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Recent Advances in Gene Therapy and Modeling of Chronic Granulomatous Disease

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ABSTRACT

The Chronic granulomatous disease (CGD) is a primary immunodeficiency that characterized by mutations in phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in deficient antimicrobial activity of phagocytic cells and recurrent childhood infections. Hematopoietic stem cell transplantation (HSCT) is a curative option for patients with human leukocyte antigen (HLA) matched donor, when conventional cares and therapies fail. However, in many cases when the patients have not an HLA-matched donor, they need to a method to recapitulate the function of the affected gene within the patient's own cells. Gene therapy is a promising approach for CGD. While, the success of retroviral or lentiviral vectors in gene therapy for CGD has been hampered by random integration and insertional activation of proto-oncogenes. These serious adverse events led to improvement and generations of viral vectors with increased safety characteristics. Gene therapy continues to progress and the advent of new technologies, such as engineered endonucleases that have shown a great promise for the treatment of genetic disease. This review focuses on the application of gene therapy for the CGD, the limitations encountered in current clinical trials, advantages and disadvantages of endonucleases in gene correction and modeling with CRISPR/Cas9 approach.

Keywords: Chronic granulomatous disease; Endonucleases; Gene editing; NADPH oxidases

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INTRODUCTION

Chronic granulomatous disease (CGD) is one of the lethal primary immunodeficiencies (PIDs) with an incidence of 1:250.0001 which characterized by severe bacterial and fungal infections. CGD is caused by the mutation in the genes encoding the phagocyte NADPH oxidase that normally have critical roles in the production of Reactive oxygen species (ROS) which is required to neutralize the pathogenic microorganism and to prevent inflammatory reactions.[2-5] NADPH oxidase is a transmembrane complex enzyme with multiple components including, gp91^{phox}, p22 ^{phox}, p47 ^{phox}, p67 ^{phox}, and p40 ^{phox}. About 60% of CGD cases are X-linked (XL) and caused by mutations in the CYBB gene encoding for gp91^{phox} protein. The other form of disease is autosomal recessive (AR) CGD and most common form of AR-CGD is mutation in Neutrophil Cytosolic Factor 1 (NCF1) encoding p47- phox (about 20%) and another, mutations in NCF1 (6%) and CYBA (6%) genes encoding p67 phox and p22 phox respectively (Table 1)[6-8]. Mutation in NCF1 encoding p40-phox has been reported in one patient that has prevented activation of the NADPH oxidase at intracellular membrane without any effect on NADPH oxidase assembly.[9] Another study demonstrated a critical role of phosphatidylinositol 3-phosphate [PI(3)P] binding domain in p40^{phox} for the optimal activation of NADPH oxidase in macrophages.[10] Gene therapy of autologous hematopoietic stem cells (HSCs) or induced pluripotent stem cells (iPSCs) by delivery of a functional copy of mutated NADPH oxidase subunit is another promising approach to treat high-risk CGD patients without HLA-matched donor for HSCT. In this alternative approach, wild-type of the mutated gene transfers into autologous hematopoietic stem cells using retro or lentiviral vectors. Although, CGD patients were functionally corrected early after treatment, however, insertional activation of PRDM16 and EVI1 could lead to life-treating myelodysplasia. This demonstrates the potential of successful, however, the alternative strategies to eliminate the risk of insertional oncogenesis is needed. Genome editing technologies are powerful therapeutic strategies for gene correction at the precise location through introducing site-specific double-strand DNA break (DBS) by engineered endonucleases such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 system. The DSBs are repaired by error-prone non-homologous end joining (NHEJ) or through homologous recombination, in which repairs DNA accurately in the presence of single-stranded DNA template. Reprogramming of adult somatic cells into induced pluripotent stem cells (iPSCs) is a newly never-ending search approach for various diseases. Patient-specific disease model creations as a valuable in vitro model of CGD at the cellular level hold a great promise for correction of disease-causing mutations by gene targeting. However, future investigations are needed for the standardization of technology in clinical practicing. This review focuses on the molecular and cellular mechanism of CGD and application of gene therapy for the CGD disease and discusses the preclinical results and recent phase I/II trials. We also describe the advances in vector design and novel genome editing technologies in cell modeling and gene correction.

Molecular and Cellular Mechanism of CGD

NADPH oxidase enzyme oxidizes NADPH and reduces molecular oxygen to form superoxide ions (O^{2-}) and their derivatives in order to protect the cells from infectious pathogens such as staphylococcus and Aspergillus. NADPH oxidase system in phagocytes consists of two main subunits, a membrane-bound catalytic subunit (gp91^{phox}, p22 ^{phox} units) and a cytoplasmic soluble component (p47 ^{phox}, p67 ^{phox}, p40 ^{phox}). The assembly of various subunits of the NADPH oxidase system and their cellular activation is shown in figure 1. Activation of the NADPH oxidase system is occurred after activation of neutrophils during phagocytosis by movement of soluble cytoplasmic components, p47^{phox}, p67^{phox}, p40^{phox} and binding to cytochrome-b558 components gp91^{phox}, p22^{phox}. Formation of superoxide ions detected in the extracellular component by transfer of electrons from NADPH to oxygen and reactive oxygen. Then activation of superoxide dismutase (SOD), catalase, and myeloperoxidase (MPO) generate reactive oxygen species including hydrogen peroxide (H2O2), H2O and hypochlorous acid (HOCL), respectively. However, a defective phagocyte NADPH oxidase in CGD patients prevents the formation of superoxide ions. The most severe case of CGD (60%) is X-linked form of the disease, in which the affected gene is CYBB. The CYBB gene codes the 91-KD polypeptide as an alpha

catalytic subunit (gp91^{phox}) of the catalytic subunit of the phagocyte NADPH-oxidase which forms a heterodimer with the integral membrane protein. In autosomal recessive forms of mutations in the genes encoding p22 ^{phox}, p47 ^{phox}, p67 ^{phox} longer survival rates have been reported compared to X-CGD.[11] However, AR form of CGD is the most common form (87.1%) among Iranian families and patients while XL-CGD is only 12.9% (Table 1).[12]

Recent Gene Therapy Clinical Trials for CGD

Although bone marrow transplantation (BMT) is an attractive option to treat children with CGD, in many cases, HLA matched donors can be hard to find. Gene therapy is a promising technique that has the potential to cure monogenic disease by introducing a functional copy of the defective gene into appropriate cells.

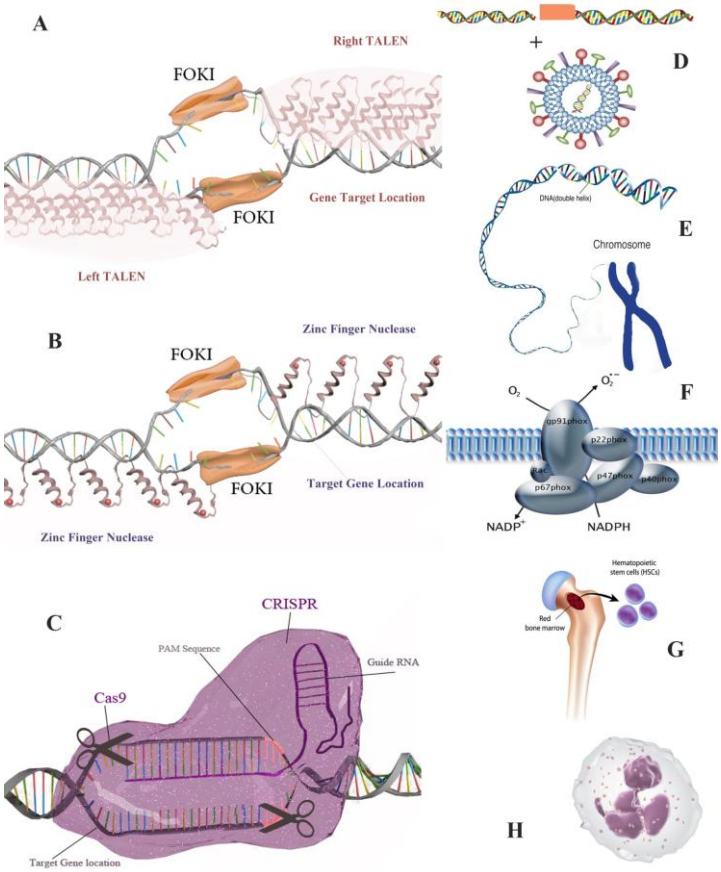

Figure 1. A. TALEN system is composed of two recognition motif which binds to 14-23 base pair of target gene location specifically, after right and left TALEN motifs attachment, the deoxyribonucleotides FOK1 which is attached to the binding Talens' motifs can cut the target gene location. B. Zinc Finger nucleases (ZFNs) are the recombination systems which consist of specific motifs containing zinc element. After ZFNs attachment on specific site of DNA, the FOK1 nucleases can cut the target DNA between two domains of ZFNs. C. CRISPR/Cas9 system containing guide RNA which can bind to the 18-24 bp of the target gene. PAM region in downstream of gRNA improves the specificity of the CRISPR system. The Cas9 nuclease after gRNA binding and three strand DNA formation can cut the target site. D. To gene editing, the double-stranded breaks must be corrected by homologous recombination. E. After gene manipulation by one of the recombination's methods and using guest DNA, the DNA footprinting can show the stable gene editing. F. A corrected gene must have a complete function of the protein and must help to complete NADPH oxidase complex formation. G. After editing by gene editing nucleases, the bone marrow would be depleted from previous stem cells. Then, transplantation can be performed by patient-specific corrected stem cells. H. After stem cell transplantation, the patients' neutrophils would be followed and screened for the correct function of oxidative burst.

Table 1. Comparison of different subtypes of CGD in Iran and large cohort studies in other countries

Patients	XL-CGD	AR-CGD			Unknown	Total
	CYBB	CYBA	NCF1	NCF2		
Iran[12]	12/93 (12.9%)	17/93 (21%)	45/93 (55.5%)	5/93 (6.2%)	14/93 (17.3)	93
Europe[16]	290/424 (68%)	22/429 (5%)	69/429 (16%)	11/429 (3%)	37/429 (9%)	429
United States[15]	259/368 (70%)	7/368 (2%)	45/368 (12%)	10/368 (3%)	28/368 (8%)	368

CGD is a suitable candidate for treatment by gene therapy and it has been undertaken for more than 15 years.[13] Gene therapy trials to treat X-CGD were initiated in late 1990 by gammaretroviral (γRV) vector with limited success. Early clinical trials were reported without bone marrow conditioning. Conditioning of the patient's marrow before gene-therapy was identified as an essential prerequisite to increase the efficiency of gene-corrected hematopoietic stem cell engraftment. Several trials, at different clinical centers worldwide, have used the non-myeloablative conditioning and γ-retroviral gp91^{phox} vector to promote long term clinical benefit.[14-16] In several patients, gp91^{phox} expression and function have been improved temporarily without clinical benefit due to very low levels of gene-marked neutrophils presence in the periphery for up to 6 months.[17] Initial preclinical studies have been reported in the mouse model of CGD using recombinant retroviral vectors. functionally normal neutrophils with an increased defense again photogenes were detected .[18] In these studies, the obtained human cell lines and primary CD34$^+$ cells from X-CGD patients were genetically manipulated to restore the NADPH oxidase activity.[19] Clinical trials performed by Kang *et al*. at the National Institute of Health, three X-CGD patients with an average age between 19-23 years were treated with a gp91^{Phox} expressing gamma retroviral vector. After transduction, initial efficiencies were between 25-73% positive cells. The cells were reinfused into the patients after bone marrow conditioning regimen consisting busulfan at a total dose of 10 mg/kg. The percentage of functionally modified cells in the peripheral blood of P1 was decreased from 24% to 1% at 7th month and in P2 decreased from a peak of 4 to 0.03% at 11th month after gene therapy. Whereas in P3 no corrected cell could be detected in peripheral blood after 4 weeks.[20] Similarly, two adult patients (25 and 26 years old) were treated in Frankfurt using myelosuppression with an 8mg/kg dose of busulfan. Initial gp91^{phox} expression level was between 40-45% at the end of 5th -day transduction period. After 5 months, the expression level of gp91^{phox} in peripheral blood neutrophils has been increased up to 15%. Also, after transduction, some retroviral insertions found in myelodysplastic syndrome 1-ecotropic viral integration 1 (MDS1-EV11), PRDM16 proto-oncogenes and monosomy observed in chromosome 7 in both patients. One of the patients died 2.5 years after gene transfer due to septic shock and myelodysplastic syndrome. While the second patients underwent an allogenic stem cell transplantation from a fully matched donor.[21] The same protocol and vector were used in Switzerland to treat two pediatric patients. Both patients suffered from a life-threatening fungal infection. In one subject an MDS was developed with monosomy 7 and in second subjects was not diagnosed. MDS and cured by early allo-HSCT and followed for 3.2 years. Finally, both subjects cured of CGD by allo-HSCT after gene therapy.[16] In a similar study conducted in London, they used gamma retroviral vector for the transduction of mobilized peripheral blood CD34$^+$ cells obtained from four patients and preconditioning with melphalan at a dose of 140 mg/m^2. In this trial, initial gp91^{phox} expression levels were less than 10%.[22.]Gene therapy trials were reported in X-CGD patients by using different protocol and vectors. On a trial performed by Kang E.M et al, two patients were treated with autologous CD34$^+$ cells transduced with long terminal repeats (LTRs) driven murine leukemia virus-based vector (MT-gp91^{phox}). After bone marrow conditioning regimen consisting of fludarabine at 40 mg/m^2/day and busulfan at a dose of 3.2 mg/kg/day, the frequency and efficacy of functionally corrected cells were investigated by NADPH oxidase assay. The level of functionally corrected cells has been decreased shortly after transplantation and three years later, it has been reached only to 0.05 (P1) and 0.21%

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(P2). No hematological malignancy was observed, although some retroviral vectors were integrated within or close to the proto-oncogenes MDS1-EV11, PRDM16, and CCND2.[20] A new generation of self-inactivating (SIN) lentiviral vectors (LVs) could be produced in response to various hematological cancers.[23] Currently, SINLVs were used in ongoing trials for different types of X-CGD and have shown effective immune reconstitution with no vector related complications.[24,25] Recently, miR-126 restricted LV vectors were used to improve transgene expression in myeloid cells and avoid ectopic expression. The use of this regulated LV vector was shown high-level expression of the transgene in myeloid cells without toxicity.[24, 26] Several early phases of clinical trials and a multicentral trial between Germany, the United Kingdom and Switzerland of CGD have been summarized in Table 2.

Advancing Viral Vectors

Success in gene therapy is dependent on the suitable and efficient vectors having high safety profile. Different types of trials in the 1990s designed to transduce retrovirus vector backbones encoding p47 phox and gp91 phox cDNA into peripheral blood mobilized autologous CD34+ (PBSC) with the various cycle of gene therapy.[17,27] In these trials neutrophils with oxidase activity were persisted for only a few months. The absence of bone marrow conditioning procedure and transient appearance of gene-corrected cells was a critical problem. Therefore, the investigators used non-myeloablative conditioning approach. They used retrovirus-derived murine spleen focus-forming virus (SFFV) vector) encoding gp91 phox to transduce autologous $CD34^+$ peripheral blood stem cell (PBSC) to treat X-CGD.[14,15] Gene-corrected neutrophils comprised more than half of the circulating neutrophils and infections of patients were cleared; however, after 2 years, loss of oxidase activity in gene-corrected cells was noticed and patients developed myelodysplasia.[15] These adverse effects of retroviral vectors were, in fact, genotoxic integration into chromosomal DNA in target cells. These events led to up-regulation of proto-oncogenes expression such as MDS-EVI1, PRDM1 or SETB1 that resulted in lethal complications. Furthermore, methylation in viral LTRs led to transgene silencing and missing in expected protein expression.[14,15,28] However, a new generation of SIN vectors such as γRV and LV vectors had been developed. Some of these vectors are now in phase I/II clinical trials for different types of PIDs and other diseases with no serious adverse effects in related to clonal expansion reported.[30] The SIN-RV vectors are the first line of efficient gene transfection vectors in PIDs gene therapies, the long term transgene expression and low genotoxicity are the most important advantages of these vectors.[34]

Some lentiviral vectors in gene therapy protocols are used to change the pattern of gene expression, for example, the over expression of some microRNAs can induce apoptosis in malignant cells and this approach is very important in targeted therapy of cancer.[36] Interesting results have been recently obtained by the use of the alternative lentiviral delivery system and special transgene cassette. In this study, miR-126 was incorporated in a lentiviral backbone for proper regulation of gp91 phox expression in transduced hematopoietic stem cells and avoiding ectopic expression transgene.[24,26] Another alternative strategy

Table 2. Characteristic of chronic granulomatous disease genes

Gene symbol	Chromosomal location	Gene name	Protein	Mutational total
CYBA	16q24.2	Cytochrome b-245, alpha polypeptide (p22-PHOX)	Cytochrome b-245 light chain	63
CYBB	Xp21.1	Cytochrome b-245, beta polypeptide	Cytochrome b-245 heavy chain	684
NCF1	7q11.23	Neutrophil cytosolic factor 1, 47 kDa	Neutrophil cytosol factor 1	34
NCF2	1q25	Neutrophil cytosolic factor 2, 67 kDa	Neutrophil cytosol factor 2	62
NCF4	22q13.1	Neutrophil cytosolic factor 4, 40kDa	Neutrophil cytosol factor 4	4

Data were acquired from HMG (the Human Gene Mutations) and OMIM database. Proteins were collected from The Universal Protein Resource (UniProt)

Table 3. Chronic granulomatous disease (CGD) gene therapy using HSCs in clinical trials

Vectors	Promoter	Patients treated	Centre	Target cells	Conditioning	Recurring since Identifier
SIN-LV	Chimeric	1	US	CD34+ cells	MAC busulfan	NCT02234934
SIN -γRV	Myeloid specific	0	Germany	CD34+ cells	Busulfan	NCT01906541
SIN-LV	Chimeric	1	Switzerland	CD34+ cells	MAC busulfan	NCT01855685
SIN-γRV	Chimeric	2	Switzerland	CD34+ cells	Busulfan	NCT00927134
RV	Chimeric	14	US	CD34+ cells	None	NCT00001476
RV	SFFV	3	US	CD34+ cells	Busulfan	NCT00394316
SIN-LV	Chimeric	5	Germany Switzerland UK US	CD34+ cells	Busulfan	NCT01855685

Table 4. Targeted repairs of chronic granulomatous disease (CGD)

Tools	CGD subtype	Target	Strategy	Edited cells	Condition	Ref
CRISR/Cas9	X-CGD	CYBB	NHEJ	CD34+ cells	In-vivo	(61)
ZFN	Five CGD genotype	CYBA CYBB NCF1 NCF2 NCF4	NHEJ	iPS cells	In-vitro	(57)
ZFN	X-CGD	CYBB	NHEJ	iPS cells	In-vitro	(56)
CRISR/Cas9	X-CGD	CYBB	NHEJ	iPS cells	In-vitro	(60)
TALEN or CRISR/Cas9	X-CGD	CYBB	NHEJ	iPS cells	In-vitro	(59)
CRISR/Cas9	X-CGD	cybb	NHEJ	NSG mice	In-vivo	(63)
TALEN and ZFN	X-CGD	CYBB	NHEJ	iPS cells	In-vitro	(58)

to produce a lentiviral vector with the myeloid specific regulatory element is Cathepsin G and c-FES that designed by multicenter trials. This synthetic chimeric promoter helps transgene expression in myeloid cells and differentiated granulocyte.[37] Phase I/II clinical trial using a SIN-lentiviral vector was currently approved in the UK for the treatment of CGD. In this case, the vector contains myeloid specific promoters from human MRP8, c-FES or miR-223 genes. However, weak activity results were produced in vivo.[38] Today, regulated transgene expression in CGD gene therapy is necessary for efficiency increment, the miRNA recruitment with cDNA transferring arise the efficiency of gene therapy by lentiviral vectors. [39]

Gene Editing Strategies and Results in Correction of Genes Causing CGD

Getting the human genome editing tool has been a great human dream. The discovery of prokaryotic defense mechanisms against viruses promotes the development of gene editing systems in mammalian cells. The genome manipulations methods in mammalian cells are based on DNA repair and recombination pathways.[40] There are generally two pathways of DNA repair: HDR and NHEJ repair systems. Both of these systems are based on DNA cutting by endonucleases and oligonucleotide replacement. In a targeted gene therapy, HDR system is very efficient because this system is pattern dependent.

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Non-homologous end-joining repair system is an error-prone DNA repair system which is based on blunt-end cutting of DNA and gap or nick generation. Today, most gene editing system is based on the HDR system and most genes shutting down mechanisms based on NHEJ system.[41] Gene editing methods comprise many phases: The target gene location finding, to eliminate the previous version of the gene and to replace the desired sequence into the incision site. The first step is necessary for gene location manipulation and the second phase is essential for targeted editing. Second phase pathways in many gene editing systems are comprised of some recombinant nucleases which are modulated by many factors.[42] There are three important gene editing systems: ZFNs, TALENs, and CRISPR/Cas9. These systems can edit and modify the mutated gene sequence to wild type. The main mechanism of these systems is based on homologous recombination which is characterized by two steps: mutated site incision and using correct sequence of the mutated site of the gene as an oligonucleotides pattern for gene modification. The ZFNs, TALENs and CRISPR/Cas9 systems' structure comprise some nucleases which can target some sequences in genome according to amino acid motifs of recombinase enzymes or based on guide oligonucleotides identify regions of the genome that are very similar to the target sequence by these systems termed off-targeting which are the main disadvantage of gene editing tools. So the determination of the off-target activity, off-target frequency and the probability of recognition by editing systems are very important for a successful gene editing prognosis. Today, there are some soft wares and databases that can detect the frequency and sites of off-targets in the whole genome and estimate the probability of nucleases recognition.[43] Off-target site recognition probability by CRISPR/Cas9 system is more than other editing systems and the guide RNA designing without off-target or low probability in CRISPR/Cas9 system is very important. TALENs and ZFN have two identifying motifs which bind to DNA with double checkpoints and these type of attachments reduce the possibility of off-target DNA binding sites.[44] The ZFNs are metalo-enzymes which have zinc ions in their structures (Figure 1B).The DNA-binding domains of ZFNs comprise of the pair of 3-zinc finger motifs and FokI nucleases that can recognize 18 base pairs of sense or antisense DNA. These domains can be engineered according to specific target DNA sequences in mammalian genomes. Specific targeting is mediated by engineering of six zinc-finger tandem repeats, and each of the zinc-fingers recognizes primarily three bases of the DNA target site.[45] The nuclease motif or DNA-cleavage domain (Fokl) should be fused to T-terminus of each ZF domain. Since double strand breakage requires for ZFN/FokI dimerization and both strands coverage, therefore, each part of the ZFN is linked by a linker containing 5-7 base pair and bound to 5' of the target sequence. The ZFN/FokI or SucI (other endonucleases used in some ZFN systems) attachment on soluble stranded DNA cause to 18 base pairs incision, create a gap in the DNA molecule leads to the activation of the repair system. If there is no pattern of oligonucleotides in the breakage location of the DNA, the NHEJ repair system fills the gap and it may cause gene knocking down by stop codon generation.[46] The TALENs is one of the most important defensive systems against bacteriophages in herbal bacteria especially Xanthomonas. In this system, the foreign sequence of bacteriophages would be detected by TAL recognition domain which can bind to 18-20 bp of double-stranded DNA.[47] The important domain of TALEN for specific recognition is Repeat Variable Di-residue (RVD) which contains 12-25 residues with the ability of target sequences in genome recognition (figure 1A). The nucleases domain which comprises of FokI nucleases domains attached to the main construct of TALEN by a linker. The endonucleases of TALEN after target sequence recognition can cut the double strands of DNA and make a gap. TAL effectors and TALENs were created by Golden Gate assembly system.[48] The off-targeting of TALEN is not so much but the prediction of protein-DNA interaction rate can be useful for the risk estimation.[49] CRISPR/Cas9 is the latest series of genome editing tools that hold the promise to accelerate this function. Cas9 was the best type of endonucleases which can attach to CRISPR and studies showed CRISPR attachment and Cas9 co-ordination need the especial three base pairs with NGG sequence upstream of guide RNA region. The crRNA serve as sequence-specific guides for the Cas proteins/nucleases which act as a specific defense system against invading elements. The crRNA and the trans-activating crRNA (tracrRNA) after target DNA recognition, form a double strand RNA structure that directs the Cas9 to generate DSBs in the target DNA.[50] In this method, after crRNA binding to the DNA, the Cas9 HNH nuclease domain cleaves the

complementary strand, whereas the Cas9 RuvC-like domain cleaves the non-complementary strand. The RNA hybrid of tracrRNA: crRNA, when engineered as a single RNA chimera, named as gRNA, can bind up to 20 base pair of target sequences and then it directs sequence-specific double strand DNA and cleavage by Cas9.[51] Eventually, the CRISPR –Cas9 system defined as a DNA editing system at 2009 which can be used in eukaryotic cells genome edition.[52-54] CRISPR variation is dependent on the number of repeats which is different in some organisms.[55] Nowadays, Gene therapy is pioneered by CRISPR-Cas9 technique. In this method, after the mutated site incision by the CRISPR-Cas9 system, the correct oligonucleotides would be added and the cell homologous recombination system can reconstitute the gap by the inserted sequence. Gene correction in HSC or iPSCs has been demonstrated in preclinical studies for X-CGD and other forms of PIDs. In the context of CGD, different editing systems have been used to correct disease by introducing different functional genes. The ZNFs have been used for correction of $gp91^{phox}$ mutation in patient-specific iPSCs by introducing up to five different genes into AAVS1 safe harbor. The ZNFs system integrated a therapeutic $gp91^{phox}$ expression cassette with CAG promoter to functional correction of a point mutation in patient-specific iPSCs. After in vitro differentiation, normal granulocytes were generated from corrected iPSCs and targeted gene transfer into one allele of the AAVS1 safe harbor was without off targets and colons containing an insert at only a single AAVS1 allele were selected successfully. However, clinical applications require patents specific iPSCs production under GMP condition and virus-free approach.[56] In another study, ZFNs was used for targeted insertion of CYBB minigene into the AAVS1 safe harbor in five genetic forms of CGD under the control of a chimeric "CAG" promoter.[57] Constitutive expression of gp91phox and ROS production could be detected in differentiated neutrophils and macrophages from patient-specific iPSCs, but ectopic expression of the gp91phox protein and aberrant production of ROS before myeloid differentiation was in expected results. Successful in vitro differentiation of human iPSCs into neutrophils has been recently reported.[58] In this report TALENs used to target the insertion of the gp91phox cassette with the miR-223 promoter to AAVS1 in X-CGD patient-derived iPSCs. They showed the up-regulated levels of gp91 phox mRNA levels and in differentiated granulocytes ROS production and neutrophils, extracellular trap (NET) formation detected. They suggested TALENs as a safe and efficient system to produce functionally corrected granulocytes. A recent study demonstrated TALENs or CRISPR mediated correction of CYBB exon 5 mutations.[59] They used iPSCs from patients to transfer of CYBB minigenes to the start sites of exon 1 or exon 2 of the CYBB locus. The results showed the targeted insertion of exon 1-13 minigenes into exon 1 of the CYBB locus could not restore $gp91^{phox}$ expression or ROS activity in iPSCs. In contrast, insertion of an exon 2-13 minigene into exon 2 results in normal expression of gp91phox and restoration of ROS activity. The results of this study highlighted the intronic elements roles insufficient expression of CYBB minigene. A number of studies have demonstrated the feasibility of the CRISPR/Cas9 editing system to encourage repair of point mutations in the CYBB gene. The CRISPR/Cas9 nuclease system was used to correct a single intronic mutation in the CYBB gene.[60] In this approach using iPSCs from a patient with a single point mutation (T>G) in CYBB gene, they reported therapeutic levels of gene correction and restoration of oxidative burst function in differentiated phagocytes from patient iPSCs. In another study, the CRISPR/Cas9 single guide RNA delivered into $CD34^{+}$ HSPCs by electroporation system that resulted in repair of 20% HSPCs from patient and functional restoration of gp91phox was achieved in differentiated myeloid cells from gene corrected progenitor cells in vitro.[61] 20 weeks after transplant clinical efficient levels of gp91phox could be detected in peripheral blood of NSG mice. Productions of functional mature myeloid and lymphoid progenies were detected in peripheral blood (PB) up to 6.5 months.

Modeling CGD using Genome Editing

Human somatic cells reprogramming to iPSCs are mediated by ectopic expression of four transcription factors Oct3/4, Sox2, Klf4 and c-Myc and in this regard, these self- renewing human iPSCs can be differentiated into different cell type.[62] A growing number of researchers have employed chemical and viral hiPSCs for *in vitro* modeling specific disease, including neurological, cardiac and blood disorders. Generation of iPSCs from patient-specific sources provides great promise for drug discovery and precision medicine. The development of custom-

designed nucleases (CDNs), including ZFNs, TALENs and meganucleases has been successfully employed for disease modeling and gene editing. However, more recently CRISPR/Cas9 technology has been shown to be more efficient than CDNs in genome editing, regulation, and modeling in cellular and animal levels. CRISPR/Cas9 system utilized for establishment of phagocyte defective NSG-Cybb knockout mouse model.[63] In that case, knock out of Cybb on X-chromosome achieved by zygotic microinjection of Cas9 mRNA and CRISPR single-guide RNA. NSG generated mice had the characteristic of CGD and were deficient in ROS production. Encouraging results were also obtained using the enzyme replacement therapy approach in patient's specific iPSCs.[64] X^0-CGD- iPSCs derived macrophages used as target cells to deliver recombinant cytochrome b558 containing liposomes. They demonstrated the capacity of recombinant NOX2/P22 ^{phox} liposomes in deliver human cytochrome b558 to differentiated macrophages from patient iPSCs without any toxicity. They introduced liposomal enzyme replacement therapy as a new promising technology for the future treatment of acute and life-threatening lung infections in CGD patients. In recent years, the researchers introduced iPSCs as a useful tool for modeling and studying of different genetic defects. Merling et al generated iPSCs from peripheral blood $CD34^+$ hematopoietic stem cells of a patient with five genetic subtypes of CGD.[57] They achieved a targeted correction of 70-80% of selected iPSCs colons by ZFN mediated safe harbor targeting. Differentiated neutrophils from corrected CGD iPSCs were oxidase positive in DHR assay. The iPSCs modeling is a promising approach to create personalized models of monogenic and polygenic disease and it is a worthwhile tool for investigation of the loss and gain of function genetic manipulations to understand disease mechanisms, cellular and molecular phenotyping.[65] Therefore, iPSCs generation by genome editing technologies provides valuable information about understanding and treating diseases.

CONCLUSION

Over the last two decades, gene therapy had significant progress. The improvement in gene editing technologies has helped to overcome some of the obstacles in the field of gene therapy. However, many challenges still remain before clinical applications. Safety and effective delivery are two main challenges in this approach. Precise engineering in viral vector design will enable vectors for efficient gene delivery that closely mimics the endogenous pattern and avoids activating the innate and adaptive immune system. Beside gene editing tools, iPSCs is a strong tool for modeling disease and is a promising approach to overcome the issue of limited availability of biopsy samples. Therefore, iPSCs based model of disease on modern medicine may help to identify the underlying cellular and molecular mechanisms. In CGD, gene corrected iPSCs could be used to generate functional neutrophils and to manage the infections in subjects. Gene delivery systems have the main function on the stability and amplification of delivered genes. Lentiviral systems have been shown the mainstay of gene delivery especially to deliver therapeutic genes for PIDs in preclinical and clinical stages. Although, long term follow-up treated patients will be required to monitor potential long-term adverse events.

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