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REVIEW

Genome Editing Technologies: From Bench Side to Bedside

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ABSTRACT

The development of genome editing technologies has given the chance to researchers to manipulate any genomic sequences precisely. This ability is very useful for creating animal models to study human diseases in vivo; for easy creation of isogenic cell lines to study in vitro and most importantly for overcoming many disadvantages that the researchers faced during the human gene therapy trials. Here we review the basic mechanisms of genome editing technology and the four genome-editing platforms. We also discuss the applications of these novel technologies in preclinical and clinical studies in four groups according to the mechanism used, and lastly, summarize the problems in these technologies.

Key words: Genome, genome-editing technology, genome-editing platform.

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INTRODUCTION

The use of genome editing technologies has provided researchers a powerful, economic and rapid way of precise modification of the genome in any living cell that is useful in solving research questions as well as developing novel treatment methods for not only single gene disorders but also for complex disorders and infectious diseases. Conventional gene therapy, which is known as the addition of new genes to human cells, has not met the expectations of the patients and clinicians due to many challenges such as stability of expression, unpredictable effects, addressing large genes and dominant mutations [1]. However, the genome editing technology has brought the ability to overcome the challenges of the conventional gene therapy methods and has been acknowledged as more promising than the previous one.

I. Mechanisms of genome-editing

The basic mechanism of genome-editing technologies depends on the induction of DNA double strand breaks (DSBs) on the target sequence by nucleases which stimulate the repair mechanisms of the cell and give a chance to introduce site-specific genomic modifications [2].

IA. Genome-editing platforms

Genome editing technology uses different genome editing platforms namely targeted nucleases to create site-specific DSBs. There exists four major genome editing platforms:

Meganucleases

Meganucleases were the firstly described as targeted nucleases. LADLIDADG family is the largest class of this group of naturally occurring enzymes. It is possible to change the target specificity of meganucleases by protein re-engineering mechanisms that made this platform useful in studies. Relatively small size (20-37 kDA) and ability to recognize longer target sequences (14-40bp) are the main advantages

of meganuclease platform [3]. For the gene delivery purposes, it could be easy to package this platform as a monomer into a single viral vector. On the other hand, difficulty in separating the DNA-binding and cleavage domains of meganucleases, and relative difficulty of protein engineering to generate monomeric enzymes having new target sequences, limited the use of them as genome-editing tools [1].

Zinc-finger nucleases

Zinc finger nucleases (ZFNs) are the hybrid proteins containing a DNA-cleavage domain from Fok1 and a DNA-binding domain composed of Cys2-His2 domains [4]. These domains in the presence of Zinc atom get into contact with 3-4 bp in the major groove of DNA specifically. ZFNs consist of a tandem array of three to six of these domains recognizing 9-18 bp of DNA sequences and only active in a dimerized conformation. As a result, each ZFN target site includes two ZFP binding sites on either site of a spacer region of 5-7 bp within which the dimerized FokI cleaves. Additionally, usage of heterodimeric FokI containing ELD/KKR mutations in the dimerization interface increases the specificity of ZFNs by prevention of homodimerization and creation of undesired breaks in the DNA [5]. Although the need for denovo protein design for ZFNs such as meganucleases limits their use, ZFNs have been used in genome-editing applications in plants, animals including zebrafish, rats and numerous mammalian cell lines. ZFNs have been also used to modify human somatic and pluripotent stem cells for treatment purposes which we will mention in detail in the next sections.

Transcription activator-like effector (TALE) nucleases (TALENs)

TALENs are chimeric nucleases like ZFNs which are composed of a nuclease domain, namely FokI endonuclease and a designable DNA-binding domain. TALE proteins were discovered in the plant pathogens, Xanthomas bacteria and they contain highly conserved 33 to 35 repetitive amino acid residues. The DNA specificity of TALE repeats based on the repeat variable residues (RVDs) which are the 12th and 13th residues in the conserved TALE sequence. The understanding of the RVD code gave the ability to generate engineered TALENs to target any sequence in the genome [6]. There exist many platforms for engineering TALE arrays: Standard cloning technique, Golden Gate cloning system, solid phase assembly and ligation-independent techniques [1]. As the nuclease domain of TALENs consists of FokI, they can only result in DSBs as dimers and to increase their specificity they can be designed as obligate heterodimers like ZFNs. The target sequence of TALENs consists of two adjacent TALE binding sites at opposite strands that are separated by a spacer sequence of 12-20 bp which introduces a DSB on FokI dimerization. Since the design-assembly of TALENs is easier than that of ZFNs and the targeting range of TALENs is nearly unlimited, they become an attractive platform of genome-editing. The main disadvantage of TALENs is their large size which creates a difficulty for in vivo delivery systems, especially the viral vectors. There are also hybrid systems such as megaTALs that combine TALENs and meganucleases to improve specificity, affinity, and the ease of delivery [7].

CRISPR/Cas9 system

The latest discovered genome-editing platform is the CRISPR (Clustered regularly interspaced short palindromic repeat) and it is associated with Cas9 protein. Actually, this system is a part of adaptive immunity of prokaryotes against viruses and plasmids. Among these systems, type II system from Streptococcus pyogenes has 3 essential components: the nuclease (Cas9), a DNA-binding CRISPR RNA (crRNA) including a 20 nt guide-RNA (gRNA) sequence with precise complementarity to its DNA target and an auxiliary trans-activating crRNA(tracrRNA) bridging crRNA to Cas9 [8]. The two components of this system (crRNA and tracrRNA) were fused into a single component namely single guide RNA (sgRNA) and this system was shown to recognize different sequences by only changing the sgRNA [9]. This two-component system has been widely introduced to eukaryotic organisms such as yeasts plants and mammals successfully. Recognition of a target sequence by SpCas9 requires the presence of a protospacer adjacent motif (PAM) sequence (NGG) downstream of the gRNA target sequence. DNA double starnd break occurs 3 bp upstream of this PAM sequence. Additionally, many naturally occurring Cas9 orthologues have been used for genome editing of human cells to date. For example, non-Cas9 based RNA guided-endonuclease Cpf1 has a different PAM sequence (TTN) and introduces a staggered rather than blunt DSB [10,11]. Staphylococcus aureus Cas9 with a different PAM sequence (NNGRRT), smaller than SpCas9 that provides advantages in terms of cellular delivery [12]. Additionally, the ability to engineer Cas9 to develop Cas9 variants with different PAMs can overcome the specific sequence limitation of the previous CRISPR systems [13].

There are two important advantages of CRISPR/Cas9 system: First, there is no need to engineer proteins for different DNA target sequences; synthesizing a

new sgRNA is enough for directing the Cas9 protein to the desired new target that is much easier and quicker. Second, this system can be used to make multiple DSBs simultaneously by using multiple sgRNAs since the nuclease protein is not fused to gRNA unlike the other genome editing platforms.

IB. Endogenous cell repair mechanisms

There exist two major DNA repair mechanisms to protect the integrity of the genome: 1) Homology-directed repair (HDR) and 2) Nonhomologous end-joining(NHEJ) [14]. NHEJ occurs by rejoining the two broken ends of the genome in a process that often results in small insertions and/or deletions namely indels at the cleavage site. These indels may lead to functional disruption of target genes by producing missense or frameshift mutations or interruptions of splice sites or transcription factor binding sites [15]. When two simultaneous DSBs are achieved on the same chromosome but on different sites, this may lead to large interstitial deletions or inversions. If simultaneous DSBs are on different chromosomes, translocations may occur [16-18]. If there exists a donor template for repair, HDR takes place. Normally, when living cells are exposed to genotoxic injury, homologous sister chromatids are used as a template for HDR in replicating cells. In the case of genome editing of an extrachromosomal donor sequence, plasmid or single strand oligonucleotide can be used to integrate sequences of choice which are adjacent to induced DSBs. In cells, NHEJ pathway is much more efficient than HDR which makes it difficult to achieve precise changes in the genome [19]. It was shown that the efficiency of HDR could be increased by using different ways to suppress key enzymes of NHEJ in the cell [20].

II. Applications of genome-editing

Genome editing platforms use targeted nucleases, lead to DSBs and stimulate two different endogenous cell repair mechanisms. These platforms allow us to make precise changes in any genomic sequence by one of the four mechanisms: Gene disruption, gene excision, gene correction, and gene insertion. Here, we are going to mention about the pre-clinical studies and clinical trials which use any one of these as a potential treatment method.

IIA) Gene knockout via mutation (Gene disruption)

Gene disruption or knockout via mutation can occur when the DSB is repaired by using NHEJ pathway. NHEJ is active during the whole cell cycle and it does not need a donor template DNA so it is the simplest and the more efficient way of genome editing. This repair pathway results in the formation of insertions and/or deletions at the site of the cleavage and when this occurs in the coding region of a gene it may result in a frameshift and can disrupt the gene function.

Gene knockout mechanism has been widely used in research for creating in vitro and in vivo models to analyze the newly described gene functions in human disorders as well as solving the unknown disease mechanisms of previously known disease-causing genes. Additionally, this mechanism results in rapid and easy development of larger animal models such as rats, pigs and primates for drug development studies that are more representative of human diseases and gives more reliable results to determine efficiency of therapeutic agents.

Gene knockout mechanism has also been used for therapeutic purposes in preclinical studies and clinical trials. Permanent disruption of deleterious sequences in case of dominant gain of function mutations has been one of the ways to treat human disorders. Huntington disease, a neurodegenerative disease caused by trinucleotide (CAG) repeats in the Huntingtin gene (HTT), was a good example of this group of disorders. Suppression of the mutant HTT expression could be an effective treatment method in these patients before the deposits in the neuronal tissue cause symptoms. In a preclinical study in mutant HTT-expressing transgenic mice, it was reported that permanent suppression of endogenous mutant HTT expression using allele specific gRNA and Cas9 effectively depleted HTT aggregates by creating small indels in the first exon of mutant allele [21].

Gene knock-out mechanism has also become an important therapeutic strategy in cancer. Genome-editing tools can be used to increase the efficiency of T-cell immunotherapy: Knock-out of check point inhibitor receptor protein PD-1 by using sgRNA and Cas9 on T-cells has been shown to be applicable [22]. There exists a completed Phase 1 clinical trial (NCT01082926) originated from a preclinical study in which knock-out of T-cell receptor protein was achieved by genome editing and glucocorticoid-resistant T-cell source was created that was used in malignant glioma therapy in mouse models succesfully [23].

Additionally, gene knock-out mechanisms was shown to be useful in case of infectious diseases by destroying infectious agents' or hosts' genes that favor those agents to invade, replicate and kill human cells. There exists many preclinical studies and also clinical trials especially in the area of HIV infection. Ex-vivo modification of T-cells or CD34+ human stem cells (HSCs) to disrupt the CCR5

coreceptor gene is the most advanced genome-editing strategy used for primary HIV infection. These studies showed that the viral load decreased CD4+ T-cell counts increased in HIV-infected mice engrafted with immune cells in which the CCR5 gene had been disrupted by zinc finger nuclease [24,25]. These studies have resulted in several clinical trials evaluating this approach in HIV-infected human patients (NCT00842634, NCT01044654, NCT01252641, NCT02225665, NCT01543152, NCT02500849). The trials have shown promising results that was achieved by ex-vivo genome editing approach in humans [26].

II-B) Gene excision (Gene deletion)

Gene deletion can occur when two simultaneous targeted DSBs are created in the flanking DNA sequence by using a pair of TALENs or gRNAs with Cas9. These DSBs will be repaired by NHEJ mechanism creating large deletions in the DNA up to several mega-bases in size or even genomic inversions [27]. This mechanism could be used for removal of a deleterious gene, removal of any other genomic element such as an enhancer or promoter, correction of reading frame of a gene by deletion of mutated exons or deletion of multiple neighboring genes or gene clusters at once. Additionally, in case of a single coding gene, frameshift mutations created by the gene disruption mechanism mentioned above cannot eliminate the function of the target gene totally because of existing splice variants, multiple transcripts, unexpected alternative start codons. These problems can be overcome by using gene excision mechanism [28].

Gene deletion mechanism has been used in many studies for research and therapeutic purposes to date. Duschenne Muscular Dystrophy (DMD) is one of the mostly studied diseases for therapeutic purposes. DMD is caused by mutations, mainly deletions causing frameshift and unfunctional protein product, of the dystrophin gene which have a coding sequence of nearly 14 kb. Due to its large size, it cannot be packaged as a whole into viral delivery vectors for gene therapy or genome editing therapy by gene insertion. In one of the recent studies about DMD, deletion of >300 kb of dystrophin gene including exons 45–55 was achieved by multiplexed Cas9. This treatment strategy could address most of the patient population because this has the ability to restore dystrophin expression in 62% of DMD patients [29].

Sickle cell anemia (SCA) and Beta-thalassemia were other examples that could be treated by using gene deletion mechanism. BothSCA and β-thalassemia are caused by mutations on the HBB gene which encodes the β-globin chain. More than 200 different mutations in HBB locus are known to be responsible for β-thalassemias. In contrast, SCA is caused by a missense mutation at codon 6 of HBB. γ-globin which is upregulated in fetus but then down regulated after birth can compensate the disrupted function of β-globin in SCA and β-thalassemia. The expression of γ-globin is suppressed by the transcriptional regulator, namely BCL11A. An erythroid lineage specific enhancer of BCL11A was discovered [30] and by using Cas9 mediated gene deletion mechanism, BCL11A was suppressed and upregulation of γ-globin was achieved [31]. This genome editing strategy could be an effective treatment method both for SCA and β- thalassemia.

For the infectious diseases, gene deletion mechanism could be an option as in HIV infection studies. HIV genome could be completely deleted by nucleases from the infected cells by targetting long terminal repeats (LTRs) at both ends of the viral genome [32].

II-C) Gene correction

Gene correction can occur when the DSB is repaired by HDR instead of NHEJ. An exogenously supplied donor template such as plasmid or single stranded oligonucleotides (ssODNs) must be used with the targeted nuclease to induce HDR in the cell. This approach gives the ability to precisely correct the mutation (missense, nonsense, small indels) in the target gene as opposed to the unpredictable changes seen as a result of NHEJ. Another newly reported method of gene correction is the programmable base editing by using modified CRISPR/Cas9 system [33]. In this system, cytidine deaminase is fused to Cas9 nickase, and by this modification, up to 28% of site-specific single-base changes were achieved in multiple gene loci [34].

Although gene correction methods are not as efficient and easy as the previous ones, there have been studies to achieve gene correction in the target sequence because of its therapeutic potential. SCA is an example of these studies: As previously mentioned, it is a hemoglobinopathy due to a missense mutation in HBB gene. It was shown that when a donor template and ZFN mRNA were delivered simultaneously to CD34+ HSC progenitor cells derived from patients with SCA, %18 HDR was achieved. Additionally, this correction led to production of wild type β-globin in vitro that could reach to therapeutic levels in patients [35].

Another example is the hereditary tyrosinemia type I (HTT1). Point mutations of FAH gene that encode the last enzyme in the tyrosine catabolic pathway cause this disease. The rescue of a mouse model of

HTT1 is the first example of in vivo genome editing by using CRISPR/Cas9. Although tail vein injection of the SpCas9 enzyme and gRNA and short ssODN was shown to result in relatively low gene-editing efficiency (~0.4%), lower amount of corrected cells repopulated the liver and corrrected the disease phenotype [36].

II-D) Gene insertion

Gene insertion shares the same methodology as conventional gene therapy procedures with one and very advantageous exception which is the ability to control the integration site of the gene. These mechanisms can occur by using both HDR and NHEJ repair pathways. For HDR, we have to use a donor template especially a plasmid including the gene to be inserted and the homology arms carrying the sequences identical to the ones around the DSB site. For the targeted integration of the genetic insert, NHEJ can also be used as an alternative pathway by creating compatible overhangs both on the donor template and the targeted endogenous site [1].

Gene insertion can be used for therapeutic purposes in three ways: Firstly, insertion of a group of adjacent exons into the endogenous locus can be used. The mutations in interleukin-2 receptor commonγ-chain (IL2RG) gene lead to X-linked severe combined immunodeficiency (SCID-X1). ZFN was used to knock-in of cDNA of exons 5-8 in the IL2RG endogenous locus to correct mutations downstream of exon 4 in HSCs. This method corrected the IL2GR expression in HSCs and shown to be therapeutic in mice model of SCID [37].

Secondly, knock-in of a full cDNA into the endogenous locus can be used as an alternative strategy. β-globin full-length cDNA was inserted into endogenous β-globin locus in K562 erythroleukemia cells by using TALEN genome editing platform [38]. This strategy could be therapeutic for both β-thalassemia and SCA patients.

Thirdly, another strategy is the knock-in of a full cDNA into a genomic safe harbor locus. Genomic safe harbor is theDNA region where inserted genes integrate with an anticipated expression level and without insertional modification or disturbance to other gene functions [39]. Haemophilia, a group of bleeding disorders has been the most promising disease for this strategy. Haemophilia B is caused by the mutations in the factor IX gene, a clotting factor important in the coagulation cascade. By using ZFN genome editing platform, Factor IX therapeutic transgene without its promoter was inserted into the locus of albumin gene which has a high expression level [40]. This study showed that AAV8 and ZFN mediated integration of Factor IX into a safe harbor locus could be therapeutic in mouse model of Hemophilia B and this achievement made a progress towards clinical application [41].

III. Problems and recent advances in research and clinical applications

III-A) Problems in efficiency

The most efficient mechanism in genome editing is the gene disruption method where a targeted nuclease can achieve this mission by itself via NHEJ [42]. The researchers mainly face the problems of efficiency in precise sequence modification methods such as gene insertion or gene correction where HDR is the main rate-limiting step [43]. However, as genome-wide association studies and the next-generation sequencing has identified many candidate genes with missense mutations associated with diseases, researchers have to model these mutations precisely to validate the associations or for therapeutic purposes it will be much better to make precise changes in the genome rather than imprecise indel mutations. There have been many studies to solve this problem: One group of studies focuses on ways to increase HDR and decrease NHEJ efficiency [20,44]. Another group of study focuses on the properties of donor template to increase efficiency [45]. Others focus on modifying nucleases: Using base-editing [34], nickase [46] or other type of nucleases such as Cpf1 [11]. Lastly, instead of HDR there have been studies to achieve knock-in process through NHEJ [47]. These efforts have increased the knock-in efficiency considerably, but it cannot catch up the efficiency of knockout systems.

III-B) Problems in targeting

There are two main problems of targeting for researchers to deal with: First one is the limits of targeting and the second one is the off-target effects. Targeting limits of different genome editing platforms are different. ZFNs and meganucleases need protein engineering and it is difficult and time consuming to direct these platforms to a new specific target sequence. However, higher specificity and accumulative knowledge about ZFNs make this genome editing platform the most clinically advanced one [48-50]. TALENs are relatively easy to design for newer target sequences when compared with ZFNs and the only restraint on targeting is the need for 5′ T, specified by the constant N-terminal domain [6]. Although among all these four platforms CRIPSR/Cas9 system is the easiest to design to target new sequences, the range of sequences that Cas9 can recognize is limited by the need for a specific PAM [51]. Therefore, it is not possible to target the sequence of interest precisely to achieve the

exact editing application in some cases. Cas9 orthologues that is discovered from different bacterial species other than Streptococcus pyogenes such as Streptococcus thermophilus and Staphylococcus aureus or Neisseria meningitides with different PAM specificities can broaden these limits [12, 52, 53]. Additionally, the efforts to engineer these Cas9 orthologues by either bacterial selection-based directed evolution or through structure based rational design further relax these PAM limitations and these studies showed the utility of engineering a wide range of Cas9s to acquire more specific and more efficient Cas9s with different PAM specificities [13,54].

Off-target activity, the occurrence of DSBs in unintended sites of the genome, is the foremost problem of genome editing platforms before widely applicable in clinical trials. The specificity of editing depends on three factors: 1) nuclease platform itself, 2) feature of target site, mainly chromatin context that determines the accessibility and 3) nuclease delivery method to target cell [18]. Struggling with this problem, various solutions have been reported to decrease and to screen off-target activity of a nuclease platform. The first group of solutions is to design the nuclease platforms with the lowest possibility for off-target cleavages. There have been many bioinformatics tools for each nuclease platform to search for off-target effects of nucleases. The second group of solutions is to engineer nuclease platforms to promote their specificity. To maximize the specificity of ZFNs and TALENs their DNA recognition, linker and dimerization domains can be engineered [55]. Addition of TALE RVD recognition modules to megaTALs is reported to increase their specificity [56]. Using shorter sgRNAs (17-18 nt), having mutations in the Cas9 nuclease domains to obtain nickase or creating Cas9-FokI fusions both of which require paired gRNAs targeting opposite strands [46, 57, 58] and producing Cas9 variants that has reduced interaction strength with the target DNA-gRNA heteroduplex [13, 59], all of which were reported to increase the specificity of CRISPR/Cas9 system. Resolution of the crystal structure and molecular interaction mechanisms between gRNA-Cas9 protein and DNA target gives the ability to researchers to alter right amino acid combinations to generate more specific Cas9 variants [54]. The third group of solutions is more general and applicable for all nuclease platforms: To minimize the duration and level of the genome is exposed to the nuclease. Nonintegrating viral vector usage, delivering the nucleases in mRNA or protein form can limit the exposure time and level in the cell [60-62].

Once a nuclease is designed and produced as specific as possible by the methods mentioned above, in-vitro screening is a requirement for researchers to define the specificity of a nuclease more precisely in order to use it for therapeutic purposes in humans. One way is to deeply sequence the potential off-target sites that is detected in in silico tools. But none of these tools cannot identify all potential off-target sites. To compensate the biased approach of these tools, there are newly developed unbiased assays to evaluate the specificity of nucleases. Unbiased off-target analysis methods need to define off-target DNA cleavage sites on live cells in an unrestricted way [63]. These include Integrative-Deficient Lentiviral Vectors (IDLV) Capture [64], chromatin immunoprecipitation sequencing (ChIP-seq) [65], Genome-wide Unbiased Identification of DSBs Enabled by Sequencing (GUIDE-Seq) [66], LAM-PCR-Based High-Throughput Genome-Wide Translocation Sequencing (LAM-HTGTS) [67] and Digenome-seq [68]. And lastly, whole genome sequencing (WGS) is useful for the analysis of single cells, clones, and F1 genome-edited animals [69] but not for the analysis in bulk populations [65].

The last approach to assess the specificity of a nuclease is the classical functional approach that is used in pharmacology and toxicology. In this strategy after genome editing process the cells are evaluated if they perform their normal functions, if they transform into cancer cells, or if they survive.

These biased, unbiased and functional methods are important to carry genome editing approaches from bench side to bedside as therapeutic methods, but this problem is still in need of improvement especially for in vivo genome editing therapies.

III-C) Problems in delivery

Classical gene therapy and the recently advanced genome editing therapeutic approaches both share the same problems of the delivery to target cells or tissues. An ideal delivery system should carry the nuclease platform to the target cells or tissues efficiently without immunological or toxic reactions, insertional mutagenesis or off target-activity. In case of genome editing, the duration of nuclease expression is very critical for the efficiency without causing off-target effects. According to the therapeutic approach the choice of delivery systems can change. If the therapeutic approach is ex-vivo the nuclease platform can be delivered as plasmid based (DNA-based), RNA-based, protein-based or with viral vectors. In vivo therapeutic approaches can use either viral delivery systems or non-viral delivery systems such as plasmid with or without electroporation, cationic lipid complex or nanoparticles [70].

Ex-vivo therapeutic approaches

Plasmid-based delivery: The most widely reported method to date is the transfection of plasmid carrying nuclease platform in the form of DNA. The efficiency of this method is moderate, but it causes DNA related toxicity to the cell, contains bacterial DNA in the backbone that increases its immunogenicity, and the most importantly, it carries random integration risk.

RNA-based delivery: This can be achieved by electroporation of mRNA encoding nuclease or in case of CRISPR/Cas9 system mRNA of Cas9 with the sgRNA. This delivery method could be used efficiently in T cells, HSCs and IPSCs [18, 61]. It was reported that clinical scale gene editing could be achieved by ZFN mRNA delivery to T cells [71, 72]. In this method, cellular toxicity is low, there is no risk of integration, immunogenicity and also off-target activity is relatively low.

Protein-based delivery: In this method purified nuclease proteins or in case of CRISPR/Cas9, ribonucleoprotein (RNP) complex is delivered to the cells via electroporation or via cell penetrating peptides. CRISPR/Cas9 RNP complex was reported to be delivered to T cells, HSCs and IPSCs [1, 73, 74]. Normally Cas9 protein has positively charged and the gRNA is negatively charged so it is impossible to pack them together in the same nanoparticle delivery vehicle. Rotello et al, added positive charges to Cas9 protein and packed RNP complex in positively charged gold nanoparticles and showed that it worked in vitro [70]. This delivery method is reported to be highly efficient, without risk of integration, low-immunogenicity.

Viral delivery: Integrase deficient lentiviral vectors are the widely used viral vectors for ex-vivo genome editing lately. Their efficiency was reported to be high and they do not carry integration risk. Additionally, they have sufficient packaging capacity for various nucleases. They were used successfully in genome editing of T cells and HSCs [37, 75, 76].

In-vivo therapeutic approaches

In case of in vivo therapeutic approaches, tissue-specific targeting, vector distribution in the organism, immune reactions and biocompatibility of carriers become a concern unlike the ex vivo approaches. There are two groups of vectors used in this approach:

1) Viral vectors: Adenoviruses are efficient delivery vectors and were used for in vivo genome editing of PCSK9 gene in adult mouse [77]. They can deliver the package to both dividing and non-dividing cells, but they cause significant immune reaction in the organism. Adeno associated viruses (AAV) have been the most widely used viral vectors for in vivo studies. These vectors were reported to be non-immunogenic and have vast range of serotypes that made them useful to target specific tissues and cells such as liver, eye, nervous system, skeletal and cardiac muscle [78]. The packaging capacity of AAV is relatively small (less than 4.8 kb). Therefore, each TALEN (each 4kb) and SpCas9 (4.2 kb) with gRNA needs two separate vectors for packaging that was reported to reduce the efficiency of genome editing. SaCas9 is smaller (3.1 kb), so single AAV can carry the Cas9 and gRNA together [12]. There were reported successful preclinical studies of in vivo genome editing with AAVs. ZFN delivery with AAV to mouse model of hemophilia could achieve a therapeutic effect both in Hemophilia A and B [40, 79, 80]. Cas9 and gRNA delivery to the mouse model of DMD could achieve exon deletion in dystrophin gene that saved the protein and resulted in improved muscle function [81].

2) Nonviral vectors: Plasmids could be used for in vivo delivery of genome editing platforms. Hydrodynamic tail vein injection of plasmid carrying CRISPR/Cas9 and donor template was shown to be effective in hereditary tyrosinemia mouse model [36]. It was also shown that HBV replication could be inhibited by targeting HBsAg using plasmid carrying CRISPR/Cas9 expression cassette in HBV infected mouse model [82]. Cationic lipid complexes and nanoparticles have also been studied recently for in vivo delivery of genome editing platforms [83, 84]. Recently, the delivery of Cas9 ribonucleoprotein and donor DNA conjugated via gold nanoparticles (CRISPR-Gold) was shown to induce HDR and correct a point mutation in DMD mouse model via intramuscular injection more efficiently than ribonucleoprotein and donor DNA without particles [85]. CRISPR/Cas9 RNP delivery system seems to be better than other systems because it results in transient delivery, no insertional mutagenesis, low off target effect and low immunogenicity. However, there are few in vivo studies with this method, so it also needs to be improved before translation into clinics.

Each delivery system has its advantages and disadvantages but none of these systems has been ideal yet and they are still in need of improvement before wide clinical applications.

III-D) Problems in ethical and regulatory framework

Although genome editing technology has been developing rapidly, development of ethical and regulatory frameworks which ensure their safe and

effective clinical implementation are not as rapid as the technology itself. In case of implementation, genome editing has two areas of concern: Firstly, germline cells or somatic cells will be edited and secondly, the purpose of editing is therapy for a disease or enhancement of human properties such as memory, height, etc. According to a public survey conducted in more than 10 countries, the public view favors genome-editing in adults for therapeutic purposes and disapproves this technology especially in prenatal-life for enhancement purposes [86]. This study showed us that the public participation should be taken into consideration during policy making process for human genome editing and it is important to define internationally accepted regulatory issues to avoid different regulations on the uses of this technology.

Besides moral considerations about editing human germline, genome editing technology cannot be applied ethically in pluripotent/germline cells due to the lack of enough knowledge about its safety issues at present level of development. However, it is a rapidly developing area. Therefore, regulations about it should be developed as early as possible. In contrast, genome editing technology can be applied to human somatic cells as safety and efficacy assessments are done extensively and according to the standards defined and the risk to benefit ratio is acceptable. In this context, very rare diseases become a concern because it is impossible for them to pass every detailed safety and efficacy assessment and they need some exceptions [87].

In case of editing in human embryos, there exists the guidelines of US National Academy of Sciences (NAS; Washington, DC) and the International Society for Stem Cell Research (ISSCR), which give permission to germline editing of nuclear DNA only for embryo research without implantation for reproduction [88]. An important example of this kind of permitted study came from Britain: By using CRISPR genome editing in human embryos, researchers could reveal the role of OCT4 gene in early human development [89]. This knowledge and further studies can be used to improve infertility treatment procedures in future. Additionally, the NAS defines ten necessary criteria for future in vitro germline editing research that might be permitted to cross over into first-in-human clinical trials for reproduction. These are important to guide and motivate researchers for choosing their special disease to pursue their research to pass all preclinical safety and efficacy assessments and be ready for approval processes. In contrast to the early attempts of embryo editing that resulted in mosaic outcomes and off-target effects [90-92], a recent study from US reported that MYBPC3 gene mutation which causes hypertrophic cardiomyopathy was successfully corrected in viable human embryos by using CRISPR/Cas9 without off-target effects and with very low level of mosaicism [93]. This success shows that genome-editing in human embryos is not far from being used in fertility clinics as a complementary approach to preimplantation genetic diagnoses. This situation raised a concern to be discussed about safety assessment of this implementation which requires a long term follow up, not only in the original trial but over generations. There exist no ethical or regulatory guidelines for this concern. Thus, ethical guidelines of germline editing should be newly designed [94].

III-E) Clinical applications

In addition to previously mentioned ongoing and completed ex-vivo clinical trials in HIV infection and cancer, there exist planned clinical trials using genome-editing technology [26]. Therapy for Beta-thalassemia and Sickle Cell Disease by using CRISPR are the ones, permissions for clinical trials were asked for relevant institutions. Also, treatment of Leber Congenital Amaurosis type 10 is also one of the planned clinical trials by using CRISPR genome editing [49]. In addition to planned CRISPR trials, genome editing clinical trials with Zinc-finger nucleases have already started for the treatment of hereditary metabolic diseases such as MPSI and MPSII, hemophilia B [26, 50]. In near future, as the preclinical studies continue to improve our knowledge about safety and efficacy issues, the number of these trials will definitely increase to cover more diseases.

CONCLUSION

Genome-editing technology is rapidly developing and seems to overcome the problems mentioned above in near future. This technology is very helpful in understanding the function of newly described genes, the role of the genome in diseases by easy in vitro and in vivo modeling and lastly this technology gives us the chance to cure diseases that do not have a therapeutic option now.

Hacettepe University is a major reference center for rare disorders with high in-out patient capacity. As Medical Biology Department in collaboration with the other departments of the university used these availability of diversified patient phenotypes as an advantage and conducted a TUBITAK project in order to establish a Zebrafish Research Laboratory to investigate effects of new mutations on zebrafish (Project No: 214S174). The first step of the project is to create homozygous knock-out desmin a and desmin b zebrafish models that was completed in

February 2016. Then as a second step, we mimicked a desmin frameshift mutation in zebrafish that destroys desmin-lamin B interaction [95] and causes LGMD2R (MIM 615325) in humans, using TALEN genome-editing platform. This mutation was shown to cause muscle degeneration in zebrafish. Now as a third step, we are studying the effects of this mutation in mechanotransduction. In the long run, we aim to investigate the role of different muscular dystrophy genes identified by our group and mutations in different genetic diseases. Furthermore, we have planned to form a catalogue of mutant zebrafish stocks consist of specific mutations that we hope to contribute to future studies of drug, treatment and more. For this purpose, we had an infrastructure grant from the Hacettepe University Scientific Research Projects Council for Modelling human rare diseases in zebrafish by using CRISPR/Cas9 editing platforms and maintaining sustainability (Project No: 12735). With this project the capacity of our Zebrafish Research Laboratory will be increased, and our genome-editing research infrastructure will be improved for modelling various rare genetic diseases. Finally, we will have a chance to maintain and promote our competetive research capability in the genome-editing era.

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