Problematique Of Use Of The Crispr/Cas9 System To Create And Research Cellular Models Of Cardiovascular Inherited Human Diseases

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Abstract: The genome editing technology has become a powerful method for creating genetically modified cells and organisms, necessary for the genes function and mechanisms of human disease determination. The clustered system with regularly interspaced short palindromic repeats (CRiSPR-), associated with9 (Cas9), quickly became one of the world's major approaches to genome editing in basic biomedical research in recent years as a result of its simplicity and adaptability.

Genome editing CRiSPR/Cas9 werebeing used for correction mutating people's DNA from one base pair to large deletions both in vitro and in vivo. Model systems CRISPR/Cas9 are usedfor a better understanding of many aspects of cardiovascular disease, including lipid metabolic, electrophysiology and genetic heritage. CRiSPR/Cas9 technology has been proven to be effective for editing induced pluripotent stem cells. In spite of these gains, some biological, technical and ethical problems limit therapeutic capacity to edit the genome by cardiovascular diseases. The purpose of the study is to analyze the applications of CRiSPR/Cas9 genome editing in the cardiovascular system, for both disease research and treatment prospects by editing the genome in vivo in the future.
Key words: CRISPR/Cas9, heredity, cardiovascular system, genes, genome.

1. INTRODUCTION

Ability to conduct gene therapy, correcting certain pathogen mutations in the embryonal stage, appeared more than 40 years ago. In the 1980-s, when the transduction capabilities of retroviruses became better understood, scientists have investigated the clinical efficiency of these viruses, for use in gene therapy. It has been demonstrated that retroviruses can correct phenotypes in patients' cells. However, the retroviruses use presents some serious obstacles such as their instability, toxicity, and inability to infect nonproliferating cells.

For those reasons there were developed other delivery tools for viral and innocent genes, which include adenoassociated virus vectors (AAV), herpes and liposome vectors. The first clinical trial of cardiovascular gene therapy was gene delivery, gene coding for the vessel

endothelium growth factor (VEGF), for patients suffering from peripherical disease, but it was not considered the ideal treatment, since many patients had lesions, making it impossible to deliver genes.

During the first decade of the century efforts focused on developing effective and safe tools to deliver genes into patient's cells. By mutagenesis with insertion and replacement were developed modernized vectors AAV, which are safer and more tolerable. Recently, genome editing technology became a new powerful method of determining the genes function and correcting variants, causing human diseases, which allows to produce genetically modified cells and organisms. The main features of genome editing tools are presented by the fact, that they rely on nucleases that introduce DNA double-strand break to trigger a gene mutation, stimulating endogenous reparation mechanisms. The most important part is introduction of DSB in a specific target area.

This specificity of action is influenced differently by different instruments, the most commonly used are zinc finger nucleases (ZFNs), effector nucleases like transcription activator (TALEN) and interspaced short palindromic repeats at regular intervals (CRiSPR-), coupled systems 9 (Cas9). Of which CRiSPR/Cas9 system which was first described in 2012 by Jinek et al., made significant progress in this area [1].

This system is simple and efficient enough to edit genes in virtually any organism and cell types, and due to its simplicity and adaptability, CRiSPR/Cas9 has quickly become one of the most popular genome editing approaches.

In the context of a basic understanding of the disease molecular mechanisms and its treatment, the CRiSPR/Cas9 system is just beginning to reach its potential, being an extremely powerful tool for the continuation of basic research, and applying for new clinical techniques. The clinical trials are ongoing testing use CRiSPR/Ca9-edited human cells, for example to cure cancer.

Cardiovascular diseases continue to be a serious health problemimproving knowledge of mechanisms, underlying development, both common and less common causes of cardiovascular disease and deaths remains a challenge [2].

Cardiovascular diseases are divided into several types of diseases, affecting the blood vessels and the heart. It's mostly coronary heart disease (CHD), cerebrovascular disease (stroke), peripheral artery disease (PAD), cardiomyopathy, rheumatic heart disease, arrhythmia, hypertensive heart disease and congenital heart defects. Owing to genetic tests and bioinformatics analysis, modern medicine makes it possible to identify potential patients prone to certain heart diseases more and more rapidly. However, further mechanistic research aimed at understanding the causes of diseases, limited by the fact that the assignment and cultivation of primary human cardiomyocytes for cardiovascular research, extremely difficult [4].

In this context, researchers around the world have invested time and resources to improve animal and human disease cell models. CRiSPR technology / Cas9 was used to create mouse models of genetic diseases, such as severe cardiomyopathy, in a shorter time than traditional homologous recombination techniques. In addition, it is now possible to inject CRiSPR/Cas9 into cell embryos of rats, rabbits and primates used to study cardiovascular diseases [5].

With advent of induced pluripotent stem cells (iPSC) researchers have also been able to create alternative cell models for the study of the disease's molecular mechanisms. In that regard, CRiSPR/Cas9 technology provides a simple mechanism to find out how cells behave incorrectly, by allowing reversion of the causal mutation. Finally, in the area of the cardiovascular system important progressive steps have been taken towards therapeutic application.

This work focuses on different applications of the CRiSPR/Cas9 genome editing tool in the region of hearth, from its application to human and animal models to his therapeutic potential, with a description of the advantages and potential disadvantages of the system.

2. MATERIALS AND METHODS

By analysing the data presented in the scientific literature, we had reviewed the mechanism and application of CRiSPR/Cas9 technology for the management of hereditary diseases in particular the cardiovascular system.

CRiSPR/Cas9 technology.CRiSPR/Cas9 technology is based on the adaptive immunity of Streptococcus pyogenes.

Nuclease Cas9 mediates anti-phage activity thanks to its combination with locus clusters of short palindromic repeats (CRiSPR) at regular intervals. These loci are short repetitive sequences consisting of 30-40 p.a. and intercalated spacer sequences relevant viral genomes. CRISPR loci transcribe into long RNA which is subsequently split by CRiSPR-associated endorionucleases (Cas) with release of small RnasCRiSPR (crRNA). These crRNAs then form the Cas-RNA complex, which recognizes the genome of the virus and begins to cleave it [6].

In its modified form for genome editing, the CRiSPR/Cas9 system consists of a sequence of RNA guides (gRNA), aimed at Cas9 nucleases at a specific location in the genome.gRNA consists of a short sequence of RNA, necessary for binding cas9 plus nucleotide sequence, is calledspaceprotor, which defines DNA-targets for modifying [7].

CRiSPR/Cas9 system has many other functions, in addition to gene editing, for example, regulation the expression of endogenous genes. Nucleasedeficient Cas9, called «dead cas9» (dCas9), was developed to create inactive fused proteins [10].

Binding dCas9 to transcription repressor, such as KRAB (CRiSPRi) can reduce the transcription of human endogenous genes. This approach was described for progressive reduction of gene expression in eukaryotic models. Most recently, it has been demonstrated that it suppresses gene expression also in bacteria and man-induced pluripotential stem cells. Similarly, merging dCas9 with the transcription activation domain, such as VP64 or p65 (CRiSPRa), may increase the expression of human endogenous genes. CRISPRa also provides the activation of multiplex genes, using single gRNAs (sgRNA), targeting more genes at the same time.dCas9 can also be fused with functional domains of methylation enzymes or DNA demethylation or histone modifiers for editing the epigenome [11].

3. RESULTS AND DISCUSSION

One of the tasks of genetic engineering is to rid humanity of hereditary diseases. In this regard, the existing methodologies are constantly being improved, in order to achieve as close as possible to the desired result. Advances in science have also led to improvements in genome modification techniques CRISPR/Cas9.

Nuclease Cas9 contains HNH-nuclease and RuvC like nuclease regions. After binding protospacer-adjacent motif, Cas9 of its HNH and Ruvc catalytic regions, generate a blunt double-stranded break (DSB), which can be repaired either by a nonhomologous ends junction (NHEJ), or by means of a system of homologous reparation (HDR).

NHEJ recovery occurs mainly during G1 phase, while HDR is most visible during phases S and G2. NHEJ does not require a reparations matrix or extensive DNA synthesis, and it is faster than HDR in DSB repair. Sometimes it provides small inserts or deletions (indels) into the site of splitting [8].

If an indel occurs in a gene coding sequence it leads to a shift of the reading frame, which could lead to gene infusion, which makes it unsuitable for gene correction.

On the contrary, HDR uses a DNA matrix or non-mutant homologous chromosome to achieve precision reparations and can be used for injection of an exact mutation or insertion by

recombination. HDR efficiency in DSB recovery is relatively low and less often than NHEJ in human proliferating cells.

HDR efficiency can be facilitated by temporary inhibition of NHEJ. It should be noted that there are a limited number of PAM spaces (protospacer adjacent motif) in the genome of eukaryotes, a restriction for the accuracy of the initial genome editing points.

To solve this problem, the number of target sites in the genome was enhanced by the CRiSPR/Cas system profile in different bacteria with different PAM-locations [9].

Several studies used the advantages of the HDR recovery apparatus for precision point mutations or noquins in the target gene providing a homologous matrix of restoration. Repair mechanism NHEJ is the preferred method of injection inserts and/or deletions, that could disrupt the target locus.

It is also possible to generate large deletions or genomic alterations such as inversions or translocations, using a pair of Cas9 nucleases directed towards gRNA [10].

Cas9 nucleases were created to modify nuclease activity or selectivity of binding. Cas9d10a, mutant CRiSPR-nikase, was obtained for sampling single-stranded DNA in target sequence. Thanks to this double-click approach, Cas9d10a creates «sticky ends» that guarantee that a fragment of DNA is inserted into the genome in the correct orientation. CRISPR / Cas9 was also used to mark living cells, dCas9 binding to fluorescent proteins to visualize specific genomic loci.

CRiSPR/Cas9 system for heart disease in human cells. The introduction of genome editing has revolutionized basic and translation research with the innovative discovery of induced pluripotent stem cells.

Due to their close resemblance to embryonic stem cells (ESC) and to that fact, that they are not burdened with the same ethical issues of induced pluripotent stem cell (iPSC), are currently the most appropriate model for studying cardiomyogenesis in human cells.

In addition, iPSC are an almost infinite source of human cardiomyocytes and constitute an indispensable tool in the field of cardiology, especially in the absence of human models in vitro [12].

Given that the iPSC show phenotype, which is still far from the phenotype of adult cardiomyocytes, the scientific community put a lot of effort into developing protocols, capable of improving their maturation in vitro, to create better models of heart pathologies.

To date, several heart diseases, which proved to be a good model for hereditary arrhythmia disorders, were investigated with induced pluripotent stem cells.

Specific iPSC received from people and families affected by long-range QT syndrome type 1, and induced to differentiation into functional cardiac myocytes [13].

These cells showed longer action potential, altered activation and deactivation of potassium rectification current (IK) with delay and an anomalous response to catecholamine, which is an electrophysiological feature of the disease. Accordingly, iPSC-CM received from a patient with catecholaminergic polymorphic ventricular tachycardia (CPVT), showed abnormal electrophysiological characteristics, including delayed postdepolarization.

Besides iPSC-CM were created to study single-cell changes in structural heart defects, that are detected in dilated cardiomyopathy (DCM) and by hypertrophic cardiomyopathy (HCM) [14].

Generated cardiomyocytes from iPSC patients with DCMP, carrying a mutation in the gene encoding the sarcomeric protein cardiotroponin T, and showed that the iPSC-CM reproduce morphological and functional phenotypes of the affected heart, such as the modified processing of Ca2+, inotropism reduction and anomalous distribution of sarcoma in alpha-actinin.

Thus, it is clear that parallel advances in genome editing and iPSC technologies now offer opportunity to explore the pathophysiological mechanisms of hereditary heart diseases directly on human cell models.

In this case, the CRISPR/Cas9 tool allows relatively efficiently, and easily creates isogenic cell lines, DNA sequence of interest only, thereby eliminating other factors affecting genetic background and epigenetic memory [15].

It has now been proven that the CRISPR/Cas9 system is an effective and useful approach to create the KO gene, or a caged in human cells, and in particular the iPSC.

Moreover, the system has the potential to correct genetic mutations in models, diseases, connected with iPSC and has already begun to be applied to the study of various heart diseases, such as Bart's syndrome, X-linked genetic heart disease.

In particular, an excellent example of the potential provides a combination of CRiSPR/Cas9 and iPSC technologies [16].They created the patient's iPSC with Bart's syndrome and characterized the mitochondrial CM anomalies, related to this pathology. By injecting mutation into the Tafazine (TAZ) gene in the iPSC from healthy donors through Cas9-mediated genome editing, they also demonstrated a causal relation between TAZ gene mutation and mitochondrial phenotype [17]. It is important to note that the mitoTEMPO-antioxidant injection by Bart induced iPSC-CM syndrome was effective in suppressing excess mitochondrial AFC products has resulted in the normalization of sarcomer organization and contraction.

More recently, the CRiSPR/Cas9 system was used to evaluate the pathogenicity of taitine gene mutations by dilated cardiomyopathy.

Missense mutations ortaitin mutation withreading frame shift were introduced into the IPSC and the contractile deficit was subsequently estimated in the iPSC-CM [18]. In addition, iPSC-KM was received of two independent patients groups with Jerwell and Lange-Nielsen syndrome (JLNS), which is one of the most severe cardiac disorders and heart arrhythmia. The cardiomyocytes showed the characteristics of JLNS, including IKs extension validity potential [19].

Most recently, Yamamoto et al. established a disease-specific clone of iPSCFrom an individual with 3 QT Long Stem Cells International (LQTS), carrying heterozygous CALM2 mutation, as a model of in vitro disease reproducing the phenotype of disease. They also cut out a mutant allele using the CRiSPR/Cas9 system and restored abnormal electrophysiological properties.It is important to note that CRiSPR/Cas9 can also be used to make DNA changes to noncoding areas. This approach was successfully used in the human iPSCto remove the sequence adjoining the intron single-nucleotide polymorphism, in the gene PHACTR1, strongly related to premature myocardial infarction [20].

*Strengths and weaknesses of CRiSPR/Cas9 system.*CRiSPR/Cas9system has many advantages compared to other existing technologies. Cas9 enzyme goes to a certain DNA sequence with a single RNA guide (gRNA) that can be easily cloned. On the contrary, ZFN and TALEN are used proteins of constructed DNA-binding sequences and the fission points of Fok I. Since the Fok I domain must be dimerized, to be an active, two proteins are needed for genome editing experiments compared to one gRNA of the CRiSPR/Cas9 system [21].

Using several gRNA we can affect several genomic loci, simultaneously introducing mutations into several genes.CRiSPR/Cas9 has demonstrated its effectiveness in editing various human cells. Moreover, CRiSPR/Cas9 can be used to screen non-coding areas of DNA to identify regulatory elements to understand how genetic variations are related to human diseases. For example 9 Dietary cell transplants were identified using CRiSPRi, Fulco et al. approach [22].

There are two possible approaches to genome editing by cardiovascular disease. Somatic cells can be isolated from patients and reprogrammed into IPS cells. Patient-specific

iPSC can be modified ex vivo, and after editing, they can be differentiated and transplanted back to the patient.

Also mutations can be directly edited in vivo, delivering the CRiSPR/Cas9 integrated delivery tool to the desired genomic place in a particular tissue [23].

Despite the huge progress achieved in our genome editing capabilities, thanks to the innovations of CRiSPR/Cas9, some problems remain.

Therapeutic genome editing capability is still limited by biological and technical problems by cardiovascular diseases, and it's just beginning to work.The main limitation of CRiSPR/Cas9 is that PAM sequences are needed to target and bind nucleases [24].

Two approaches to the problem include: increasing the number of PAM sequences using sequences from different bacteria and change the specificity of the PAM S. pyogenes Cas9 sequence. Another major problem related to application of the CRiSPR/Cas9 system for mutagenesis of animal embryos, related to non-earmarked effects, which were described with high frequency in human cells. Non-targeted effects are the result of non-specific activity of Cas nuclease in non-target genome regions due to incorrect binding of sgRNA (single guide RNA).

Various groups estimated the frequency of non-target events in projected non-earmarked locations. In one study editing the genes CCR5 and HBB, some structures generated up to 58% of the mutation rate in secondary but related DNA sequences CCR2 and HBD in cells, transfected CRISPR/Cas9. However, it was demonstrated that non-target events differ for different cell types.

In fact, other studies have shown the human iPSC, that the system provides effective tools for editing the high-level genome.

In addition, studies on whole organisms show lower deviation frequencies compared to previous studies on cancer cell lines. These studies on mice and monkeys did not reveal any mutations in predicted sites outside the target with use CRiSPR/Cas9 in genome editing for zygotes. Some bands attempted to reduce non-target effects by modifying the Cas9 binding site. Breaking some Cas9 binding sites, they were able to cut DNA on the targets, which led to low or no connection to the target.

Another attempt to reduce the non-targeted effects were reports about use of paired Cas9 nicase instead of one Cas9, which significantly reduces unearmarked splitting by 50-1000 times. Although there may be a few non-targeted events, they should not be underestimated, because other genes can mutate with potentially dangerous outcomes. In particular for the clinical use of genome-modified cells or tissue, it's important to completely avoid the unintended effects.

Another problem is the variability in the efficiency of genome editing across tissues, especially in vivo. The effectiveness of CRiSPR/Cas9 in skeletal and cardiac muscles is lower than in the liver. Even in cardiospheric transgenic mice Cas9's editing efficiency in heart cells is rather low. Further concerns about the CRiSPR/Cas9 system relates to its editing efficiency: sgRNAs induce Cas9-mediated DSB in the desired destination site. DSB stimulates DNA recovery through HDR. However, an alternative NHEJ DNA repair mechanism can occur at lower frequencies causing unpredictable consequences of small inserts and deletions [25].

4. CONCLUSIONS

Currently editing the genome became a powerful tool for modifying cell lines and organisms to study biology and pathophysiological mechanisms of various genetic illnesses. Genome editing tools have recently begun to be used also in the area of the cardiovascular system to create new cellular and animal models of cardiovascular disease.

Therapeutic potential of genome editing continue to have biological and technical barriers. Another important issue is ethical concern about the use of CRISPR technology in

humans. Progress in genome editing will allow us to better understand the development and pathogenesis of diseases, and also try to treat cardiovascular diseases.

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