

KNOCK OUT ARYL HYDROCARBON RECEPTOR GENE IN ADIPOSE-DERIVED MESENCHYMAL STEM CELL LINE (PT-5006) BY CRISPR/CAS9

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ABSTRACT

Mesenchymal stem cells (MSCs) have garnered significant attention in biomedical studies due to their remarkable properties, such as self-renewal, differentiation into diverse cell types and immune responses. The proliferation and differentiation of MSCs are significantly influenced by the ligand-dependent transcription factor known as the aryl hydrocarbon receptor (*Ahr*). In order to understand the roles of *Ahr* in adipose-derived MSCs (AD-MSCs), we disrupted the *Ahr* gene in this work using CRISPR/Cas9 gene editing technology. The gRNA/Cas9 dual vector and donor vector were introduced into the AD-MSC cell line (PT-5006). Green fluorescent protein (GFP) expression and puromycin resistance were used to identify the transfected cells. *Ahr*-KO cells were cloned and confirmed by PCR and sequencing. By using RT-qPCR, the expression levels of *Ahr* and the *Ahr*-related gene *Cyp1B1* were investigated. The results showed that the knocked out of *Ahr* using CRISPR/Cas9, resulting significantly decreased expression of 7.69-fold for *Ahr* and 3.70-fold for *Cyp1B1* in the cells. These cell clones and CRISPR/Cas9 vectors could be used as tools to investigate the functions of *Ahr* in both AD-MSCs and other cell types.

Keywords: Adipose-derived MSCs, aryl hydrocarbon receptor, knockout gene, CRISPR/Cas9, puromycin selection.

INTRODUCTION

Mesenchymal stem cells (MSCs) are a specific type of stromal cell that have the ability to renew themselves, differentiate into other types of cells, and regulate immunological responses. (Pittenger *et al.*, 1999). MSCs have been studied in

biomedical research and cell treatments targeting immune-mediated inflammatory illnesses over during the past several years due to their immunomodulatory properties. (Saadh *et al.*, 2023; Müller *et al.*, 2021). MSCs balance the inflammatory responses by releasing various anti-inflammatory agents, such as indoleamine 2,3-

dioxygenase (IDO), interleukin-10 (IL-10), transforming growth factor beta (TGF- β), prostaglandin E2 (PGE2), and TNF-stimulated gene 6 (TSG-6) (Markov *et al.*, 2021). Adipose-derived stem cells (AD-MSCs) are mesenchymal stem cells (MSCs) that can be harvested through a minimally invasive lipectomy procedure (Gimble *et al.*, 2007). AD-MSCs are the most commonly utilized stem cells in cellular therapy due to their wide accessibility and unique features, including immunosuppressive and immunomodulatory properties (Wang *et al.*, 2018; Zhang *et al.*, 2020).

The aryl hydrocarbon receptor (AhR) acts as a transcription factor that is stimulated by ligands. It is known for its ability to control cellular reactions to environmental contaminants, including dioxins, polycyclic aromatic hydrocarbons (PAHs), and various phytochemicals (Stockinger *et al.*, 2014). Numerous studies have demonstrated that AhR is involved in many cellular processes, such as cell proliferation, metabolism, and immunomodulation (Nguyen *et al.*, 2013; Baricza *et al.*, 2016; Riaz *et al.*, 2022). Furthermore, recent studies suggest that the anti-inflammatory and immunomodulatory effects of MSCs can be regulated by ligand-activated AhR (de Almeida *et al.*, 2017; Zhang *et al.*, 2019; Lkhagva-Yondon *et al.*, 2023). Activating AhR with specific agonists, including TCDD, FICZ, tryptophan derivatives, and benzo(a)pyrene, enhances the expression of the *Cyp1a1* and *Cyp1b1* genes in MSCs (Podechard *et al.*, 2009; Lewis *et al.*, 2017). Hinden *et al.* showed that pretreatment of MSCs with IFN- γ , TGF- β , and kynurenine increased the expression of several immunomodulatory genes, such as inducible nitric oxide synthase (NOS), indoleamine 2,3-dioxygenase (IDO), cyclooxygenase-2

(COX2), heme oxygenase 1 (Hmox1), leukemia inhibitory factor (LIF), and programmed death ligand 1 (PD-L1) (Hinden *et al.*, 2015).

The utilization of CRISPR-Cas9 has led to an important evolution in the field of genome engineering, since it allows for accurate and effective editing of DNA sequences. (Prasad *et al.*, 2021). This method can be utilized to disrupt, knock out (KO), or knock in genes (Nishiga *et al.*, 2021). The CRISPR/Cas9 technology has been employed to genetically modify several types of human stem cells, therefore enhancing their therapeutic capacity (Hazrati *et al.*, 2022; Han *et al.*, 2024). Given the multiple functions of AhR and its effects on MSCs, employing CRISPR/Cas9 technology to knock out or knock in the *AhR* gene could be beneficial for further exploring the precise roles of *AhR* in MSCs.

This work employed CRISPR/Cas9 technology to disrupt the *AhR* gene (*AhR*-KO) in AD-MSCs, creating model cell lines for further investigations into the consequences of *AhR* depletion in MSCs.

MATERIALS AND METHODS

Cell culture

The AD-MSC cell line PT-5006 was purchased from Lonza (USA). AD-MSCs were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). The cells were seeded in a 6-well plate (2×10^5 cells) with 3 mL of medium per well and stabilized for 24 hours in an incubator at 37 °C and 5% CO₂. The cells were cultured and harvested for subsequent experiments as described previously (Tien *et al.*, 2023).

Generation of AD-MSC AhR-KO cell clones

The *AhR* gene was knocked out in AD-MSCs using the pCas-Guide CRISPR vector carrying aryl hydrocarbon receptor gRNA (target sequence: CCTACGCCAGTCGCAAGCGG in exon 1 of the AhR gene, NCBI Reference Sequence: NM_001621), a donor vector containing the left and right homologous arms and GFP-Puro functional cassette, and a scramble sequence in the pCas-Guide vector (Origene, USA). The protocol is based on the manufacturer's recommendation. AD-MSCs were transfected with the gRNA and scramble control vectors using TurboFectin (Origene, USA). After 48 hours of transfection, the cells were divided at a ratio of 1:10 and cultured for 3 days. The process was repeated for a total of seven splits based on the manufacturer's protocol. At 20 days posttransfection, the AhR-KO cells were selected with 5 µg/mL puromycin. Subsequently, individual cell colonies were isolated by diluting and seeding approximately 1-2 cells per well in a 96-well

plate. After 14 days, cell colonies were formed and observed under a microscope. Only the wells containing one cell colony were selected. The cell clones were then expanded in 6-well plates.

Analysis of AhR-KO cells

The transfected cells were confirmed by the detection of green fluorescence protein (GFP) using the ImageXpress® Pico Automated Cell Imaging System (Molecular Devices, USA) via automated digital microscopy. Following puromycin selection, genomic DNA was extracted from cells 23 days post-transfection. Specific primers were designed for PCR to verify GFP-puromycin integration in the genome. The forward primer was located on the *AhR* gene, and the reverse primer was located on the GFP gene (Table 1). The presence of products of the expected size (approximately 1 kb) was confirmed using 1% agarose gel electrophoresis. The PCR products were then subjected to Sanger sequencing to confirm integration (1st BASE, Singapore).

Table 1. Sequences of primers used in this study.

Primers	Sequence (5'-3')	Purpose
<i>AhR</i> g-F	CCCAGGCCAGGATTCTAAATA	Detection of transfected cells
<i>GFP</i> g-R	CGGATGATCTTGTCGGTGAA	
<i>AhR</i> -F	ACATCACCTACGCCAGTCGC	Analysis of gene expression levels
<i>AhR</i> -R	TCTATGCCGCTTGAAGGAT	
<i>Cyp1B1</i> -F	CACTGCCAACACCTCTGTCTT	Analysis of gene expression levels
<i>Cyp1B1</i> -R	CAAGGAGCTCCATGGACTCT	
β -actin-F	TCATGAAGTGTGACGTGGACATC	Analysis of gene expression levels
β -actin-R	CAGGAGGAGCAATGATCTTGATCT	

Quantitative real-time PCR (RT-qPCR) was conducted to confirm the knockout of *AhR* by measuring the gene expression of *AhR*

and the downstream gene *Cyp1B1*. Total RNA was isolated from cell lines using TRIzol Reagent (Thermo Fisher Scientific,

USA) according to the manufacturer's instructions. cDNA was synthesized from 1.0 µg of total RNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). RT-qPCR analysis was performed using primers (Table 1) and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA) in a QuantStudio™ 6 Pro Real-Time PCR System. The relative expression levels of the genes were calculated based on the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) after normalization of the mRNA levels of the target genes to those of the endogenous housekeeping gene β -actin. The graphs and data were analyzed using Microsoft Excel, and the *p* values were calculated using the t test.

Statistical analysis

The data are presented as the mean \pm standard deviation of triplicate samples. $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

The integration of the GFP-Puro cassette in AhR-KO cell clones

The CRISPR/Cas9 technology has been extensively employed in stem cell research to induce gene knockout (Zhang *et al.*, 2017). In this work, we employed CRISPR/Cas9 technology to disable the *AhR* gene (AhR-KO) in a cell line called AD-MSC (PT-5006). A schematic of the AhR-KO process is shown in Figure 1A. pCas-guide vectors have been successfully used in previous studies to knock out genes in

human stem cells (Kim *et al.*, 2020; Lee *et al.*, 2022, Disse *et al.*, 2023). The target sequences used in previous studies were employed to generate AhR-KO cells (Rothhammer *et al.*, 2018). By transfecting the pCas-Guide vector, which contains an AhR target sequence, along with a donor vector carrying a GFP-Puro cassette encoding a green fluorescent protein and puromycin resistance, AD-MSC clones with AhR-KO cells were obtained through optical and puromycin selection. The presence of the PGK promoter in the puromycin resistance gene of the donor vector enables the plasmid donor DNA to be resistant to puromycin before it is integrated into the genome. The goal of cultivating cells for a period of 20 days before puromycin selection is to reduce the number of cells containing the donor in its episomal form by dilution. Cells expressing the GFP-Puro cassette were visualized using fluorescence microscopy, as depicted in Figure 1B. Genomic PCR results, shown in Figure 1C, confirmed the integration of the functional cassette into the genome in three cell clones. Agarose gel analysis revealed successful integration of the GFP-Puro cassette into the *AhR* gene, facilitated by the gRNA used, resulting in a PCR product size of approximately 1 kb.

To investigate the type of integration, the PCR products from cell clone 1 were subjected to Sanger sequencing. As shown in Figure 2, cell clone 1, transduced with the pCas-guide vector and donor vector, exhibited homozygous integration of the *AhR* gene.

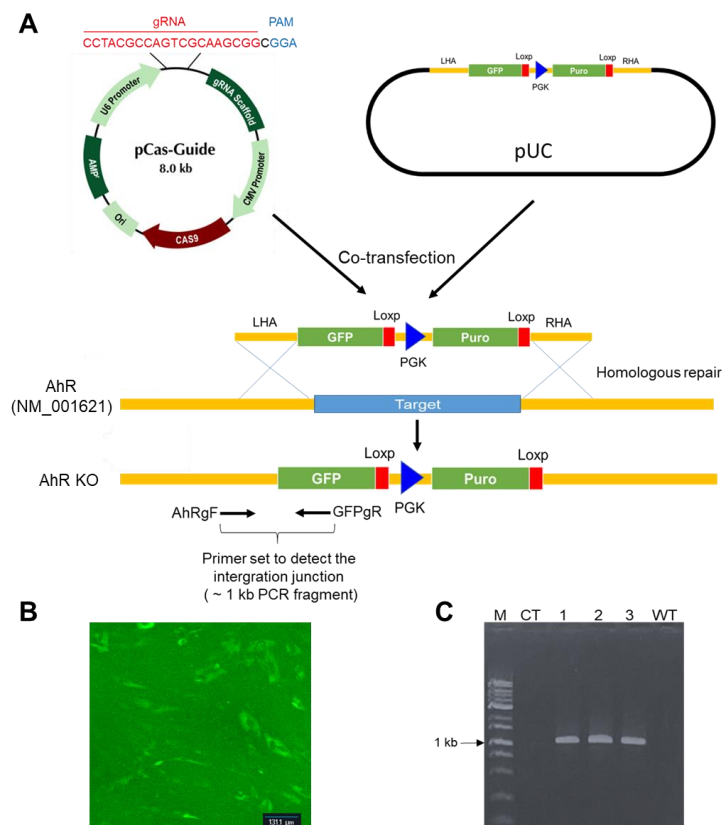


Figure 1. Diagram of the use of CRISPR/Cas9 to knock out the *AhR* gene in the AD-MSC (PT-5006) cell line. **A.** pCas-guide, a dual-function vector containing both guide RNA and Cas9, and a donor vector carrying the functional cassette (GFP-Loxp-Puro-Loxp) were cotransfected into AD-MSCs. **B.** GFP expression was analyzed in transfected cells using automated digital microscopy. **C.** PCR detection of the integrated GFP-Puro cassette in transfected cell lines. Clones 1, 2, and 3 contained an amplified integrated fragment (approximately 1 kb). CT: cells were transfected with a scramble control or the donor vector.



Figure 2. Sanger sequencing confirmation of the integration of the GFP-Puro cassette in the AhR-KO cell clone.

Expression levels of genes in AhR-KO cell clones

AhR is a transcription factor that is activated by ligands and is responsible for controlling the transcription of several target genes, such as *Cyp1B1* (Jacob *et al.*, 2011). The AhR/ARNT heterodimer, consisting of AhR and aryl hydrocarbon nuclear translocator (ARNT), forms an active transcriptional complex. This complex binds to xenobiotic responsive elements (XREs) located in the *Cyp1B1* promoter. As a result, the transcription of *Cyp1B1* is stimulated (Mohamed *et al.*, 2018). Downregulation of *AhR* expression led to a decrease in *Cyp1B1* expression (Do *et al.*, 2014).

In order to determine the impact of *AhR* deletion in AD-MSCs on the expression of

AhR and *Cyp1B1* at the mRNA level, RT-qPCR analysis was used to investigate the gene expression of the two genes in cell clone 1. *AhR* and its downstream gene *Cyp1B1* were expressed in the wild-type AD-MSC (PT-5006) cell line. However, when *AhR* was knocked out using CRISPR/Cas9, there was a significant decrease in the expression of both genes in the cells, with fold changes of 7.69 for *AhR* and 3.70 for *Cyp1B1*. The scramble control-transfected cells did not exhibit any significant difference in *AhR* or *Cyp1B1* expression compared to the wild-type cells (Figure 3). These findings were similar to previous study that found a decrease in the expression of *AhR* and its downstream genes in KO cells utilizing CRISPR/Cas9 technology (Zgarbová & Vrzal, 2022).

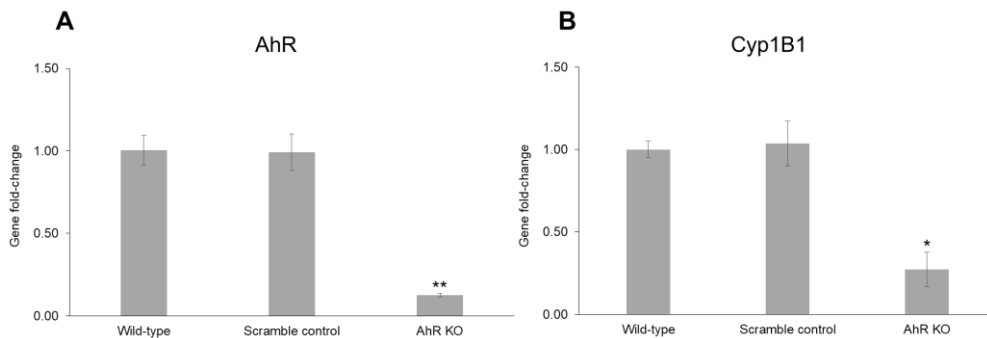


Figure 3. *AhR* (A) and *Cyp1B1* (B) expression in transfected cell clone 1. The data obtained were normalized to the levels of the housekeeping gene β -actin. Error bars represent the mean \pm SD of three biological replicates from one representative experiment. * $p < 0.05$; ** $p < 0.01$.

Disrupting specific genes in stem cells through knockout techniques is a highly effective method for investigating their roles in differentiation, proliferation, and other essential cellular functions (Mandl *et al.*, 2020). Several genome editing methods have been developed to generate KO stem cells, with CRISPR/Cas9 is one of the most powerful systems (Kim *et al.*, 2020; Lee *et al.*, 2022). Mammalian somatic cells have traditionally been shown to be resistant to

genetic modification (Komor *et al.*, 2017). The efficacy of CRISPR/Cas9 knockout varies depending on the complexity of the experimental procedure and the natural features of the stem cells. In this research, we utilized commercially available pCas-Guide vectors carrying *AhR* gRNA and donor vectors containing left and right homologous arms and a GFP-Puro functional cassette. These vectors and gRNA sequences were extensively used in previous studies to

generate KO stem cells and AhR-KO cells (Kim *et al.*, 2020; Lee *et al.*, 2022; Disse *et al.*, 2023; Rothhammer *et al.*, 2018). After cotransfection into commercialized AD-MSCs (PT-5006), genome editing was achieved via homology-directed repair. Using CRISPR/Cas9 technology, our results demonstrated that the *AhR* gene was successfully knocked out in AD-MSCs. However, transfections with more than one gRNA and optimization of the puromycin selection concentration will also be necessary to improve the efficacy of CRISPR/Cas9 in AhR-KO cells.

CONCLUSION

This study utilized the CRISPR/Cas9 system to knock out the AhR gene in AD-MSCs (PT-5006). By employing the pCas-guide vector in conjunction with a donor vector carrying a functional cassette, followed by GFP and puromycin selection, AhR-KO cell clones were successfully generated. Further investigations are needed to explore the effects of AhR knockout on the functions of AD-MSCs using these AhR-KO cells.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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