii) CD52 gene - targeted molecule for therapy with Campath that the patient was receiving. Campath contains of mononuclear antibodies which bind a CD52 antigen on T cells, which allow the immune system to recognize and destroy labelled cancer cells. This way, functional donor T-cells became invisible to the therapy. Three months later patient received bone marrow transplantation and significant recovery was noted. In a report made 18 months after therapy, the patient showed no signs of illness.<sup>64, 65</sup> In December 2015 a similar form of treatment was applied to another 16-month-old infant girl with a diagnosis of acute lymphoblastic leukemia. Twelve months later, the patient showed no signs of disease.<sup>65</sup>

The first official clinical trial using CRISPR-Cas9 technology, will be the one led by a partnership between USA company Vertex Pharmaceuticals and European company CRISPR Therapeutics. They developed the CTX001 *ex vivo* autologous therapy intended for treating one of the most common monogenic diseases,  $\beta$ -thalassemia. It has been shown that the presence of fetal hemoglobin in patients with  $\beta$ -thalassemia can replace the function of adult one.<sup>66</sup> Therefore, this therapy is not focused on replacing the mutated form of *HBB* gene with a functional one, but it is directed to encourage re-production of fetal hemoglobin. CTX001 therapy with CRISPR-Cas9 technology will stimulate re-production of fetal hemoglobin by mutating the coding sequence of *BCL11A* transcription factor, a negative regulator of fetal hemoglobin expression. Hematopoietic progenitor cells will be harvested and modified *ex vivo* by this technology and then reinfused to the patient's body. It is expected that FDA will soon give final approval for the start of Phase1/2 of this clinical trial.<sup>67, 68</sup>

New studies in the field of developing innovative techniques and therapies for other disorders using gene editing will surely continue. Besides that, these tools have also a promising potential in precise epigenetic manipulations. By removing their nuclease activity and fusing them with proteins such as transcription factors and enzymes for epigenetic remodeling, they can be directed to any genomic site to change its epigenetic status. We presume that new tools like CRISPR-Cas9 system for targeted DNA methylation.<sup>69</sup>

demethylation<sup>70</sup> and targeted histone modification<sup>71</sup> as well as other epigenome editing tools which are using modified ZFNs<sup>72</sup> and TALENs<sup>73, 74</sup> will soon emerge and more preclinical trials will follow.

Despite many benefits these tools bring to the improvement of gene therapy, this treatment is still not widely accepted.

## CONCLUSION

Taken together, some of the main obstacles to a successful gene therapy are: bringing therapeutic genes into targeted cells without disturbing regulatory and transcriptionally active regions in the cell genome; development of adequately large and non-immunogenic vectors; and high cost of treatment. Nevertheless, gene therapy is a revolutionary method directed to the treatment of genetic diseases at the very cause of the disease itself. It allows the treatment of various inherited diseases as well as diseases acquired during life. New developments in the field of genome editing tools may soon be a starting point for cure of all diseases caused by a change in gene function, not only on genetic but as well as on epigenetic level. This technological advancement must be accompanied by solving issues that genome editing brings: delivery of treatment in the aimed cells, quality of preclinical research, ethical standards and most of all - consequences on the human society.

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