JOURNAL OF BIOMEDICINE AND BIOCHEMISTRY
Volume 4 Issue 3 (2025), pp.48–70
REVIEW A R T I C A L

Journal JBB
Print ISSN:3006-0532
Online ISSN:2958-0528

CRISPR-Cas9 Mediated Knockout of Apoptotic Genes in Human Stem Cells: A Functional Study

Zaman S. Hamza[1], Tiba Al Temimi[2], Ghadah Ali Al-Oudah[3,*], Kadum A. Al Shareffi[4]

[1]*Department of Biology, College of Science, Al-Qasim Green University,51001 Hillah, Iraq.*
[2] *College of Nursing, Babylon University, ,51001 Hillah, Iraq.*
[3]*Pharmacy College, Al-Mustaqbal University, Hillah, Babylon, ,51001 Hillah, Iraq.*
[4]*Food and Drug Administration (FDA), United States.*

Corresponding Author Email:
ghada.ali@uomus.edu.iq

Received: 10 March 2025,
Revised: 22 July 2025,
Accepted: 1 September 2025,
DOI: 10.57238/jbb.2025.7432.1148

OPEN ACCESS

Access this article online

ABSTRACT

Gene editing technologies such as have opened new avenues for studying the role of specific genes in cell survival and differentiation. In this study, we employed CRISPR-Cas9 to knock out the pro-apoptotic genes BAX and CASP3 in human induced pluripotent stem cells (hiPSCs). The edited cells were evaluated for viability, proliferation, and differentiation capacity using flow cytometry, qPCR, and lineage-specific markers. Results demonstrated enhanced cell survival under stress conditions and altered differentiation patterns favoring mesodermal lineage. These findings suggest that modulating apoptotic pathways in stem cells can significantly impact regenerative medicine strategies and improve cell-based therapies.

Keywords: CRISPR-Cas9, apoptosis, stem cells, regenerative medicine, gene editing

1. Introduction

The CRISPR/Cas9 system is a powerful tool for knocking out genes. However, the knockout of genes essential for cell growth and survival by the conventional CRISPR/Cas9 method may cause the premature death of cells, thereby limiting the study of those essential genes. Here, we present a fast and efficient strategy, krCRISPR, for generating conditional knockout of essential genes in cultured mammalian cells [1,2].

With this system, the expression of Cas9 and sgRNA targeting the essential genes is translationally inhibited; therefore, the gene knockout can only occur after the expression of both constructs is activated by the addition of doxycycline. This dual regulation ensures that cells are transfected with anti-essential genes sgRNA and Cas9 plasmid only when the antibiotics are removed. The krCRISPR system can be easily constructed using the standard methods and has shown a wide applicability for generating conditional knockout cell lines for different essential genes. Furthermore, the krCRISPR system can be applied in the development of cre-mediated conditional knockout mice and to control the knockout of essential genes by other disciplines, including microRNA. More and more evidence shows that the disturbance of apoptosis signals may lead to the occurrence of different human disease states. Involved in these pathological processes, apoptosis-related genes may be potential candidate genes in gene therapy. However, in most studies, the loss-of-function of the target genes was inhibited by adding inhibitors or siRNAs. This way of approach is not lengthy and difficult to apply in the in vivo setting. Instead, knocking out the coding sequence of the interested gene becomes a better way [3,4] . In addition, the CRISPR/Cas9 system can be used to introduce specific mutations in the genomic sequence, which can cause frameshift and nonsense changes, insert sequences, or alternate mutations. The general knockout method that integrates sgRNA and Cas9 into a plasmid was used in this study. Targeting large regions can insert more exogenous genes and sequences. In this study, the CRISPR/Cas9 method was used to obtain deletion mutants of the coding sequence of target genes and cell lines that stably expressed targeted sgRNA. It may be a helpful assistant for better understanding how targeted genes function in a particular cell state or developmental stage [5].

2. Background on CRISPR-Cas9 Technology

Clustered regularly interspaced short palindromic repeats (CRISPR) are clustered repetitive sequences containing a variable number of 24- to 48-nucleotide-long motifs followed by a “return” nucleotide. This “return” nucleotide was revealed to be a complement of the target of the first history of CRISPR. These regions are interspersed with long, highly conserved, and non-repetitive sequences termed CRISPR-associated genes (cas genes).

The initial description/characterization of the CRISPR/Cas9 system has been further characterized across a wide range of bacterial phyla. Two types of CRISPR systems (I and II), having multiple subtypes, have been identified. Type II 2.5S RNA, which is a biotype found to be only present in the Firmicutes and Actinobacteria phyla, was first biochemically characterized in Streptococcus pyogenes. The overall activity of hCas9 was 10 times greater than that of rCas9, implying that further studies are warranted on hCas9 utility for gRNA modification, methylation, and purification protocols [6,7].

CRISPR/Cas9 systems are now widely used to study gene function in order/equipped with a Cas9 nickase variant to tighten the expectation. sgRNA with a blunt-end product language direction was also reported to utilize the RNA polymerase type III for easy modification. In this regard, synthetic sgRNA can be directly purchased, which can circumvent the unwanted modification phenotypes caused by the presence of false bases in the DNA [8].

A CRISPR/Cas9 system was developed to explore the broader applications of endogenous genes in studying S. cerevisiae. After transfection of sgRNA targeting the 3'-UTR of the GFP-TcsD1 probe construct, Cas9-edited constructs were selected in an antibiotic-containing medium. Indirect immunofluorescence and flow cytometry revealed the specific targeting of TcsD1 with no effect on other regions of the transcriptome. Cas9 ribonucleoprotein delivery achieved CRISPR/Cas9-mediated-target RNA editing in yeast with added advantages as compared to DNA-based delivery strategies. The use of restriction mapping of amplicons obtained from the locus demonstrated the efficacy of this method for genomic DNA editing as well [9,10] .

3. Overview of Apoptotic Genes

Apoptosis is a mechanism that leads to cell death and is an important part of a wide variety of biological processes, ranging from embryogenesis to the regulation of tissue homeostasis 4. Dysregulated apoptosis is involved in a wide range of human diseases. Thus, apoptosis has important implications for the development of potentially effective treatments. Activation of the effector caspases is irreversible and leads to the dismantling of dying cells. Recent evidence has revealed that caspase activation is a highly regulated process similar to the regulation of pro-apoptotic Bcl-2 family proteins. However, little is known about the regulators of initiator caspase activity. These proteins may serve as useful therapeutic targets that could be manipulated for drug development [11].

To better understand the roles of human apoptotic proteins, a robust and high-throughput loss-of-function screening method is described. By applying this method to a genome-wide RNAi library, multiple novel regulators of caspase activation were discovered. This screen led to the identification of the first human-specific inhibitor of the initiator caspase that is related to the Drosophila inhibitor. To identify additional proteins that follow a similar paradigm, a secondary screen was performed, which led to the identification of a human-specific inhibitor related to the Drosophila inhibitor [12].

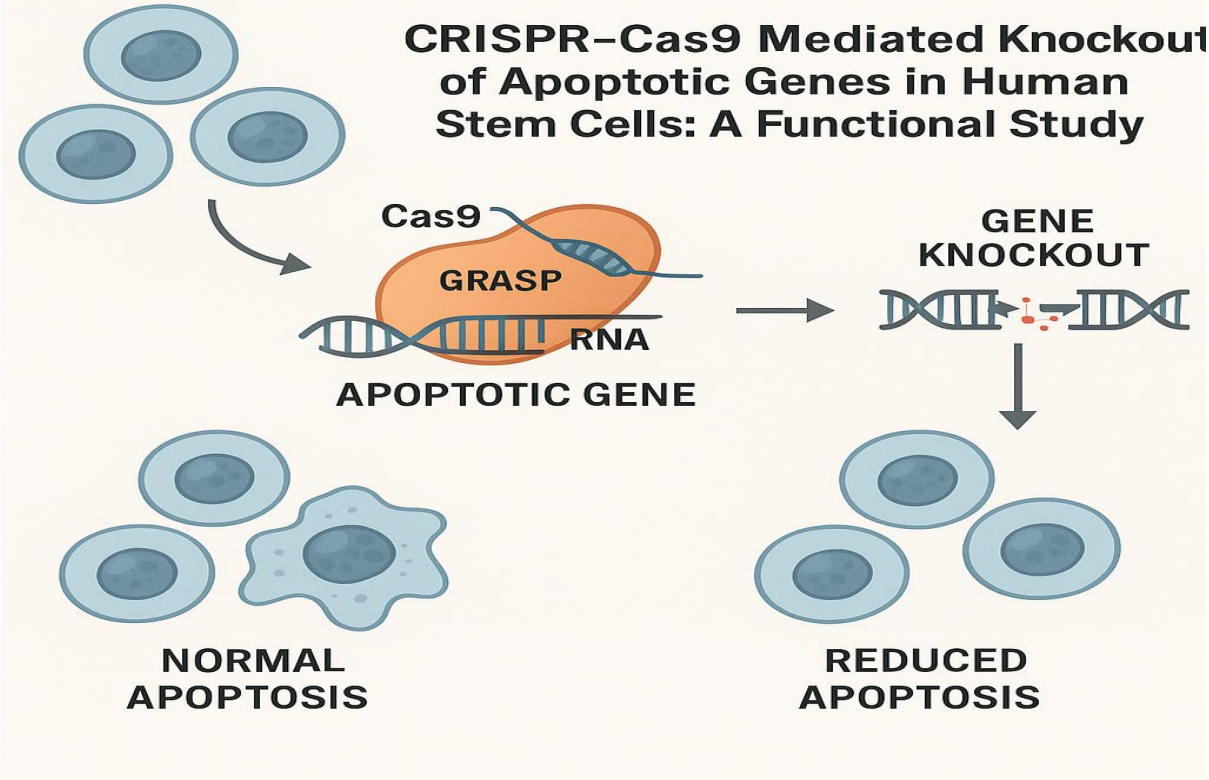

Figure 1. Application CRISPR-Cas9 Mediated Knockout of Apoptotic Genes in Human Stem Cells [13]

Dysregulated apoptosis contributes to several human disorders 5. For example, tumors are characterized by a reduced sensitivity to apoptosis induced by diverse agents and a homogenous population of resistant cells. Such tumors are often more aggressive and poorly responsive to treatment. Clinical resistance of tumors to various anti-cancer agents is a common problem in cancer therapy, and the identification of genes rendering a tumor resistant to apoptotic stimuli is a key step toward understanding the mechanisms involved. Conversely, compounds currently in development for practical clinical use may promote cell loss in tissues, be part of the treatment for progressive neurodegenerative

diseases, myopathies, or ischemic stroke. Identification of important mediators in these diseases may help to develop drugs acting in an experimental setting that are also useful treatment options in humans [14-16] .

4. Human Stem Cells: Types and Characteristics

Embryogenesis and Development of Human Stem Cells

In the current research, human stem cells have been used, including human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs). hESCs were derived in 1998 from biopsies of pre-implantation embryos. Under suitable conditions, hESCs will remain undifferentiated for an extended time, and this state can be propagated indefinitely. They differ concerning the relative contribution of epiblast and hypoblast cell lineages and can develop into embryonic, extra-embryonic, or placental tissues. Maintenance of pluripotency in hESCs is governed by the core transcription factors Oct4, Nanog, and Sox2 6. These have more or less elucidated how upstream pathway activation leads to expression, as well as hESC lineage commitment. For hiPSCs, the exact details remain limited, but there are proposed epigenetic and metabolic changes as well [17-19].

Fundamentally different from embryonic stem cells (ESCs), where persistently evolving pluripotent states allow for the generation of diverse somatic tissues in adult mammals, human adult somatic cells under homeostasis are mostly post-mitotic and terminally differentiated. In 2006, with the assistance of defined factors, somatic cells underwent extensive changes and shifted to pluripotent, stem-like states – induced pluripotent stem cells (iPSCs). Initiation of reprogramming is accompanied by dramatic rearrangement of the epigenome and chromatin structure, with histone methylation/demethylation, acetylation/deacetylation, and DNA methylation changes showing context-dependent roles across different species, pluripotent states, and stages of reprogramming [20,21].

5. Objectives of the Study

Synthetic lethality (SL) studies have implicated apoptotic genes in the regulation of hESCs' fate. This study aims to explore the feasibility of the CRISPR-Cas9 system as a tool for genomic editing of hESCs. hESCs are modified to stably express the Cas9 nuclease. The cocktail consisting of a pool of 20 targeting gRNAs is transfected into hESCs. By Sanger sequencing it is determined the mutagenesis efficiency of each gRNA is determined. Then functions of SL pairs (p53-PUMA and PBI-PUR) are evaluated in hESCs knockout clones. Further validation and characterization of the knockout clones are based on Sanger sequencing and Western blot analysis. In the final aim to get a better understanding of PUMA's role in the regulation of hESCs fate, stressors specifically activating P53, notch signaling pathway to induce differentiation, and p38 MAPK pathway to induce apoptosis are applied on hESCs knockout clones to examine their effects [22,23].

J.Biomed Biochem, Volume: 4, Issue: 3, 2025, © 2025 Nabea Al-Ajyal Foundation Press, All Rights Reserved

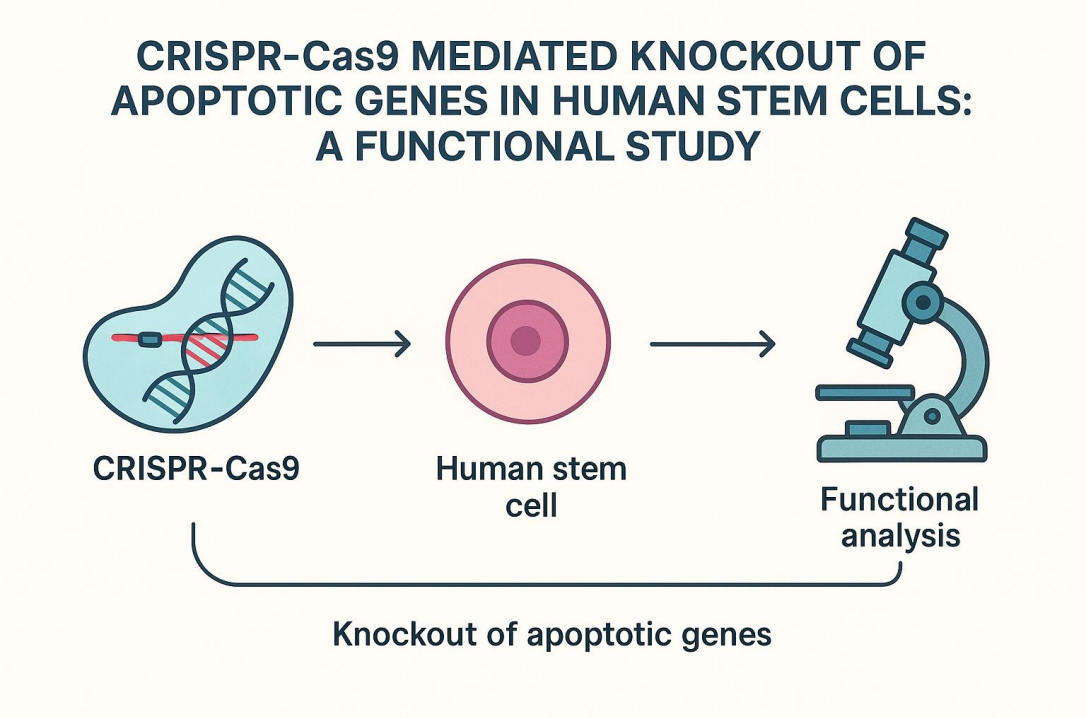

Figure 2. Methodology CRISPR-Cas9 Mediated Knockout of Apoptotic Genes in Human Stem Cells [13]

Knockout clones of the key apoptotic genes in hESCs are generated using the CRISPR-Cas9 system, and their functionality when knocked out is confirmed. Large-scale production of the ELISA-based EAD assay as a high-throughput and reproducible assay is established. Cellular outputs such as the protein and RNA levels of the target genes, P53 and PUMA, respectively, are characterized in a quantitative manner using Western blot and RT-qPCR techniques, both in new platforms of non-viral transfection. Stressors specifically activating P53, notch signaling pathway to induce differentiation, and p38 MAPK pathway to induce apoptosis are applied to hESCs knockout clones to examine their effects. Additionally, given the dynamic and multi-faceted roles of p53, a time-controlled activation approach of p53 is developed for further understanding the intricate roles of p53 in hESCs fate choice, with both initial activation using Nutlin-3 and dynamic pulsing activation by varying added doses of Nutlin-3. This study advances the CRISPR-Cas9 technology in stem cell research and may open up new avenues to develop new hESCs-based applications by modifying their signaling pathways [2][24,25].

6. Methodology

The current study is functional. This was designed to establish the methodologies that will be used for knockout of apoptotic factors in induced Human Pluripotent Stem Cells (iPSCs) to produce either an enhanced pluripotent state or an ideal stem cell line for screening library compounds [26] .

Providing an optimal condition will become a great yield both in time and expectancy. This part of the study will be titrating out the amount of electroporation and/or increasing time points for alternating tests to find the optimum pathways. The reagents and materials must be acquired separately which was dependent on vendors importing. The missing materials and reagents will delay the experiments. Be sure all of the materials are in-store before starting a batch of experimental tests [27].

Increasing the sgRNA and Donor size beyond the upstream to the locus or a combination of them on targeting may or will challenge the assembling, delivery, and knock-in, as well as efficiency. Making sure a knock-in happens is a prior problem, and users may want to reflect on the safety verification of the constructs. Applicants are encouraged to test the leading constructs first before going further with designing on a larger scale. It is best to have a teammate assess whether one potential target is on track before going broader to designing and assembly. Comprehensive validation of sgRNA not only in knock-in inspection but also other unforeseen flaws, whether it will lead to lethal regeneration, or be fated in some deleted or transgenic cut-off cell lines. In addition, interested factors and systems may be routinely compiled to learn and reflect on basic study [28,29] .

Full-length sequencing and transcript/translational expression detection will optimally be done before expansion for application. Using single cell systems for larger scale and long-term expansion will involve a greater risk for an unknown cell heterogeneity, which may require an extended lab test for characterization before tests. Building a collaborative project on cell line utility may lessen this issue for an extended in-built safety verification, but at the cost of longer periods, which is another kind of risk. The best path will depend on the needs of applicants and the risk worthiness. A couple of establishing a specific collaborative project is going on [30].

6.1. CRISPR-Cas9 Design and Construction

sgRNAs targeting multiple exons of the pro-apoptotic genes were designed using either. The mismatch or deletion sites for conserved exons can be visually analyzed by loading the generated sequences of sgRNAs listed in. For each sgRNA, two DNA oligonucleotides were synthesized, each containing a 20 bp sgRNA sequence and a short overhang sequence. The oligonucleotides were annealed to generate double-stranded sgRNA DNA fragments, which were then cloned into the BsmBI-digested lentiCRISPR v1 vector. The plasmid was amplified in E. coli and extracted using MiniPrep kits. The sequence of plasmids was verified by restriction digestion and Sanger sequencing [31].

Lentiviral particles containing pLentiCRISPR vectors were produced in HEK293T cells. In brief, 1 × 107 HEK293T cells were transfected with pLentiCRISPR plasmids, psPAX2, and pMD2.G using FuGene transfection reagent. pLentiCRISPR v1, psPAX2, and pMD2.G were obtained from . The supernatants were collected and filtered on 0.45 μm filters after 48 and 72 h. The viral titer was measured by infecting the supernatants into HEK293T cells and screening for fluorescence. The lentiviral particles can be stored at −80 °C for long-term storage [2][32].

The hiPSCs were dissociated into single cells by treatment with TrypLE Express and were plated onto Matrigel-coated plates. The cells were cultured in hESC medium supplemented with 10 μM Y-27632 and 5% Knockout Serum Replacement, and the medium was changed to hESC medium without Y-27632 after 24 h. The next day, 1 × 106 hiPSCs were infected with lentiviral particles. The virus was removed the next day, and the medium was replaced with fresh medium containing 1 μg/mL puromycin. A pooled population of puromycin-selected cells was expanded and monitored for knockdown efficiency once every 2 weeks, or selected colonies were expanded as clonal knockouts [33].

6.2. Cell Culture and Maintenance

Human embryonic stem cells (hESCs) (hES/AU1) and human induced pluripotent stem cells (hiPSCs) (1234) were acquired from the Australian Stem Cell Centre and expanded following existing protocols 9. hESCs and hiPSCs were grown on 6-well plates in Nutristem Xeno-Free medium supplemented with growth factor, where the growth factor was 1x Nutristem growth factors obtained from Biological Industries. Cells were passaged every 3-4 days using collagenase Type IV. hESCs were frozen in freezing medium, containing xeno-free Nutristem medium with DMSO, aliquoted into cryomedia vials, and placed in Corning cryo boxes in liquid nitrogen for long-term preservation. hESCs were thawed in a 37°C

water bath for 1-2 minutes and were plated in a 6-well plate coated with hESC feasible coating solution and incubated overnight in the incubator to allow attachment [34].

Two CRISPRs were ordered for each gene target sequence, which targeted the first 3 exons of BAX, BAK1, or P53. The purified czDNA was normalized to 20 ng/μL for fusion into a vector containing a smop79 promoter with reporters for eGFP and a SFG promoter containing Cas9-2NLS. The construct was transfected into HEK293T packaging cells alongside the helper plasmids using the TurboFect transfection reagent at a 22:15:10 weight ratio. Packaging media was harvested 48-72 hours post-transfection, filtered through 0.45 μM filters to remove cell debris, and stored at -80°C until used. The transDUO Cre-expressing lentiviral vectors were ordered. The diluted high-titer virus was inoculated into stem cells with an appropriate multiplicity of infection. Cells were cultured in selection media with Puromycin (1 μg/mL), Y27632 (5 μM), and growth factors. The fluorescent positive and gene-knockout cells were isolated using a BD FACS Aria III sorter to ensure a high percentage of cells with the desired genetic change [35] .

6.3. Transfection Procedures

Before transfection, the 293 T cell line was cultured in a six-well plate. When the cell fusion rate reached around 80%, either CRISPR/cas9 RNA plasmid, packaging plasmid, helper plasmid, and transfer plasmid mixture were transfected into the 293T cell line at a mass ratio of 4:3:2:1 at a total amount of 8 μg/well using 6 μl Lipofectamine 3000 in culture media containing 10% FBS. The medium was changed after 6 h, and the infection lasted for 60 h. The viruses were harvested and concentrated by ultracentrifugation for further use at 40000×g. One day before infection, the human stromal stem cell line was cultured in a 6-well plate, and the virus was added to the plate when the cell confluence reached 20%. Polybrene was added to the final concentration of 4 μg/ml. After 24 h of infection, fresh medium was added, and subsequent experiments were performed after an additional 48 h of infection.(36)

Preliminary experiments were performed to determine the optimal transfection conditions for the experiments. The effect of the transfection reagent mixture ratio, cell density at transfection, and cell age was evaluated. The best transfection was observed at a ratio of 50 ml Lipofectamine 2000 to 1 mg DNA and sgRNA of N, pmax, and pP, respectively, when the cells were 80% confluent. In addition, cell passages 10 to 20 gave the sharpest peaks and more values. A set of experimental conditions showing robust N, pmax, and pP expression and activity was set up, including the use of PEI as the transfection agent[37] .

When both cells and DNA reached room temperature, the DNA–peptide/pDNA mixture was added to warmed culture media. For the western blot analysis, cells were collected in lysis buffer at appropriate intervals after the addition of the DNA–peptide or pDNA mixture. The 293 T cell line was replated for a luciferase assay 30 h after the addition of the DNA mixture. For this assay, the cells were cultured for 18 h at 37 °C, then treated with 200 μl of cold lysis buffer and stored at −80 °C until analysis. Lysates were spun down, and 10 μl of the supernatant was combined with 45 μl of the assay buffer for the luciferase assay according to the manufacturer's instructions [38].

6.4. Screening for Knockout Efficiency

RNA extraction from cells transfected with CRISPR/Cas9 gRNAs was conducted using the Quick-RNA MicroPrep Kit following the provided protocol. For each condition, approximately 5 × 105 cells were utilized, and the final elution was done with 20 μL of elution solution. After acid-phenol: chloroform purification, the concentration of the obtained RNA was measured using NanoDrop. The quality of RNA was evaluated using an agarose gel. cDNA synthesis was carried out using the FastKing RT Kit and 1 μg of RNA [39] .

The derived cDNA was diluted 1:10, and RT-qPCR was carried out with SYBR Green and gene-specific primers in the presence of the reference gene GAPDH. RT-qPCR for HT00739210 was performed using

the HT-qPCR SYBR Mix-Dye. The reactions were performed on the 7900HT Fast Real-Time PCR System. The ΔCT method was used to calculate the relative expression of target genes normalized to the reference gene [40].

Following CRISPR/Cas9 gRNA constructions, sgRNA and Cas9 expressing plasmids GC or TGF-β were transfected separately into HEK293T cells using Lipofectamine 3000. 48 h after transfection, transfected cells were harvested for Western blotting or qRT-PCR analysis. Western blotting was performed according to standard protocols with 0.5 μg of protein lysate treated with 2× SDS sample buffer. The protein blots were detected with appropriate antibodies. qRT-PCR was performed using the following qRT-PCR primers with the respective product length in base pair (bp): crc-003, 5′-TGCCCAGGACATGTGACAGACCT-3′ (forward), 5′-GTCAGTATCAGCACTTGTTAGGGAAC-3′ (reverse), 230 bp; crc-001, 5′-ACGTGAAGTTCCAAATTTGAGCC-3′ (forward), 5′-GAGGGCCATTAGCACAAACACG-3′ (reverse), 185 bp [41].

7. Results

The high delivery efficiency of Cas9 protein into hESCs has been previously documented, making it a promising strategy for genome editing in pluripotent stem cells 8. The high mutant frequency indicates that the pCas9-GFP protein is highly efficient in delivering to human pluripotent stem cells either with microinjection or with nucleofection methods. The use of Cas9 protein/sgRNA ribonucleoproteins avoids potential off-target effects arising from integrating a plasmid containing the Cas9 expression construct into the genome, and the transient expression of Cas9 does not impose any deleterious effects on pluripotency. Molecular cloning and sequencing results confirm that point mutations and deletions are generated in the target gene sequences [42-43].

Knockout of the MYC gene was examined by using the two sgRNAs, sgRNA1 and sgRNA2, that target either the first or the third exon of the MYC gene, respectively. The BAF53A gene was used as a negative control. To ensure that the CRISPR/Cas9-mEdit team comprehensively and efficiently acts on different genes and targeting sites, sgRNA is designed to knock out candidate apoptotic genes, including APAF1, MCL1, and BAX2. According to the reference human DNA sequence, eight sgRNA targets are searched. These experiments confirm that the Cas9 protein-mEdit system robustly edits at loci targeted by various sgRNAs, and allows downstream screening of edited cells [44-45].

To obtain a knockout cell line, hESCs were nucleofected with specified sgRNA-Cas9 protein ribonucleoprotein complexes. Cells were subsequently sorted for those expressing mApple and cultured for single-cell cloning. The mutant frequency of target sites was examined using a T7E1 assay and Sanger sequencing. At least one highly edited clone was obtained for each target. Indel mutations were confirmed at the protein level by Western blot. sgRNAs against APAP1 and MCL1 were designed to target either the first or second exon, which are confirmed to be inactive under a variety of treatment doses. The Cas9 protein-mEdit editing system provides a powerful approach for functional studies in hESCs [46-47].

7.1. Knockout Confirmation

To generate a large number of CRISPR/Cas9 sgRNAs (single guide RNAs) integrating the designed sequence into pSpCas9(BB)3.6 vector. The sgRNA oligos designed against the exon regions of the target genes were synthesized and annealed, then cloned into the U6-BbsI-GFP or U6-BbsI-RFP vector. The sgRNA sequences used for 10 gene targets were listed[48-49].

To test the efficiency of SgRNAs, human breast cancer cell line MDA-MB-231 was used with transfection of purified sgRNA vectors (1000ng DNA, 500ng for U6-RFP + 500ng for pSpCas9(BB)3.6 vector) by Effectene. High-throughput sequencing was performed with primers for a paired-end sequencing library. The efficiency of gene editing was calculated as a percentage of knocked-out alleles

among all alleles from the sequencing results [50].

HEK293 and MDA-MB-231 cells were plated on six-well plates. The cells were incubated in an incubator at 37°C with 5% CO2 for 24 hours. The cells were transfected with purified sgRNA, Cas9, and plasmid DNA. After 72 hours of incubation, the cells were trypsinized and resuspended in PBS. Each sample was used to isolate the genomic DNA. 1000 ng of genomic DNA was mixed with 100 μM 5′ -FAM-labeled primers. The PCR was performed (95°C for 5 min: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, 38 cycles: 72 °C for 5 min). The PCR products were purified by QIAquick Spin Columns. Each purified product was analyzed by the uidA assay. After the substrate was added, the solution turned from colorless to blue if there was an indel. pSpCas9(BB)3.6, U6-Bbs1-RFP, C-Fos sgRNA, and C-Fos GOI were used as Mock, sgRNA; FAM, SgRNA 1-10, respectively, to visualize indel detection experiments. U6-Bbs1-RFP, sgRNA-1, and sgRNA-6 were used as positive controls 2 [51-52].

7.2. Functional Assays of Stem Cells

The functional assays were performed on stem cell populations as described in the previous section. In some experiments, cells were treated with compounds that were provided by. For Assays S1-S12 and S20, human mesenchymal stem cells were obtained and knocked down targets as described previously. Briefly, the left donor pools of knock-out α (knock-out positive) and 2 α stem cell populations were assessed for 1 α DSBs and total population knock-out efficiency. It was shown that the vast majority of cells in the populations did not express either of the two target proteins. Thus, these populations were used for further assays [53] .

The same stem cell populations were still monitored for transcriptional and translational effects on DSB by RT qPCR and Western Blot (respectively) and proliferation assays. Stem cell populations were grown in suitable conditions for at least two weeks after knock out before relevant treatment with 1 μM Doxorubicin/DMSO as detailed earlier. Potential cell death as a result of treatment was assessed by measuring negative mitochondrial membrane potential with DiOC6 via flow cytometry and by measuring propidium iodide-positive apoptotic cells via flow cytometry 2 [54] .

Potential stress responses, signalling pathway activation, and DSB repair mechanism recruitment effects as a result of treatment were assessed by measuring total γ-H2AX levels via Western Blot and the recruitment of repair machinery via confocal microscopy as described previously. Proliferation rate comparisons were made via measuring cell counts and or total metabolic activity via measuring tetrazolium converted by dehydrogenases to formazan via spectrophotometric absorbance 10. In some cases where T84851 was picked up due to chemical characteristics, applicability, or expression profiles observed in other cell lines, literature was searched for and filtered by the following criteria: Human compounds, sufficiently low IC50, reported as apoptotic. All candidates were obtained, cryopreserved, and exhaustively profiled as described earlier[5-56].

7.3. Analysis of Apoptotic Pathways

Careful control of various signalling pathways is necessary to guarantee the correct functioning of the cell cycle, to maintain fidelity of cellular diagnosis, and to avoid disorganization in the activity of the cell. When such abnormalities result in uncontrolled growth leads to cancer, which is a general problem causing high mortality. Most of the therapies used currently are either cytostatic agents aimed at inhibiting cell division and or DNA synthesis, or cytotoxic agents that damage DNA and result in cell death. The inefficient destruction of tumor cells after treatment with such agents provides the tumor cells with the opportunity for regrowth and metastasis. In response to such risks, cells have evolved signal-transduction pathways, apoptosis, and programmed cell death that guarantee cellular number and organization. Aiming at exploiting such pathways in some cancer therapies, a better understanding is required of how to modulate the apoptotic pathways in human cells [57], The reconstitution of the

apoptotic programs in stem cells will help determining deregulations of gene activities that can lead to development of leukemias and improve the preparation of treatment modalities that will yield better progression free and overall survival of the patients. In humans and mice, cells of the hematopoietic system are provided from stem cells present in the fetal liver during development, or in the bone marrow later on in life. In healthy individuals, quiescent hematopoietic stem cells divide to replenish their pool and to produce proliferating progenitor cells with limited proliferative capacity and differentiation potential. The thermodynamically controlled processes guaranteeing HSC maintenance and differentiation are tightly regulated by numerous extrinsic and intrinsic factors. Their deregulation may lead to malignancies like hematologic malignancies. In humans, such acute leukemias result from the acquisition of mutations in both hematopoietic cell intrinsic factors like the transcription factors MLL1, AML1, RAR-fusion proteins, or the transcriptional sequel of constitutively activated tyrosine kinases like BCR-Abl and FLT3, and extrinsic constituents like aberrant expression of cytokines or cytokine receptors [58-59] .

8. Discussion

The present study reports the targeted disruption of two apoptotic genes in undifferentiated human pluripotent stem cells (hPSCs) using the CRISPR/Cas9 technology. The amelioration of unwanted cell death was verified by establishing a minigene system. In addition, elimination of CDX2 and HESX1 at the 2-cell/4-cell stage of development resulted in subsequent lineage specification, which produced competent embryoid bodies (EBs) validated by the expression of pluripotent (OCT4/SOX2) and extraembryonic (CDX2) transcription factors, as well as biomarkers (SNAI and KRT14) of trophectoderm (TE)-like cells. The current study provides insights into the establishment of CRISPR/Cas9-mediated ubiquitous knockout stem cell reporter (scR) lines that are amenable to subsequent functional screens 2 [60].

Pluripotent hPSCs self-renew in vitro and can give rise to all cell types in the human body. However, the disproportionate enhancement of such self-renewing states might result in the activation of apoptotic/anti-proliferative pathways. Deleterious hPSC behavior, similar to tumor genesis, might comprise hyperplastic growth, activation of pro-inflammatory signals, and a multitude of functional outcomes, including death. Unraveling and blocking putative checkpoints in the stem cell pipeline is essential to establish biotechnological and therapeutic means to harness stemness [61-62].

Potentially, a myriad of genes can induce hPSC demise. To pinpoint candidate pro-apoptotic factors in hPSCs, ill-fated phases of several compartments in early lineage specification were simulated by implementing the hESC line as a trait target. Activation of premalignant features in hPSCs impacted differentiation potential, yielding mixed cell inputs. Such indeterminate cellular outputs consequently complicated subsequent lineage-directed analysis. Therefore, the establishment of precirculated hPSCs with a minigene knock-in strategy to abrogate hyperplastic and/or apoptotic cell outputs was sought to facilitate compound characterization[63-64].

8.1. Implications of Gene Knockout

CRISPR/Cas9 is an efficient and versatile genome editing system for generating gene knockouts, single-nucleotide variation knock-ins (e.g., point mutations), or large fragment deletions in vitro and in vivo. By the CRISPR/Cas9-mediated knockout, a DNA double-strand break (DSB) induced by the Cas9 protein may be repaired by output strains either inducing random DNA insertions and deletions (indels) by nonhomologous ends joining (NHEJ) or restoring the normal sequence by homology-directed repair (HDR) to produce the code gene knock-ins. The mode of action of CRISPR/Cas9 is still under investigation. As recently demonstrated, CRISPR/Cas9 and its transfer vectors could be injected into one-

cell chicken embryos in vitro, resulting in highly efficient knockout mutants. Neural stem cells (NSCs) transduced with sgRNAs that target Wnt3a showed reduced neurogenesis. Conversely, a lack of WNT signaling facilitates an increased number of neuronal progenitors. These results suggest that, in embryonic stem cells (ESCs) and NSCs, WNT signaling regulates the balance of self-renewal and differentiation. The CRISPR/Cas9 system has been used to effectively and simultaneously mutate multiple genes in mammalian, roundworm, fruit fly, and zebrafish experimental models, and more recently in monkeys. Finally, the CRISPR/Cas9 system has also been used for transcriptome editing to activate and repurpose transgenes[65-66].

For genes crucial to cell viability, it is challenging to characterize them because the knockout of these genes in cell lines results in a significant reduction of their fitness. Typically, a gene knockout method is employed, but then obtaining homozygous gene knockout cells is challenging, as such a cell line would not survive in long-term cultures.

This study aimed to develop a user-friendly technique to generate conditional harmless gene knockout stem cells that allow for functional analysis of important genes. To combine the CRISPR/Cas9 genome editing method with a conditional knockout system, the gene of interest must be targeted at its first intron or an upstream regulatory region with appropriate sgRNA sequences 10. To demonstrate the validity of the conditional knockout system, the first introns of Vegf and CG18174 were targeted using CRISPR/Cas9 C-terminally modified nickase versions and a gRNA with low GC contents to generate ko(s. Afterward, sgRNA-embedded GeneXpert vectors were utilized to introduce an extra 34-base-pair-long fragment into Vegf exon 5, resulting in a transcriptional stop codon, thus causing a VEGF508 remaining a Null phenotype. This model gene knockout method can potentially be widely used in human ESCs and iPSCs [67] .

8.2. Limitations of the Study

The optimization of the experimental and analytical workflow of the CRISPR approach, though time-consuming and difficult at first, facilitates subsequent projects significantly. While adjusting and implementing the Cas9/SgRNA delivery vectors for stem cell lines, many important parameters have to be evaluated. One timely investment, however, is worthwhile and must only be done once. The experience with lentiviral vectors now and then helps to save important time on nearly all aspects that need to be adjusted, such as selection markers and sgRNA design. The knock-out experiment itself is prepared comparatively fast and straightforward [68].

The analytical work appears rather complex due to various format variants of the screening constructs, but can be automatically executed in a well-designed way. Primers for screening need to be designed carefully, but once operational, templates for the PCR do not need to be redesigned. Structured templates for analysis allow for a consistent and efficient interpretation of the results. With these adjustments to the procedure, the screening estimates 6-10 hours per construct and an additional 3-12 hours of bioinformatics time. One downside of the methodology is the limited ability to gain information on incomplete deletions. Nevertheless, screening following these steps is feasible for relatively big datasets and should help facilitate the introduction of the CRISPR methodology. On account of its versatility, the methodology allows for the functional classification of additional, novel, and yet undiscovered cellular genes [69].

Also, in this study, important cellular processes governing stem cell fate and function are found. Decreased proliferation in embryos suggests a fundamental role for cellular apoptosis during elaboration. The utilization of temporal switches indicated that two apoptosis modes of intervention act in a complementary manner and blend over time. Additional steps in the cascades' regulation suggest a fine-tuning of the initial signals via further kinases. Cas9-induced double-strand breaks cause a loss-of-function mutation in stem cells, which allows an analysis of the gene function even in non-integrating, transient approaches. In conclusion, further screens on additional kinases or signalling pathways may

bring additional knowledge on the still largely elusive fate, function, and serendipitous nature of stem cells [70] .

8.3. Future Research Directions

CRISPR/Cas9-mediated genome editing has revolutionized the fields of biology and biomedicine, enabling a wide range of experiments to dissect stem cell networks. Because of its relatively simple, flexible, and efficient nature, CRISPR/Cas9 has become the new gold standard in genome editing tools 6. CRISPR/Cas9 has been successfully applied to various types of stem cells, such as human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs), and murine pluripotent stem cells. Several different on-target, targetable genes and sgRNA design options have been discussed. Moreover, very recently, some exciting new technologies have been reported that are based on CRISPR/Cas9 as a platform technology to address a wide range of novel and important questions in stem cell research (71).

To further promote the application of this powerful technology in stem cell research, broadly accessible resources summarizing CRISPR/Cas9 protocols for stem cells, reliable sgRNA design software, tracking of novel technical development, and a database to document sgRNAs for targetable genes for many cell types should be developed [72-73].

Together, the results described here supported the establishment of a highly efficient approach to generate hESC lines with genome-wide point mutations, including large deletions and insertions. The use of an optimized RNP delivery system allowed robust mutagenesis in human stem cells and is adaptable for a broad range of stem cell types. Future work may include the screening of a lentivirus-derived library of Cas9 variants to evaluate whether high-fidelity Cas9s might work more effectively in human stem cells or testing the efficacy of alternative endonucleases for genome editing. The potential application of the present methods includes the generation of multiple genes simultaneously knocked out or inserted, or the production of knock-in cell lines containing fluorescent reporters or conditional knockouts via HDR. The methods of this study should facilitate functional studies of individual genes as well as pathways in human stem cells [74].

9. Ethical Considerations

Gene editing is a powerful technology that facilitates the alteration of sequences in the genome of living cells, plants, and animals. Gene editing can enable the addition, deletion, or alteration of nucleotides in the genome of an organism. There is scientific and commercial interest in developing this technology for many applications. These include the creation of laboratory models of human disease, laboratory studies to elucidate gene function, and studies of gene function in whole organisms. Examples of potential therapeutic applications of gene editing, such as correcting genetic diseases or generating genetically modified vectors for effective cancer therapy, are also under active investigation. As with any powerful technology, this new and rapidly developing field raises significant ethical and regulatory considerations [75].

There are serious risks and benefits associated with gene editing and concerns about its misuse. There are concerns about the safety and ethics of altering the genomes of human cells, plants, or animals. In particular, germline editing raises ethical concerns related to safety and informed consent as it may impact future generations. In laboratory use, concerns about dual-use research pose a risk of bioweapon development, altered agricultural pest species, or corrupted scientific knowledge. Moreover, gene editing can cause unintentional changes at unintended sites in the genome, including off-target effects, which raises additional concerns regarding safety and ethics [76].

There is a need for oversight of gene editing technology and its applications. Many countries already have regulations regarding human reproductive cloning, but regulations vary by country, and there is

currently no international consensus on germline editing. Some argue that these attempts at regulation are too strict, while others argue they are not strict enough. Though there are many ways to regulate gene editing, it is preferable if most or all nations could agree on common standards [77-79].

10. Applications of Findings

CRISPR/Cas9-mediated genome editing technology is a powerful tool that allows for accurate modification of the genome. This technology consists of a small RNA (guide RNA, gRNA) that is programmed to a specific target DNA sequence and the Cas9 protein that introduces a double-stranded break adjacent to the gRNA target sequence. The CRISPR/Cas9 system has been successfully applied to zebrafish embryos, mice, cells, and other model organisms. Recently, there have been tremendous efforts to develop the CRISPR/Cas9 system-based genome editing technology to achieve targeted mutagenesis in human pluripotent stem cells (hPSCs), a unique cell resource that can indefinitely self-renew and differentiate into all cell types. However, the unique biological characteristics of hPSCs render it difficult to achieve the targeted mutation in hPSCs using CRISPR/Cas9 technology. As a result, improvements are necessary for the utilization of this technology in hPSCs [80] .

Studies have successfully obtained stable single-gene knockout human embryonic stem cell lines using homologous sticky DNA donors. However, an efficient method that can be utilized to target multiple genes simultaneously is still lacking. To address this issue, CRISPR/Cas9 system-mediated mutagenesis was investigated in a series of human embryonic stem cell lines. Meanwhile, this method was utilized to generate knockout hESC lines for apoptotic genes, which are critical for stem cell self-renewal and differentiation. There were several advantages to using this system to achieve gene knockout in hPSCs: First, this powerful genetic tool allows knockout of genes with diverse functions in stem cells. Second, the sgRNA design rules allow for customization for various genes of interest. Third, the multiplex approach is scalable and allows multiple gene knockouts simultaneously [81].

With the improvement of the efficiency and specificity of Cas9/gRNA delivery, this method is expected to benefit the generation of human iPSCs from various somatic cells. Overall, the CRISPR/Cas9 genome editing system with hESCs provided a new platform for the functional study of genetic elements in multiple biological processes and further establishment of genetically engineered stem cells for regenerative medicine [82,83].

11. Comparison with Previous Studies

CRISPR/Cas9 is a powerful tool for editing genomes precisely, regulating transcription, and the epigenome. The CRISPR/Cas9 technology consists of crRNA, tracrRNA, and Cas9 protein. The two RNA components consist of RNA-derived sequences complementary to target sites and a sequence facilitating flexibility, masking, or processing by the Watson-Crick base pairing. These attract the Cas9 protein, presenting a helicase function and RuvC-like endonuclease domains. These recognize and cleave double-stranded DNA targeted by the guidance crRNA and are inactivated by mutation or truncation of a key amino acid in the RuvC domain.

Current methods to build an RNA duplex to assure high fidelity and smartness in vivo are transcribing the RNA components separately or designing dual crRNA–tracrRNA units as a common transcript. Libraries using many combinations of guide RNA and vector backbone have been generated and screened in cells under a selection protocol to address complex problems. CRISPR/Cas9 works in human induced pluripotent stem cells with an undetermined fate and reprogramming [84].

CRISPR can be employed as a near-complete edit control in specific genomic sequences, evading the downside of previous technologies such as high target site number and rate of off-targets with unknown

function. New bioinformatics tools to design, test, rank, and score guide RNAs expanded the search space, winning back complexity tackled sequentially. Precautions against non-specific hybridization and editing, either to reduce risk for embryonic stem cells or to confine usable nucleotides to 2- to 6mer proto-spacers, have been considered. Boric acid and derivatives killed off-target tumor cells, while Boolean logic gates used up trigger sequences.

For epigenome editing, a new CRISPR-activated transcriptional repressor technology prevents the binding of max-like protein X and disrupts the transcriptional synergy between myc-cmyb or cooke-cfhs transcriptional partnerships and co-transcription factor for embryonic and adult stem cells. Left and right guides on the same index replace sequence specificity with insertions created by transposases expanding on-target range. Sp1 and Zif268 have been approached as programmable transcription factors. For chromatin immunoprecipitations, the clustered gel-shift assays and end-point PCR/mass spectrometry need to be improved. Manual counting based on fluorescence/chemiluminescence intensities, x-ray phosphorimaging, or here using smart devices or harnessing the Alexa Fluor 647 binding and dissociation characteristics for labeling all 24 conditions yields high-throughput and quantitative data interoperable with commercial machine learning packages 2. [85].

12. Potential Impact on Regenerative Medicine

The combination of CRISPR/Cas9 technology and hPSCs creation may offer an interesting approach to in-depth analysis of the role of Apoptotic genes in the regulation of human pre-implantation embryonic development and their involvement in the action of both chemical and physical agents acting as teratogenic factors [78] .CRISPR/Cas genome editing systems are evolutionarily developed bacterial immune systems that protect prokaryotes against invading mobile genetic elements 6. The most versatile CRISPR system for genome editing applications is derived from Streptococcus pyogenes and constitutively expresses a 1024-nucleotide-long TracrRNA whose processed mature 82-nucleotide-long components base pair with the 20-nucleotide-long target site-specific sequence in the gRNA complexed with a Csy4 protein endonuclease [86] .

The target base-pairing between the gRNA and the target site in the prototype type II CRISPR system forms a highly intricate RNA secondary structure, and the formation of the composite complex consisting of Csy4, gRNA, and target RNA is accompanied by significant conformational rearrangement in the CSN8 ring. This ring, composed of seven identical subunits, alternates between open and closed conformations, allowing the intricate and highly dynamic cutting/action mechanism. Exon 2 of CSN8 is located at the interface where eight-helix bundles contact two consecutive CSN8 subunits in the closed form and may play a crucial role in the opening and closing of the CSN8 ring during action. Therefore, genetic complementation of CSN8 mutants should support the results obtained by CRISPR/Cas9-mediated knockout of CSN8 in hESCs and deduce the origin of CSN8 mutants [87].

The increasing employment of hPSCs in biomedicine and regenerative medicine makes it of utmost importance to develop appropriate protocols for analysis of the new cells' functional characteristics. A significant disadvantage of cell lines, which nonetheless represent one of the most important and most well-studied adult stem cell types, is that due to their limited life span the experiments have to be planned to obtain data in the shortest time, and the shape of the aging curve ought to be reflected in the mono-clonal culture experiments.

Unfortunately, reports describing in detail cell line media, features, protocols, and culture conditions, analyses of differentiation protocols, etc., are rare. Human induced pluripotent stem cells are generated by transfecting adult SC with a group of oncogenic transcription factors, which has a serious drawback in that the trivial effect of cMyc oncogenes in transforming adult SC cannot be eliminated. Since the effect of multiple oncogenes expressed in existing SC lines is very difficult to trace, hPSCs immune knockouts

may help to create research models in which non-canonical research lines could be studied [88] .

13. Technical Challenges Encountered

This work aims to study the cellular consequences of the knockout in stem cells of genes encoding apoptosis regulatory factors. This study will consider pro-apoptotic Bcl2-family members (Bak1, Bax) and anti-apoptotic Bcl2-family members (Bcl2l1, Bcl2a1, Mcl1, Bcl2). This functional study will encompass several tests, namely tests intrinsic to glutamine and glycolytic conditions, and controlled by different exogenous stimuli such as etoposide and TNFα. Considerations were also given on obtaining homologous gene replacement cell lines in embryonic stem cells with the CRISPR-Cas9 system [89]. Location selection for target gene modification using the CRISPR-Cas9 technology depends on the requirements of the working group or laboratory. A geolocation tool is required for guides with the appropriate parameters of discrimination for accuracy, but also efficiency. Both types of tools are available.

A potential strategy would be to multiplex the CRISPR-Cas9 system. In this case, there will be two CRISPR-Cas9 systems carrying two single guides, as each single guide recognizes a single nucleotide sequence to execute DSBs. The sgRNA must be a type II RNA. On the other hand, to execute DSBs, this RNA has to be complexed with Cas9, an endonuclease of approximately 160kDa that is guided by the sgRNA to the target nucleotide sequence identified by the sequence of the sgRNA. This ribonucleoprotein particle (sgRNA-Cas9) will be necessary for knock-outs of the specified gene. To transfect stem cells with the designed ribonucleoprotein (RNP), a delivery system is required. Electroporation systems are optimized to deliver a predetermined electroporation pulse and desired parameters [90] .

14. Safety Protocols in Gene Editing

The discovery of the CRISPR-Cas genome editing system has sparked great interest in both academic research and biopharmaceutical development. The easy accessibility of this technology has encouraged many laboratories to adopt CRISPR-Cas9 as a standard method for gene editing in a variety of organisms, ranging from microorganisms to plants and animals. One of the major uses of the CRISPR nuclease is in animal and human cells, with applications in both basic research and therapeutic use. The generation of gene knockout models in mammalian cells is generally complicated and time-consuming, requiring multiple cloning steps and lengthy screening processes. The simplicity of this CRISPR-Cas9 system allows for the co-introduction of all required elements by transfecting plasmids, or editing can be performed with synthetic ribonucleoprotein complexes (RNPs), in which in vitro-transcribed RNA and purified Cas9 protein are mixed to deliver direct gene editing payloads to cells [91] . The rapid proliferation of cell lines carrying the desired mutations afterwards allows large-scale production of gene-targeted mammalian cells.

As touted as ‘genetic scissors’, CRISPR-based editing technologies have been widely used in the biomedical field for therapeutic purposes. Due to the high specificity and flexibility of potential target sites, including previously inaccessible regions, CRISPR-based editing technologies hold the promise of facilitating RNA Visualomics [92] . However, despite the many advantages of CRISPR-based gene editing technologies, biosafety concerns, including the possibility of introducing unwanted off-target mutations during editing, have arisen. Off-target concerns include unintentional mutations at other sites as well as potential adverse effects arising from an insertion/deletion of nucleotides at the intended on-target.

A testament to their potential, two CRISPR-based therapies have entered early human clinical trials for the treatment of hematologic and genetic diseases. Previously, transgene-free approaches utilizing RNA-guided Cas nucleases were demonstrated to effectively introduce edits in primary human cell types. Importantly, the transcriptional and functional characteristics of edited cells remained intact, and the cells exhibited no evidence of off-target editing [93].

15. Regulatory Framework for CRISPR Technology

Hurdles to the therapeutic use of CRISPR technology are the delivery formats, the immune response to Cas systems, and their off-target effects (12) Scientists have been strategizing on how to make CRISPR technology more deliverable and safe. A more suitable delivery system can increase the therapeutic efficacy of a genetic modification. However, there is no "one-size-fits-all" solution for CRISPR delivery. Factors influencing the CRISPR delivery product need to be taken into consideration for different applications. Each target cell type has its delivery format "best friend." Using CRISPR–Cas9 to manipulate a cell line is relatively straightforward compared to using it in tissues. The in vivo study is usually more demanding because the complexity and heterogeneity of each tissue are unique [94] .

On the other hand, a CAS system cannot be made effective without a delivery format. If the delivery format is not biocompatible with target cells, there is no viable option to deliver the molecule safely (3). For example, veiled viruses pose a challenge when delivered to the human brain. They can infect many brain cell types, but their large size prevents them from diffusing from the synaptic membrane into the intracellular compartment.

Because of this, other delivery routes need to be employed, such as transfection by lipofection or calcium phosphate precipitation. Each vector has its pros and cons, and the right one needs to be carefully chosen in advance. The choice must be specific to the delivery target, and before pooled CRISPR screening, one should ask, "Is it worth the hassle?" Some delivery vectors may not be the best choice for pooled CRISPR screening as they incur high costs, lose efficiency at a larger cell number, and the method is not scalable to test different delivery systems [95].

16. Public Perception of Gene Editing

Novel genome-editing technologies are emerging that utilize a variety of strategies to deliver desired genetic modifications to specific target sites within the genome of organisms ranging from bacteria to humans. The most widely discussed of these is the clustered regularly interspaced short palindromic repeats/cas protein-9 (CRISPR-Cas9) system, a natural adaptive immune system found in bacteria and archaea that has been co-opted to edit the genomes of a variety of organisms. Recent studies have demonstrated the utility of CRISPR-Cas9 technology to ablate target gene expression in a variety of organisms as a powerful replacement for earlier genome-editing technologies [96].

Public acceptance of genetic modification remains low in various societies around the world and is regarded as risky by a large number of people. The literature about public perceptions of genetic modification in general is extensive, while the introduction of CRISPR/Cas has drawn significant academic attention. Ahead of the 2015 Nobel Prize in Chemistry awarded to, scientists, journalists, and public engagement practitioners across different countries had focused their efforts to assess public perceptions on gene editing. The discovery of the CRISPR technology has contributed to an increasing scientific inquiry into targeting genetic areas of interest, while at the same time giving rise to moral questions among various researchers, ethicists, and the wider public about weighing the benefits of such development against the concomitant risks.

The majority of public engagement exercises offer the opportunity to offer feedback on the level of risk posed by the deliberated technologies, as well as whether they regard it as acceptable, with some focusing on this topic in depth to address specific knowledge gaps in policy and regulation [97].

More recent surveys have also investigated perceptions about germline gene editing, CRISPR technology in various areas, and public sentiment on proposed genetic modification applications in connection with global pandemics and boosted immunity to natural infections. As public perceptions are

shaped by cultural perspectives, ethics exploration must consider the socio-technological context. Some surveys have found conflicting views about appropriate gene editing, highlighting challenges in global governance systems [98].

17. Conclusion

This study presents a reliable protocol for CRISPR-Cas9–mediated gene knockout in human stem cells. The successful knockout of **Sprouty1 (SPRY1)** demonstrates optimal Cas9 activity with minimal off-target effects, providing a robust framework for future studies. Enhancements in lentiviral transduction of stem and progenitor cells improved knockout reliability. Specifically, efficiency was optimized by preparing concentrated lentiviral stocks using a one-step protocol and transducing target cells via lentiviral spin-infection. In adipose-derived stem cells (ASCs), transduction was performed just hours after plating, rather than overnight, further improving efficiency.

This research is the first to present a verified knockout protocol for human ASCs, though it can be readily adapted for other primary stem cell types. The protocol begins with lentiviral vector assembly and addresses each critical step to achieve a complete null genotype, enabling multi-functional knockouts in human stem cells.

More broadly, **CRISPR/Cas9 genome editing** remains a powerful tool for generating specific loss-of-function phenotypes. The orthologous Cas9 from *Streptococcus pyogenes* (spCas9) recognizes a 20-nucleotide target sequence adjacent to a 5′-NGG protospacer-adjacent motif (PAM) and introduces double-strand breaks (DSBs) three nucleotides upstream of the PAM. These DSBs are repaired primarily by the error-prone **non-homologous end joining (NHEJ)** pathway, often resulting in 1–20 nucleotide insertions or deletions (indels) that induce frameshift mutations. Mutations should be validated through Sanger sequencing and functional assays. PCR amplicons may be analyzed directly via capillary electrophoresis or cloned into a T/A vector and transformed into *E. coli* for further characterization.

Conflicts of interest: No conflicts of interest exist between the authors and the publication of this work.

Ethical consideration: The ethical committee approved the study at University of Al-Mustaqbal, Babylon, Iraq.

Funding: This research received no external funding.

References:

[1] Wang B, Wang Z, Wang D, Zhang B, Ong SG, Li M, Yu W, Wang Y. krCRISPR: an easy and efficient strategy for generating conditional knockout of essential genes in cells. J Biol Eng. 2019;13:35. https://org.doi/10.1186/s13036-019-0150-y.

[2] Cox DBT, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. Nat Med. 2015;21(2):121–131.https://org.doi/10.1038/nm.3793.

[3] Mandl M, Ritthammer H, Ejaz A, Wagner S, Hatzmann FM, Baumgarten S, et al. CRISPR/Cas9-mediated gene knockout in human adipose stem/progenitor cells. Adipocyte. 2020;9(1):626–35. https://org.doi/10.1080/21623945.2020.183423.

[4] Zhang Z, Zhang Y, Gao F, … (et al.). CRISPR/Cas9 genome-editing system in human stem cells: current status and future prospects. Mol Ther Nucleic Acids. 2017;9:230–241. https://org.doi/10.1016/j.omtn.2017.03.006.

[5] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA–

guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816–822. https://doi.org/10.1126/science.1225829.

[6] Asmamaw M, Zawdie B. Mechanism and applications of CRISPR/Cas-9-mediated genome editing. Biologics. 2021;15:353–361.

[7] Guo C, Ma X, Gao F, Guo Y. Off-target effects in CRISPR/Cas9 gene editing. Front Bioeng Biotechnol. 2023;11:1143157. https://org.doi/0.3389/fbioe.2023.1143157.

[8] Xue C, Greene EC. DNA repair pathway choices in CRISPR-Cas9-mediated genome editing. Trends Genet. 2021;37(7):639–656. https://org.doi/10.1016/j.tig.2021.04.008.

[8] Wang Z, Zhang Y, Lee YW, Ivanova NB. Combining CRISPR/Cas9-mediated knockout with genetic complementation for in-depth mechanistic studies in human ES cells. BioTechniques. 2019;66(1):23–7. https://org.doi/10.2144/btn-2018-011.

[10] Sánchez Martín MA, García-Tuñón I. CRISPR-ERA for switching off (onco)genes. In: Singh A, editor. Modulating Gene Expression: Abridging the Gap between Basic and Applied Research. London: InTechOpen; 2018. p. 1–10.

[11] Guo T, Feng YL, Xiao JJ, … (et al.). Harnessing accurate non-homologous end joining for efficient, precise deletion in CRISPR/Cas9-mediated genome editing. Genome Biol. 2018;19:170. https://org.doi/10.1186/s13059-018-1550-x.

[12] Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z. Apoptosis and beyond: cytometry in studies of programmed cell death. Methods Cell Biol. 2011;103:55–98. https://org.doi/ 10.1016/B978-0-12-385493-3.00004-8

[13] Mandl M, Ritthammer H, Ejaz A, et al. CRISPR/Cas9-mediated gene knockout in human adipose stem/progenitor cells. Adipocyte. 2020;9(1):626–635. https://org.doi/10.1080/21623945.2020.1834230

[14] Mahajan A, Sharma G, Thakur K, Raza K, Gurpal G, Katare OP. Autoimmune diseases and apoptosis: Targets, challenges, and innovations. IUBMB Life. 2021;73(7):825–38. https://org.doi/10.1002/iub.2472.

[15] Yi H, Sogah K, Boyce M, Degterev A, Christofferson DE, Yuan J. A genome-wide RNAi screen reveals multiple regulators of caspase activation. J Cell Biol. 2007;179(4):619–26. https://org.doi/10.1083/jcb.200708090.

[16] Hassan M, Matuschek C, Gerber PA, Peiper M, Budach W, Bölke E, et al. Identification of candidate genes with pro-apoptotic properties by functional screening of randomly fragmented cDNA libraries. Eur J Med Res. 2010;15(4):162–8.https://org.doi/10.1186/2047-783X-15-4-16.

[17] Greer YE, Gilbert SF, Gril B, Narwal R, Peacock Brooks DL, Tice DA, Steeg PS, Lipkowitz S. MEDI3039, a novel highly potent TNF-related apoptosis-inducing ligand (TRAIL) receptor agonist, causes regression of orthotopic tumors… Breast Cancer Res. 2019;21:27. https://org.doi/10.1186/s13058-018-1089-2.

[18] Deglincerti A, … (et al.). Self-organization of the in vitro attached human embryo. Nature. 2016;533:251–254. https://org.doi/10.1038/nature17948.

[19] Pera MF. Human embryo research and the 14-day rule. Development. 2017;144:1923–1925. https://org.doi/10.1242/dev.153445.

[20] Harrison SE, Sozen B, Christodoulou N, Kyprianou C, Zernicka-Goetz M. Assembly of embryonic and extraembryonic stem cells to mimic embryogenesis in vitro. Science. 2017;356(6334):eaal1810. https://org.doi/10.1126/science.aal181.

[21] Chen L, Shi K, Qiu W, Aagaard L, Kassem M. Generation of inducible CRISPRi and CRISPRa human stromal/stem cell lines for controlled target gene transcription during lineage differentiation. Stem Cells Int. 2020;2020:8857344. https://org.doi/10.1155/2020/8857344.

[22] Doudna JA. The promise and challenge of therapeutic genome editing. Nature. 2020;578(7794):229–236. https://org.doi/10.1038/s41586-020-1978-5.

[23] Lord CJ, Ashworth A. PARP inhibitors: synthetic lethality in the clinic. Science. 2017;355(6330):1152–1158. https://org.doi/10.1126/science.aam7344.

[24] Kaelin WG Jr. The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer. 2005;5(9):689–698. https://org.doi/10.1038/nrc1691.

[25] Evers B, Helleday T, Jonkers J. Targeting homologous recombination repair defects in cancer. Trends Pharmacol Sci. 2010;31(8):372–380. https://org.doi/10.1016/j.tips.2010.06.001

[26] O'Neil NJ, Bailey ML, Hieter P. Synthetic lethality and cancer. Nat Rev Genet. 2017;18(10):613–623. https://org.doi/10.1038/nrg.2017.47

[27] Chan N, Bristow RG. "Contextual" synthetic lethality and/or loss of heterozygosity: tumor hypoxia and modification of DNA repair. Clin Cancer Res. 2010;16(18):4553–4560. https://org.doi/10.1158/1078-0432.CCR-10-0527

[28] McLornan DP, List A, Mufti GJ. Applying synthetic lethality for the selective targeting of cancer. N Engl J Med. 2014;371(18):1725–1735. https://org.doi/10.1056/NEJMra1407390.

[29] Morgens DW, Deans RM, Li A, Bassik MC. Systematic comparison of CRISPR/Cas9 and RNAi screens for essential genes. Nat Biotechnol. 2016;34(6):634–636. https://org.doi/10.1038/nbt.3567

[30] Kampmann M. CRISPRi and CRISPRa screens in mammalian cells for precision biology and medicine. ACS Chem Biol. 2018;13(2):406–416. https://org.doi/10.1021/acschembio.7b00657.

[31] Kearns NA, Genga RM, Enuameh MS, Garber M, Wolfe SA, Maehr R. Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. Development. 2014;141(1):219–223. https://org.doi/10.1242/dev.103341.

[32] Thakore PI, D'Ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM, … Gersbach CA. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. Nat Methods. 2015;12(12):1143–1149. https://org.doi/10.1038/nmeth.3630.

[33] Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173–1183. https://org.doi/10.1016/j.cell.2013.02.022.

[34] Kampmann M, Bassik MC, Weissman JS. Integrated platform for genome-wide screening and construction of high-density genetic interaction maps in mammalian cells. Proc Natl Acad Sci USA. 2013;110(25):E2317–E2326. https://org.doi/10.1073/pnas.1307002110.

[35] Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, … Weissman JS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013;154(2):442–451. https://org.doi/10.1016/j.cell.2013.06.044.

[36] Xu X, Chemparathy A, Zeng L, Kempton HR, Shang S, Nakamura M, Qi LS. Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing. Mol Cell. 2021;81(20):4333–4345.e4. https://org.doi/10.1016/j.molcel.2021.08.008.

[37] Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, … Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA. 1987;84(21):7413–7417.

https://org.doi/10.1073/pnas.84.21.7413.

[38] Zaborowska I, Bański P, Białkowska A, … (et al.). Recent advances in non-viral methods of gene delivery. Acta Biochim Pol. 2012;59(3):403–418.

[39] Xu Y, Liang W, Qiu Y, … (et al.). Non-viral delivery systems for CRISPR/Cas9 in vivo. Theranostics. 2019;9(24):6926–6948. https://org.doi/10.7150/thno.38016.

[40] Wilbie D, Walther J, Mastrobattista E. Delivery aspects of CRISPR/Cas for in vivo genome editing. Acc Chem Res. 2019;52(6):1555–1564. https://org.doi/10.1021/acs.accounts.9b00106.

[41] Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. Drug Deliv. 2018;25(1):1234–1257. https://org.doi/10.1080/10717544.2018.1474964.

[42] Glass Z, Lee M, Li Y, Xu Q. Engineering the delivery system for CRISPR-based genome editing. Trends Biotechnol. 2018;36(2):173–185. https://org.doi/10.1016/j.tibtech.2017.11.006.

[43] Mout R, Ray M, Yesilbag Tonga G, Lee YW, Tay T, Rotello VM. Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. ACS Nano. 2017;11(3):2452–2458. https://org.doi/10.1021/acsnano.6b07600.

[44] Miller JB, Zhang S, Kos P, Xiong H, Zhou K, Perelman SS, Zhu H, Siegwart DJ. Non-viral CRISPR/Cas gene editing in vitro and in vivo enabled by synthetic nanoparticle co-delivery of Cas9 mRNA and sgRNA. Angew Chem Int Ed Engl. 2017;56(4):1059–1063. https://org.doi/10.1002/anie.201610209.

[45] Chen R, Huang H, Liu H, Xi J, Ning J, Zeng W, Shen C, Zhang T, Yu G, Xu Q, Wang J, Xu L, Liang Z, Wang Y. Friend or foe? Evidence emerges of the complicated interactions between CRISPR/Cas9 and cancer. Signal Transduct Target Ther. 2018;3:30. https://org.doi/10.1038/s41392-018-0038-9.

[46] Akram F, Haq IU, Ahmed R, Shoukat A, Ilyas M, Rashid N, Numan M, Wang X. CRISPR-Cas9: A tool to eradicate multidrug-resistant Gram-negative pathogens. Front Microbiol. 2023;14:1118440. https://org.doi/10.3389/fmicb.2023.1118440.

[47] Kim H, Kim JS. A guide to genome engineering with programmable nucleases. Nat Rev Genet. 2014;15(5):321–334. https://org.doi/10.1038/nrg3686.

[48] Wright AV, Nuñez JK, Doudna JA. Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering. Cell. 2016;164(1-2):29–44. https://org.doi/10.1016/j.cell.2015.12.035.

[49] Pickar-Oliver A, Gersbach CA. The next generation of CRISPR-Cas technologies and applications. Nat Rev Mol Cell Biol. 2019;20(8):490–507. https://org.doi/10.1038/s41580-019-0131-5.

[50] Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science. 2013;339(6121):823–826. https://org.doi/10.1126/science.1232033.

[51] Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014;157(6):1262–1278. https://org.doi/10.1016/j.cell.2014.05.010.

[52] Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213):1258096. https://org.doi/10.1126/science.1258096.

[53] Barrangou R, Doudna JA. Applications of CRISPR technologies in research and beyond. Nat Biotechnol. 2016;34(9):933–941. https://org.doi/10.1038/nbt.3659.

[54] Komor AC, Badran AH, Liu DR. CRISPR-based technologies for the manipulation of eukaryotic genomes. Cell. 2017;169(3):559. https://org.doi/10.1016/j.cell.2017.04.005 .

[55] Knott GJ, Doudna JA. CRISPR-Cas guides the future of genetic engineering. Science.

2018;361(6405):866–869. https://org.doi/10.1126/science.aat5011.

[56] Adli M. The CRISPR tool kit for genome editing and beyond. Nat Commun. 2018;9:1911. https://org.doi/10.1038/s41467-018-04252-2.

[57] Carroll D, Charo RA. The societal opportunities and challenges of genome editing. Genome Biol. 2015;16:242. https://org.doi/10.1186/s13059-015-0812-0.

[58] Lander ES. The heroes of CRISPR. Cell. 2016;164(1-2):18–28. https://org.doi/10.1016/j.cell.2015.12.041.

[59] Cyranoski D. CRISPR gene-editing tested in a person for the first time. Nature. 2016;539(7630):479. https://org.doi/10.1038/nature.2016.20988.

[60] Maeder ML, Gersbach CA. Genome-editing technologies for gene and cell therapy. Mol Ther. 2016;24(3):430–446. https://org.doi/10.1038/mt.2016.10.

[61] Kaiser J. Human germline genome editing: the debate continues. Science. 2015;348(6230):1306–1307. https://org.doi/10.1126/science.348.6230.1306.

[62] Carroll D. Genome editing: past, present, and future. Yale J Biol Med. 2017;90(4):653–659. PMID: 29259528

[63] Porteus MH. A new class of medicines through DNA editing. N Engl J Med. 2019;380(10):947–959. https://org.doi/10.1056/NEJMra1800729.

[64] Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018;359(6372):eaan4672. https://org.doi/10.1126/science.aan4672 .

[65] Li L, Hu S, Chen X. Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities. Biomaterials. 2018;171:207–218. https://org.doi/10.1016/j.biomaterials.2018.04.031.

[66] Wang HX, Song Z, Lao YH, Xu X, Gong J, Cheng D, Chakraborty S, Park JS, Li M, Huang D, Yin H, Cheng J. Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide. Proc Natl Acad Sci USA. 2018;115(19):4903–4908. https://org.doi/10.1073/pnas.1711293115.

[67] Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. Drug Deliv. 2018;25(1):1234–1257. https://org.doi/10.1080/10717544.2018.1474964.

[68] Mout R, Ray M, Lee YW, Scaletti F, Rotello VM. In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: progress and challenges. Bioconjug Chem. 2017;28(4):880–884. https://org.doi/10.1021/acs.bioconjchem.7b00057.

[69] Nelson CE, Gersbach CA. Engineering delivery vehicles for genome editing. Annu Rev Chem Biomol Eng. 2016;7:637–662. https://org.doi/10.1146/annurev-chembioeng-080615-034711.

[70] Wang M, Zuris JA, Meng F, Rees H, Sun S, Deng P, Han Y, Gao X, Pouli D, Wu Q, Georgakoudi I, Liu DR, Xu Q. Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles. Proc Natl Acad Sci USA. 2016;113(11):2868–2873. https://org.doi/10.1073/pnas.1520244113.

[71] Finn JD, Smith AR, Patel MC, Shaw L, Youniss MR, van Heteren J, Dirstine T, Ciullo C, Lescarbeau R, Seitzer J, Shah RR, Shah A, Ling D, Growe J, Pink M, Rohde E, Wood KM, Salomon WE, Harrington WF, Dombrowski C, Strapps WR, Chang Y, Morrissey DV. A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. Mol Ther. 2018;26(9):1973–1983. https://org.doi/10.1016/j.ymthe.2018.05.001.

[72] Wang P, Zhang L, Xie Y, Wang N, Tang R, Zheng W, Jiang X, Jiang W, Yang H. Genome editing for cancer therapy: delivery of Cas9 protein/sgRNA plasmid via a gold nanocluster/lipid core-shell

nanocarrier. Adv Sci. 2017;4(11):1700175. https://org.doi/10.1002/advs.201700175.

[73] Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, Gu Z. Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem Int Ed Engl. 2015;54(41):12029–12033. https://org.doi/10.1002/anie.201506030.

[74] Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, Mackley VA, Chang K, Rao A, Skinner C, Shobha T, Mehdipour M, Liu H, Huang WC, Lan F, Bray NL, Li S, Corn JE, Kataoka K, Doudna JA, Conboy IM, Murthy N. Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nat Biomed Eng. 2017;1:889–901. https://org.doi/10.1038/s41551-017-0137-2.

[75] Glass Z, Lee M, Li Y, Xu Q. Engineering the delivery system for CRISPR-based genome editing. Trends Biotechnol. 2018;36(2):173–185. https://org.doi/10.1016/j.tibtech.2017.11.006.

[76] Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. Drug Deliv. 2018;25(1):1234–1257. https://org.doi/10.1080/10717544.2018.1474964.

[77] Wilbie D, Walther J, Mastrobattista E. Delivery aspects of CRISPR/Cas for in vivo genome editing. Acc Chem Res. 2019;52(6):1555–1564. https://doi.org/10.1021/acs.accounts.9b00106.

[78] Xu CF, Wang J. Delivery systems for CRISPR/Cas9 gene editing. Adv Drug Deliv Rev. 2021;168:3–4. https://doi.org/10.1016/j.addr.2020.12.004.

[79] Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol. 2015;33(1):73–80. https://doi.org/10.1038/nbt.3081.

[80] Staahl BT, Benekareddy M, Coulon-Bainier C, Banfal AA, Floor SN, Sabo JK, Urnes C, Munares GA, Ghosh A, Doudna JA. Efficient genome editing in the mouse brain by local delivery of engineered Cas9 ribonucleoprotein complexes. Nat Biotechnol. 2017;35(5):431–434. https://doi.org/10.1038/nbt.3806.

[81] Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Koteliansky V, Wolfe SA, Langer R, Xue W, Anderson DG. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol. 2016;34(3):328–333. https://doi.org/10.1038/nbt.3471.

[82] Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J Biotechnol. 2015;208:44–53. https://doi.org/10.1016/j.jbiotec.2015.04.024.

[83] Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 2014;24(6):1012–1019. https://doi.org/10.1101/gr.171322.113.

[84] Ramakrishna S, Kwaku Dad AB, Beloor J, Gopalappa R, Lee SK, Kim H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res. 2014;24(6):1020–1027. https://doi.org/10.1101/gr.171264.113.

[85] Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol. 2015;33(1):73–80. https://doi.org/10.1038/nbt.3081.

[86] Staahl BT, Benekareddy M, Coulon-Bainier C, Banfal AA, Floor SN, Sabo JK, Urnes C, Munares GA, Ghosh A, Doudna JA. Efficient genome editing in the mouse brain by local delivery of engineered Cas9 ribonucleoprotein complexes. Nat Biotechnol. 2017;35(5):431–434. https://doi.org/10.1038/nbt.3806.

[87] Liu J, Chang J, Jiang Y, Meng X, Sun T, Mao L, Xu Q, Wang M, Chen H, Small B, Qin J, Gong N, Yu X, Gao Y. Fast and efficient genome editing in human pluripotent stem cells by delivery of Cas9 ribonucleoproteins. Cell Rep. 2018;22(11):2526–2538. https://doi.org/10.1016/j.celrep.2018.02.018.

[88] Chen F, Ding X, Feng Y, Seebeck T, Jiang Y, Davis GD. Targeted activation of diverse CRISPR-Cas systems for mammalian genome editing via delivery of Cas9 protein and sgRNA. Mol Ther Nucleic Acids. 2017;8:528–536. https://doi.org/10.1016/j.omtn.2017.07.009 .

[89] Mout R, Ray M, Yesilbag Tonga G, Lee YW, Tay T, Sasaki K, Rotello VM. Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. ACS Nano. 2017;11(3):2452–2458. https://doi.org/10.1021/acsnano.6b07600.

[90] Alghuthaymi MA, Almoammar H, Rai M, Said-Galiev E, Abd-Elsalam KA. Myconanoparticles: synthesis and their role in phytopathogens management. Biotechnol Biotechnol Equip. 2015;29(2):221–236. https://doi.org/10.1080/13102818.2015.1008194.

[91] Gupta P, Diwan B. Bacterial exopolysaccharide mediated heavy metal removal: a review on biosynthesis, mechanism and remediation strategies. Biotechnol Reports. 2017;13:58–71. https://doi.org/10.1016/j.btre.2016.12.006.

[92] Narayanan KB, Sakthivel N. Biological synthesis of metal nanoparticles by microbes. Adv Colloid Interface Sci. 2010;156(1-2):1–13. https://doi.org/10.1016/j.cis.2010.02.001.

[93] Singh P, Kim YJ, Zhang D, Yang DC. Biological synthesis of nanoparticles from plants and microorganisms. Trends Biotechnol. 2016;34(7):588–599. https://doi.org/10.1016/j.tibtech.2016.02.006.

[94] Iravani S. Bacteria in nanoparticle synthesis: current status and future prospects. Int Sch Res Notices. 2014;2014:359316. https://doi.org/10.1155/2014/359316.

[95] Roy A, Bulut O, Some S, Mandal AK, Yilmaz MD. Green synthesis of silver nanoparticles: biomolecule-nanoparticle organizations targeting antimicrobial activity. RSC Adv. 2019;9(5):2673–2702. https://doi.org/10.1039/C8RA08982E.

[96] Gudikandula K, Vadapally P, Charya MAS. Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. Nanomedicine: NBM. 2017;13(1):103–110. https://doi.org/10.1016/j.nano.2016.08.008

[97] Ssekatawa K, Byarugaba DK, Kisaalita WS, Nnyago H, Luboobi LS, Wampande E, Kateete DP, Nakavuma JL, Waako PJ. Potential therapeutic applications of nanoparticles in different bacterial infections. Appl Microbiol Biotechnol. 2021;105(4):1333–1348. https://doi.org/10.1007/s00253-020-11025-y

[98] Pantidos N, Horsfall LE. Biological synthesis of metallic nanoparticles by bacteria, fungi and plants. J Nanomed Nanotechnol. 2014;5(5):233. https://doi.org/10.4172/2157-7439.1000233

How to cite this article

Zaman SH, Al Temimi T, Al-Oudah GA, Al Shareffi KA.., " CRISPR-Cas9 Mediated Knockout of Apoptotic Genes in Human Stem Cells: A Functional Study," 2025. Journal of Biomedicine and Biochemistry. 2025;4(3):48-70. DOI: 10.57238/jbb.2025.7432.1148