Rapid identification of genome-edited mesenchymal stem cell colonies via Cas9

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ABSTRACT

Mesenchymal stem cells (MSCs) have been intensively investigated and widely applied in regenerative medicine and immune modulation. However, their efficacy declines during the aging or disease process. Thus, genome-edited MSCs with overexpression or inhibition of specific genes hold a great deal of promise in terms of their therapeutic application. Here we optimized the direct PCR approach for rapid identification of genome-edited MSCs with only ten cells, which reduces the time and labor required to expand the MSC colonies. Combining with our previously optimized guide RNA structure and plasmid construction strategy for Cas9, we successfully identified MSC colonies overexpressing IL-10 in the AAVS1 locus.

METHOD SUMMARY

We optimized a direct PCR approach for rapid identification of genome-edited Mesenchymal stem cells (MSCs) with only ten cells, which reduces the time and labor required to expand MSC colonies.

KEYWORDS:

AAVS1 • Cas9 • direct PCR • genome editing • IL-10 • IL-37 • mesenchymal stem cells • MSC • MSC colony • rapid identification

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Mesenchymal stem cells (MSCs) have multipotent (chondrocyte, osteoblast and adipocyte) and self-renewal abilities [1,2]. They have been proposed as an effective and safe cell source for stem cell therapy, with low immunogenicity and without major ethical concerns [3]. However, their efficacy declines during the aging or disease process [2,4]. Thus, genome-edited MSCs with overexpression or inhibition of specific genes hold great promise for therapeutic application.

Normally, it is necessary to expand a single cell colony to reach a sufficient cell number for DNA extraction after genome editing, which is both time- and labor-intensive. Thus, the development and optimization of a direct cell lysis and PCR approach is necessary for rapid characterization of genome-edited MSC colonies with low cell number. Indeed, the direct cell lysis and PCR approach has been widely applied in many fields for rapid DNA characterization [5–15]. However, its application in characterizing genome-edited MSCs has not been demonstrated.

In the current study, we first compared different approaches of direct cell lysis and PCR analysis. We found that lysis buffer containing Tween 20 and proteinase K (PK) was the most efficient approach to release DNA from only ten cells, which was sensitive enough for PCR detection. Previously, we had identified one optimized guide RNA structure with higher genome-editing efficiency and established one more efficient plasmid construction strategy for Cas9-gRNA [16,17]. Then we combined these techniques together to insert the immune suppressive gene IL-10 in the AAVS1 site, the safe harbor locus of human genome [18,19].

MATERIALS & METHODS

Cell culture

Human MSCs were isolated from umbilical cord. The umbilical cords were collected 10 min after the birth and informed consents were obtained. The umbilical cords were

stored in DMEM/high glucose (Gibco, USA) with antibiotics (500 units/ml of penicillin and 500 µg/ml of streptomycin) on ice and delivered to the lab. Cell isolation should be performed within 8 h. The umbilical cords were minced into 1–3 mm³ fragments and incubated in 1 mg/ml collagenase B (STEMCELL Technologies, diluted in DMEM/high glucose) for 12 h at 37°C. Then, the isolated single cells were expanded in DMEM/high glucose (Gibco, USA) plus 5% platelet lysate (Sigma), 2 U/ml heparin, and antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin). The human MSCs were passaged with 0.05% Trypsin-EDTA (Thermo Scientific). The MSCs were characterized with flow cytometry (CD90-FITC mouse anti-human, BD Biosciences 555595; CD73-FITC mouse anti-human, BD Biosciences 561254; CD105-FITC mouse anti-human, BD Biosciences 561443; HLADR-PE mouse anti-human, BD Biosciences 562304; CD45-PE mouse anti-human, BD Biosciences 555483; CD34-PE mouse anti-human, BD Biosciences 550761; CD19-PE mouse anti-human, BD Biosciences 555413; CD11b-PE mouse anti-human, BD Biosciences 555388). The Differentiation was performed with the StemPro® Chondrogenesis Differentiation Kit (Gibco), StemPro Adipogenesis Differentiation Kit (Gibco), and StemPro Osteogenesis Differentiation Kit (Gibco).

Direct cell lysis & PCR

12 different direct cell lysis methods were compared. The procedures were presented below:

(I) Direct lysis method: the cells were suspended in 15 µl PBS and incubated at 95°C for 15 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(II) PK method: this method was performed as described before with modifications [12,14]. Briefly, the cells were suspended in 15 µl lysis buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA pH ▶

Figure 1. Methods comparison of direct cell lysis and PCR. Mesenchymal stem cells were plated onto a p96 plate with 1, 10, 10² and 10³ cells per well. After overnight incubation, cells were directly lysed with the indicated cell lysis methods. I: direct lysis method; II: PK method; III: PK-T20 method; IV: PK-SDS-1 method; V: PK-SDS-0.5 method; VI: PK-SDS-0.1 method; VII: NaOH method; VIII: KOH method; IX: NL method; X: NaOH-T20 method; XI: PK-Igepal-T20 method; XII: Amp (Extract-N-Amp[™] Blood PCR Kits) method. PCR was performed with 2 x PCR mix (Thermo Scientific) with or without 0.1% BSA and 1% PVP40.

M: DNA ladder; arrows show DNA size with 1.0 or 1.5 kb.

► 8.0, 0.2M NaCl and 1 mg/ml proteinase K), followed by 55°C for 60 min and then 95°C for 15 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(III) PK-T20 (Proteinase K-Tween 20) method: this method was performed as described before with modifications [11]. Briefly, the cells were suspended in 15 µl lysis buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 2% Tween 20, and 1 mg/ml proteinase K), followed by 55°C for 60 min and then 95°C for 15 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(IV) PK-SDS-1 (Proteinase K-SDS 1%) method: this method was performed as described before with modifications [6]. Briefly, the cells were suspended in 15 µl lysis buffer (1% SDS + 1 mg/ml PK, 5% Tween 20, 5% NP-40, 0.1% BSA and 1% PVP 40), followed by 55°C 60 min and then 95°C 15 min; the supernatant was used for PCR

directly after centrifugation at 12,000 rpm for 1 min.

(V) PK-SDS-0.5 (Proteinase K-SDS 0.5%) method: the cells were suspended in 15 µl lysis buffer (0.5% SDS + 1 mg/ml PK, 5% Tween 20, 5% NP-40, 0.1% BSA and 1% PVP 40), followed by 55°C for 60 min and then 95°C for 15 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(VI) PK-SDS-0.1 (Proteinase K-SDS 0.1%) method: the cells were suspended in 15 µl lysis buffer (0.1% SDS + 1 mg/ml PK, 5% Tween 20, 5% NP-40, 0.1% BSA and 1% PVP 40), followed by 55°C for 60 min and then 95°C for 15 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(VII) NaOH method: this method was performed as described before with modifications [10,15]. Briefly, the cells were suspended in 10 µl 50 mM NaOH, followed by 95°C for 10 min and neutralization by

5 µl (1/10 volume) 1M Tris-HCl pH 8.0; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(VIII) KOH method: this method was performed as described before with modifications [5,7]. Briefly, the cells were suspended in 10 µl lysis buffer (200 mM KOH, 50 mM DTT), followed by incubation at 65°C for 10 min and neutralization by 5 µl neutralization buffer (300 mM KCl, 900 mM Tris-HCl pH 8.3, 200 mM HCl); the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(IX) NL (*N*-Lauroylsarcosine salt) method: this method was performed as described before with modifications [13]. Briefly, the cells were suspended in 15 µl lysis buffer (3 µl H₂O, 3 µl 250 ng/µl polyadenylic acid, 3 µl 10 mM EDTA, 3 µl 250 mM DTT, 3 µl 0.5% *N*-Lauroylsarcosine salt solution); followed by incubation at 95°C for 10 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(X) NaOH-T20 (NaOH-Tween 20) method: the cells were suspended in 10 µl lysis (100 mM NaOH and 2% Tween 20); followed by incubation at 95°C for 10 min and neutralizing by 5 µl neutralization buffer (100 mM Tris-HCl, 2 mM EDTA); the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(XI) PK-Igepal-T20 (Proteinase K-Igepal-Tween 20) method: this method was performed as described before with modifications [8]. Briefly, the cells were suspended in 15 µl lysis buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 1% Tween 20, 1% Igepal and 1 mg/ml proteinase K), followed by 55°C for 60 min and then 95°C for 15 min; the supernatant was used for PCR directly after centrifugation at 12,000 rpm for 1 min.

(XII) Amp (Extract-N-Amp[™] Blood PCR Kits) method: the cells were lysed according to the manufacturer's instructions (Sigma).

PCR was performed in a total volume of 25 µl containing 2x PCR Master Mix (Thermo Scientific), 10 µM forward and reverse primers, and 12 µl cell lysis solution obtained from the above methods. Primers were used for amplifying one 1106 bp fragment from the AASV1 site. Forward: 5'-CCCAACCCCATGCCGTCTTCACTCG-3' and reverse: 5'-CTAGGACGCACCATCTCACAAAGG-3'. 40 PCR cycles were performed.

Gene targeting

The donor plasmid was purchased from SBI (System Biosciences). Human IL-10 was PCR amplified and cloned into the donor plasmid according to the manufacturer's instructions. The gRNA target sequence (5'-GGGGC-CACTAGGACAGGAT-3') was cloned into our Cas9-gRNA system as described before [16,17]. Plasmids were purified by PureYield™ Plasmid Maxiprep System (Promega). 1 µg donor plasmid, plus 500 ng gRNA plasmid and 500 ng hCas9 plasmid were mixed with 2 µl Lipofectamine3000 (Thermo Scientific) to transfect 20×10^4 human MSCs/well in a p6 plate. 48 h later, cells were trypsinized and diluted into 0.5 cells per well in a p96 plate after neutralization with MSC culture medium. 2 weeks later, cells were directly lysed with the optimized method and characterized with PCR according to the manufacturer's instruction (SBI, System Biosciences). The IL-10 protein secretion was validated by human IL-10 Quantikine ELISA Kit (D1000B, R&D Systems).

Real-time PCR

RNA extraction and real-time PCR were performed as described previously [20]. The primers used for amplifying the IL-10 were: forward 5'-GCC TAA CAT GCT TCG AGA TC-3'; reverse 5'-TGA TGT CTG GGT CTT GGT TC-3' [21]; IL-37: forward 5'-TCA GCT GAA GAA GGA GAA ACT G-3'; reverse 5'-TTA TCT GTC ACC CCA ACA GG-3' [22].

RESULTS & DISCUSSION

To determine the most efficient approach for direct cell lysis and PCR characterization of the genome-edited MSCs, a total of 12 different methods were compared using the wildtype MSCs, including previously demonstrated methods [5–15] and some modified methods. The MSCs were firstly plated onto a p96 plate with 1, 10, 10^2 and 10^3 cells per well. After overnight incubation, cells were directly lysed with the indicated cell lysis methods. One 1106 bp DNA fragment amplified by PCR was used to assess the efficiency of DNA release from a different number of MSCs. PCR was performed with 2 × PCR mix (Thermo Scientific) with or without 0.1% BSA and 1% PVP40, which could enhance the PCR efficiency and release polymerase inhibition. Data showed that the method III

Figure 2. Target inserting the IL-10 into AASV1 site. (A) Donor and gRNA structures. (B) PCR characterization of genome-edited mesenchymal stem cell (MSC) colonies by amplifying the left arm. Arrow shows the predicted PCR product size. (C) PCR characterization of genome-edited MSC colonies by amplifying the right arm. Arrow shows the predicted PCR product size. (D) mRNA and protein levels of IL-10 among the colony 16 (C16) without donor insertion, colony 17 (C17) with correct insertion, and the wild-type of MSCs (NC). (E) MSC colony C17 was characterized with MSC markers by flow cytometry. (F) MSC colony C17 could differentiate into adipocytes, osteocytes and chondrocytes. M: DNA ladder; arrows show DNA size with 750 bp, 1.0 or 2 kb.

(PK-T20) and XI (PK-Igpal-T20) were the most efficient approaches, successfully amplifying the target DNA fragments from only ten cells (Figure 1). Furthermore, the DNA fragment could be successfully amplified from 10 to 10^3 cells, making them suitable for both low and high cell number (Figure 1), as the proliferation ability may differ from different MSC colonies. However, the BSA and PVP40 addition did not improve efficiency.

Method III (PK-T20) was applied to characterize the MSC colonies by correctly inserting IL-10 into the AASV1 site via Cas9. IL-10 is an important anti-inflammatory factor with immune suppressive functions [23,24]. Although MSCs have been widely studied to treat autoimmune diseases, such as systemic lupus erythematosus [2]. Their therapeutic efficacy should be enhanced with overexpression of the immune suppressor IL-10. Thus, we ►

► have tried to introduce IL-10 to the human genome safe harbor AASV1 site [19]. The gRNA structure and Cas9 system have been demonstrated before (Figure 2A) [16,17]. The donor structure and target sequence were obtained from SBI (Figure 2A). Data showed that method III (PK-T20) could successfully identify the colonies characterized with left-arm PCR (Figure 2B). And this was further confirmed with right-arm PCR (Figure 2C). Then, the mRNA level of IL-10 was measured by real-time PCR among the colony 16 (C16) without donor insertion, colony 17 (C17) with correct insertion, and the wildtype MSCs. Data showed that the mRNA and protein levels of IL-10 in C17 were much higher than the wildtype and C16 (Figure 2D). The genome editing procedures have not affected the phenotype of the MSC, including the cell surface marker expression (Figure 2E) and differentiation abilities (Figure 2F), which was also confirmed prior to genome editing (Supplementary Figure 1). Furthermore, this was also validated by inserting another immune suppressor gene, IL-37, into the same locus [25] (Supplementary Figure 2).

We demonstrated here that the PK-T20 method could be used for characterizing MSC colonies with cell number ranging from 10 to 1000. By using this method, we have successfully established a MSC colony overexpressing IL-10 or IL-37 expression in the AASV1 site, which might hold big promises for therapeutic applications in the future.

AUTHOR CONTRIBUTIONS

Wei Jiang, flow cytometry analysis; Wei Lian, cell culture and PCR analysis; Jieting Chen, MSC isolation; Wenlei Li, MSC isolation; Jieyong Huang, PCR characterization; Baoyu Lai, PCR characterization; Lingyun Li, plasmid construction; Zhong Huang, qPCR and ELISA; Jianyong Xu, Gene targeting and MSC differentiation; project design and manuscript preparation

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SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0183

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