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Generation of CCR5-ablated Human Induced Pluripotent Stem Cells as a Therapeutic Approach for Immune-mediated Diseases

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ABSTRACT

C-C chemokine receptor type 5 (CCR5) is a receptor for some pro-inflammatory chemokines which plays important roles in immunological disorder and host responses to infectious agents. Additionally, the prognosis of some immune-mediated diseases in the people who are naturally carrying the *CCR5* 32bp deletions is optimistic. However, the clinical application of *CCR5* 32bp mutant cells is very limited due to the rare availability of donors who are homozygous for *CCR5* Δ32.

The transfection efficiency of nucleofected placental mesenchymal stem cells derived - human induced pluripotent stem cells (PMSC-hiPSCs) was examined through the evaluation of green fluorescent protein (GFP) expression using flow cytometry. The nucleofected clonal populations were selected using colony picking. The *CCR5* gene disrupted clonal populations were evaluated and confirmed by PCR and Sanger sequencing methods. Also, off-target sites were evaluated by the "Loss of a primer binding site" technique.

The results of the flow cytometry revealed that among the six applied nucleofection programs for PMSC-iPSCs, the program of A-033 has achieved the best transfection efficiency (27.7%). PCR and then sequencing results confirmed the *CCR5* gene was disrupted in two clonal populations of 16 (Δ6) and 62 (Δ20) by the Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease 9 (CRISPR/Cas9) system. The "Loss of a primer binding site" technique showed that no exonic off-target mutations were induced in both *CCR5* gene disrupted clonal populations.

We establish a CRISPR/Cas9 mediated *CCR5* ablated PMSC-hiPSCs without detectable off-target damage. This approach can provide a stable supply of autologous/allogeneic *CCR5*-disrupted PMSC-hiPSCs that might be a feasible approach for the treatment of immune-mediated diseases.

Keywords: Chemokines; CRISPR-cas systems; Flow cytometry; Gene editing; Transfection

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INTRODUCTION

CCR5 is a receptor for some chemokines such as RANTES/CCL5, MIP-1β/CCL4, and MIP-1α/CCL3.[1] Altered *CCR5* expression influences the onset and clinical progression of autoimmune and infectious diseases such as Human Immunodeficiency Virus (HIV),[2] Hepatitis B Virus (HBV), Multiple Sclerosis (MS), Rheumatoid Arthritis (RA) and also immunological phenomenon such as Graft-versus-Host Disease (GvHD).[3] More than ten years after the recognition of HIV-1 as the infectious agent of acquired immunodeficiency syndrome (AIDS) in 1996, CCR5 (CD195) was introduced as one of the pivotal co-receptors for the entry of HIV-1 into the immune cells.[4] A 32-base-pair deletion within the coding region of the *CCR5* gene causes the change in a frameshift that results in a complete loss of the receptor function in homozygous individuals. The people who naturally carry a homozygous form of the *CCR5-Δ32* mutation have shown strong resistant to HIV-1 strains.[5] There is still no reliable strategy for functional HIV/AIDS cure. With one confirmed exception – Timothy Ray Brown, known as the Berlin patient – no one has been cured of HIV, functionally. He received the bone marrow stem cell transplantations from a donor who was HLA matched and homozygous for the *CCR5-Δ32* mutation.[6] To succeed in a stem cell transplantation, it first and foremost dependents on how closely the donor and recipient immunogenetically match. Although, GvHD is less common among the patients whose donors were *CCR5-Δ32* homozygous compared to patients with the wild-type or heterozygous donors. Generally, it can be very difficult to find the *CCR5-Δ32* allele carrying individuals who are compatible with unrelated donors for stem cell transplantation. The gene frequency of the homozygous and heterozygous form of the CCR5-Δ32 allele in the European population is 1% and 10%, respectively.[7] Previous epidemiologic data in the Iranian population have shown that an HLA-matched donor with *CCR5-Δ32/Δ32* genotype cannot be found.[8] Using human induced pluripotent stem cells (hiPSCs) technology, it is now possible to produce patient-specific pluripotent stem cells from any cell type of individuals. The pluripotency characteristics and of morphology human iPSCs are similar to human embryonic stem cells (hESCs) in their potential to differentiate into the primary germ layers i.e. the endoderm, mesoderm, and ectoderm.[9] Various cell types can derive from iPSCs e.g. mesenchymal stem cells and hematopoietic cells.[10] Consequently, iPSCs have the great capacity as a powerful source of autologous/allogeneic cells for preventive and therapeutic approaches. CRISPR/Cas9 gene modification system was first introduced in 2013, which has resulted in a revolution of the gene targeting.[11] This type of genome surgery technique has shown great promise for the treatment of congenital diseases. In the present study, we have used hiPSCs from Placental mesenchymal stem cells. PMSC-hiPSCs are the attractive sources for cell therapy due to avoidance of ethical issues and being readily available.[12] We engineered CRISPR/Cas9 directed homologous recombination to knock out the *CCR5* gene within the PMSC-hiPSCs with no off-target damage.

According to the best of our knowledge, our work is the first to generate *CCR5*-ablated PMSC-hiPSCs.

By scaling up and transferring the *CCR5*-ablated PMSC-hiPSCs, these cells may complete reconstituting the immune system of the people who are at a high risk of immune-mediated diseases. Finally, our results suggest the potential of using *CCR5*-knocked out CRISPR/CAS9-modified PMSC-hiPSCs for prevention/treating immune-mediated disease with/without genetic disposition such as MS and HIV, respectively.

MATERIALS AND METHODS

PMSC-hiPSCs Culture

PMSC-hiPSCs were the gift from Prof. Masoud Soleimani of Stem Cells Technology Research Center. The PMSC-hiPSCs were cultured on mitotically inactivated mouse embryonic fibroblast (iMEF) in the culture plates.[13] (Figure 1). Approval for the research study was obtained from the National Institute for Medical Research Development (NIMAD) ethics board (N. IR NIMAD REC 1396 389).

Guide RNA Design and Plasmid Preparation

Design of the guide RNA (gRNA) sequence is a very important step in gene modification procedure based on CRISPR/Cas9 system. The gRNAs were designed by Benchling online software (https://benchling.com). Again, the efficacy of the selected gRNA was checked through (crispr.mit.edu) (Figure 2). The best gRNA was synthesized and

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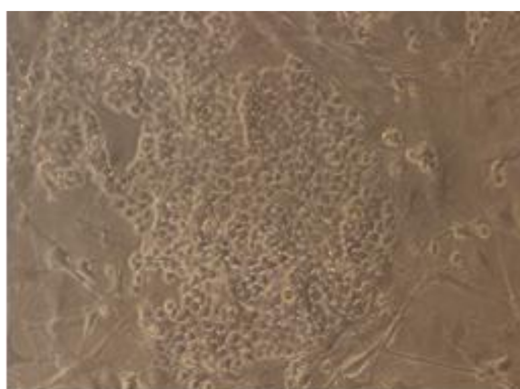

Figure 1: Typical morphology of a PMSC-hiPSCs colony. The PMSC-hiPSCs were maintained on iMEF in the culture plates.

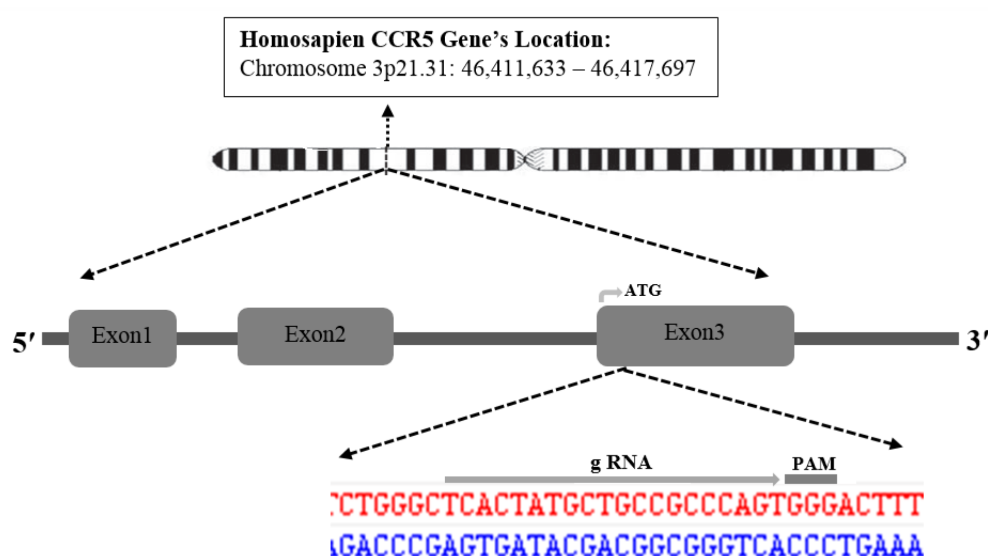

Figure 2. A schematic figure of human CCR5 gene locus showing the location of the gRNA designed for CCR5. In human, the *CCR5* gene located on the short arm at position 21 on chromosome 3. To disrupt the *CCR5* locus in the PMSC-hiPSCs, the CRISPR/CAS9 technique was employed. The single gRNA was designed by Benchling online software. PAM: Protospacer adjacent motif.

inserted into the pSpCas9 (BB)-2A-GFP (PX458) plasmid by CloneEZ (GenScript USA, Inc.) (Figure 3).

The PX458 plasmid containing designed gRNA was propagated in DH5α strain (*E.Coli*) and the plasmid was extracted by Qiagen Endo-free Plasmid Maxi Kit according to the manufacturer's protocol. The concentration of extracted plasmid was more than 2 mg/ml that was measured by Thermo Scientific Nanodrop Spectrophotometer.

PMSC-hiPSCs *CCR5* Gene Modification

The nucleofection procedure was performed based on the manufacturer's instruction [Human Stem Cell Nucleofector Kit 1 (Lonza, cat. no. VPH-5012)]. According to the manufacturer manual: the efficiency of nucleofection is drastically influenced by the cell type, the cell source, the cell viability, confluence and number of cell passage. Thus, providing a unique optimized protocol with predefined conditions i.e. one program (electrical parameter) which is guaranteed to work with any hiPSCs clone is not possible. For our PMSC-hiPSCs clones, it is recommended to first determine the optimal program. In brief, on the day of nucleofection, PMSC-hiPSCs were pre-treated with 10 µmol/L Rho kinase inhibitor (Y-27632) (R&D Systems, EMD Millipore) for 2-3 hours before

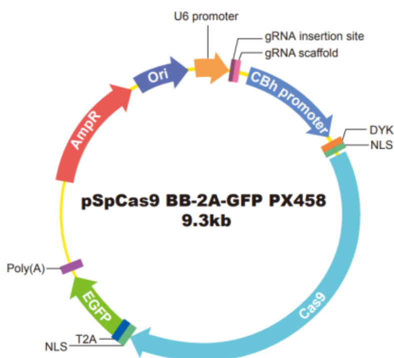

Figure 3. Schematic diagram showing components and arrangement of the PX458 Plasmid. The selected gRNA for human *CCR5* gene was inserted into the cloning site of the pSpCas9 (BB)-2A-GFP (PX458) plasmid by CloneEZ. (GenScript USA, Inc.).

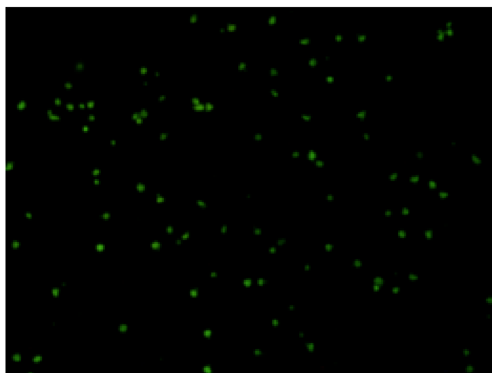

Figure 4: Evaluation of PMSC-hiPSCs GFP expression post-nucleofection. PMSC-hiPSC colonies were passaged enzymatically using 0.1 % Collagenase type IV (Invitrogen), and a single-cell suspension was prepared and Nucleofected by [Human Stem Cell Nucleofector Kit 1 (Lonza, cat. no. VPH-5012)] based on the manufacturer's instruction. About 12 hours after delivery procedure of PX458 plasmid, the expression of GFP was examined by fluorescence microscope to find and mark the nucleofected PMSC-hiPSCs.

nucleofection. To prepare a single-cell suspension, the culture was treated with collagenase IV (Sigma). Detached PMSC-hiPSCs were collected and centrifuged at 100 x *g* for 3 min at RT. The hiPSCs pellet was suspended and dispensed (8 x 10^5 cells) into the seven separated tubes, and spin down in low-speed centrifugation. Then, ready-to-use nucleofection solutions and 7 µg of fluorescent protein-expressing plasmid DNA were added into the Nucleocuvette containing the cell suspension and mixed by tapping gently. Finally, to find the best nucleofection program, six different programs of A-012, A-13, A-023, A-027, A-033, and B-016 were applied. Nucleofected PMSC-hiPSCs were recovered and transferred onto the feeder layers. On the following day, nucleofection efficiency was monitored using Flow cytometry analysis. Also, the expression of GFP was examined to find and mark the nucleofected PMSC-hiPSCs by fluorescence microscope (Figure 4). Then, PMSC-hiPSCs were cultured at 37°C until distinct colonies appear large enough for colony picking. About 70 clear single colonies were picked under sterile conditions. The picked up single colonies were cultured and propagated, separatel.

Evaluation of Nucleofection Efficiency Based on Flow Cytometry Analysis

About 12 hours after nucleofection, the GFP expression of nucleofected PMSC-hiPSCs was examined by Flow cytometry (Attune™ Acoustic Focusing Cytometer). A sample of the un-transfected PMSC-hiPSCs was served as negative control. The results were analyzed using the FlowJo software (Figure 5).

DNA Extraction and PCR Amplification

The genomic DNA of the 70 clonal populations was extracted by the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's protocol. The genomic DNA of PMSC-hiPSCs were amplified using two pairs of primers. The first pair of primers spanning the CRISPR/Cas9 target region. The size of the first-round PCR product was 351bp. The forward primer of the second-round PCR was designed exactly on the gRNA target position (Table1). The templates were amplified by ampliqon PCR master mix according to the following steps, predenaturation 95°C for 3min and 40 cycles: denaturation at 94°C for 30 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 60 seconds. The first-round PCR products were used as the templates for the second-round PCR. The second round PCR was performed under the above-mentioned conditions. The product of the second-round PCR was 234bp. Finally, the PCR products of the first-round PCR with negative second-round PCR were chosen and electrophoresed on a 1.5% gel agarose and then cleaned up using a gel extraction kit (Qiagen, Germany). The gel extracted PCR products were analyzed using the Sanger sequencing method (Macrogen, Seoul, South Korea).

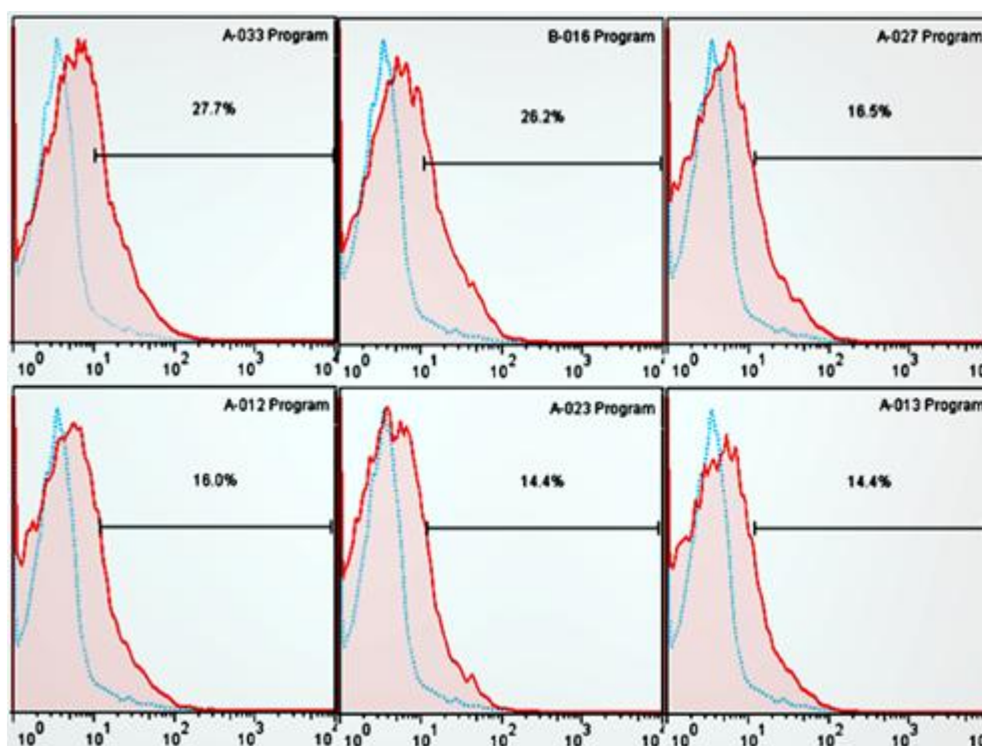

Figure 5. Evaluation of transfection efficiency in PMSC-hiPSCs. To find the best Nucleofection program, six different programs of A-012, A-13, A-023, A-027, A-033, and B-016 were applied according to the manufacturer's instructions. About 12 hours after delivery procedure of PX458 plasmid, the transfection efficiency (GFP expression) of nucleofected PMSC-hiPSCs were examined by flow cytometry.

In Silico Prediction of gRNAs Off-Target

The potential off-target sites of designed gRNA were predicted by searching the human genome (GRCh38/hg38) using different web-based tools: Benchling (https://benchling.com), TagScan (http://www.isrec.isb-sib.ch/tagger), and the CRISPR design web tool (http://crispr.mit.edu/).

In vitro Assessment of gRNA Off-target Sites

In order to evaluate the predicted off-targets, the "Loss of a primer binding site" technique was performed.[14] In brief, the genomic DNA was amplified with a pair of primers. The forward primer was designed for the predicted off-targets site, exactly. PCR was performed for 40 cycles by the following conditions: 95ºC for 3min, 95ºC for 30 sec, 60ºC for 30 sec, 72ºC for 35 sec, and final extension 72ºC for 2 min. Finally, PCR products were resolved by electrophoresis on 1.5% gel agarose. The size of the PCR product was 297 bp (Table 1).

RESULTS

In Silico Analysis of gRNA Off-Targets

To minimize the chromosomal damage, just a single gRNA with maximum on-target and minimum off-target was selected and ordered. The off-target sites were predicted by using several online tools (Table 2).

Evaluation of Nucleofection Efficacy by Flow Cytometry Analysis

The Flow cytometry results revealed the efficacy of the six applied nucleofection programs for PMSC-hiPSCs was: A-012:16.0%, A-13:14.4%, A-023:14.4%, A-027:16.5%, A-033: 27.7% and B-016:26.2%. The nucleofection program of A-033 has achieved the best transfection efficiency (27.7%). Therefore, the A-033 program was applied in subsequent experiments (Figure 4).

Table 1. The list of the primer sequences and size of their products used in a study in order to evaluation of CCR5 gene deletion and off-target analysis of nucleofected PMSC-hiPSCs, respectively.

The first-round PCR		**Product size**
Forward primer1	5′-CAACATGCTGGTCATCCTCATCCTG-3′	351bp
Reverse primer	5′-ATTCCTGGGAGAGACGCAAACAC-3′	
The second-round PCR		
Forward primer2	5′-GGCTCACTATGCTGCCGCCCA-3′	234bp
Reverse primer	5′-ATTCCTGGGAGAGACGCAAACAC-3′	
Off-target primer sequences		
Forward primer	5′-CTCCATGCTGCCGCCAGTAT-3′	296bp
Reverse primer	5′-CTCCCTGATCTTGAGATGCCTTG-3′	

Table 2. Off-target analysis of gRNA designed for CCR5 gene. The online tool for off-targets prediction was detected five mismatches for the designed gRNA when compared to the target sequence. The mismatch positions are bolded and underlined in the predicted off-target sequence

gRNA Sequence	**PAM**	**Score**	**Gene**	**Genomic coordinates (GRCh38/hg38)**
TCACTATGCTGCCGCCCAGT	GGG	100	CCR5 (ENSG00000160791)	chr3:+46414671
Exonic off-target sequences				
TCAC**C**A**G**GCTGCCG**G**CCAGT	TGG	0.6	FRMPD3 (ENSG00000147234)	chr-X:-106847195
TC**G**CTAT**TT**TGC**A**GCCCAGT	AGG	0.4	PDE4 (ENSG00000065989)	chr19:+10569917
TCAC**A**AT**T**CTGC**A**GCCC**C**GT	GGG	0.1	RNF123 (ENSG00000164068)	chr3:+49742360
TCAA**GA**TGCTG**G**C**C**CCCAGT	GGG	0.1	CD93 (ENSG00000125810)	chr20:-23065219
TCA**T**T**T**TGCTGCC**A**CCCAG**G**	GAG	0.1	TRPA1 (ENSG00000104321)	chr8:-72973960

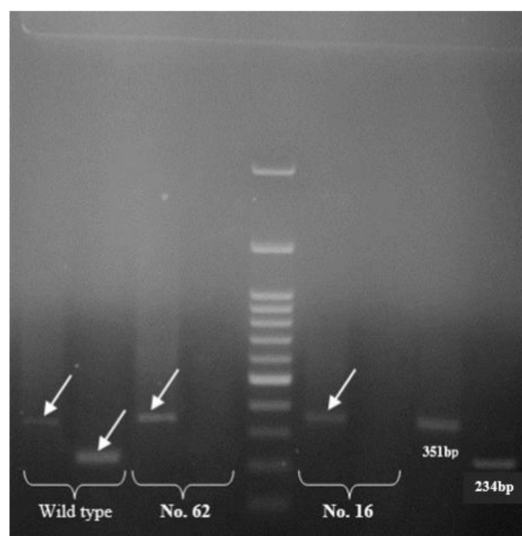

Figure 6. Evaluation of the CCR5 gene disruption in PMSC-hiPSC by PCR. Electrophoresis of PCR products on 1.5% gel agarose in order to find the CCR5 gene-disrupted PMSC-hiPSC clonal populations. To select the PMSC-hiPSC clonal populations that gene disruption was performed, a two-step RCR was designed. The first round PCR product was used as the template for second round PCR. The second forward primer was overlapped with the putative CRISPR/Cas9 target site, deletions at the target site will prevent the latter reverse primer annealing and the second PCR will not be produced. In the clonal population No. 16 and 62 only the first round PCR that spanning the entire target site was produced and the second round was not. 1: round 1 PCR product (351bp), 2: round 2 PCR product (234bp), and 3: 100bp ladder.

Evaluation of the *CCR5* Gene Editing by PCR

70 double round PCR reactions were performed and the products were electrophoresed on 1.5% gel agarose. Among 70 clonal populations, just two clonal populations (No. 16 and 62) had the positive first-round PCR and the negative second-round PCR (Figure 6). The gel extracted second-round PCR products were analyzed by using the Sanger sequencing method (Macrogen, Seoul, South Korea). The sequencing results (Normal and gene disrupted clonal populations) beside reference sequence (https://www.ncbi.nlm.nih.gov/gene) were aligned using the Mega6 software (Figure 7). In the clonal population No. 16 and 62, six (Δ6) and twenty base pairs (Δ20) were deleted, respectively. In fact, gene editing efficiency was about 3%. The results of the Sanger sequencing and DNA sequence alignment confirm that the *CCR5* gene was disrupted.

Species/Abbrv	
1. CCR5-Clon 16	CCCCTTCTGGGCTCACTATGCTGCCGCC------GACTTTGGAAATACAA
2. CCR5-Clon 62	CCCCTTCTGGGCTCACTATG--------------------GGAAATACAA
3. NORMAL	CCCCTTCTGGGCTCACTATGCTGCCGCCCAGTGGGACTTTGGAAATACAA
4. AH005786.2 Homo sapiens	CCCCTTCTGGGCTCACTATGCTGCCGCCCAGTGGGACTTTGGAAATACAA

Figure 7. The sequencing results showing the clonal population No. 16 and 62, six (Δ6) and twenty base pairs (Δ20) were deleted. The Sanger sequencing results of the CCR5 gene disrupted PMSC-hiPSC clonal populations besides normal (wild-type) and also reference sequence of the human CCR5 gene [downloaded from genome database (https://www.ncbi.nlm.nih.gov/gene)] were aligned using the Mega6 software.

Figure 8. Evaluation of predicted off-target site of the gRNA designed for CCR5 gene in the CCR5 gene disrupted clonal population No. 16 and 62. The gel electrophoresis results of highest score off-target PCR product (The 296 bp band) on 1.5% gel agarose showed no exotic off-target mutation was induced into the FRMPD3 genomic location (ENSG00000147234) in two PMSC-hiPSC clonal populations of 16 and 62 (CCR5 gene disrupted populations) by CRISPR/Cas9 system. 1: PCR product of clonal population No. 16, 2: PCR product of clonal population No. 62, 3: 1 kb DNA ladder (Fermentas), 4: PCR product of wild type clonal population, and 5: negative control.

In vitro Analysis of gRNA Off Targets

The experimental analysis of exonic off targets (five off-target) was performed (Table 2) on two clonal populations No. 16 and 62. These two clonal populations were validated for the *CCR5* specific gene editing using PCR. The "Loss of a primer binding site" technique showed that no exonic off-target mutation was induced in two clonal population No. 16 and 62. The gel electrophoresis results of the highest score off-target (0.6) are shown in Figure 8. The electrophoresis results of low score off-target are not shown.

DISCUSSION

As a first step toward utilizing iPSCs for the treatment of immune-mediated diseases, we have developed *CCR5*-ablated PMSC-hiPSCs. The PCR results revealed that there is no unwanted chromosomal damage in the *CCR5* ablated PMSC-hiPSCs. Different "starter cells" have been used to generate hiPSC including hematopoietic stem cells and lymphocyte. In the current study, PMSCs were chosen as the origin of hiPSCs because of their immunomodulatory effects and availability.[12] In the other study, homozygous *CCR5Δ32* genotype was generated using the transcription activator-like effector nucleases (TALENs) and *piggyBac* technology. *PiggyBac* is a transposon that randomly integrates into the genome

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which is not suitable for clinical use.[15] The researchers, compare the editing capability of CRISPR-Cas9 and TALEN when targeting the human *CCR5* locus. Their results showed that the CRISPR-Cas9 based gene editing system was about five times more efficient than the TALEN based system.[16] In another study, the *CCR5* gene of hESCs was disrupted using zinc finger nucleases (ZFNs).[17] ZFNs are not specific to their target site and may damage the chromosomes. Because of the simplicity and high cleavage efficiencies, the CRISPR/Cas9 rapidly replace other genome-editing techniques. Kambal et al. generated anti-HIV iPSCs from cord blood CD34+. They used a CCR5 short hairpin RNA which has a temporary effect.[18]

Chemokines and chemokine receptors play the central role in regulation, migration, and activation of leukocytes.[19] The *CCR5-Δ32* homozygous patients have a great therapeutic response to antiretroviral therapy (ART). Additionally, HIV progression to AIDS is mild in *CCR5Δ32* heterozygous individuals.[20] *CCR5Δ32* mutation accelerates and improves the treatment process of an HBV infection. The individuals who have a heterogenic form of *CCR5-Δ32* are more likely to recover from HBV infection.[21] Moreover, *CCR5Δ32* heterozygous mutation can block the progress of cerebral malaria from human malaria parasites of the genus Plasmodium.[22] Additionally, *CCR5Δ32* heterozygous mutation can slow the progression of immune diseases such as MS.[23] Inflammation appears related to almost every autoimmune and infectious diseases.[24] Immune system cells are the most important sources of inflammatory factors in the body.[25] Removing the unwanted source of inflammation is the key point of inflammation reduction. HiPSCs have the potential to produce key cellular subsets which, given to patients, may alter their disease process and suppress the inflammation by providing complete replacement of autoreactive cells, hyperactive or even lost cells.[26] This approach can reduce the patient's complexities through immune-modulation or even immune system replacement.[27] Also, the transfusion of differentiated hematopoietic precursor cells from hiPSCs to the people who are at a high risk of autoimmune and infectious diseases may complete reconstituting their immune system. For instance, MS is an autoimmune inflammatory disease that is associated with an HLA-DRB*1501,[28] which in concert with environmental factor exposure,[29] such as viral infections and vitamin D deficiency.[30] Therefore, a person who has the above-mentioned conditions may be a suitable candidate for gene edited autologous iPSCs and derived progeny transplantation. It seems, autologous transplantation using genetically modified hiPSCs potentially offers a good therapeutic method.[31] However, application of this procedure is limited due to the complicated condition of some patients and rare availability of high technology medical centers, in some region especially developing countries such as Iran. Allogeneic genetically modified hiPSCs transplantation is another alternative therapeutic procedure for many immune-mediated disorders and hematologic malignancies. The first clinical trial of allogeneic iPSCs-derived products is in progress.[9] However, GvHD is the main problem in the allogeneic transplantation (SCT) recipients.[32] The *CCR5Δ32* mutation is an immune response modulator. GvHD is less common among the recipients whose donors are *CCR5Δ32* homozygous compared to patients with heterozygous or wild-type donors. The *CCR5Δ32* mutation is important in renal allograft survival. In fact, *CCR5Δ32* homozygous allograft are increased survival in the HLA mismatched laboratory models.[33]

Nevertheless, cell therapy and gene targeting are a double-edged sword. Despite the protective effects, the *CCR5* deficiency is associated with increased susceptibility to a number of infectious diseases including *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Herpes simplex virus*, *Listeria*, *Toxoplasma gondii*, *Cryptococcus neoformans*, and *Trypanosoma cruzi*.[34] Therefore, in order to translate gene targeting and also cell therapy to the clinic, different aspects should be considered.

Herein, the best gRNA with the least off-target effect was chosen to edit the *CCR5* gene in the PMSC-hiPSCs. Our study illustrates that the *CCR5* gene targeting can be successfully and efficiently accomplished in the PMSC-hiPSCs. Importantly, we demonstrated that the "colony pick up" technique could be applied to select the transfected hiPSCs to ensure homogeneity of the CRISPR/ Cas9 genomic editing system.

Some issues need to be addressed before *CCR5*-ablated PMSC-hiPSCs can advance to clinical trials. Scaling up is the single most important criterion for the success of stem cell-based therapy projects. Unfortunately, conventional hiPSCs culture protocols do not have the ability to produce large-scale hiPSCs. Maintenance of high-quality hiPSCs in culture will be

crucial for therapeutic goals. HiPSCs maintain pluripotency and self-renewing capacity under time-consuming and carefully monitored culture conditions. So, the effective and fully automated process should be considered for the large-scale culture and maintenance of iPSCs in the optimal conditions. Despite the current limitations, the results of this study suggest that our artificially *CCR5*-ablated PMSC-hiPSCs may use as a very attractive source for allogeneic stem cell transplantation and regenerative medicine due to the promising effects of *CCR5* ablation on GvHD, immunomodulatory effects of PMSCs as the origin of hiPSCs, and various effects of *CCR5* disrupted cells on the immune-mediated diseases. Unfortunately, we did not explore the immunomodulatory property and differential capacity of our artificially *CCR5*-ablated PMSC-hiPSCs in the present study. It could be an attractive topic for future investigations. This study was approved and supported financially by a grant from Tehran University of Medical Sciences (No: 28230-30-02-94) and National Institute for Medical Research Development (NIMAD) (No: 962781).

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