임 동 현 교수 지도
석사학위 청구논문

Development of cell-based therapeutics for diabetes using genome-editing technologies

2024

성신여자대학교 대학원
미래응용과학학과
신 시 윤

Development of cell-based therapeutics for diabetes using genome-editing technologies

Adviser: Donghyun Lim, Ph.D.

Submitted in partial fulfillment
of the requirements for the degree of master.

Nov, 2023

Department of Next Generation Applied Sciences
The Graduate School of Sungshin University
Siyoon Shin

Certificate of Committee Approval

Be accepted partial fulfillment

of the requirements for the degree of

: Master of Engineering

Signatures:

Commitee Chairman ..... 이 지 연 .....

Commitee Member ..... 임 동 헌 .....

Commitee Member ..... 김 다 현 .....

The Graduate School of Sungshin University

ABSTRACT

Development of cell-based therapeutics for diabetes using genome-editing technologies

Siyoon Shin
Department of Next Generation Applied Sciences
Graduate School
Sungshin University

The current stem cell-derived beta cells for the ultimate treatment of diabetes face several limitations. 1) They exhibit insufficient glucose-stimulated insulin secretion (GSIS) compared to human islets. 2) Although cells are encapsulated in a macroencapsulation device to eliminate immune rejection, foreign body reaction (FBR) occurs in response to the device, leading to the formation of a fibrotic capsule around the device. This capsule obstructs substance transport, resulting in cell death. 3) Cells within the device experience insufficient oxygen supply, especially problematic for beta cells with high oxygen consumption, making them prone to apoptosis under hypoxic conditions. Research aims to overcome these limitations. Using CRISPR/Cas9 technology, functional genes are introduced into stem cells, which are then differentiated to produce enhanced stem cell-derived beta cells.

Editing was attempted not only at the existing safe harbor loci but also in the exploration of new candidate safe harbor genes to introduce various

functional genes. A system was established by fusing a secretion peptide to nanoluciferase, enabling the external secretion of proteins. This laid the groundwork for the external secretion of anti-fibrosis proteins and GSIS-enhancing proteins. Additionally, a hypoxia-responsive protein expression system was developed using the hypoxia response element (HRE). Based on this system, anti-apoptotic functions of XIAP and BCL-xL were confirmed under hypoxia.

Furthermore, an efficient edited cell sorting system using GFP fragment complementation was established, minimizing genetic footprint while effectively isolating edited cells with approximately 20% efficiency. Presently, these method is employed for the generation of therapeutic cells. These findings are expected to provide new perspectives for the development of next-generation diabetes cell therapies.

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Introduction

Diabetes, a chronic metabolic disorder characterized by difficulty in maintaining normal blood glucose levels, stands as a significant global health challenge. According to the World Health Organization (WHO), there were over 420 million individuals worldwide diagnosed with diabetes in 2014, reflecting a high prevalence. Type 1 diabetes results from an autoimmune response disrupting insulin production, while some Type 2 diabetes involve compromised beta cell function, resulting in challenging blood glucose regulation. Conventional treatments rely on sustained insulin administration based on blood glucose levels, providing symptomatic relief but lacking a fundamental cure. Moreover, these treatments may lead to side effects such as hypoglycemia and various complications[1]. To achieve a fundamental cure for diabetes, the transplantation of beta cells capable of insulin production becomes imperative. However, the current source of beta cells is limited to organ donors, presenting limitations in supply and requiring immunosuppression[2]. Fortunately, there is promising research exploring the production of beta cells derived from indefinitely renewable stem cells[3,4]. Nonetheless, current stem cell-derived beta cells, while proficient in insulin secretion, exhibit limitations in glucose-stimulated insulin secretion (GSIS) compared to human islets[5,6].

With the advancement of stem cell-derived beta cells, various protocols are under investigation for their *in vivo* transplantation. One notable approach involves encapsulating the cells in a macroencapsulation device

before transplantation[7]. This device is constructed with selectively permeable membranes, providing protection for the cells against acute host responses while facilitating the transfer of insulin and nutrients. While this method offers advantages such as immune response mitigation and device retrievability, there are some limitations in maintaining cell survival rates.

Although cells are encapsulated in devices to prevent immune responses, this approach triggers a foreign body reaction (FBR) to the device. FBR results in the formation of a fibrotic capsule around the device, impeding the diffusion of substances and hindering the long-term efficacy of cell therapies, ultimately leading to cell death. To counteract this, strategies to prevent fibrosis in devices that encapsulate cells are imperative[8,9].

Another major challenge following device transplantation is hypoxia[10], where the absence of vascularization around the device restricts oxygen delivery[11]. Beta cells, in particular, exhibit heightened sensitivity to oxygen due to their substantial ATP consumption for insulin production[12]. Such hypoxic conditions detrimentally impact cell survival and function[13-16]. Hence, maintaining the viability and functionality of encapsulated beta cells under hypoxic conditions becomes an essential challenge.

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas technology stands as an innovative genome editing tool widely employed in biology[17]. This technique involves the precise targeting of specific gene locus using guide RNA and the induction of a double-stranded break (DSB) at the targeted DNA site by the CRISPR-associated protein 9 (Cas9) protein. When a DSB occurs at the targeted gene location, it undergoes repair through DNA repair mechanisms

such as non-homologous end-joining (NHEJ) or homology-directed repair (HDR)[18]. NHEJ, a routine method for repairing DNA, induces small insertions or deletions (indel) leading to gene disruption or knockout. On the other hand, HDR, primarily utilized for gene knock-in at specific locations, relies on homologous sequences. However, HDR is limited by its lower efficiency compared to NHEJ[19].

Despite performing gene editing, the proportion of edited cells within a cell mixture is low. Hence, selecting cells with introduced genes becomes essential. Selection markers, typically antibiotics like puromycin, are commonly used for this purpose[20,21]. Through these selection markers, cells with the desired genes can be effectively separated. However, for precise control of the expression of a specific gene under desired conditions, minimizing the genetic footprint is crucial. The genetic footprint represents the additional effects on gene regulation caused by specific gene editing or the introduction of selection markers. Minimizing the genetic footprint is a critical task to enhance the desired traits and minimize unintended side effects within biological processes[22].

The safe harbor locus refers to a specific genomic location within gene editing technology where genes can be edited safely[23]. Typically selected for specific gene insertions, this locus allows the integration of genes at that point without significantly affecting the cell's function or viability, ensuring stable protein expression[24].

The primary objective of this study is to overcome barriers to the potential use of stem cell-derived beta cells as a therapeutic intervention (Figure 1), with the focus on safely introducing various functional genes

into cells using CRISPR/Cas9 technology to address multiple challenges. Throughout the research process, the necessary gene expression systems was established and the functionality of genes inhibiting apoptosis was verified. Additionally, a system for efficiently sorting edited cells was established using a short sequence of GFP11 as an tag. We anticipate that these efforts will contribute to the exploration of the potential utilization of stem cell-derived beta cells as a promising therapeutic option.

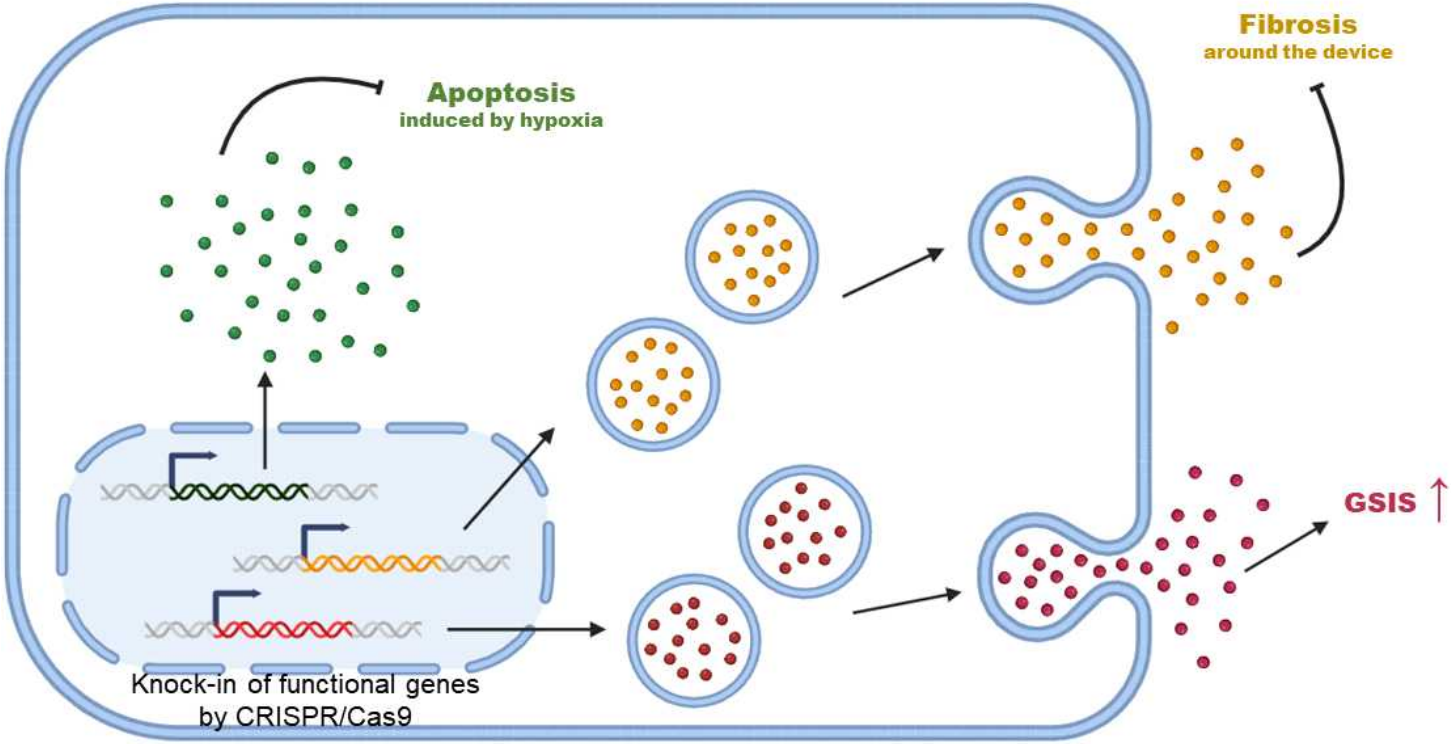

Figure 1. Comprehensive scheme of research

To address the limitations of current stem cell-derived beta cells in diabetes, CRISPR/Cas9 technology was employed. This enabled the introduction of functional genes aimed at: 1) enhancing Glucose-Stimulated Insulin Secretion (GSIS), 2) secreting an anti-fibrosis factor to prevent fibrotic capsule formation around the cell encapsulating device, and 3) sustaining viability under hypoxic conditions to counteract hypoxia-induced apoptosis.

MATERIALS AND METHODS

Cloning

1) gRNA and Cas9 expression plasmids

The genomic DNA sequences of all genes were exported from NCBI (https://www.ncbi.nlm.nih.gov/gene/). Spacer sequences of 20 nucleotides preceding the PAM ('5-NGG-3') sequence recognized and bound by Cas9 were selected. The distance between the desired editing site and the cutting site by Cas9 was set within 10 base pairs. To minimize potential off-target editing, sequences with three or more mismatches were selected using IDT's CRISPR-Cas9 guide RNA design checker (https://sg.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE). The selected sgRNAs (Table 2) were cloned into the SpCas9 plasmid (pX330; Addgene #42230) based on previous studies[25], and the sequences were verified through Sanger sequencing.

2) Plasmids for nanoluciferase secretion

The IL-6-derived signal peptide was purchased as a synthetic ssDNA oligo and cloned into the plasmid expressing nanoluciferase. And adding the linker(GSG)-2A sequence was performed as needed for the donor DNA of the target gene. The homology arms of *GAPDH* and *ACTB* were connected to the origin and ampicillin resistance gene portion of Addgene plasmid #68375. Plasmids containing homology arms for *AAVS1* (Addgene #68375) and *CLYBL* (Addgene #112499) were purchased. Between the homology

arms of each gene, the previously cloned linker-2A and IL-6 secretion signal peptide-nanoluciferase combinations were inserted.

3) Plasmids for hypoxia-responsive system

A plasmid expressing VEGF HRE and luciferase (Addgene #128096) was purchased. ssDNA oligos for PGK and iNOS HRE were synthesized and exchanged with VEGF HRE using the restriction enzymes KPnI and HindIII. XIAP and BCL-xL cDNA were obtained from the Korean Human Gene Bank. Donor DNA for integration into *AAVS1* was constructed using Addgene plasmid #68375.

4) Plasmid for GFP11 tagging

In addition to the donor DNA of *GAPDH* designed for nanoluciferase secretion, GFP11 synthetic ssDNA oligo was purchased and cloned for GFP11 tagging.

Cell maintenance

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)(Welgene, LM001-01), while Jurkat cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 media(Welgene, LM011-05). Both media were supplemented with 10% FBS(Gibco, 12483020), Anti-Anti(Gibco, 15240-062), Glutamax(Gibco, 35050-061), and 1mM pyruvate(Welgene, LS013-01). All cells were maintained in a humidified 5% CO_2, 37° C incubator. Centrifugation conditions were set at 200 RCF for 3 minutes.

Transfection

HEK293T cells were seeded at poly-lysine(Sigma, P1149)-treated multi-well plates. Once they reached 50% confluence the next day, transfection was carried out using Lipofectamine 3000(Thermo, L3000008) following the protocol.

Jurkat cells were washed twice with Opti-MEM(Thermo, 31985070). A total of 10^6 cells are mixed with 5μg of DNA. Jurkat cells were then electroporated with NEPA 21 using poring pulse conditions of 300V and 1ms.

During co-transfection of gRNA and Cas9 expression plasmid, and donor DNA, the DNA quantity was maintained at a 1:1 ratio.

T7E1 assay

Genomic DNA was extracted from harvested cells using genomic DNA extraction kit(Bioneer, K-3032). The regions with indel were amplified by PCR (Table 3). T7E1 assay was performed according to the protocol(NEB, M0302L). Subsequently, size confirmation and analysis were performed through electrophoresis (1% agarose(Biosesang, AR1200-500-00), 1X TAE(CellSTAR, CTB-TA5001-1)).

Clonal isolation of cell lines

Poly-lysine-treated 96-well plates were used for seeding via serial dilution to achieve a concentration of 1000 cells/well. After approximately 5 days, the media were replenished, and the next day, 50 μl of media were collected for luciferase assay. The wells with the highest

luminescence intensity were then transferred to 12-well plate and cultured. This cell mixture was diluted to a concentration of 100 cells/well, and the process was repeated. Single-cell seeding was achieved by further dilution to 1 cell/well. After about 7 days, cells were observed under a microscope, and wells with a single clone were selected. After another 7 days, luciferase assays were conducted and single-cell clones secreting luciferase were obtained.

Western blot

After lysing cells with RIPA buffer(CellSTAR, CTB-RP010) containing protease inhibitor(GenDEPOT, P3100-001), centrifugation was performed at 4° C for 30 minutes, and the supernatant was collected for a BCA assay(Thermo, 23225). The samples were mixed with SDS(Biosesang, SF2002-110-00) and denatured at 85° C for 8 minutes. The prepared samples were loaded onto a 12.5% SDS-PAGE gel for stacking at 90 V and running at 120 V. Transfer to a PVDF membrane(Merck, IPVH00010 was carried out at 110 V for 1 hour and 40 minutes. Blocking was done with 3% BSA(GenDEPOT, A0100-010), followed by incubation with the desired protein's antibody according to the protocol. Detection was achieved using an ECL solution(DoGen, DG-WPAL120).

Quantification and statistical analysis

All data were analyzed utilizing GraphPad PRISM software. Quantification of the Western blot results was performed using Image Lab software.

RESULTS

Selection of Gene Editing Target Sites

This study aims to develop a multifunctional cellular therapy by effectively introducing various functional genes into a single cell. To achieve this, careful selection of a “safe harbor locus“ that allows safe and stable protein expression is crucial. In addition to targeting well-known safe harbor loci such as *AAVS1* and *CLYBL*, housekeeping genes *GAPDH* and *ACTB* were identified as new candidates for safe harbor loci.

The sgRNAs were designed to minimize off-target effects (Table 2) and were cloned. HEK293T cells were transfected with the plasmids, and cells were harvested two days post-transfection. The T7 endonuclease 1 (T7E1) mismatch detection assay were conducted to confirm the occurrence of NHEJ (Figure 2, Table 3). The evaluation of gene editing at the selected loci contributed to the development of a safe and effective multifunctional cell therapy.

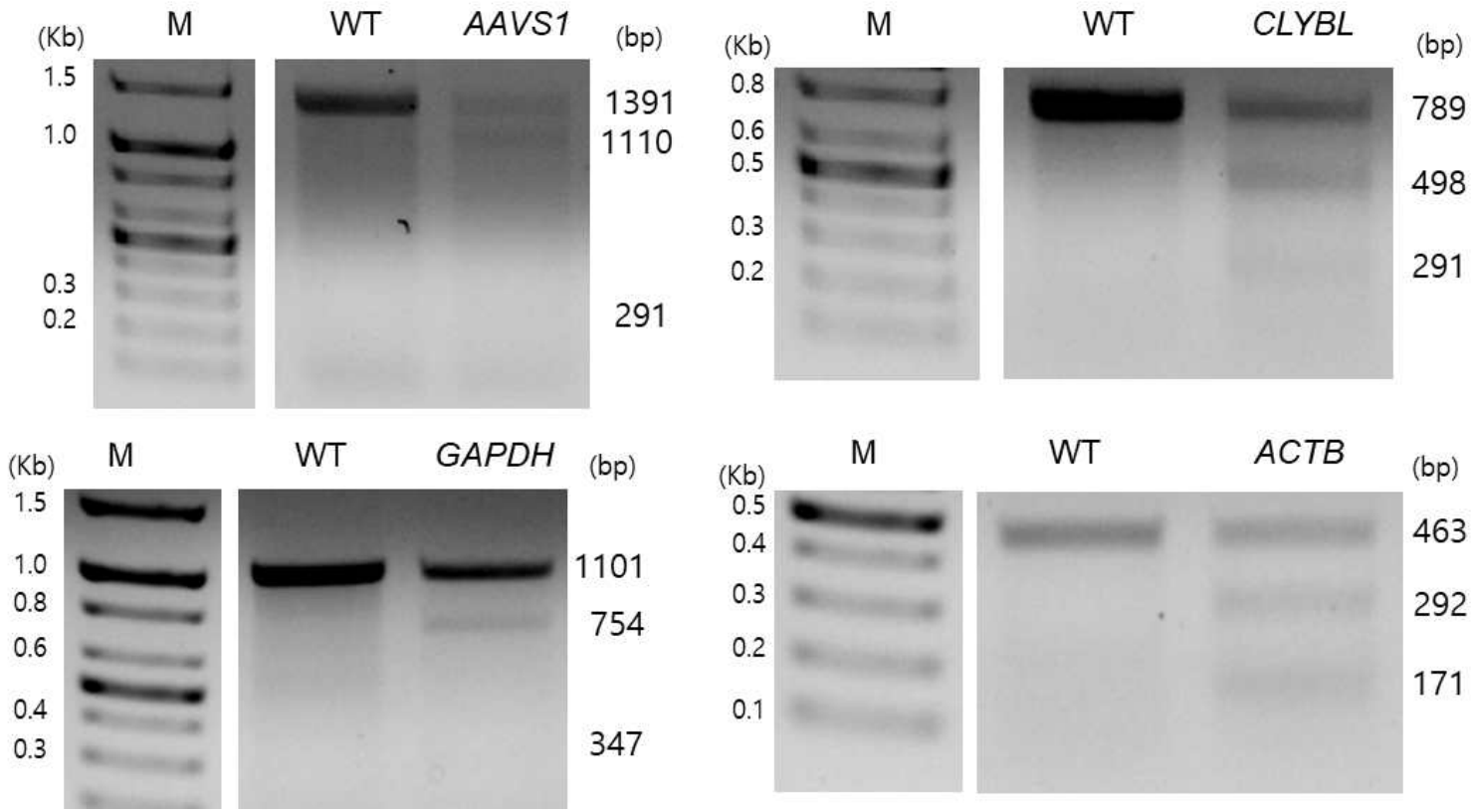

Figure 2. Confirmation of genome editing of *AAVS1*, *CLYBL*, *GAPDH* and *ACTB*

T7E1 assay for confirming cleavage and NHEJ events for each gene, indicating the potential for gene introduction into *AAVS1*, *CLYBL*, *GAPDH* and *ACTB*.

Establishing a System for Extracellular Protein Secretion

Envisioning the suppression of fibrosis in cell-encapsulating devices, the strategy involved introducing an anti-fibrosis factor into cells and facilitating its continuous extracellular secretion. Additionally, an increase in GSIS was anticipated through the introduction of a substance that promotes GSIS into cells and allowing for its extracellular secretion with alongside insulin. Establishing a system for the extracellular protein secretion was imperative for these goals. The construction of the secretion system involved fusing a signal peptide to the protein.

Using the Gibson assembly cloning method, nanoluciferase was fused with the 28-amino acid IL-6-derived secretion peptide[26] (Figure 3a). Upon transfection into HEK293T cells, a Luciferase assay of the cell supernatant confirmed the successful extracellular secretion of luciferase (Figure 3b). Subsequently, donor DNAs for editing at *AAVS1, CLYBL, GAPDH, ACTB* were cloned (Figure 3c-e). Gene editing was executed in HEK293T cells through co-transfection with plasmid expressing sgRNA and Cas9. Luciferase assays on both cell lysates and supernatant, based on cell viability, demonstrated successful luciferase secretion in *AAVS1*, *CLYBL*, and *GAPDH*. However, in the case of *ACTB*, luciferase was observed to be expressed only intracellularly, leading to the conclusion that *ACTB* is inappropriate as the insertion site for secreting proteins (Figure 3f).

To isolate gene-edited cells from cell mixtures, single-cell sorting was conducted. After culturing gene-edited cells of *AAVS1* and *CLYBL* in a medium with puromycin at a concentration of 5 μg/ml, single-cell sorting

was performed. However, anticipating a low ratio of edited cells at *GAPDH* without a selection marker, an intermediate step of single-cell sorting was implemented. Sequential sorting with 1000 cells/well, 10 cells/well, and 1 cell/well resulted in the successful isolation of single-cell clones exhibiting effective secretion. Subsequent PCR analyses for each clone confirmed the precise insertion of the gene at the intended location (Figure 4).

Editing has been successfully carried out at both existing and newly identified safe harbor loci. Ongoing assessment involves confirming the sustained and stable expression of nanoluciferase. Future plans include editing at these loci to introduce factors inhibiting fibrosis and enhancing GSIS, with the intention of validating their effectiveness.

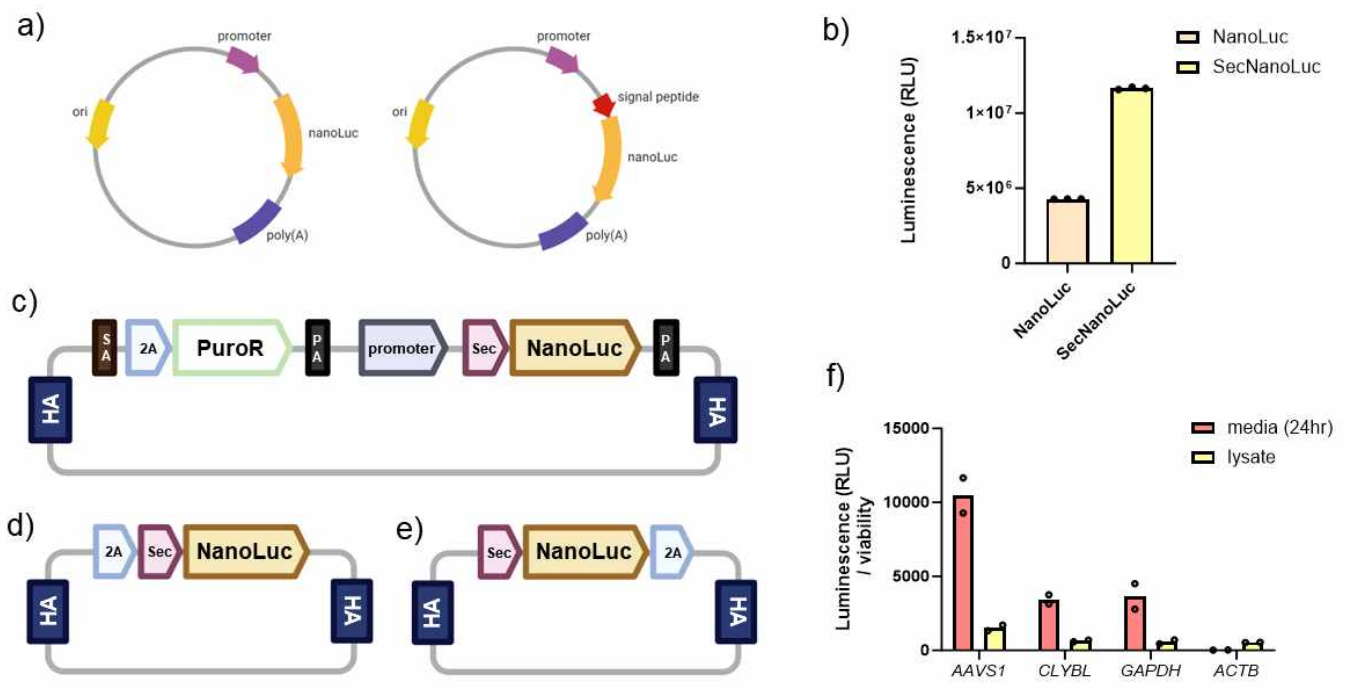

Figure 3. Establishing a system for extracellular nanoluciferase secretion

a) To establish a secretion system, nanoluciferase (NanoLuc) was fused with the IL-6-derived secretion signal peptide. b) Media from cells transfected with plasmids expressing NanoLuc and NanoLuc with the secretion tag (SecNanoLuc) were subjected to luciferase assay. Successful secretion of luciferase indicated the functionality of the signal peptide. Donor plasmids for introducing SecNanoLuc into the c) intron region of *AAVS1* and *CLYBL*, d) C-terminus of *GAPDH*, and e) N-terminus of *ACTB* were constructed. f) Editing was performed in HEK293T cells, and luciferase assays were conducted on cell lysates and media for each gene. Efficient secretion was observed in *AAVS1*, *CLYBL*, and *GAPDH*, whereas *ACTB* exhibited only intracellular expression.

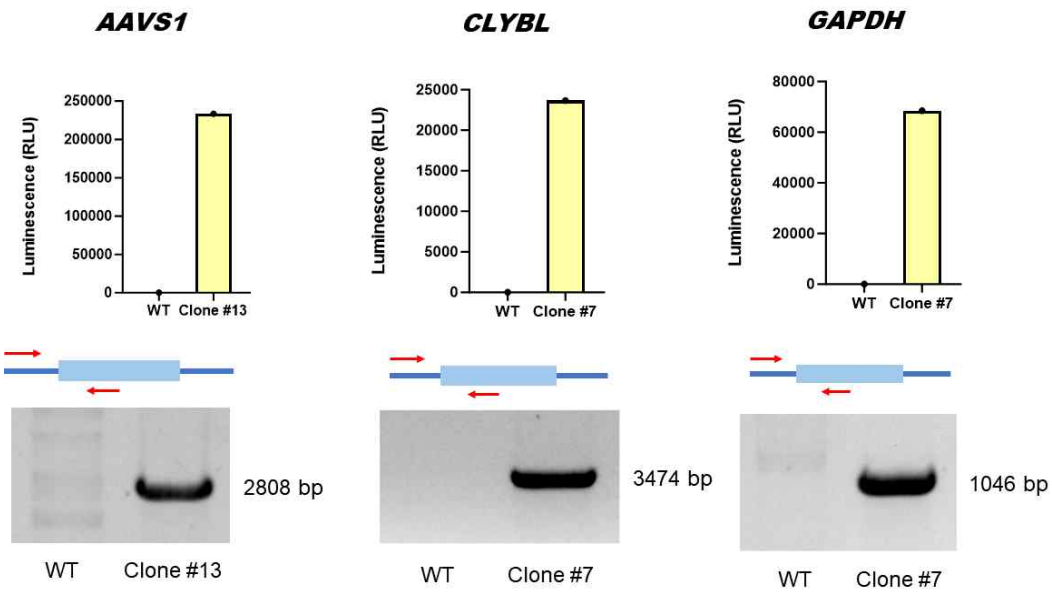

Figure 4. Isolation of single clones secreting nanoluciferase

To separate gene-edited cells from the editing cell mixture, single-cell sorting was performed. Successfully obtained clones showed nanoluciferase secretion, and subsequent PCR confirmed precise editing at the desired loci.

Establishing a System for Hypoxia-Responsive Protein Expression

To address hypoxia-induced apoptosis, a strategy of introducing an anti-apoptosis factor into cells was employed. However, due to the potential risk of continuous expression of the anti-apoptosis factor transforming cells into tumors, it was crucial for its expression to occur exclusively under hypoxic conditions. Therefore, a protein expression system inactive in normoxia but activated specifically in hypoxia was established, utilizing hypoxia response elements (HREs). The DNA circuit incoporated HREs-vascular endothelial growth factor A (VEGF)[27], phosphoglycerate kinase 1 (PGK)[27] and induible nitric oxide synthase (iNOS) [28]-with firefly luciferase as the model protein. Jurkat cells were used for establishment, and hypoxia was induced by 500 μM CoCl2 for 24 hours[29].

Using a restriction enzyme-based cloning method, PGK and iNOS HRE sequences were cloned using plasmid expressing luciferase based on VEGF HRE (Addgene, no.128096) (Figure 7). After transfecting these into Jurkat cells, hypoxia was induced, and the cells were harvested. Luciferase assays were conducted to compare the expression levels of luciferase driven by VEGF, PGK, and iNOS HREs under normoxic and hypoxic conditions. The results revealed that all three HREs exhibited minimal expression in normoxia, while demonstrating robust expression under hypoxic conditions. Notably, VEGF HRE demonstrated the highest responsiveness, and PGK HRE showed the highest expression levels.

Subsequently, donor DNAs for editing at the *AAVS1* locus was constructed. After performing gene editing, cells were selectively cultured in media treated with 2.5 μg/ml puromycin. The luciferase expression levels of each HRE under normoxic and hypoxic conditions were also compared in gene-edited cells, exhibiting trends similar to transient expression experiments. Particularly, the use of VEGF HRE resulted in even higher expression levels.

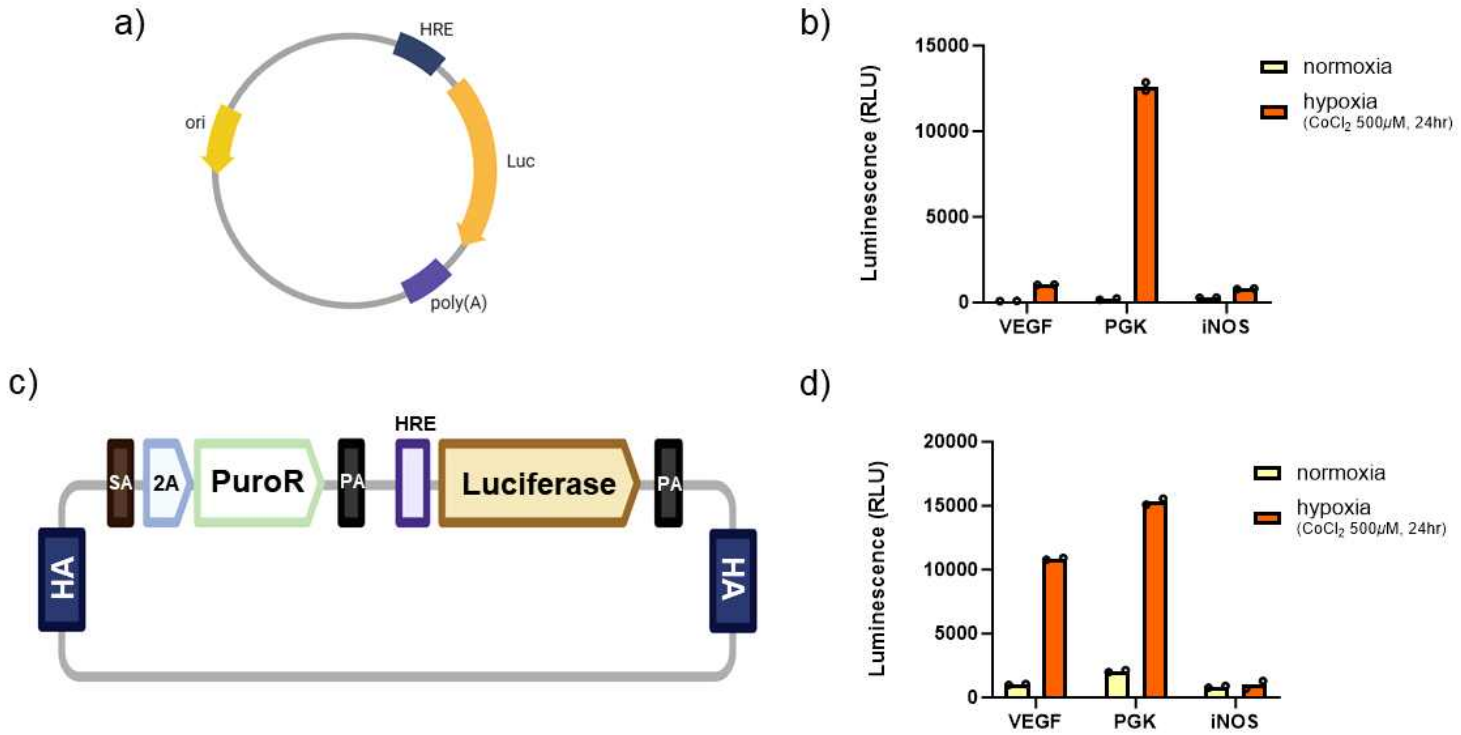

Figure 5. Establishment of hypoxia-responsive luciferase expression system

a) A hypoxia-responsive luciferase expression system was established using hypoxia response elements (HREs),- VEGF, PGK and iNOS. b) Comparison of luciferase expression between normoxia and hypoxia through luciferase assays. The functionality of HREs under hypoxia conditions is demonstrated. c) Donor plasmids were created for editing *AAVS1* locus, incorporating each HRE and luciferase. d) Editing was performed in Jurkat cells, and luciferase assays were conducted to compare luciferase expression levels between normoxia and hypoxia. The result indicated that the HREs function as desired even in edited cells.

Validation of the Functionality of Anti-Apoptotic Proteins: XIAP and BCL-xL

In the subsequent phase, anti-apoptotic potential of selected proteins were evaluated. XIAP[30,31] and BCL-xL[32], renowned for their anti-apoptotic properties, were chosen for this investigation. Their functionality within the established hypoxia-responsive DNA circuit was ensured by employing the highly expressive PGK HRE. Plasmids expressing XIAP and BCL-xL were cloned based on the previously developed PGK HRE-controlled luciferase-expressing plasmid. Simultaneously, donor DNAs for *AAVS1* integration were generated (Figure 6a). After gene editing, cells were cultured in puromycin media, followed by induction of hypoxia and subsequent harvest.

Subsequent Western blot analysis was conducted using a PARP antibody (CST. #9542) as an apoptotic marker. Comparing PARP (116kDa), representing normal cells, with cleaved PARP (89kDa), indicating apoptosis, both proteins exhibited a significant reduction in cleaved PARP levels compared to the control protein, luciferase (Figure 6b,c). This reduction underscores the efficacy of XIAP and BCL-xL in inhibiting apoptosis under hypoxic conditions, emphasizing their potential as valuable contributors to anti-apoptotic interventions in hypoxia-related scenarios.

Applying apoptosis markers other than PARP, the plan is to dual-confirm the anti-apoptotic function of functional genes and conduct an apoptosis detection assay using FACS. The established system is currently being introduced to stem cells, with ongoing research aiming to minimize background expression under normoxia.

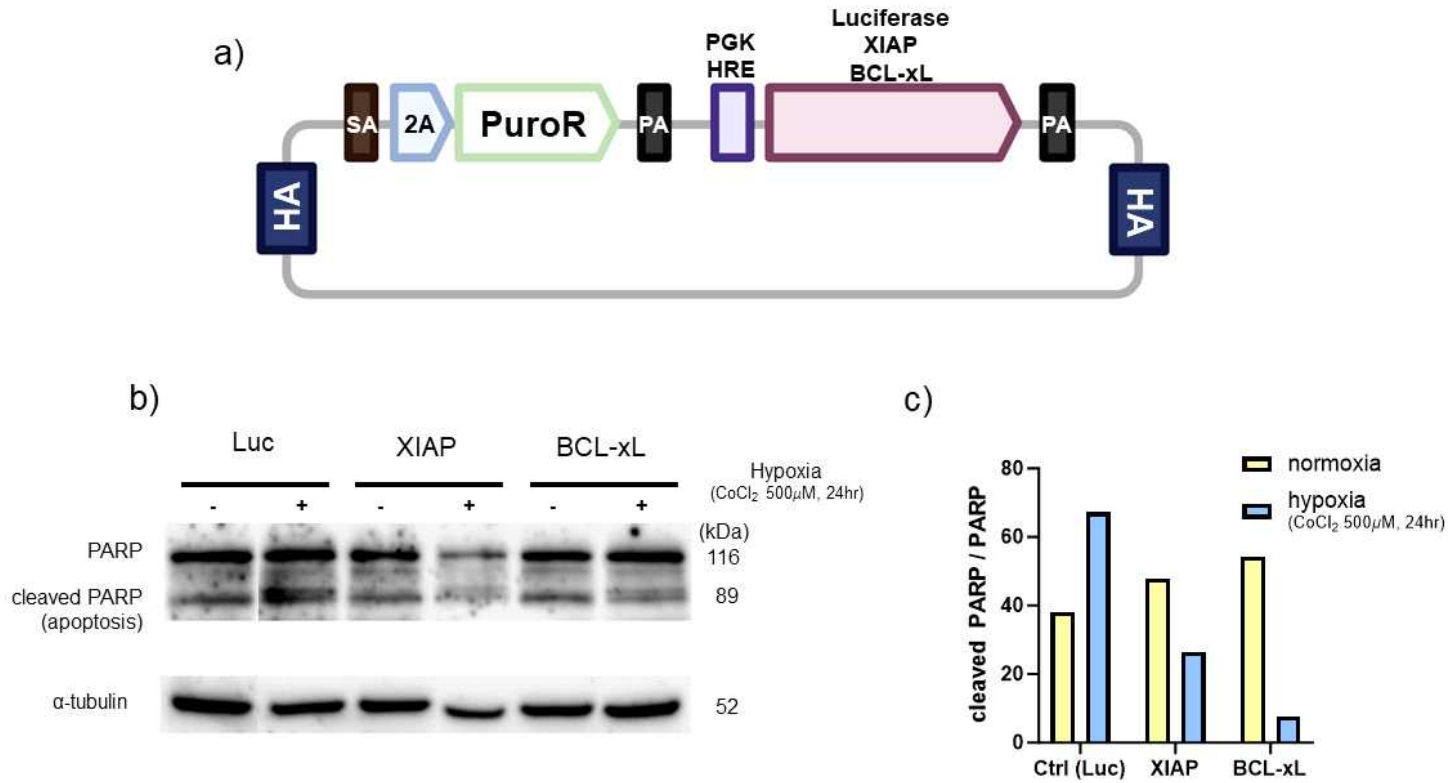

Figure 6. Validation of anti-apoptotic proteins: XIAP and BCL-xL

a) Donor plasmids were constructed for editing *AAVS1* locus, incorporating PGK HRE, Luciferase, XIAP, and BCL-xL were constructed. b) Editing was conducted in Jurkat cells, followed by induction of hypoxia. The inhibition of apoptosis under hypoxia was verified through western blot analysis using PARP antibody as an apoptosis marker. c) Quantification of the western blot revealed that both XIAP and BCL-xL effectively suppressed apoptosis compared to the control protein, luciferase.

Efficient Cell Selection with GFP11 Tag

Green Fluorescent Protein (GFP) can be divided into two fragments, GFP1-10 and GFP11. These fragments individually do not produce fluorescence, but when complemented, they emit fluorescence. Previous studies utilized 16-amino acid. GFP11 as an tag fused with the target protein, followed by the introduction of GFP1-10 fragment, leading to fluorescence upon complementation.

In this study, this principle was applied and efficient selection of edited cells was anticipated by using Fluorescence-Activated Cell Sorting (FACS) to isolate cells expressing GFP. Nanoluciferase was employed as a model protein, and editing was performed at the *GAPDH* (Figure 7a). Subsequent transfection with GFP1-10 plasmid (Addgene #70219) and the GFP-positive cell sorting was conducted the next day (Figure 7b). Further, single-cell sorting of these cells revealed luciferase expression in 7 out of 40 clones, demonstrating a successful selection of edited cells with an efficiency of approximately 20%.

Our research team is currently applying this strategy to create therapeutic cells. This strategy, applicable not only to GFP but also to fluorescent proteins such as RFP and BFP, is intended to be used as a selection marker for multiplex genome editing.

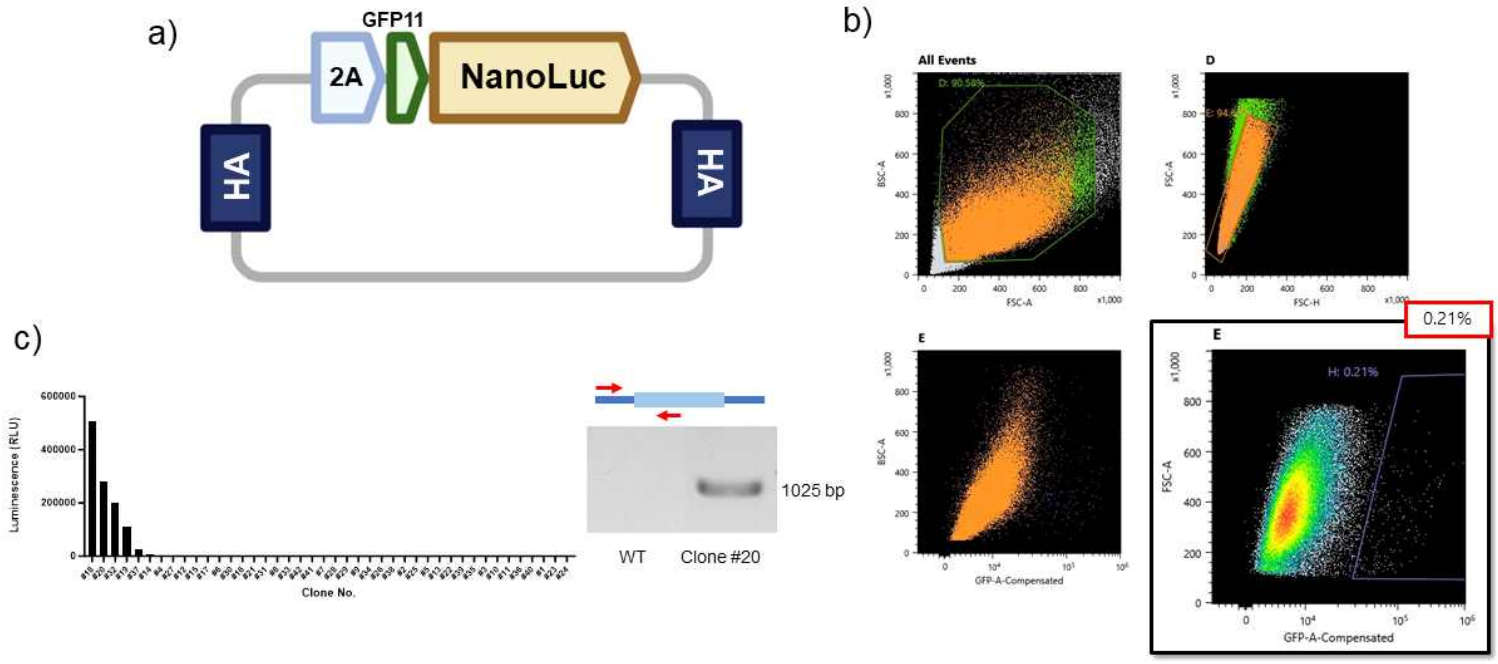

Figure 7. Efficient selection of edited cells using GFP11 tag

a) Donor plasmids were constructed for editing the *GAPDH* C-terminus, incorporating GFP11 and nanoluciferase. b) Editing was conducted in HEK293T cells. Subsequently, a plasmid expressing GFP 1-10 was delivered, and cells exhibiting GFP fluorescence were sorted using FACS. c) Single-cell sorting was performed on the sorted cell mixture. A single clone expressing luciferase was obtained, and PCR confirmed the precise editing at the desired location.

Discussion

Our research aims to utilize CRISPR/Cas9 technology to introduce functional genes into stem cells, induce differentiation to generate enhanced stem cell-derived beta cells, and ultimately develop next-generation cell therapies. Throughout the research process, various strategies and technologies were employed to overcome several challenges.

Firstly, multiple safe harbor loci were explored to successfully introduce various functional genes. In addition to *AAVS1* and *CLYBL*, *GAPDH* and *ACTB* were investigated as new candidates. NHEJ was induced using CRISPR/Cas9 at these loci and the possibility of effective editing was confirmed through the T7E1 assay.

Next, an external protein secretion system was optimized by fusing the IL-6-derived signal peptide with nanoluciferase. Successful establishment of a protein secretion system was achieved in *AAVS1*, *CLYBL*, and *GAPDH*. However, an issue was identified in *ACTB*, where the protein was not secreted externally but expressed only in the cell lysate. Based on this system, the introduction of functional proteins such as anti-fibrosis factors or enhancing GSIS factors is anticipated to enable external protein secretion. However, further research is needed to verify sustained and non-silenced protein expression.

Moreover, hypoxia-responsive protein expression system was developed. Utilizing hypoxia-activated HREs (VEGF, HRE, iNOS) and luciferase as an model protein. According to differences in expression levels based on HRE,

it is expected to allow adjustable protein expression under hypoxic conditions. Additionally, by introducing anti-apoptosis factors XIAP and BCL-xL into cells, Inhibition of apoptosis under hypoxia was observed through western blot analysis.

Lastly, for efficient gene-edited cell selection, a fluorescent-based sorting system was established using the GFP fragment, GFP11. Following the gene editing process by fusing luciferase with GFP11, GFP1-10 was tranfsected. Cells emitting fluorescence were sorted using FACS. This method effectively separates a low percentage of edited cells. Using a short 16-amino acid sequence as an tag minimizes the genetic footprint. We currently apply these techniques to produce therapeutic cells. This system, adaptable to other fluorescent proteins, holds promise for the multiple genome editing goals envisioned by our research team.

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Table 1. Key DNA sequences

name	sequence
IL6-derived signal peptide	atgaactccttctccacaagcgccttcggtccagttgccttctccctgggcctgctcctggtgttgcctgctgccttccctgccc ca
VEGF HRE (x5)	tcgagccacagtgcatacgtgggctccaacaggtcctcttg
PGK HRE (x3)	tgtcacgtcctgcacgactctagt
iNOS HRE (x4)	tgactacgtgctgcctag
XIAP	atgacttttaacagttttgaaggatctaaaacttgtgtacctgcagacatcaataaggaagaagaatttgtagaagagtttaata gattaaaaactttgctaattttccaagtggtagtcctgtttcagcatcaacactggcacgagcagggtttctttatactggtgaa ggagataccgtgcggtgctttagttgtcatgcagctgtagatagatggcaatatggagactcagcagttggaagacacaggaa agtatccccaaattgcagatttatcaacggcttttatcttgaaaatagtgccacgcagtctacaaattctggtatccagaatggt cagtacaaagttgaaaactatctgggaagcagagatcattttgccttagacaggccatctgagacacatgcagactatcttttg agaactgggcaggttgtagatatatcagacaccatatacccgaggaaccctgccatgtatagtgaagaagctagattaaagt cctttcagaactggccagactatgctcacctaaccccaagagagttagcaagtgctggactctactacacaggtattggtgac caagtgcagtgcttttgttgtggtggaaaactgaaaaattgggaaccttgtgatcgtgcctggtcagaacacaggcgacacttt cctaattgcttctttgttttgggccggaatcttaatattcgaagtgaatctgatgctgtgagttctgataggaatttcccaaattcaa caaatcttccaagaaatccatccatggcagattatgaagcacggatctttacttttgggacatggatatactcagttaacaagg agcagcttgcaagagctggattttatgctttaggtgaaggtgataaagtaaagtgctttcactgtggaggagggctaactgattg gaagcccagtgaagacccttgggaacaacatgctaaatggtatccagggtgcaaatatctgttagaacagaagggacaaga atatataaacaatattcatttaactcattcacttgaggagtgtctggtaagaactactgagaaaacaccatcactaactagaag aattgatgataccatcttccaaaatcctatggtacaagaagctatacgaatggggttcagtttcaaggacattaagaaaataat ggaggaaaaaattcagatatctgggagcaactataaatcacttgaggttctggttgcagatctagtgaatgctcagaaagaca gtatgcaagatgagtcaagtcagacttcattacagaaagagattagtactgaagagcagctaaggcgcctgcaagaggaga agctttgcaaaatctgtatggatagaaatattgctatcgtttttgttccttgtggacatctagtcacttgtaaacaatgtgctgaag

	cagttgacaagtgtcccatgtgctacacagtcattactttcaagcaaaaaatttttatgtcttaa
BCL-xL	atgtctcagagcaaccgggagctggtggttgactttctctcctacaagctttcccagaaaggatacagctggagtcagtttagt gatgtggaagagaacaggactgaggccccagaagggactgaatcggagatggagacccccagtgccatcaatggcaacc catcctggcacctggcagacagccccgcggtgaatggagccactggccacagcagcagtttggatgcccgggaggtgatc cccatggcagcagtaaagcaagcgctgagggaggcaggcgacgagtttgaactgcggtaccggcgggcattcagtgacct gacatcccagctccacatcaccccagggacagcatatcagagctttgaacaggtagtgaatgaactcttccgggatggggta aactggggtcgcattgtggccttttctccttcggcggggcactgtgcgtggaaagcgtagacaaggagatgcaggtattggtg agtcggatcgcagcttggatggccacttacctgaatgaccacctagagccttggatccaggagaacggcggctgggatactt ttgtggaactctatgggaacaatgcagcagccgagagccgaaagggccaggaacgcttcaaccgctggttcctgacgggca tgactgtggccggcgtggttctgctgggctcactcttcagtcggaaatga
GFP11 (codon optimized)	atgagagatcacatggtgctccatgagtatgtgaatgccgctggcattact

Table 2. sgRNA sequence

Gene	Symbol	spacer (20nt)	PAM
adeno-associated virus integration site 1	*AAVS1*	ggggccactagggacaggat	TGG
citramalyl-CoA lyase	*CLYBL*	atatttatgttggaaggatg	AGG
glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	cctccaaggagtaagacccc	TGG
actin beta	*ACTB*	gctattctcgcagctcacca	TGG

Table 3. Primers for T7E1 assay

Gene	primer		length (bp)	cleaved length (bp)
AAVS1	Fwd	tcgacttcccctcttccgatg	1391	1110 / 291
	Rev	ctcaggttctgggagagggta		
CLYBL	Fwd	actgtgccccaaagtgttga	789	498 / 291
	Rev	gctcatggaggcaggatgaa		
GAPDH	Fwd	ggctcccacctttctcatcc	1101	754 / 347
	Rev	cgaagcaagcaaggctgttt		
ACTB	Fwd	ctccgaccagtgtttgcctt	463	291 / 171
	Rev	ggaatccttctgacccatgc		

Table 4. Primers for validating precise gene insertion at target loci

Gene	primer		length (bp)
AAVS1	Fwd	tcgacttcccctcttccgatg	2808
	Rev	cgctcaggacaatcctttgg	
CLYBL	Fwd	tcctcgacttggctttgagc	3474
	Rev	cgctcaggacaatcctttgg	
GAPDH	Fwd	gaagacgggcggagagaaac	1046
	Rev	cgctcaggacaatcctttgg	

논문개요

현재 줄기세포 유래 베타세포를 활용한 세포치료제 개발 과정에는 몇 가지의 한계점이 존재한다. 1) 사람의 섬유세포에 비해 포도당 자극 인슐린 분비 (glucose-stimulated insulin secretion) 기능이 떨어진다. 2) 세포는 면역거부반응을 없애기 위해 macroencapsulation 디바이스에 담겨 이식되지만, 디바이스에 대한 이물반응 (foreign body reaction)로 인해 디바이스 주위에 섬유화가 일어나고 이는 물질수송을 막아 세포가 사멸한다. 3) 디바이스에 담긴 세포에게는 산소 공급이 충분히 이루어지지 않는데, 특히 베타세포는 산소 소모량이 많아 산소가 부족한 환경에서 쉽게 사멸한다. 본 연구에서는 이러한 한계점을 극복하기 위해 CRISPR/Cas9 기술을 활용하여 기능성 유전자를 줄기세포에 도입한 후, 이를 분화시켜 기능이 향상된 줄기세포유래 베타세포를 제작하는 것을 목표로 한다.

우리는 다양한 기능성 유전자를 도입하기 위해 기존의 safe harbor locus 뿐만 아니라 새로운 후보 safe harbor 유전자들을 편집했다. 모델 단백질인 nanoluciferase에 시그널 펩타이드를 퓨전하는 전략을 사용한 단백질을 세포 외부로 분비시키는 시스템을 구축하여 항섬유화 단백질과 GSIS를 증가시키는 단백질을 세포 외부로 분비시키기 위한 기반을 마련했다. 뿐만 아니라, hypoxia response element (HRE)를 활용하여 저산소 환경에서 반응하는 단백질 발현 시스템을 개발했으며, 이를 기반으로 XIAP와 BCL-xL과 같은 세포 사멸 억제 단백질의 기능도 확인했다. 추가적으로, GFP fragment complementation을 활용하여 genetic footprint를 최소화하면서도 약 20%의 높은 효율로 효과적으로 편집된 세포를 분리할 수 있는 시스템을 수립했다. 이러한 연구 결과는 차세대 당뇨병 세포치료제를 제작하는데 새로운 전망을 제시할 수 있을 것으로 기대된다.