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HLA-A gene knockout using CRISPR/Cas9 system toward overcoming transplantation concerns

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Abstract

Background: The treatment of many cancers and genetic diseases relies on novel engraftment approaches such as cell therapy and hematopoietic stem cell transplantation (HSCT). However, these methods are hindered by the alloreactive immune responses triggered by incompatible human leukocyte antigen (HLA) molecules. A successful HSCT procedure requires the eradication of donor and recipient HLA alloimmunization. Eliminating *HLA-A* gene expression using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 nuclease (CRISPR/Cas9) could be a great approach to increase the possibility of a successful HSCT through extending pool of unrelated donors.

Results: Our dual gRNA approach introduced a large deletion in the *HLA-A* gene. Among 22 single-cloned cells, two clones (9.09%) and 11 clones (50%) received homozygous and heterozygous large deletions, respectively. Finally, the real-time PCR results also revealed that *HLA-A* gene expression was diminished significantly.

Conclusion: The results suggested that CRISPR/Cas9 could be used as an efficient technique to introduce *HLA-A* gene knockout; thus, it can considerably lessen the burden of finding a fully matched donor by lowering the alleles required for a successful HSCT.

Keywords: HLA-A, HSCT, CRISPR/Cas9

Background

HSCT is a widely accepted approach for the treatment of many hematological malignancies and non-neoplastic disorders. However, serious challenges including stringent matching, donor insufficiency, engraftment rejection, and graft-versus-host disease (GVHD) may hinder the widespread clinical applications of this approach [1]. In this context, the major underlying issue is the incompatibility of HLA molecule expressed on the surface of various cells. Being located on chromosome 6p21, the HLA gene family is among the most polymorphic

regions in human genome with a significantly low rate of linkage disequilibrium; thus, HLA compatibility between two individuals is considered a bottleneck in the field of HSCT.

HLA disparity between donor and recipient of HSCT leads to provoking the immune system [2] which consequently urges the patients to take immunosuppressive drugs. However, due to their immense adverse effects, the persistent usage of immunosuppressive drugs is limited. According to the National Marrow Donor Program guideline [3], in a safe allogeneic HSCT, donor and recipient alleles need to be matched at HLA-A/-B/-C/-DRB1. Previous studies have revealed promising solutions to the above challenges by eliminating HLA complex, complete or partially. These studies suggest that even the partial ablation of HLA expression can reduce the transplantation prerequisites and increase the chance

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of finding suitable donors for a certain patient. Until now, multiple attempts were made to eliminate the expression of HLA complex through targeting beta-2 microglobulin (B2M), by employing various approaches such as small interfering RNA (siRNA) [4–6] and zinc-finger nuclease (ZFN) [7, 8].

CRISPR/Cas9 is an emerging strategy for genome editing and an efficient and promising technique revolutionizing this field [9–11]. A double-strand break (DSB) can be made easily by Cas9 nuclease protein with the presence of a 20-nucleotide single guide RNA (gRNA), which recognizes a specific DNA region in the genome. Non-homologous end joining (NHEJ) repair system can lead to a random insertion-deletion following DSB, while these mutations may eliminate the expression of the coding region of the target gene. In this study, we implemented CRISPR/Cas9 system to introduce DSB in the *HLA-A* gene as a part of HLA class I to reduce minimum requirements for donor-recipient matching. We used double gRNA as a satisfactory approach to induce a large deletion and guarantee the disruption of the intended gene. The results from real-time PCR revealed a significant decrease in the expression of *HLA-A* genes, suggesting a targeted gene knockout in HEK293T cells.

Methods

Construction and cloning of gRNAs

The online CRISPR tool available at <http://crispor.tefor.net/> [12] was used to design three gRNAs to target *HLA-A*. Since the HEK293T cell line is homozygous for *HLA-A2*, a combination of A-gRNA1 and A-gRNA3 was employed to expel an estimated 459 bp fragment between exons 2 and 3. In addition, A-gRNA2 was solitarily designed for a single guide approach, which in association with A-gRNA1 will be able to knockout *HLA-A* gene regardless of allele type (Table 1). These oligonucleotides were inserted into PX458 (addgene, plasmid #48148) expression plasmid to create PX458-A-gRNA1 and PX458-A-gRNA3.

Cell culture and transfection of HEK293T cell lines

HEK293T cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 500 µl penicillin-streptomycin (Gibco, USA). Twenty-four hours prior to transfection, 5×10^5 cells were seeded in 6-well plates in a 2-ml cell growth medium without antibiotics. Equal concentration of

PX458-A-gRNA1 and PX458-A-gRNA3 were co-transfected to HEK293T using lipofectamine 2000 (Invitrogen, USA).

Isolation of transfected cells by FACS and establishment of single-cell clones

Forty-eight hours post-transfection, the GFP⁺ cells were sorted using fluorescence-activated cell sorting (FACS). Afterwards, the cells were cultured in complete growth media for 3–4 days and allowed to reach 70–80% confluency. Then, single cells were obtained from the heterogeneous population of transfected cells by performing serial dilution. Briefly, the cell suspension was prepared in a concentration of 10^6 cells per ml of complete growth media, and the serial dilution was carried out to reach the concentration of 100 cells/ml. The concentration of a single cell per 100 µl was obtained by adding 9-ml growth media to the cell suspension. Finally, 100 µl of cell suspension was divided into each well of the 96-well plate to establish single cell clones (Fig. 1).

DNA extraction, PCR, and sequencing to analyze editing

After expanding clones in 96-well plates, genomic DNA of each clone was extracted using a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instruction. The following primers were used to amplify the intended target of genomic DNA extraction: forward: 5'-GCCCTGACCCAGACCTGG-3' and reverse: 5'-ATTGAGTGTGAAGCAGAGAACAAGG-3'. PCR was carried out using 30 cycles of heating at 95 °C for 30 s, 65.5 °C for 25 s, and 72°C for 50 s, and PCR products were evaluated by gel electrophoresis. Next, PCR products were purified and integrated into the TA vector using the TOPO-TA cloning kit (Thermo Fisher Scientific, USA), and were further analyzed by Sanger sequencing.

Real-time PCR

To evaluate the impact of gene knockout on *HLA-A* gene expression, real-time PCR was carried out in treated target cells. HEK293T cells were harvested by trypsin, and RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized, using Prime-Script 1st strand cDNA Synthesis Kit (Takara, Japan), according to the manufacturer's instructions. Real-time PCR was performed on QuantStudio 3 Real-Time PCR System (Applied Biosystem, USA), using high-ROX RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) and specific primers for *HLA-A* and *Beta-2-microglobulin* (*B2M*) genes (forward: 5'-ACCGTC CAGAGGATGTATG-3', reverse: 5'-CTTTCAGGGC-GATGTAATC-3'). The experiments were performed

Table 1 gRNA sequences

Name of gRNA	gRNA sequence
A-gRNA1	ACAGCGACGCCGCGAGCCAG
A-gRNA2	TACCGGCAGGACGCCTACGA
A-gRNA3	TGCCGTCGTAGGCGTACTGG

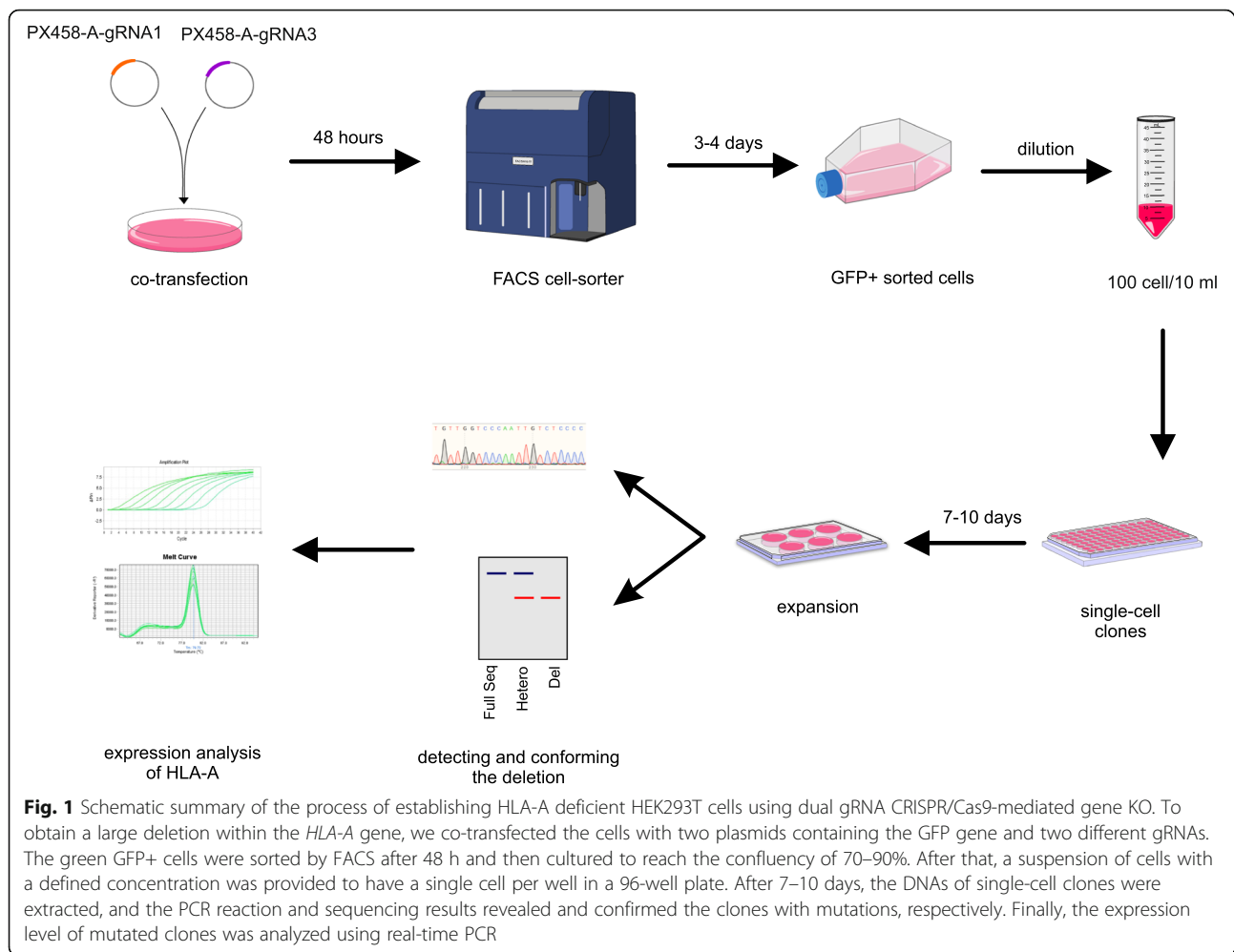


Fig. 1 Schematic summary of the process of establishing HLA-A deficient HEK293T cells using dual gRNA CRISPR/Cas9-mediated gene KO. To obtain a large deletion within the *HLA-A* gene, we co-transfected the cells with two plasmids containing the GFP gene and two different gRNAs. The green GFP+ cells were sorted by FACS after 48 h and then cultured to reach the confluency of 70–90%. After that, a suspension of cells with a defined concentration was provided to have a single cell per well in a 96-well plate. After 7–10 days, the DNAs of single-cell clones were extracted, and the PCR reaction and sequencing results revealed and confirmed the clones with mutations, respectively. Finally, the expression level of mutated clones was analyzed using real-time PCR

independently in a duplicate manner, and the relative expression of genes was analyzed using the $2^{-\Delta\Delta Ct}$ method [13].

Results

Designing a universal gRNAs for *HLA-A* alleles

Since HLA genes are among the most polymorphic region of human genome, finding a consensus region among various alleles of an HLA gene was extremely difficult. We collected all *HLA-A* allele sequences of ethnic groups, which cover >99.9% of the Iranian population according to the Royan Public Umbilical Cord Blood Bank registry [14]. The coding sequences which were elicited from IPD-IMGT/HLA [15] were aligned simultaneously, and the appropriated gRNAs were picked out among the consensus regions. The criteria for designing the optimal gRNA were considered, and the most efficient gRNAs with the lowest off-target were selected (supplementary table). Initially, A-gRNA1 was picked as a universal gRNA. A-gRNA1 could target most of the *HLA-A* alleles, except

*HLA-A*01*, which includes a significant portion (11.1%) of the Iranian allelic population. Consequently, we committed to designing A-gRNA2, which has less allelic coverage (42.3%) compared to A-gRNA1 (88.9%), but it can cover *HLA-A*01* (Fig. 2). Thus, A-gRNA1 and A-gRNA2 could be suggested to be used for ablation of *HLA-A* gene universally, regardless of the type of the allele.

Transfection of vectors into the HEK293T cell line

HEK293T cell line was selected as a model for the intended manipulations due to its high transfection efficiency, simple culturing, and a homologous allele for *HLA-A* (A*02:01). The cells were simultaneously transfected with two plasmids (PX458-A-gRNA1 and PX458-A-gRNA3), and after that, a remarkable GFP signal was observed in transfected cells after 48 h. Consequently, the GFP+ cells were isolated from the transfected cell pool using FACS Aria III™ (BD Biosciences, US) cell sorter (Fig. 3).

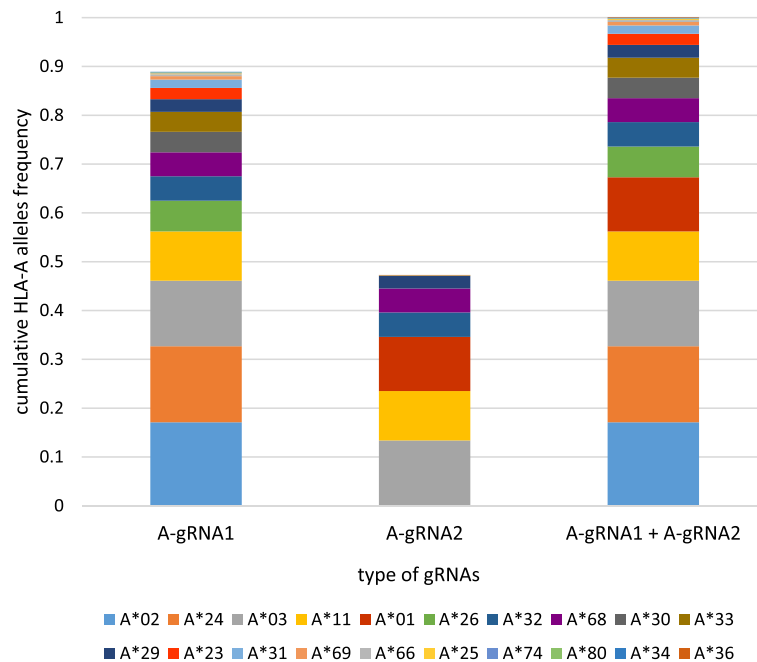


Fig. 2 Coverage of gRNAs for HLA-A alleles based on the frequencies in the Iranian population. Each column represents the possibility of each gRNA binding to its complementarity among HLA-A alleles. Each gRNA cannot be implemented for all ethnicities, so the last bar shows that all HLA-A alleles seem to be covered when both gRNAs are included

Serial dilution and single-cell isolation

Following the separation of GFP+ HEK293T cells, three types of cells were expected based on their HLA-A allele status: first, the cells without any alteration in the sequence of HLA-A gene; second, heterozygous cells with a deletion in single HLA-A allele; third, the homozygous cells in which both alleles completely abolished (Fig. 1). In order to isolate and detect homozygous and heterozygous cells, serial dilution was carried out to isolate single cells per well in 96-well plates. As a result, 22 wells out of 192 wells demonstrated to generate single-cell clones. The single-cell clones were further expanded for subsequent mutation analysis.

Analysis of HLA-A null HEK293T cell lines by PCR and sequencing

The PCR reaction was carried out on the genomic DNA of all of the expanded clones to determine the HLA-A allele status in each clone. PCR products on 2% agarose gel illustrated clones with a deletion in one HLA-A allele (heterozygous), deletion in both HLA-A alleles (homozygous), or without any mutation (wild-type). As demonstrated in Fig. 4, two clones (9.09%) showed homozygous deletion in *HLA-A*, and 11 clones (50%) showed heterozygous deletion in *HLA-A*. Moreover, analysis of the PCR products by Sanger sequencing further validated the deletions in genetically homogeneous clones.

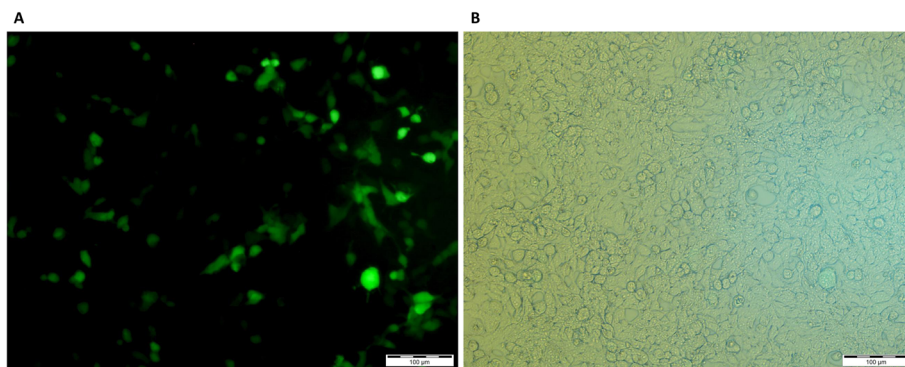
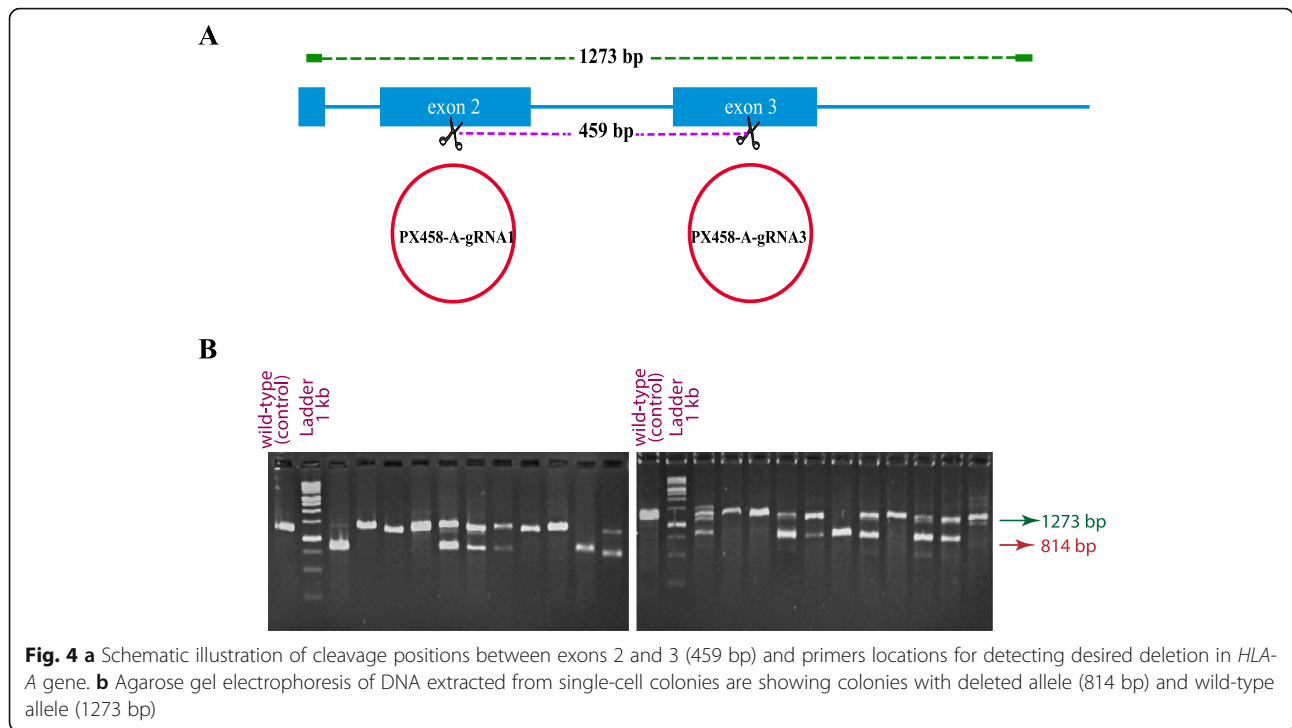


Fig. 3 Efficient transfection of pX458 plasmids into pX458 cells. **a** represents the GFP+ cells visualized using fluorescent microscopy, and **b** shows the same cells with normal visible light (scale bars 100 µm)



Sequencing results revealed dual-Cas9-mediated double-strand break (DSB) precisely in both alleles. Notably, both homozygote clones demonstrated cleavage three nucleotides upstream of PAM (Fig. 5).

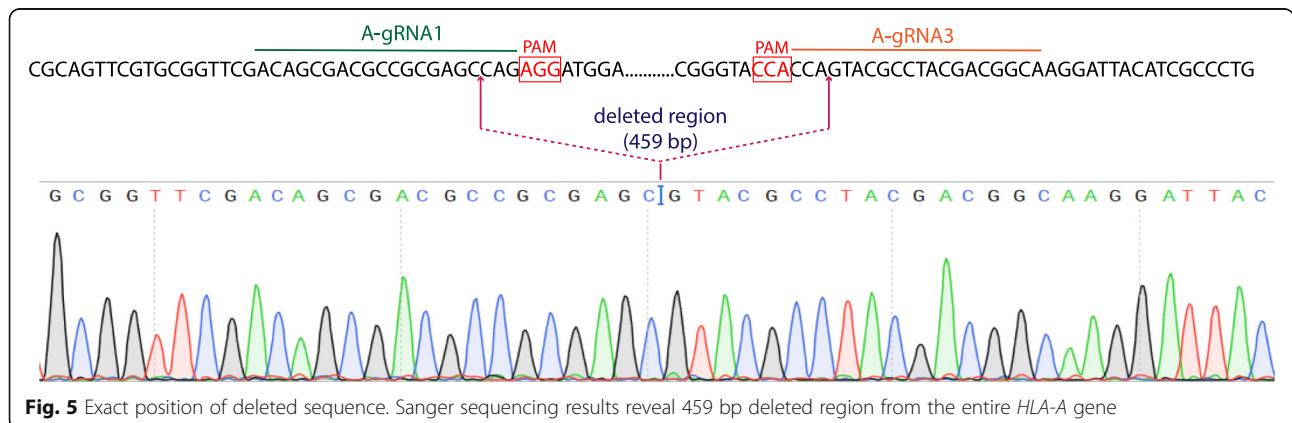
Expression of HLA-A in HEK293T

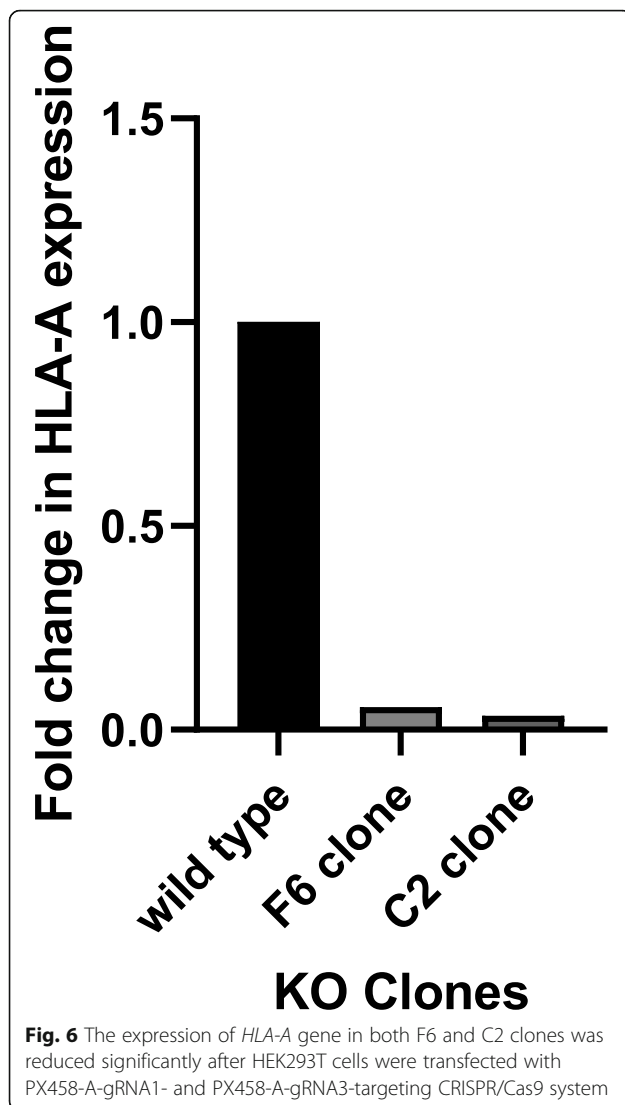
Real-time PCR was performed to identify the expression levels of HLA-A in the HEK293T cell line. The results demonstrated that the levels of HLA-A mRNA expression in the two homozygous KO clones, namely F6 and C2, were diminished almost completely (Fig. 6).

Discussion

The minimum requirement for a successful engraftment between donor and recipient in HSCT is based on HLA-

A/-B/-C/-DRB1 matching, nominated as 8/8 HLA-matching. However, regarding the high allelic diversity for each locus, it is blatantly obvious that the probability of finding a potentially fully matched allogeneic donor is grossly small. In the absence of a fully matched donor, the alternative way is to refer to the unrelated donor through a local or global donor registry or receive transplantation from a donor with only a single mismatch for these loci (7/8 HLA match). However, it is associated with higher mortality and 1-year survival compared with 8/8 matched pairs [16]. Furthermore, umbilical cord blood is not a reliable source for HSCT due to the need for having sufficient recoverable cells besides matching of HLA-A, HLA-B (low resolution), and HLA-DRB1 (high resolution) [17–19]. Typically, the best situation





for finding a suitable donor is fully matched siblings. Unfortunately, the chance of finding a matched sibling is 15 to 30%, depending on the number of children in a family [20–23]. Due to a lack of HLA registries and cord blood banks in many countries, consanguineous marriages provide the only available resource for a suitable donor [24, 25]. In recent years, scientists have been looking for a way through genetic manipulation to address donor insufficiency and the following problems of allogeneic transplantation. With the emergence of RNAi, many researchers sought to knockdown the HLA class I [4–6, 26–28]; however, the significant challenge of RNAi technology is the transient silencing of the target gene. Although the problem of temporary expression was resolved with the advent of ZFN and transcription activator-like effector nuclease (TALEN) technologies, laborious engineering and cloning a novel protein is required to target the desired region on a gene, which

restrains these technologies from being put to use for high-throughput applications.

Thus, eliminating one of the *HLA* gene families, regarding maintaining immunological manner, may facilitate finding a suitable donor. *HLA-A* and *HLA-B* were our candidates, which are more reliable to be knockout. However, designing a unique gRNA for *HLA-B* is almost impossible because of massive allelic diversity in populations [14, 29]. The main reason for not choosing *HLA-C* as a target gene was its inhibitory effects on NK cell receptors [30]. *HLA-DRB1* was not chosen due to the linkage disequilibrium with *HLA-DQB1*; therefore, they may appear as a haplotype [31, 32].

Torikai et al. generated *HLA-A* deficient on engineered CD19 CAR T cells [7] and hematopoietic stem cells [8] using ZFN technology. They successfully demonstrated that eliminating the expression of *HLA-A* can maintain the hematopoiesis ability as well as increasing the probability of identifying a fitting donor; however, the complexity of designing a specific nuclease for the intended target remained a prominent challenge. Recently, different approaches have been implemented using CRISPR/Cas9 as an efficient and easier way of improving transplantation rather than previous technologies. For instance, thorough knockout of HLA class II β -chain genes was accomplished using a single guide toward producing HLA-II-compatible HSCs for transplantation [33]. HLA class I (*B2M* gene) and II (*CIITA* gene) were targeted in human-induced pluripotent stem cells (iPSCs) and differentiated into cardiomyocytes for generating universal donor of allogeneic transplantation [34]. Another engineered iPSC was successfully developed by abolishing *HLA-A* and *HLA-B* to overcome immune rejection induced by HLA disparity [35].

We hired CRISPR/Cas9 system to generate *HLA-A* null cells. With considering the frequency of Iranian *HLA* alleles [14], we applied dual gRNAs (A-gRNA1 and A-gRNA3) among few conserved sequences to make a large deletion (Fig. 3) as a straightforward and cost-benefit method rather than single gRNA/Cas9 [36]. Although the efficiency for co-transfection of two vectors is less compared to the single-guide approach, introducing a large deletion could guarantee the ablation of the target gene. The possibility of A-gRNA1 for targeting 88.9% of allelic diversity in the Iranian population will make it to be used widely in future clinical trials; nevertheless, we designed another gRNA (A-gRNA2) to cover other alleles where A-gRNA1 cannot cover (Fig. 2). Even though we did not use A-gRNA2, it can be suggested for further studies. In this study, two clones (F6 and C2) received a large deletion in both alleles, and the sequencing results revealed the precise cleavage of Cas9 nuclease (Fig. 5). The real-time PCR further indicated that the expression of the *HLA-A* gene was suppressed

following gene disruption (Fig. 6). Albeit the efficiency of homozygous deletion was almost similar to Hong et al. [37], the efficiency of heterozygous deletion was higher (50% in comparison with 7.5% in Hong et al.'s study), regarding that they analyzed a wider range of single cells.

Conclusion

We demonstrated that the dual-gRNA approach in CRISPR/Cas9 could lead to the elimination of the *HLA-A* gene. Despite the fact that the HLA gene family is highly polymorphic, we established universal gRNAs, which potentially can be used in clinical settings to knockout the *HLA-A* gene. While HLA allele varieties are distributed with different frequencies around the world, our proposed universal gRNAs can be used in diverse ethnicities. Therefore, *HLA-A* gene elimination can diminish transplantation requirement from 8/8 allele match to the 6/6 or 5/6 considering one allele disparity between donor and recipient; as a result, finding a fully matched donor will be much more accessible. Further studies need to be conducted to demonstrate the applicability of gRNAs in human-derived hematopoietic stem cells with subsequent functional and immune assays.

Abbreviations

B2M: Beta-2 microglobulin; Cas9: CRISPR-associated protein 9 nuclease; CRISPR: Clustered regularly interspaced short palindromic repeats; DNA: Deoxyribonucleic acid; DSB: Double-strand break; FACS: Fluorescence-activated cell sorting; GFP: Green fluorescent protein; gRNA: Single-guide RNA; GVHD: Graft-versus-host disease; HEK293T: Human embryonic kidney; HLA: Human leukocyte antigen; HSCT: Hematopoietic stem cell transplantation; iPSC: Induced pluripotent stem cells; KO: Knockout; NHEJ: Non-homologous end joining; NK cell: Natural killer cell; PCR: Polymerase chain reaction; RNA: Ribonucleic acid; siRNA: Small interfering RNA; TALEN: Transcription activator-like effector nucleases; ZFN: Zinc-finger nuclease

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43042-021-00155-y>.

Additional file 1. Supplementary table

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Authors' contributions

FA carried out all of the experiment with the support from MR. FA and MP wrote the manuscript. MN contributed in carrying out and the interpretation of RT-PCR. MD designed and supervised the study. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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