REVIEW | GENOME EDITING

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The rapidly evolving genome editing techniques are steadily moving from research laboratories to clinical practice. Fundamentally new methods of editing the genome of human embryos in the early stages of development have been developed. Tools for correction of genetic disorders in people of any age have also been created. In fact, the doctor is becoming a corrector of genetic instructions on construction and functioning of the human body. This review generalizes the data on the current state of genome editing techniques and existing approaches to applying them in clinical practice.

Keywords: genome editing, gene therapy, hereditary diseases, oncology, HIV

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Genome changes occur constantly in living organisms, determining the course of their evolution. Man started interfering in this process thousands of years ago, selecting successfully cultivated plants and producing breeds of pets. Genetic engineering, which emerged a little more than half a century ago, made it possible to create transgenic organisms: gene transfer between genomes or gene manipulation within a single genome. The idea of using DNA imported from the outside for treatment of human genetic diseases originated in the early 1970s [1]. In the 1980s, improvements in gene handling techniques and creation of eukaryotic vectors opened up a real opportunity for genome changes. The point is that gene therapy may not have anything to do with chromosome — the delivered gene may operate as a variant of ‘gene therapy’ in which nuclear genome (chromosomal DNA) changes. The point is that gene therapy may not have anything to do with chromosome — the delivered gene may operate as a variant of ‘gene therapy’ in which nuclear genome (chromosomal DNA) changes. The point is that gene therapy may not have anything to do with chromosome — the delivered gene may operate as a variant of ‘gene therapy’ in which nuclear genome (chromosomal DNA) changes.

For the past few years, with the emergence of qualitatively new directed genome change techniques (ZFNs, TALENs, CRISPR/Cas9), areas of applications for clinical trials of gene therapy drugs have skyrocketed in number like an avalanche. Thanks to the simplicity and accuracy of new techniques.
deployed to introduce changes in the genomic DNA of eukaryotic cells, a new term ‘genome editing’ even arose — after all, DNA change may in the future be used not only for therapeutic purposes but also for less important tasks.

Genomic therapy can be applied for: treatment of hereditary (usually monogenic) diseases, treatment of diseases caused by somatic mutations (mostly cancer), and attempts to treat HIV infections by destroying the copies of the virus integrated into the genome or receptor genes allowing the virus to enter the cell. Genomic therapy is one of the variants of personalized medicine, when the approach used is selected individually to the patient’s disease (and sometimes even to his genome).

Russia’s Federal Law No. 86-FZ dated 5th July 1996 “On State Regulation in the Field of Genetic Engineering” defines gene therapy as a set of genetic engineering (biotechnological) and medical techniques aimed at making changes in the genetic apparatus of human somatic cells for the purpose of treating diseases. With the advent of Federal Law No. 180-FZ dated 23 June 2016 “On Biomedical Cell Products”, the number of clinical trials of innovative gene therapy products in Russia is expected to increase.

### Genome editing techniques

Although there are a variety of methods for directed change of complex eukaryotic genomes, only a few of the methods are currently used in practice:

- a) non-break induced homologous recombination [33];
- b) site-specific recombination (recombinase and transposase) [34, 35];
- c) repair induced by site-specific nuclease, where the following are used as the nuclease:
  - artificial (hybrid, designer) nucleases with zinc finger nucleases (ZFNs) [36–38],
  - natural or hybrid endonucleases of gene conversion or meganucleases (homing endonucleases, HEs) [39],
  - artificial (hybrid, designer) nucleases with transcription activator-like effector nucleases (TALENs) [40],
  - natural RNA-guided nucleases (RGNs), in particular, clustered regularly interspaced short palindromic repeats / CRISPR-associated nuclease 9 (CRISPR / Cas9) with designer ‘guide’ RNA [41],
  - a combination of various nucleases [42–44].

Fig. 1 shows a timeline indicating the evolvement of genome editing techniques [45].

To date, the most promising techniques are those based on the use of artificial (so-called hybrid or designer) site-specific nucleases: ZFNs, TALENs and CRISPR/Cas9 [46]. Although, the term ‘hybrid’ (or ‘designer’) nuclease was initially applied to ‘protein’ techniques ZFN and Talen, today the CRISPR/Cas9 technique can be confidently categorized under the same class since the RNA in this system is the designer component (similarly to the ‘guide’ blocks of the ZF or TALE domain) (fig. 2) [47].

In general, each of these genome editing tools consists of three components: ‘guide’ specific to DNA sequence (indicating where to cut), cutting DNA ‘scissors’ (endonuclease) and the actually introduced DNA sequence (not always necessary). Delivery into cells a ‘genetic patch’ (a DNA fragment to be substituted) is required in those cases where it is necessary to add or substitute a genomic fragment. However, in some cases, only part of the sequence needs to be removed.

Table 1 shows the features of the most popular genome editing systems.

We use the ‘guide’ to indicate endonuclease, where a cut needs to be made in the DNA molecule. The cut is stitched usually through intracellular repair systems (such as double-stranded break repair or homologous recombination).

Since genome editing techniques — based on homologous recombination, recombinase and transposase — have been actively used in clinical practice for over 30 years and they are well described in literature (a Nobel Prize was even awarded in 2007 for homologous recombination techniques), we will not delve into details in this review. Rather we will elaborate on relatively new approaches based on hybrid nucleases and meganucleases, as well as directed double-stranded break repair.

![Fig. 1. Timeline indicating the evolvement of some genome editing systems (Doudna, Charpentier [45])](image-url)
Guidance of nuclease using zinc fingers (ZFNs)

Small domains stabilized by one or more zinc ions and known as ‘zinc fingers’ were identified around the middle of the 1980s (for the first time as part of Xenopus laevis transcription factor TFIIIA). These domains can bind DNA, RNA, proteins and lipids effectively and quite specifically [48]. It turned out that a zinc finger specifically binds a triplet of nucleotides. If 3–6 zinc fingers with known specificity are combined into a single protein, sufficiently accurate recognition of DNA sequence in 9–18 base pairs can be achieved. Here, any endonuclease (the most commonly used is non-specific endonuclease and FokI catalytic domain from Flavobacterium okeanokolites which introduces a single-stranded break) is added to the zinc fingers, you get targeted endonuclease. To get a double-stranded break, it is necessary to create two such enzymes recognizing neighboring regions on opposite DNA strands (fig. 2).

Since the beginning of the 2000s and up till now, zinc-finger systems had been successfully used in a wide range of practical modifications of genomes both on plant and animal models, and on therapeutic approaches (table 2). The advantage of the method is the versatility of its nuclease targeting technique. The disadvantages include the relatively high complexity of genetic engineering assembly of the enzyme gene; the need to create two enzymes for each of the DNA strands; toxicity associated with lack of specificity of this type of systems [49]; risk of immunogenicity of foreign proteins [50]. In this regard, the use of zinc-finger systems is gradually being replaced by new approaches.

Hybrid meganucleases

In 2003, Epinat et al. proposed a genomic editing technique based on the so-called meganucleases [39] (fig. 2). Meganucleases were found in archaea, bacteria, phage, yeast, algae and some plants, and they are endodeoxyribonucleolases — small proteins, mirror monomers or homodimers, characterized by a very long double-stranded DNA recognition site: from about 10 to 40 base pairs. Usually, a site of such length is seen only once in the genome or even never. For example, the I-SceI meganuclease recognition site, which is 18 base pairs in length, theoretically occurs once in the genome, exceeding the length of the human genome by 20 times. Typically, they form a part of introns or transposable elements of the genome. The biological function of meganucleases is unclear.

Representatives of the LAGLIDADG meganuclease family, found in the mitochondria and chloroplasts of unicellular eukaryotes, are the most widely used tool for genomic editing. Advantages of the technique involve the fairly high specificity and spontaneous dimer assembly. The disadvantage — high limitation on impact site selection.

TALEN (transcription activator-like effector nucleases) technique

The history of development of the TALEN system is connected with the study of the Xanthomonas bacteria genus. The reason for the long-term study of this group of bacteria was their pathogenic effects on crops, including tomatoes, peppers, rice and others. It was found that Xanthomonas secretes regulatory proteins — transcription activator-like effectors (TALE) — into the plant cell cytoplasm. These proteins increase the susceptibility of cells to the pathogen. Upon further study of the mechanisms of action of these proteins, they were found to be capable of binding to DNA and activating the expression of certain genes, mimicking host cell transcription factors [51, 52].

It was found that the TALE of a particular site in the DNA is recognized with the help of a series of small domains, each of which recognizes a single nucleotide in the site. Researchers figured out quite quickly the specificity of domains to specific nucleotides. This allowed to collect from them ‘packs’ precisely recognizing a specific sequence of bases in DNA.

Thus, the principle of use of TALEN system is similar to that of the system described above using triplet specific zinc-finger domains. The only difference is that nucleotide-specific domains (transcription activator analogs), connected in series by 12–20 pieces each are used as the ‘guide’, while the proven FokI catalytic domain is used as the nuclease. For double-stranded break, it is necessary to create two such enzymes (the target landing sites of ‘guide’ TALEs) that should be on the opposite DNA strands and separated by a site of about 20 base pairs (fig. 2). Advantages of the method: versatility of the nuclease guiding technique and versatility of the designer nuclease assembly technique. Disadvantages: high complexity of the genetic engineering assembly of the enzyme gene and the need for creation of two enzymes for each of the DNA strands.

There are attempts to cross individual elements of different techniques. For example, hybrids of ‘guide’ TALEs and meganucleases (megaTALs) are described [42]. There are attempts to attach enzymes to meganucleases (by one way or another), which process (for example, destroy) the ends of the double-stranded break in order to strengthen the mutagenic effect of this break, and achieve other effects [43, 44].

In 2012, the Nature Methods journal named high-precision genome editing methods the methodical discovery of the year. TALEN was included in the methods.

CRISPR/Cas9 (nuclease associated with regularly interspaced short palindromic repeats) technique

The CRISPR/Cas9 system — proposed just a few years after TALEN — is a fundamentally different system when it comes to mechanism for guiding a nuclease to the target. The system differs from TALEN by the fact that as a ‘guide’, it uses not
Genomic therapy algorithms

Therapeutic uses of genome editing systems can be divided into three groups: 1) changing the genome of gamete/zygote/blastomeres for the purpose of obtaining a whole organism from one modified cell (fetal gene therapy); 2) changing the genome of individual somatic cells selected from the body for the purpose of subsequently returning the modified cells to the organism (somatic cell gene therapy); 3) changing the genome of individual groups (or all) of somatic cells in a multicellular organism directly (tissue somatic gene therapy).

The first two approaches involve manipulation of cell cultures in the lab (for which the broadest technology base is currently being developed). For the third approach, special systems (preferably tissue-specific systems) should be used for delivery of genetically engineered constructs into the body’s cells.

Genetically engineered constructs

Hybrid nucleases and genetic patches (genetic material to be replaced) are typically delivered into the cell in the form of genetically engineered constructs from which the corresponding RNA and proteins are developed inside the cell. Variants of direct introduction into the mRNA cell are described, particularly for the CRISPR/Cas9 system [55].

A typical genetic construct for site-specific designer nuclease system contains a nuclear localization signal, an artificial guide unit (zinc fingers, TALE or ‘guide’ RNA), nuclease catalytic domain (for example FokI) and, if required, fragment to be replaced.

Gene delivery systems

Various viral and non-viral systems recognizing a large number of potential target tissues (skin, muscle, lung, brain, colon, spleen, liver, blood cells, and so on.) have been designed for delivery of ‘therapeutic’ genes or genetic constructs. The delivery system should ensure high efficiency of absorption of the genetic construct by the target cells, immunity to intracellular destruction during transportation to the nucleus and maintenance of necessary expression level.

Non-viral systems include direct introduction of DNA constructs into cells and tissues (e.g. electroporation), liposomes, cationic polymers, and others. Among viral systems, the most common are systems based on retroviruses, lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex virus. Targeted delivery is determined by the presence of specific molecules (recognizable by target cell receptors) on the surface of viral particles or on the liposome’s membrane. Such molecules could be viral capsid proteins, antibodies to surface cell antigens (inserted into the liposome’s membrane), folic acid molecule (strongly captured by tumor cells), and others.

There are much attempts to use viral and non-viral delivery methods to deliver vectors with hybrid nucleases [56].

Genomic therapy of genetic disorders

As mentioned above, therapeutic approaches based on addition of genetic material into a cell with the help of viral vectors have been in use since the beginning of the 1990s.
These techniques restore synthesis of protein, whose gene is dysfunctional in both copies in the chromosome. However, changing or deleting DNA regions has long been an extremely complex and non-reproducible approach. With the advent of designer nucleases, researchers began to rapidly develop methods of directed DNA change directly in the chromosome structure. At present, there are treatment options for retinitis pigmentosa, glaucoma, hemoglobinopathies, muscular dystrophies (Table 2).

Fetal gene therapy is the most actively developing direction. In 2015-2016, many laboratories in the US, China, UK and several other countries, as well as some biotech companies, namely Ovascience (USA), Editas Medicine (USA), etc announced their plans for modification of human embryo genomes using CRISPR/Cas9-mediated gene editing techniques. If for a given pair of individuals, a potentially healthy genotype cannot be selected from ‘natural’ variants of embryos, genome editing methods aimed at adding/ correcting a pathogenic allele in the zygote stage can be used.

Liang et al. published their work in April 2015, in which the CRISPR/Cas9 system was used at the zygote level to repair a mutant beta-globin gene. Of the 86 zygotes taken for experiment, only 4 cases were repaired [7].

**Somatic gene therapy**

“Chemotherapy will be obsolete within 20 years,” said Prof Jeremy Farrer, head of the Wellcome Trust Sanger Institute. “We will look back in 20 years time and the blockbuster chemotherapy drugs that gave you all those nasty side effects will be a thing of the past and we will think ‘gosh what an era that was’. Just as today, we are terrified by examples of electricity treatment at the beginning of the last century. Understanding humanity’s genetic code is going to be fundamental to the medicine of the future. In rare congenital disease, in cancer and in infections, genomic insights are already transforming diagnosis and treatment” [57].

Designer nucleases can be used to effectively and accurately alter the DNA to correct any mutations. This opens up great opportunities for their use for correction of disorders that caused tumorigenesis [29, 30]. Variants for use of the CRISPR/Cas9 system for treatment of sarcoma and lung cancer have been proposed [31, 32]. For example, correction or deletion of mutant version of the EGFR gene using the CRISPR/Cas9 system delivered by virus has been proposed for lung cancer [32].

**Antiviral therapy**

**HIV gene therapy**

The fight against HIV is another direction of therapeutic use of hybrid nucleases. There are two directions in this fight: deleting HIV copies from the genome of the HIV carrier and altering the receptor genes through which the virus penetrates the T-lymphocytes, particularly the CCR5 gene (Table 2). By destroying the proviral DNA copies in the genome, it is theoretically possible to completely neutralize the virus and

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prevent it from reactivating in the patient’s cells. Another approach — altering the receptor gene — does not allow the virus to infect the lymphocytes, thus leading to restoration of the patient’s T cell population.

One of the problems in the development of gene-editing antiviral drugs is the ability of the virus to very quickly change sequence and thereby leave from the ‘guide’, specific to a particular sequence of the attack site. However, with proper legislative regulation of the release of modifications of gene therapy drugs, the release of new ‘antivirus’ can easily overtake the pace of HIV.

Fight against non-integrated viruses

The research world is also trying to apply genome editing systems to fight against viruses that do not integrate genetic material into the cellular genome. Their destruction mechanism is the same as in the case of HIV, but hybrid nuclease attacks free viral genome. The use of CRISPR/Cas9 to fight hepatitis B virus is described in [58].

Non-therapeutic genomic editing objectives

Genetic doping

Genetic doping is a variant of non-therapeutic use of genome editing to enhance athletic performance. It is no secret that maximum sports performance is largely determined by the individual’s genetic component. Athletes from Kenya or Ethiopia almost always win marathons because genetically determined glucose metabolism pathway, which determines the ability to quickly run a marathon, is most developed in the African population of these countries.

At present, athletic success is linked with over 150 polymorphic positions in the DNA out of which 93 are associated with endurance and 62 with power load [91]. The spectrum of potential genes for effecting an influence by means of genomic editing is very wide: erythropoietin, insulin-like growth factor 1, human growth hormone, myostatin, endothelial growth factor, fibroblast growth factor, endorphins, enkephalins, cytoskeleton protein genes, etc. Approaches have already been developed for some of these genes, while clinical trials on introduction of specific alleles into the human genome have been carried out [85].

Reprogenetics

In classical interpretation, reprogenetics involves the selection of human embryos with certain properties from the resulting ‘natural’ variants. However, genome-editing technology helps to expand the features of the approach by creating variants that are impossible for a given pair of parents [88]. This raises a lot of ethical issues that mankind is yet to address [90].

Genomic editing: ethical and regulatory issues

Despite the fact that genome editing techniques via designer nucleases have enormous potential for creation of an effective therapy for patients suffering from genetic diseases, their use for therapeutic purposes is still in its infancy. In this regard, development of an ethical and legal framework that would ensure the effectiveness and safety of using genomic editing is an extremely important factor [92].

While developing such a framework, ethics committees and authorized government bodies need to establish and clarify aspects influencing the clinical implementation of genome editing techniques. These bodies should propose such a road map for development and implementation of genomic editing techniques that would allow to safely and quickly transfer the latest techniques into clinical practice.

Rapid development of innovative medical technologies does not allow the legislator to work on the legal framework for the use of these technologies in the same way as before. Presently, there is a change in the paradigm of legislative regulation on introduction of new medical technologies from research laboratory to the clinic. Globalization has made innovation to be spread around the world literally at the speed of light. New promising medical technology, no matter where it has been developed, is inevitably developed and primarily used in countries with more flexible and liberal laws. Such countries receive a head start on early introduction of innovative approaches, despite the risks involved in such approaches. Many legal restrictions on ‘research-to-medicine’ transition in certain countries do not make sense because technologies quickly spread to the rest of the world from ‘research offshores’, attracting clients to their territory.

From the very start, some countries try to prohibit the use of designer nucleases for human genome editing. However, such countries are forced to quickly change position to avoid being at the tail of technological leaders. After a Chinese team announced in 2015 that it had carried out experiments on editing of genomes of human embryos through the CRISPR/Cas9 method, a group of British scientists were in February 2016 granted permission to genetically modify human embryos through CRISPR/Cas9 and related designer nuclease methods [93].

Public opinion amidst the introduction of techniques in separate jurisdictions is changing rapidly and starting to put pressure on their own legal regulation.

CONCLUSION

Genome editing methods created over the last few years are an improvement of gene therapy approaches existing at the end of the last century. However, it can be confidently argued that today the paradigm in the field of genomic medicine is shifting. The beginning of the second decade of the 21st century witnessed several technological breakthroughs with a strong synergistic effect — improvement in directed cell differentiation techniques, considerable reduction in cost, routine application of genome sequencers and creation of the described genome editing systems. All of these combined will inevitably give birth to new-quality personalized genomic medicine in the next 3–5 years. Directed genome alteration techniques will be a new tool for doctors.
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