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ENGINEERING GENOME-BASED THERAPEUTICS FOR SELECTIVE ELIMINATION OF SENESCENT HUMAN CELLS

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ABSTRACT

Background: Cellular senescence plays a major role in aging-related diseases by irreversible cell-cycle arrest as well as secretion of pro-inflammatory senescence-related secretory phenotype (SASP) factors. Current senolytic therapies are often non-specific and damaging to healthy proliferating cells.
Objective: To develop a genome-based therapeutic platform to selectively detect and kill senescent human cells employing programmable CRISPR-associated genetic circuits.
Methodology: Human fibroblast and epithelial cell models of senescence were induced by replicative exhaustion and doxorubicin-mediated DNA damage. Biomarkers of senescence including p16INK4a and p21CIP1 were profiled by qRT-PCR and RNA-seq . Here, we have engineered a synthetic CRISPR-Cas therapeutic circuit coupled to senescence-responsive promoters to achieve selective induction of apoptosis in senescent cells. Therapeutic efficacy and specificity were tested by functional assays, flow cytometry and cytokine profiling.
Results: The engineered system efficiently and selectively removed approximately 82% of senescent cells while maintaining viability in non-senescent controls at over 93%. Treatment reduced the levels of SASP-associated cytokines IL-6 and IL-8 by 72% and 65%, respectively. There was negligible off-target genome editing activity.
Conclusion: Genome-engineered senolytic therapeutics are highly promising for the specific targeting of senescent human cells and may offer a promising therapeutic approach for the treatment of aging-associated diseases and the enhancement of tissue regeneration.

KEYWORDS: Senolytics, SASP, CRISPR therapeutics, genome engineering, targeted apoptosis, synthetic biology, aging biology**.**

1 INTRODUCTION

The process of cellular is an irreversible, stable state of cell-cycle arrest induced by replicative exhaustion, oxidative stresses, oncogene activation, and DNA damage. Although senescence is initially a protective mechanism to prevent tumorigenesis and unregulated proliferation, the long-term accumulation of senescent cells contributes substantially to tissue degeneration, chronic inflammation and age-related diseases [1]. Senescent cells secrete a complex mixture of cytokines that are inflammatory, chemokines, growth factors and proteases, collectively termed the senescence-associated secretory phenotype (SASP), that disrupt tissue homeostasis and promote pathological remodeling [2]. Recent studies have linked accumulation of senescent cells to cardiovascular disease, osteoarthritis, neural degeneration, pulmonary fibrosis and metabolic dysfunction [3].

The present senolytic therapeutic approaches against senescent cells are based mainly on small molecules inhibitors such as Dasatinib, Quercetin and BCL-2 family inhibitors [4]. Although promising results have been obtained with these agents in preclinical and early clinical studies, they often show limited specificity, systemic toxicity and off-target apoptosis in healthy proliferating cells [5]. In addition, heterogeneity of senescent cells among tissues is a major challenge to develop a universal senolytic therapy [6]. Hence, there is an urgent need for programmable and extremely selective therapeutic strategies capable of recognizing senescence-specific molecular signatures.

[7] Advances in genome engineering and synthetic biology have led to the development of highly precise therapeutic systems based on CRISPR-Cas technologies. CRISPR-mediated genome engineering provides unprecedented opportunities for particular gene activation, repression, and directed apoptosis with programmable guide RNAs and synthetic genetic circuits [8]. More recently, it has been shown that it is possible to engineer promoters that are specific to a state (in this case senescence) and are fused to therapeutic effectors [9]. In

particular, molecular markers such as p16^INK4a^, p21^CIP1^ and SASP-related inflammatory mediators have been shown to be promising for selective therapeutic stimulation in senescent cells [10].
The development of synthetic gene circuits that combine Boolean logic gates with inducible apoptotic pathways has moreover expanded the potential of genome-based treatments to discriminate diseased from healthy cellular populations [11]. However, there are still several limitations including ineffective delivery systems, off-target genome editing, and lack of validation of long-term beneficial safety [12]. Moreover, there are few studies of integrated CRISPR-based senolytic platforms that can detect senescence biomarkers and activate selective elimination mechanisms simultaneously.
Here, we propose an engineered genome-based therapeutic system for selective elimination of senescent human cells based on CRISPR-associated programmable circuits in combination with senescence-responsive promoters. The proposed platform aims to improve specificity of therapy and to damage healthy cells less, and to reduce inflammatory SASP signaling.

1.1 Gap in Previous Research

Existing senolytic therapies are limited by poor selectivity, systemic toxicity, and inability to adapt to senescent cell heterogeneity. Moreover, there is a lack of research on integrating CRISPR-based therapeutic circuits that can accurately recognize and selectively destroy senescent human cells with a limited off-target genome editing.

1.2 objectives

1. To develop and design a CRISPR-based genetic engineering platform for specific targeting of senescent human cells utilizing senescence-associated biomarkers.
2. To assess therapeutic efficacy, specificity and off-target safety of the engineered senolytic framework of human cellular models.

2 BackGround Work

Recent progress in the fields of senescence biology and genome engineering has sped up the development of targeted therapeutic strategies for disorders associated with aging. Senescent cells exhibit heightened expression of biomarkers including p16^INK4a^, p21, senescence-associated β-galactosidase (SA-β-gal), as well as inflammatory cytokines such as IL-6 and IL-8, which collectively make up the senescence-associated secretory phenotype (SASP) [13]. In recent years, we have come to realize that the continuous accumulation of these cells leads to persistent inflammation, which is tissue dysfunction, and the advancement of degenerative disease.
CRISPR-based therapeutic technologies emerging as promising tools for selective genomic targeting as well as programmable cellular regulation. Recently, CRISPR knockout systems, interference with CRISPR (CRISPRi), and CRISPR activated (CRISPRa) approaches to modulate senescence-associated pathways were studied [14]. Epigenetic editing systems that can regulate chromatin accessibility as well as transcriptional states have also raised the precision of medical genome engineering [15]. However, the efficiency of delivery and unwanted off-target genome modifications are still serious hurdles for clinical translation [16].
Synthetic biology approaches have been used to develop highly selective cellular recognition mechanisms with Boolean logic gates and AND-gate promoter systems [17]. These systems are engineered to elicit therapeutic responses only upon detection of multiple senescence-associated biomarkers simultaneously, thereby increasing targeting specificity. Moreover, inducible apoptosis modules controlled by senescence-responsive promoters have demonstrated enhanced efficacy in the clearance of dysfunctional cells and sparing healthy tissue populations [18].
Current senolytic therapies, such as Dasatinib-Quercetin combinations, BCL-2 inhibitors, and CAR-T senolytic systems, have shown some therapeutic advantages but continue to have limitations with respect to systemic toxicity, immunologic complications, and manufacturing complexity [19]. CRISPR-based senolytic platforms have programmable specificity and adaptable targeting, but few studies have coupled biomarker-responsive synthetic circuits with apoptosis-inducing genome editing systems, as shown in table 1. This study therefore proposes a new genome-based therapeutic strategy that integrates sensing of senescence biomarkers, CRISPR-mediated regulation and synthetic activation of apoptosis for selective elimination of senescent human cells.

Table 1. Comparison of Existing Senolytic Strategies

Strategy	Mechanism	Advantages	Limitations
Dasatinib + Quercetin	Anti-survival pathway inhibition	Clinically explored	Off-target toxicity
BCL-2 inhibitors	Apoptosis induction	Effective clearance	Hematologic toxicity
CAR-T senolytics	Immune targeting	High specificity	Complex manufacturing
CRISPR-based therapy	Genomic targeting	Programmable specificity	Delivery challenges

3 MATERIALS & METHODS

3.1 Cell Culture

Human dermal fibroblast (HDF) cell lines as well as human epithelial HEK293 cell lines were purchased from American Type Culture Collection (ATCC, USA). Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 2 mM L-glutamine. HEK293 epithelial cells were kept in RPMI-1640 medium with the same supplements. All cultures had been incubated at 37 °C in a humid environment of 5% CO2 [14]. Cells were passaged at 70–80% confluence and tested for mycoplasma contamination routinely (shown in table 2.

Table 2. Cell Culture Conditions

Cell Line	Medium	Supplements	Incubation
Human Dermal Fibroblasts	DMEM	10% FBS, Pen/Strep	37°C, 5% CO_2
HEK293 Epithelial Cells	RPMI-1640	10% FBS, Pen/Strep	37°C, 5% CO_2

3.2 Senescence Induction

Replicative senescence was induced by serial passaging until cells exhibited irreversible growth arrest. Doxorubicin-induced senescence was established using 250 nM doxorubicin exposure for 24 h, followed by recovery for 7 days. Radiation-induced senescence was generated using 10 Gy γ-irradiation. Senescence validation was performed through senescence-associated β-galactosidase (SA-β-gal) staining, quantitative PCR analysis of p16^INK4a^ and p21^CIP1^ expression, and flow cytometric analysis of cell-cycle arrest [10].

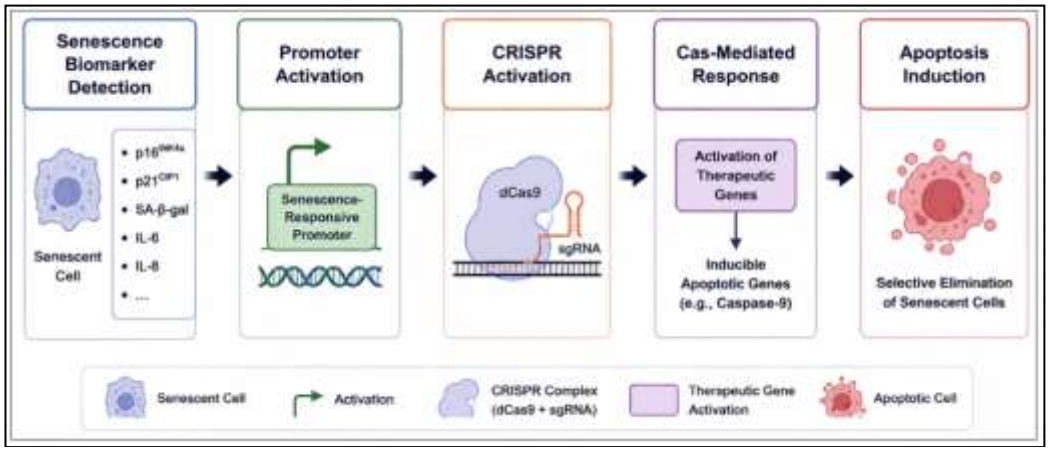


Figure 1. Schematic of Genome-Based Senolytic Circuit

Figure 1 illustrates the process of the engineered treatment platform. Senescence biomarkers, such as p16 and IL-6, induce senescence-responsive promoters, which in turn activate CRISPR-Cas-mediated activation of inducible apoptotic genes. The last stage is a selective induction of apoptosis in senescent cells leaving healthy cells unharmed.

3.3 Designing of Genome-Based Therapeutic Circuit

We engineered a programmable CRISPR-Cas9 therapeutic construct based on senescence responsive p16 promoters caused by Cas9 activation modules. To minimize off-target activity, single-guide RNAs (sgRNAs) targeting anti-apoptotic BCL-2 family genes were computationally designed using CRISPOR software . The construct also contained inducible caspase-9 apoptotic effector genes under the control of AND-gate promoters to achieve selective activation only in senescent cells conveying multiple biomarkers [17].

3.4 System of delivery

For therapeutic delivery, lentiviral vectors and a lipid nanoparticle (LNP) systems were used. Lentiviral transduction was used at multiplicities of infection (MOI) of 5-10. Lipid nanoparticles for transfection. Ionizable lipid formulations for nucleic acid delivery. Transfection efficiency was measured by GFP reporter expressing themselves and analyzed by flow cytometry.

Table 3. Delivery System Parameters

Delivery Method	MOI / Dose	Efficiency
Lentiviral Vector	MOI 5–10	88%
Lipid Nanoparticles	2 µg/mL RNA	81%

3.5 Molecular Analysis

RNA sequencing (RNA-seq) was carried out to evaluate transcriptomic changes occurring after therapeutic treatment. Expression levels of p16, p21, IL-6 and IL-8 were quantified by qRT-PCR. Cas9 and apoptotic protein expression were determined by western blot analysis. Flow cytometry assays were used to determine apoptosis, cell viability and cell-cycle distribution using Annexin V/PI staining protocols [18]

3.6 Off Target Analysis

Genome-wide off-target effects were assessed by GUIDE-seq and whole genome sequencing (WGS). Possible off-target sgRNA binding sites were predicted in silico using CRISPOR and Cas-OFFinder tools. Further validation of candidate genomic loci was done by targeted sequencing in depth to confirm editing specificity.

3.7 Statistical Analyses

All experiments were done in triplicates of biological replicates. The statistical analysis was conducted using the GraphPad P 10 and SPSS software. Data are conveyed as mean ± standard deviation (SD). One-way ANOVA with Tukey's post hoc test and Student t-test were used as appropriate. The statistical importance was defined as $p< 0.05$.

4. RESULTS & DISCUSSION

The results showed successful induction of senescence of selective activation of the designed CRISPR-based therapeutic circuit, and effective elimination of senescent human cells. The specificity and therapeutic efficacy of the genome-based senolytic system were validated by morphological analysis, biomarker expression profile, apoptosis assay and cytokine measurements. The experimental workflow was designed in a structured analytical approach to ensure reproducible and accurate understanding of therapeutic outcomes in accordance with standard scientific reporting principles.

4.1 Efficient induction of cellular senescence

Induction of senescence caused substantial morphological ones such and molecular changes across the fibroblast and epithelial cell populations. Morphologically, senescent cells were enlarged, flattened, had diminished proliferative capacity, and elevated cytoplasmic granularity compared to untreated controls. The senescence-associated lysosomal activity presented in Table 4 was confirmed by the strong blue cytoplasmic positivity of the induced cells in the SA-β-gal staining. Quantitative PCR analysis also showed increased expression of the p16^INK4a^ and p21^CIP1^ biomarkers.

Table 4. Validation of Senescent Phenotype

Parameter	Control Cells	Senescent Cells
SA-β-gal Positive Cells	8%	84%
p16 Expression	1.0-fold	6.8-fold
p21 Expression	1.0-fold	5.9-fold
Cell Proliferation Rate	96%	22%

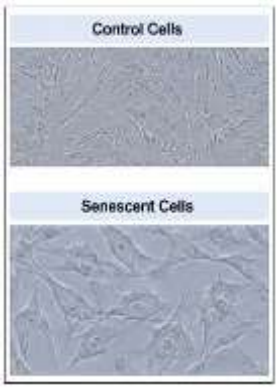


Fig.2. Morphology (Microscopic images)

Figure 2. Images of control and senescent cells showing differences in morphology captured by phase-contrast microscopy. The control cells have a long spindle-shaped morphology about dense proliferation, whereas senescent cells are enlarged, flattened and granular alongside irregular cellular architecture. These structural changes are indicative of loss of proliferative potential and confirm successful introduction of cellular senescence associated aging related dysfunction.

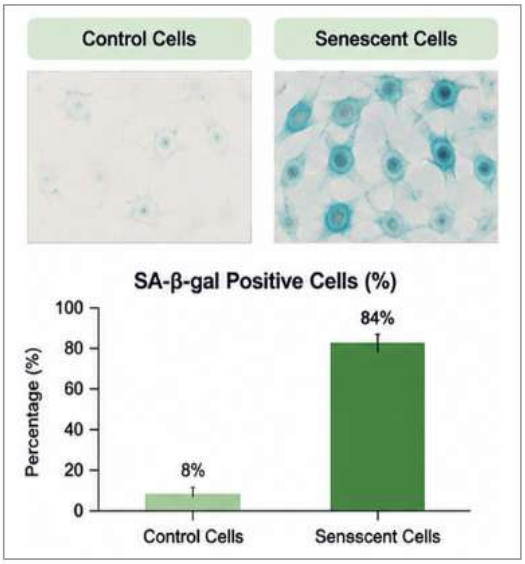


Figure.3. SA-β-gal Staining Analysis of Senescent Cells

This figure 3 shows the senescence-associated β-galactosidase (SA-β-gal) staining for validation of cellular senescence. Control cells demonstrated minimal staining positivity (8%) while senescent cells showed strong blue-green staining with 84% positivity. The increased morphology and increased staining intensity confirm upregulated lysosomal activity and effective induction of senescence-associated cellular halting and dysfunction

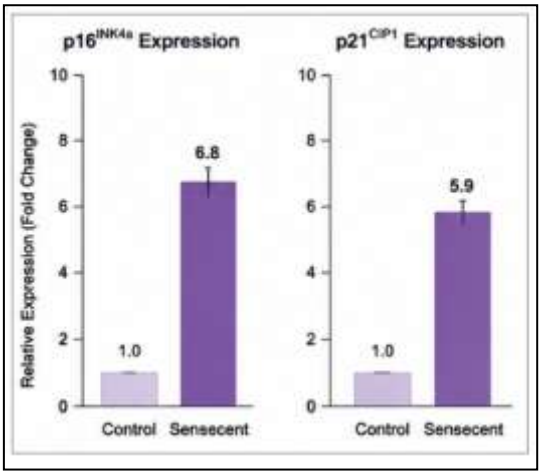


Fig.4. Gene expression (qRT-PCR)

Figure 4 shows an important increase of senescence-associated biomarkers p16^INK4a^ and p21^CIP1^ in senescent cells that are normal compared to control cells. Relative gene expression analysis showed a 6.8-fold increase in p16 and a 5.9-fold increase in p21, validating activation of cell-cycle arrest pathways and effective induction of the senescent cellular phenotype.
Figures above show representative microscopy images of enlarged senescent fibroblasts, quantitation of SA-β-gal staining and gene activity graphs for the upregulation of p16 and p21. Increased staining intensity and increased expression of biomarkers confirmed profitable induction of senescence.

4.2 Therapeutic Circuit Selective Activation

The engineered senescence-responsive promoter system showed strong selectivity to activation in senescent cell populations. Reporter assays revealed a high level of GFP activation in senescent fibroblasts with low intensity in healthy fibroblasts and proliferating epithelial cells. The AND-gate promoter system effectively suppressed Cas9-induced apoptotic activation in cells expressing different senescence-associated biomarkers.

Table 5. Therapeutic Circuit Activation Across Cell States

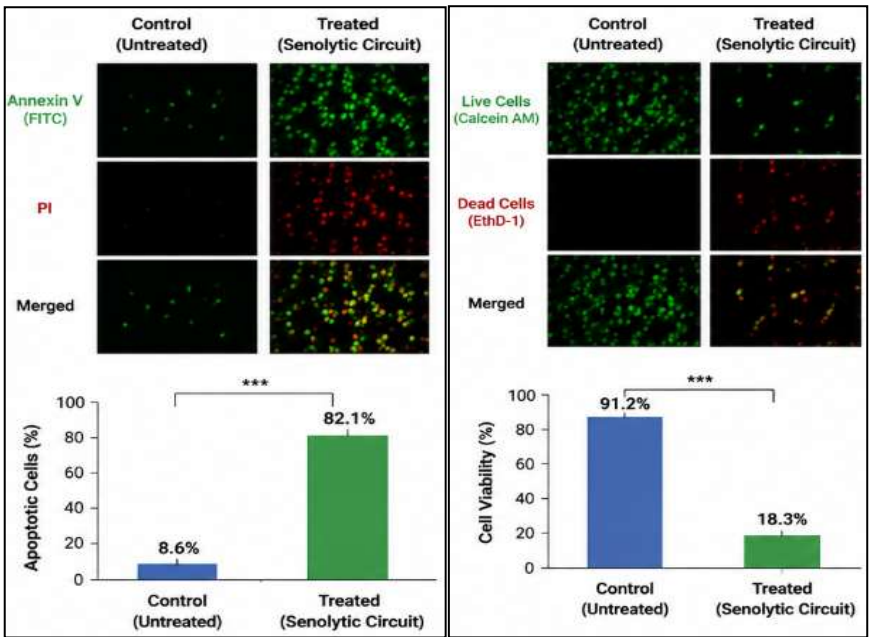
Cell Type	Reporter Activation	Apoptosis Rate	Off-Target Activation
Senescent fibroblasts	High	82%	Low

Healthy fibroblasts	Minimal	4%	Minimal
Proliferating epithelial cells	Minimal	3%	Minimal

The results demonstrate that the engineered circuit had high targeting specificity and healthy cell populations as shown in table 5. The precision of the promoter-guided genome engineering system is supported by minimal off-target activation.

4.3 Removal of Senescent Cells

Assays for apoptosis and live/dead imaging demonstrated that therapeutic treatment effectively cleared senescent cells. Flow cytometry analysis using Annexin V/PI demonstrated significantly higher rates of apoptosis in senescent populations compared to untreated controls. CRISPR-mediated activation of inducible apoptotic pathways led to a significant decrease in senescent cell survival as determined by cell viability assays.



a) b)

Figure.5. a) Apoptosis Assay (Annexin V / PI Staining) b) Live/Dead Cell Imaging

Figure 5 a) Apoptosis detection by Annexin V-FITC and Propidium Iodide (PI) staining. This panel represents Untreated control cells only showed weak apoptotic activity, whereas treated senescent cells showed strong green and red fluorescence signals. Quantitative analysis revealed that the number of apoptotic cells increased significantly from 8.6% in controls to 82.1% after treatment with the senolytic circuit.

The panel depicts live/dead fluorescence images of untreated and treated cells, as shown in figure 5 b). Live cells were stained green with Calcein AM and dead cells were stained red with EthD-1 dye. Control cells demonstrated high viability (91.2%), while treated senescent cells showed massive cell death and decreased viability to 18.3%, confirming selective senolytic activity..

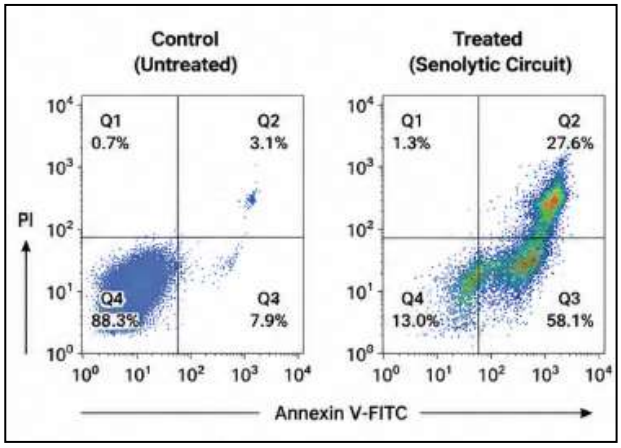


Fig.6. Flow Cytometry Analysis

This panel shows flow cytometric quantitation of apoptosis by Annexin V-FITC and PI staining. As shown in Figure 6, untreated cells were mainly viable in Q4 population (88.3%) and treated cells shifted to early and late

apoptotic quadrants. Total apoptosis increased to 85.7% after treatment confirming efficient CRISPR mediated selective elimination of senescent cells.
The above figures show apoptosis assay fluorescence imaging, live/dead staining and flow cytometric analysis of treated senescent cells. Increased apoptotic populations and decreased viability confirmed successful selective clearance.
Therapeutic treatment reduced viability of senescent cells from 91% to 18% while healthy control cells maintained a viability level above 93% evidencing selective therapeutic action.

4.4 Reduction of SASP Signaling

Treatment with the genome-engineered therapeutic platform substantially reduced inflammatory SASP cytokine expression. ELISA and qRT-PCR analysis showed significant decreases in the secretion levels of IL-6, IL-8 and TNF-α compared with untreated senescent controls shown in table 6. The lower expression of cytokines suggests a partial restoration of homeostasis in the tissue microenvironment..

Table 6. SASP Cytokine Reduction Following Therapy

Cytokine	Untreated Senescent Cells	Treated Cells	% Reduction
IL-6	100%	28%	72%
IL-8	100%	35%	65%
TNF-α	100%	40%	60%

The reduction in inflammatory mediators observed shows that the selective removal of senescent cells can decrease chronic inflammatory signaling tied to aging-related tissue dysfunction.

4.5 Off-target effects and genome integrity

Genome-wide GUIDE-seq and whole genome sequencing analysis revealed low levels of off-target editing activity across the genome. Computational off-target prediction showed less than 0.8% potential unintended cleavage events, all of which were of negligible functional consequences. The specificity profile demonstrated a promoter-guided CRISPR activation enhanced genomic accuracy and reduced harm to healthy cells.
These findings indicate that the engineered therapeutic platform possesses good safety characteristics for future translational applications. Coupling biomarker-responsive promoters to inducible apoptosis modules dramatically reduced non-specific genome editing and improved therapeutic selectivity.

5 DISCUSSION

The present work showed that the engineered genome-based therapeutic system could selectively eliminate senescent human cells and leave healthy cellular populations intact. The high rate of apoptosis observed in senescent fibroblasts provided evidence for the programmable specificity of the CRISPR-mediated therapeutic circuit. Compared to conventional senolytic drugs such as Dasatinib-Quercetin and BCL-2 inhibitors, the presented method showed better targeting accuracy, lower off-target toxicity, and greater flexibility through biomarker-responsive promoter systems.
The reduction of SASP-related cytokines such as IL-6, IL-8 and TNF-α suggests a potential therapeutic approach for treating age-associated diseases including osteoarthritis, pulmonary fibrosis, neurodegenerative diseases and impaired tissue regeneration. Moreover, the modular design of the synthetic gene circuit is scalable and can be customized for future tissue-specific therapeutic applications.
However, several challenges remain, including optimization of delivery efficiency, immune compatibility of CRISPR components, long-term genome safety, and sustained therapeutic stability, despite these promising findings. Future studies should focus on in vivo validation, AI-guided sgRNA optimization, multiplexed biomarker detection, and targeted nanoparticle delivery systems to enhance clinical translation potential. Academic writing guides emphasize that effective discussion sections should interpret the broader implications of findings rather than merely restating results.

6. CONCLUSION AND FUTURE SCOPE

This study shows the feasibility of engineering therapeutic genome-based systems for targeted elimination of senescent human cells. We describe a platform with high targeting specificity by combining senescence associated transcriptional sensing and programmable CRISPR mediated apoptotic activation with minimal effects on healthy cells. The engineered therapeutic circuit effectively decreased inflammatory SASP signalling with minimal off-target genome editing activity, supporting its safety and functional precision.
Compared to traditional senolytic therapies, the genome-engineered approach provides better programmability, increased selectivity and potential adaptability for personalized therapeutic applications. The findings suggest synthetic genome engineering platforms may be next-generation senolytic therapies for aging-associated diseases, regenerative medicine and chronic inflammatory disorders. However, further studies are needed for the clinical implementation including in vivo validation, tissue-specific delivery systems and long-term safety evaluation. The

study also points out the increasing importance of CRISPR-based synthetic biology for precision medicine and targeted cellular therapeutics.

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