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Research Article

Comparison of dCas9-activator complexes for the activation of *PDX1* and *NGN3* pancreatic genes using the CRISPR system

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Abstract: *Diabetes mellitus* is a prevalent metabolic disorder characterized by persistently high blood glucose levels due to insufficient insulin production or response. Although significant progress has been made in symptom management, a definitive cure remains unavailable. This study presents a novel approach to generate insulin-producing β cells from non-β cell sources using the CRISPR/dCas9 gene activation system. We focused on enhancing β-cell differentiation by activating *PDX1* and *NGN3*, two key transcription factors in pancreatic development. To optimize this process, we compared three activator domains (VP64, VPR, and p300) and found VPR to be the most effective. Specifically, the VPR activator led to a 19-fold increase in *PDX1* expression and a 256-fold increase in *NGN3* expression when combined with four gRNAs. This superiority is likely due to its stronger transcriptional activation capability, which enhances gene expression more efficiently than VP64 and p300. Gene and protein expression were confirmed through RT-qPCR and immunostaining, respectively. Our findings demonstrate that CRISPR/dCas9-mediated gene activation can effectively induce β-cell differentiation, offering a promising approach for type 1 diabetes therapy, where β-cell loss is a major challenge. Future studies should explore the long-term functionality and stability of these β-like cells in preclinical models to further assess their therapeutic potential. By optimizing transcription factor activation, our study provides new insights into β-cell regeneration, advancing the field of gene-based diabetes treatments.

Özet: Türkçe *Diabetes mellitus*, yetersiz insülin üretimi veya yanıtı nedeniyle kalıcı olarak yüksek kan glukoz seviyeleriyle karakterize edilen yaygın bir metabolik bozukluktur. Belirtilerin yönetimi konusunda önemli ilerlemeler kaydedilmiş olsa da kesin bir tedavi halen mevcut değildir. Bu çalışma, CRISPR/dCas9 gen aktivasyon sistemini kullanarak insülin üreten β-benzeri hücrelerin β hücresi olmayan kaynaklardan elde edilmesine yönelik yeni bir stratejiyi araştırmaktadır. Pankreas gelişiminde kilit rol oynayan iki transkripsiyon faktörü olan *PDX1* ve *NGN3*'ün aktivasyonu yoluyla β hücre farklılaşmasını artırmaya odaklandık. Bu süreci optimize etmek için üç farklı aktivatör bölgesini (VP64, VPR ve p300) karşılaştırdık ve VPR'nin en etkili aktivatör olduğunu belirledik. Özellikle VPR aktivatörü, dört gRNA ile birlikte kullanıldığında *PDX1* ekspresyonunda 19 kat, *NGN3* ekspresyonunda ise 256 kat artış sağladı. Bu üstünlüğün, VPR'nin VP64 ve p300'e kıyasla daha güçlü transkripsiyon aktivasyonu sağlamasından kaynaklandığını düşünüyoruz. Gen ve protein ekspresyonu sırasıyla RT-qPCR ve immün boyama teknikleriyle doğrulandı. Bulgularımız, CRISPR/dCas9 aracılı gen aktivasyonunun β hücre farklılaşmasını etkili bir şekilde indükleyebileceğini ve β hücre kaybının büyük bir sorun olduğu tip 1 diyabet tedavisi için umut vadeden bir yaklaşım sunduğunu göstermektedir. Gelecekteki çalışmalar, bu β-benzeri hücrelerin uzun vadeli fonksiyonelliğini ve stabilitesini preklinik modellerde inceleyerek terapötik potansiyellerini daha kapsamlı bir şekilde değerlendirmelidir. Transkripsiyon faktörü aktivasyonunu optimize eden bu çalışma, β hücre rejenerasyonu hakkında yeni içgörüler sunarak gen temelli diyabet tedavileri alanına önemli bir katkı sağlamaktadır.

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Introduction

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system is a precise genome-editing tool derived from bacterial immune defense, utilizing the Cas9 nuclease and a guide RNA (gRNA) to target specific DNA sequences. Due to its efficiency, specificity, and simplicity, CRISPR/Cas9 has become the predominant genome-editing method, enabling applications such as gene silencing and regulation. The discovery of Cas9

orthologs and variants with different protospacer adjacent motif (PAM) specificities has further expanded its targeting capabilities. To adapt CRISPR for gene regulation, (Qi *et al.* 2013) engineered a catalytically inactive Cas9 (dCas9) by mutating its HNH and RuvC-like nuclease domains. dCas9 can bind to DNA without cleaving it, allowing precise transcriptional modulation when fused to effector domains. CRISPR activation facilitates gene regulation by linking dCas9 with transcriptional activation domains, enabling targeted gene upregulation or downregulation without altering the DNA sequence (Casas-Mollano *et al.* 2020, Razavi *et al.* 2024). In the CRISPR-Cas9 system, dCas9 is preferred over Cas9 in gene regulation studies because it allows transcriptional or epigenetic control of target genes without DNA cleavage. This approach offers significant advantages for modulating gene expression without introducing permanent genomic modifications. Due to mutations in the RuvC and HNH nuclease domains (D10A/H840A), dCas9 lacks endonuclease activity but retains the ability to bind target DNA, facilitating the recruitment of transcription factors or epigenetic regulators to specific genomic loci. This property eliminates the risk of genomic instability and unintended mutations (Richter *et al.* 2020). Additionally, when fused with effector proteins such as VP64 or KRAB, dCas9 can either enhance or repress gene expression, making it ideal for dynamic control of cellular functions (Nuñez *et al.* 2021). In epigenetic regulation, dCas9 can be fused with DNA methyltransferases (e.g., DNMT3A) or histone-modifying enzymes (e.g., p300) to alter chromatin structure and achieve long-term gene expression control without permanent mutations (Dominguez *et al.* 2022). While its DNA-cleaving ability can lead to unintended mutations at off-target sites, dCas9 significantly reduces off-target effects, making it a safer option for therapeutic applications (Seo *et al.* 2023). These advantages establish dCas9 as an essential tool for gene regulation, epigenetic engineering, and therapeutic development. The CRISPR-dCas9 system utilizes effector proteins such as VPR, VP64, and p300 to regulate gene expression through transcriptional activation or epigenetic modification. These proteins, when fused with dCas9, manipulate the expression of target genes by binding to specific DNA regions. VP64 is a fusion of four VP16 transcriptional activation domains derived from the human herpesvirus and activates transcription by binding to the promoter region of the target gene. It is preferred for achieving high levels of gene expression, although its activation power is limited. VPR, a combination of VP64, human p65 (NF-κB), and Rta (from the Epstein-Barr virus), provides stronger transcriptional activation compared to VP64. This system is particularly effective for activating genes that are difficult to express, such as those located in silent chromatin regions. p300, an epigenetic regulator with histone acetyltransferase (HAT) activity, works by acetylating histones in the target gene region. This modification opens the chromatin structure, facilitating transcriptional activation. p300 is used for long-term regulation of gene expression without permanently altering the DNA sequence. These activation domains offer distinct advantages: VP64 is an efficient and straightforward tool for basic gene activation, VPR is suitable for genes requiring higher levels of activation, and p300 enables sustainable activation without permanent DNA changes (Chavez *et al.* 2015, Dominguez *et al.* 2022, Riedmayr *et al.* 2022).

Synthetic transcription factors including those used in the CRISPR-dCas9 system, play crucial roles in regulating gene expression, stimulating tissue regeneration, compensating for genetic defects, activating silenced tumor suppressors, controlling stem cell differentiation, performing genetic screening, and generating synthetic genes. These factors typically target enhancers or promoters of both endogenous genes and transgenes (Beerli *et al.* 2000, Kunii *et al.* 2018). In mammals, gRNA activation is usually moderate; however, using multiple gRNAs can help enhance activation by targeting distinct locations upstream of the transcription start site (Gilbert *et al.* 2013, Maeder *et al.* 2013). Several CRISPR activation systems, such as dCas9–VP64, dCas9–VPR, dCas9–SunTag, the gRNA-activation domain system, the SAM (Synergistic Activation Mediator) system, and the dCas9–p300 core system, employ various strategies to promote gene expression through targeted activation mechanisms. The dCas9-VP64, dCas9-VPR, and dCas9-p300 systems are distinct CRISPR activation platforms, each offering specific mechanisms for gene activation. dCas9-VP64 is the first-generation CRISPR activation system, in which dCas9 is fused with the VP64 transactivation domain (Balboa *et al.* 2015). This system can activate targeted endogenous genes using a single gRNA while minimizing off-target effects (Maeder *et al.* 2013). The use of multiple gRNAs can lead to the synergistic activation of various genes, such as *IL1RN* and *VEGFA* (Cheng *et al.* 2013, Maeder *et al.* 2013, Pablo Perez-Pinera *et al.* 2013). dCas9-VPR system enhances the activation capabilities of dCas9 by incorporating a tripartite activator domain. Compared to dCas9-VP64, the dCas9-VPR system achieves significantly higher gene expression, with up to a 320-fold increase in expression level (Chavez *et al.* 2015). This system is particularly effective when multiple gRNAs are used to activate genes related to cellular reprogramming and development (Chavez *et al.* 2015). Lastly, the dCas9-p300 system fuses dCas9 with the catalytic core of p300 acetyltransferase, which directly catalyzes histone acetylation at target sites, leading to robust transcriptional activation of genes from both promoters and enhancers. The dCas9-p300 system has been demonstrated to activate genes like IL1RN and OCT4, showcasing its ability to directly modify the epigenetic landscape (Hilton *et al.* 2015, Chen & Qi 2017). In summary, dCas9-VP64 is a foundational system for gene activation, dCas9-VPR offers enhanced activation capabilities, and dCas9-p300 provides a mechanism for direct epigenetic modification to achieve gene activation (Chen & Qi 2017, Hsu *et al.* 2019).

Recent advancements in pancreatic gene activation and β cell differentiation for diabetes treatment have achieved significant progress, particularly in stem cell-based approaches, transdifferentiation, gene therapy, and epigenetic modulation. Human pluripotent stem cells (iPSCs) have been successfully converted into functional β cells through the use of key transcription factors such as *PDX1*, *NGN3*, *MAFA*, and *NKX6.1* (Dadheech & James Shapiro 2019). Moreover, 3D organoid culture systems have further enhanced this process by enabling the maturation of these cells through matrix interactions and signaling molecules like Wnt and TGF-β (Pollock *et al.* 2023). Additionally, CRISPR-Cas9-based gene editing has proven effective in correcting genetic defects in monogenic diabetes models (Maxwell *et al.* 2020), while the dCas9-VPR system has emerged as a powerful tool for activating pancreatic genes. Studies have demonstrated that coexpressing dCas9-VPR with gRNAs targeting key transcription factors such as *PDX1*, *NGN3*, *NKX6.1*, and *MAFA* results in the substantial activation of these genes, indicating the potential of CRISPR systems in enhancing gene expression for β cell differentiation (Lee *et al.* 2023). Transdifferentiation, the process of converting non-β cells into insulin-producing cells, has also been a focus of recent research. The reprogramming of acinar cells into β-like cells has been successfully achieved through the viral delivery of transcription factors like *NGN3*, *PDX1*, and *MAFA* in mouse models (Cavelti-Weder *et al.* 2017). Further, the use of the dCas9-VP160 and dCas9-P300 systems has shown promise in activating pancreatic genes like *INS*, *PDX1*, *NGN3*, and *PAX4*, both *in vitro* and *in vivo*, indicating the potential of CRISPR-based gene activation for treating complex diseases like type 1 diabetes (Giménez *et al.* 2016). Gene therapy has also made strides, with the delivery of *PDX1* and *MAFA* genes to pancreatic cells via AAV vectors improving glucose homeostasis in mice (Guo *et al.* 2023). These findings emphasize the growing potential of gene activation and reprogramming strategies in the development of new therapeutic options for diabetes treatment. Although significant progress has been made, challenges remain in achieving a true β cell phenotype, as external genes introduced during cell reprogramming can activate both endogenous and target genes, which may continue to drive cellular programming processes even after the exogenous genes are removed (Soria 2001, Akinci *et al.* 2012, Elhanani *et al.* 2020) Nevertheless, these approaches hold great promise for future advances in regenerative medicine and diabetes treatment.

One of the main challenges in β cell reprogramming is the limited activity of endogenous genes, which often leads to inefficient reprogramming outcomes. In this study, we utilized the CRISPR-dCas9 system incorporating the VPR, VP64, and P300 activation domains to enhance the expression of *PDX1* and *NGN3*, two crucial transcription factors for pancreatic development. By targeting the promoter regions of these genes, we observed significant upregulation of both gene and protein levels, as confirmed through immunostaining and RT-qPCR analyses. Unlike previous studies that have focused on the isolated roles of *PDX1* and *NGN3*, our approach employed a multi-faceted strategy using three different dCas9 activators and multiple gRNA constructs to optimize gene expression across the -250 to +1 promoter region. Notably, our results demonstrate a synergistic effect of the VPR domain, leading to a remarkable 19- and 256-fold increase in *PDX1* and *NGN3* expression, respectively, compared to conventional methods. These findings represent a significant advancement in β cell reprogramming, providing a more efficient platform for generating insulin-producing β-like cells from non-β cell sources.

Materials and Methods

Design and cloning of gRNA expression plasmids

The PAM required for gRNA targeting was selected as the *Streptococcus pyogenes* NGG. Ten gRNA sequences for the *PDX1* gene and eight gRNA sequences for the *NGN3* gene were determined using the online CRISPR-ERA tool (CRISPR-ERA 2025). DNA oligonucleotides encoding 20-nt long gRNAs were synthesized (Supplementary Material Tables S1, S2). The synthesized DNA oligonucleotides were ligated to the *BbsI* site in the gRNA expression vector. The gRNA expression vector plasmid was obtained from Addgene pSPgRNA (# 47108) (Pablo Perez-Pinera *et al.* 2013).

Selection of dCas9-activator expression plasmids

For the activation of target genes, we used dCas9-based transcriptional activators. The following expression plasmids were obtained from Addgene (https://www.addgene.org/): dCas9-VP64 (#47107) (P. Perez-Pinera *et al.* 2013), dCas9-VP64-GFP (#61422) (Konermann *et al.* 2015), dCas9-VPR (#63798) (Chavez *et al.* 2015), and dCas9-p300 core (#61357) (Hilton *et al.* 2015). These constructs were selected based on their distinct transcriptional activation mechanisms, allowing for a comparative evaluation of their efficiency in upregulating endogenous *PDX1* and *NGN3* expression.

HEK293 cell culture and transfection

HEK293 cells (Thermo Fisher Scientific, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Capricorn, Germany) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel), 1X non-essential amino acids (Gibco, USA) and 1X Antibiotic-Antimycotic (Biowest, France). HEK293 cells were seeded into 12-well plates at a density of 25000 cells per well 1 day before transfection. The cells were cultured in medium without antibiotics prior to transfection. Twenty ng of *NGN3* or *PDX1* gRNA plasmids were transfected using Lipofectamine 2000 (Life Technologies, USA), along with 200 ng of dCas9-VP64, dCas9-VPR or dCas9-P300, according to the manufacturer's instructions. The medium was refreshed 24 h after transfection. RNA isolation was performed with TRIzol (Invitrogen, USA) 48 h after transfection, according to the manufacturer's instructions. Cells were grown for an

additional 48 h before they were harvested for immunostaining. The estimated transfection efficiency was approximately 70% using 200 ng dCas9-VP64-GFP plasmid (Fig. S1). Transformation trials were conducted using various concentrations of dCas9-VPR, dCas9-p300, and dCas9-VP64 plasmids (10, 20, 50, 75, 100, 150, and 200 ng). The highest transfection efficiency was observed at 200 ng (data not shown). Control experiments were performed using HEK293 cells that were cultured under the same conditions but without transfection.

Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from cell samples using TRIzol reagent and reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA). For each quantitative RT-qPCR reaction, 200 ng cDNA were used as a template in a 10µl reaction system with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA). Quantitative RT-PCR was performed with the LightCycler® 96 thermal cycler (Roche Applied Science, Germany), using the following conditions: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 60 s with fluorescence acquisition, followed by a standard melting curve stage. The measured transcript levels were normalized to GAPDH levels, and all samples were analyzed in triplicate. The primers used for RT-qPCR analysis are listed in Supplementary Material Table S3. RT-qPCR was performed as described above. As a negative control, HEK293 cells were used without any transfection. The data are presented as Log2 (relative expression to GAPDH), which allows for a clearer representation of fold changes in gene expression relative to the control group. This approach simplifies the visualization of gene expression changes, particularly in cases of increased or decreased expression compared to the negative control.

Immunostaining

The cell culture medium was removed 48 h after plasmid transfection. HEK293 cells were washed three times with 1X PBS. The cells were fixed with 10% (v/v) formalin for 20 min at room temperature. After incubation, the formalin was removed, and the cells were washed three times with 1X PBS, each wash lasting 5 min. Next, the cells were permeabilized by adding 0.2% (v/v) PBS-TritonX-100 and incubating for 15 min at room temperature. After incubation, the cells were washed three times with 0.1% (v/v) PBS-Tween 20, each wash lasting 5 min. Following the washing, 5% (w/v) PBS-Tween 20-BSA was added, and the cells were incubated for 2 h at room temperature. After incubation, the cells were washed three times with 0.1% (v/v) PBS-Tween 20 for 5 min each. The primary antibodies Rabbit polyclonal anti-PDX1 (1: 2000 dilution; Abcam, UK), Rabbit polyclonal anti-NGN3 (1: 200 dilutions; Abcam, UK), and Rabbit monoclonal anti-Insulin (1: 300 dilution; Abcam, UK) were used. The antibodies were incubated overnight at 4°C. Following incubation, the primary antibodies were removed by washing the cells three times with 0.1% (v/v) PBS-Tween 20 (v/v) for 5 min each. The secondary goat anti-rabbit IgG antibody was prepared in 1% PBS-Tween 20-BSA solution at a 1:400 dilution and added to each well. The nucleic acid stain, antibodies, and their dilution ratios used in immunofluorescence staining are listed in Table S4. The cells were incubated for 1 h at room temperature. After incubation, the secondary antibody was removed by washing the cells three times with 0.1% (v/v) PBS-Tween 20 for 5 min each. Hoechst dye was prepared at a 1:400 dilution in 1% PBS-Tween 20-BSA solution, added to each well, and incubated for 20 min at room temperature. After incubation, the cells were washed three times with 1X PBS. Finally, 1X PBS was added to the cells, and images were captured using a Leica DMi8 inverted microscope (Leica DMi8, LASX Software).

Statistical analysis

In the transfection experiments, HEK293 cells were transfected at three different time points to assess experimental conditions. For each time point, three independent biological replicates were performed, each with three technical replicates. Biological replicates represent separate experiments using distinct samples, while technical replicates are repeated measurements from the same sample to assess precision. This design accounted for both technical and biological variability, ensuring reliable results. Statistical analyses were performed on the averages of independent experiments. An unpaired two-tailed Student's t-test was used for comparisons between two groups, with significance set at $p < 0.05$. Data are presented as mean ± SEM (n=3).

Results

dCas9-Transactivator and gRNA Optimization for PDX1 gene activation

Each of the ten gRNAs specific to *PDX1* was introduced separately into the cells, along with the dCas9-VP64, dCas9-VPR, and Cas9-p300. The effects of each gRNA on gene expression were assessed using RT-qPCR. Fig. 1 shows that the *PDX1* gene is active in compared to the control group. Among the 10 gRNAs tested, gRNA1, gRNA2, gRNA7, and gRNA9 were most effective in activating the *PDX1*. These gRNAs also meet the criteria established in the gRNA optimization and design experiments conducted (Graf *et al.* 2019, Wang *et al.* 2019).

The four optimal gRNAs were selected for activation experiments of the *PDX1* using dCas9 activator complexes. Among these complexes, the VPR activator showed the highest activation level compared to the control group, as shown in Fig. 1d. The simultaneous application of the four gRNAs targeting *PDX1* resulted in a 19-fold increase in gene expression with VPR and a 3.9-fold increase with VP64.

The transfections were performed using 200 ng of the dCas9-activator plasmid and 20 ng of each gRNA (**$p < 0.005$, ****$p < 0.0001$).

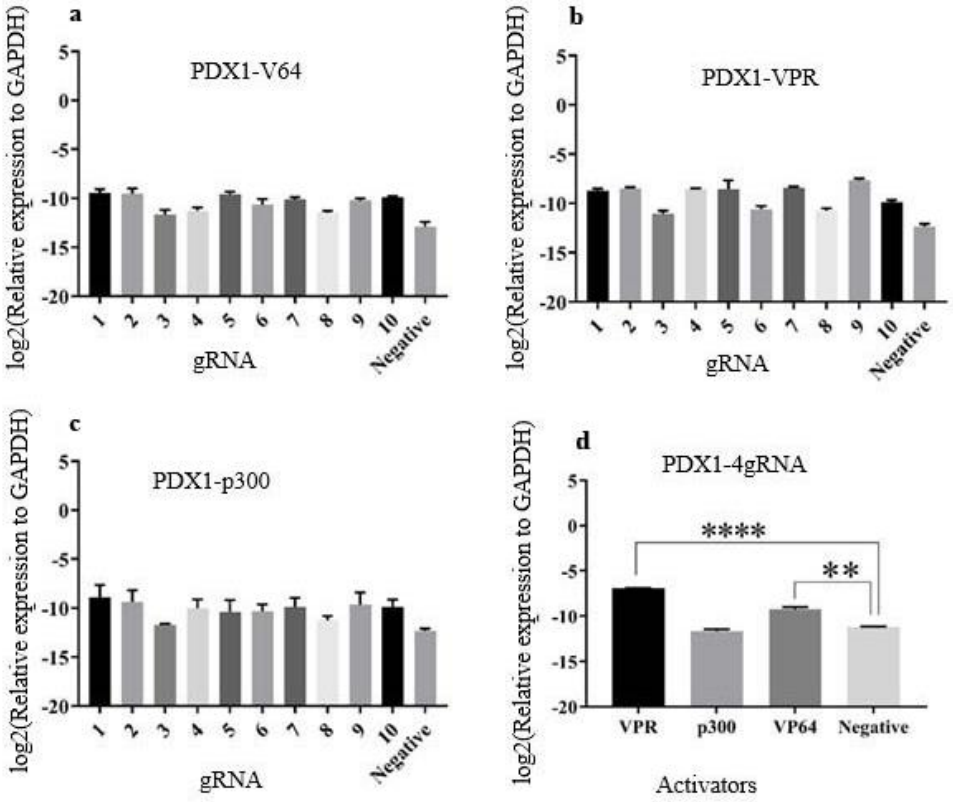

Fig. 1. Upregulation of the *PDX1* expression in HEK293 cells by **a.** dCas9-VP64, **b.** dCas9-VPR, **c.** dCas9-p300, and **d.** optimal 4 gRNA activators. The relative expression levels were measured by RT-qPCR 48 h after transfection. Data represents mean ± SEM, n=3 independent transfections.

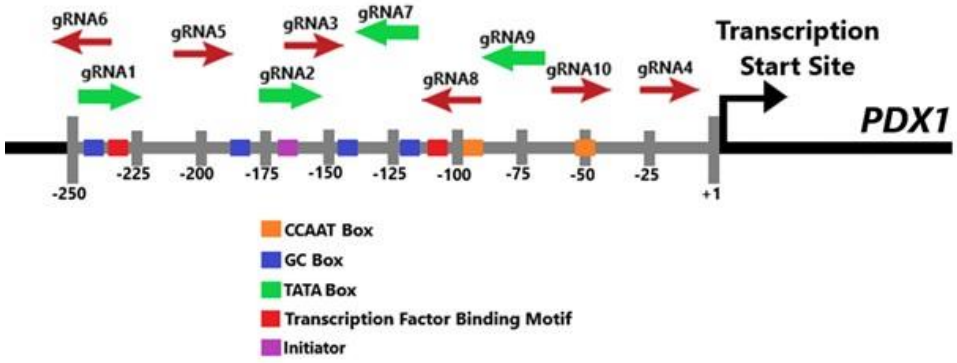

Fig. 2. The promoter region of *PDX1*, which spans from -250 to +1 base pairs, is considered crucial for the regulation of gene expression.

Once the optimal gRNAs were identified, an examination was conducted using the Eukaryotic Promoter Database (Dreos *et al.* 2017, Database 2025) to determine if any regulatory regions influencing gene activity were present near the locations of these gRNAs. Based on this investigation, we found that gRNA1 is associated with a region containing a transcription factor motif, GC box, and transcription factor binding motif. Similarly, gRNA2 is linked to a region containing the initiator motif. Furthermore, RNA7 binds to a region containing the GC box. Fig. 2 illustrates that no discernible motif or specific region was identified for gRNA9.

dCas9-Transactivator and gRNA Optimization for NGN3 gene activation

Each of the 8 gRNAs identified for the *NGN3* gene was separately introduced into the cells using dCas9-VP64, dCas9-VPR, and Cas9-p300. The effect of each gRNA on gene expression were assessed using RT-qPCR. Fig. 3 demonstrates that the *NGN3* gene is active in compared to the control group. Out of the 8 gRNAs, gRNA1, gRNA2, gRNA4, and gRNA7 were optimal for activating the *NGN3*. The simultaneous application of these four *NGN3*-targeting gRNAs resulted in a 256-fold increase in gene expression with VPR, a 35-fold increase with p300, and a 3-fold increase with VP64. The gRNAs

also met the criteria established in the gRNA optimization and design experiments conducted by Graf *et al.* (2019) and Wang *et al.* (2019). Activation assays were conducted using dCas9 activator complexes with the four optimal gRNA sequences identified to activate the *NGN3*. The results showed that the VPR activator exhibited the highest level of activation compared with the control group (Fig. 3d). The VPR activator enhanced the affinity of transcription factors for the promoter regions of *NGN3*, which is specifically recognized by four gRNAs. As a result, transcription is initiated and, gene expression is enhanced.

The absence of statistically significant activation observed for individual gRNAs implies that these gRNAs may not precisely bind to the promoter region of the target gene, or even if they do bind, they may not effectively recruit transcription factors and activate transcription. The presence of four gRNAs causing excessive activation indicates that these gRNAs have the potential to bind to distinct sections of the promoter region of their target genes.

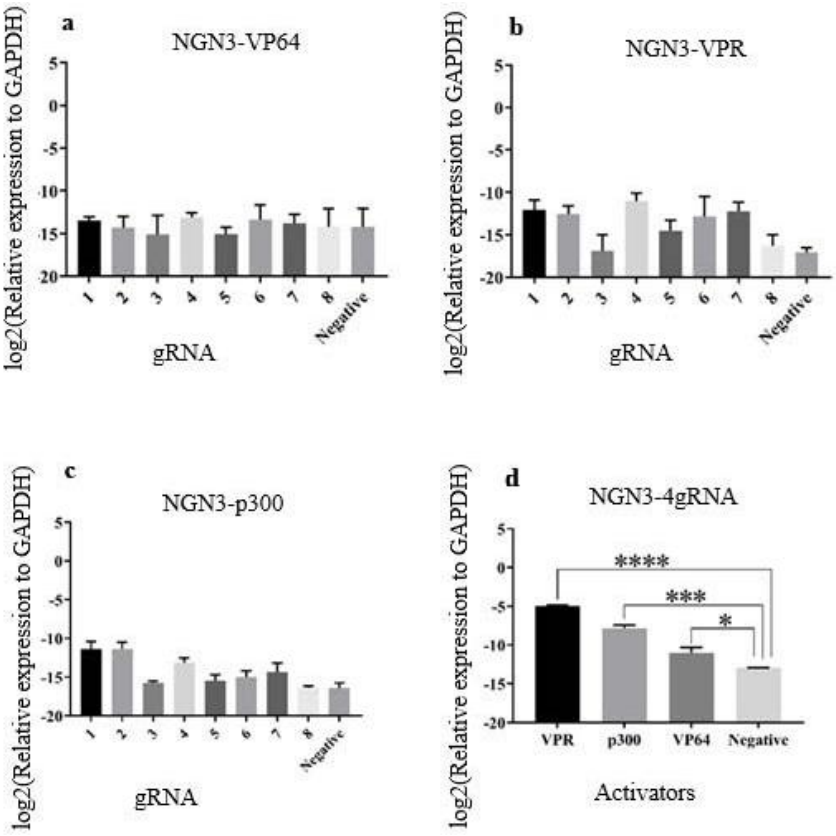

Fig. 3. Upregulation of the *NGN3* gene in HEK293 cells by **a.** dCas9-VP64, **b.** dCas9-VPR, **c.** dCas9-p300, and **d.** optimal 4 gRNA activators. The relative expression levels were measured by RT-qPCR 48 h after transfection. Data represents mean ± SEM, n=3 independent transfections. The transfections were performed using 200 ng of the dCas9-activator plasmid and 20 ng of each gRNA (*$p < 0.05$, ***$p < 0.0005$, ****$p < 0.0001$).

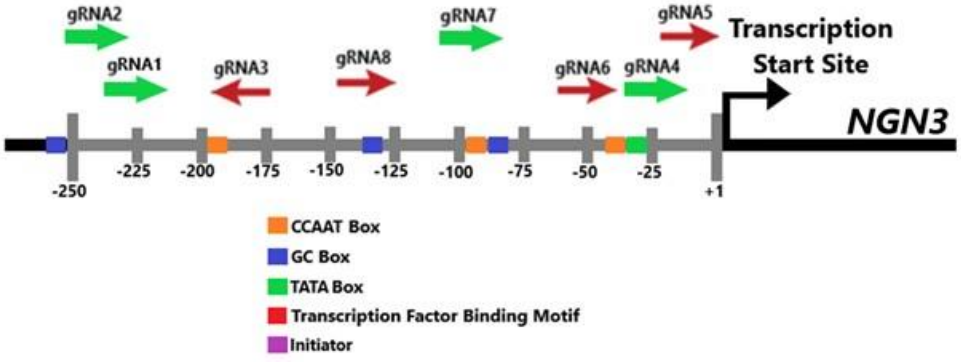

Fig. 4. The regulatory elements within the promoter region of *NGN3*, specifically spanning from -250 to +1 base pairs, are considered to play a critical role in the modulation of gene expression.

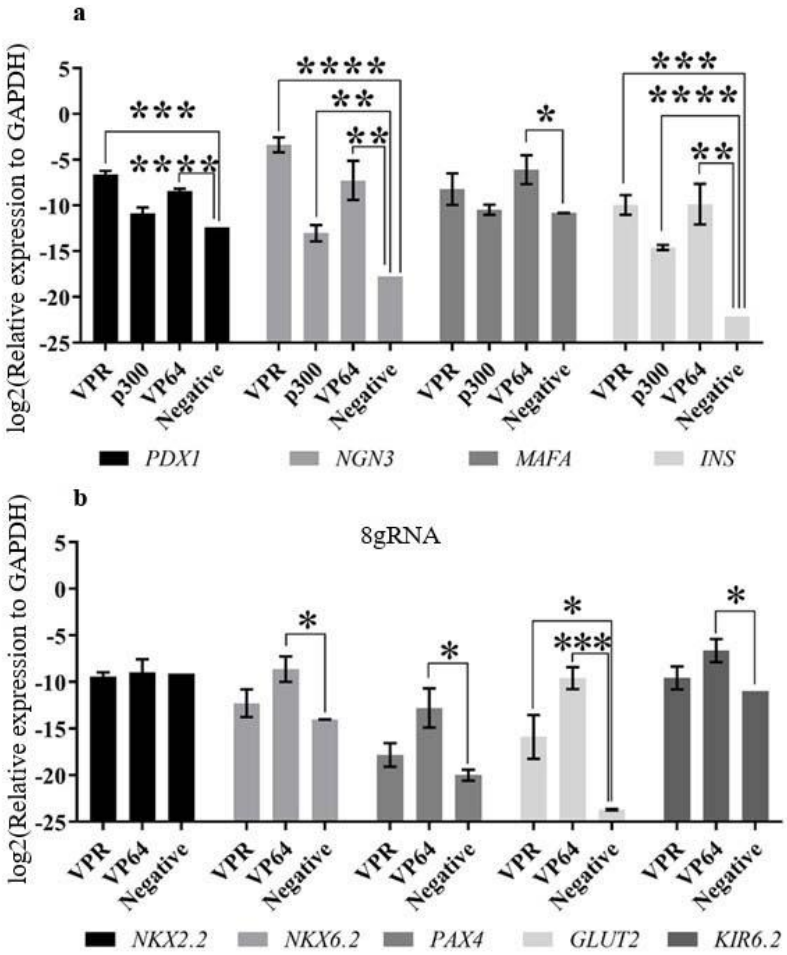

Fig. 5. Activation results of important pancreatic genes. **a.** Expression of the *PDX1*, *NGN3*, *MAFA*, and *INS* genes, **b.** activation of the *NKX2.2*, *NKX6.2*, *PAX4*, *GLUT2*, and KIR6.2 genes from important pancreatic genes. Data represents mean ± SEM, n = 3 independent transfections. The transfections were performed using 200 ng of the dCas9-activator and 20 ng of each gRNA (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$).

This leads to more efficient recruitment of transcription factors and the initiation of transcription.

Once the optimal gRNAs were identified, an examination was conducted using The Eukaryotic Promoter Database (Dreos *et al.* 2017, Database 2025) to discover if there were any regulatory areas influencing gene activity in the vicinity of the gRNAs. The results revealed that gRNA4 attaches to the region encompassing the TATA box and CCAAT box, and is located near the regions where gRNA2 binds to the GC box. Additionally, gRNA7 binds to both the CCAAT box and GC box regions (Fig. 4).

Synergistic Activation of PDX1 and NGN3 Genes Using CRISPR-dCas9 System with Optimized gRNAs

Four gRNA sequences were identified for the activation of *PDX1* and *NGN3*. These gRNA sequences were then introduced into cells along with the dCas9-VPR, dCas9-p300, and dCas9 VP64 activators. The expression levels of crucial cellular genes were subsequently analyzed. Co-transfecting these 8 gRNAs demonstrated a synergistic effect on gene activation. The combination of 8 gRNA with the VP64 activator for *PDX1*, and the VPR activator for *NGN3* yielded the highest level of activation, as shown in Fig. 5a. The *INS* gene exhibited the greatest significant activation when using 8 gRNAs and p300 activators. Activation of the *PDX1* and *NGN3* genes using 8 gRNA and VP64 activators resulted in the highest level of activation of the *MAFA*, *NKX6.2*, *PAX4*, *GLUT2*, and *KIR6.2* genes, as shown in Fig. 5b. These genes are responsible for the distinctive characteristics of pancreatic β cells. The upregulation of these genes enhances the process of cell differentiation into β cells. Fig. 5 shows a substantial increase in the expression of these genes compared with the control cells.

Immunostaining

Gene expression was demonstrated by transfecting HEK293 cells with 20 ng of gRNA plasmid and 200 ng of dCas9-activator plasmid using anti-PDX1, anti-NGN3, and anti-Insulin antibodies. The CRISPR/dCas9 activation mechanism induced the expression of endogenous PDX1 and NGN3, resulting in the localization of endogenic proteins in the nucleus (Figs 6, 7).

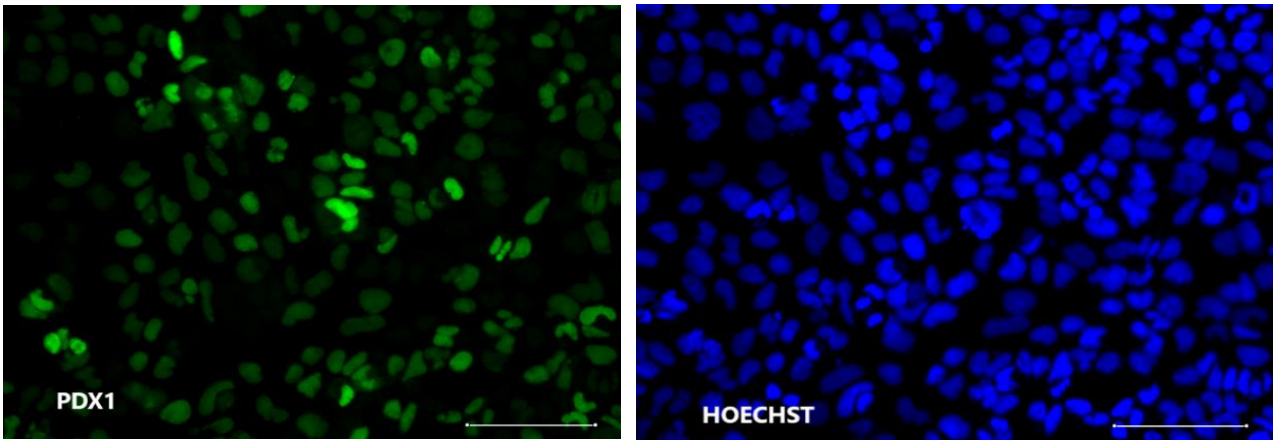

Fig. 6. Anti-PDX1 (green) and Hoechst (blue) immunostaining results after activation of the *PDX1* gene using 4 gRNA and the dCas9-VPR plasmid. Scale bars represent 100 µm.

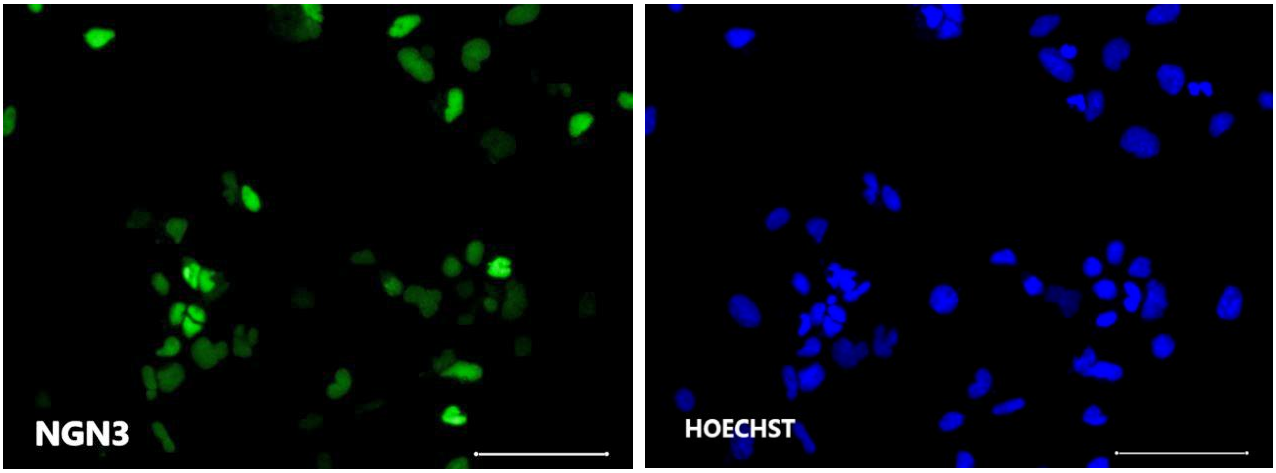

Fig. 7. Immunocytochemical analysis of NGN3 in HEK293 cells 72 h after transfection with dCas9-VPR activator and *NGN3* targeting 4 gRNAs. Anti-NGN3 (green), Hoechst (blue). Scale bars represent 100 µm.

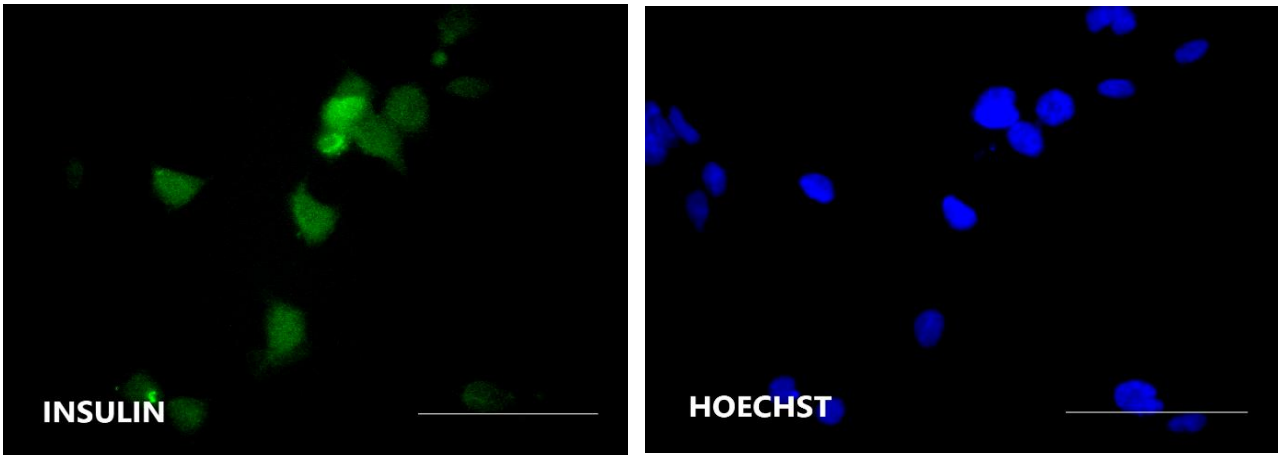

Fig. 8. Immunocytochemical analysis of INS in HEK293 cells 72 h after transfection with dCas9-VPR activator and 8 gRNAs targeting *PDX1* and *NGN3*. Insulın (green), Hoechst (blue), and scale bars represent 100 µm.

Immunocytochemical staining for insulin in HEK293 cells 72 h post-transfection using the dCas9-VPR activator system and 8 gRNAs designed to target the *PDX1* and *NGN3* genes. The cells were stained with an anti-Insulin antibody to visualize the expression of insulin. The results show significant insulin expression in cells transfected with the dCas9-VPR activator and gRNAs targeting *PDX1* and *NGN3*, compared with the control group, which exhibits minimal to no insulin expression. This indicates that the activation of the *PDX1* and *NGN3* genes via the dCas9-VPR system effectively induces insulin (INS) expression in HEK293 cells, highlighting the potential of this approach for gene activation studies aimed at generating pancreatic β cell-like cells.

Discussion

In this study, dCas9-based activator complexes were used to evaluate the activation of the *NGN3* gene using different gRNAs. The HEK293 cell line is an ideal model for CRISPR/dCas9-based gene activation due to its high transfection efficiency and sensitivity to genetic modifications. Its rapid proliferation and ease of culture ensure a stable environment for long-term experiments, allowing for effective evaluation of gene activation. Low basal expression levels facilitate clear observation of transcription factor activation, such as *PDX1* and *NGN3*, while supporting reliable results in immunofluorescence and RT-qPCR analyses. These features make HEK293 a robust model for optimizing gene regulation strategies that induce β-cell differentiation . Our findings emphasize the critical role of gRNA selection, transcription factor recruitment, and promoter structure in gene activation. Among the eight gRNAs tested, gRNA1, gRNA2, gRNA4, and gRNA7 were identified as the most effective. Activity was assessed by measuring *NGN3* gene expression levels using RT-qPCR and comparing transcriptional increases achieved with activator complexes. Additionally, the binding sites within the promoter region and transcription factor recruitment were also analyzed. When used alone, gRNAs did not result in significant transcriptional activation, but the combination of these four gRNAs, particularly when used with the VPR activator, led to a 256-fold increase in *NGN3* expression. A 35-fold increase was observed with the p300 activator, and a 3-fold increase was observed with VP64. Analysis of the binding sites within the promoter region revealed that gRNA4 was located near the TATA box and CCAAT box. These elements are critical for initiating transcription, and gRNAs binding near these regions are thought to effectively recruit transcription factors, increasing RNA polymerase II binding. In contrast, gRNA1 and gRNA2, which bind to the GC box, exhibited relatively lower activation potential. This suggests that while the GC box can initiate transcription, TATA and CCAAT boxes offer higher activation efficiency.

In studies evaluating the activation of the *PDX1* gene, the effectiveness of various gRNAs and their binding regions to transcription factors were analyzed. RT-qPCR results showed that gRNA1, gRNA2, gRNA7, and gRNA9 achieved the highest activation of *PDX1* expression. Notably, gRNA1 and gRNA2 were associated with motifs like the GC box and the promoter motif, which play significant roles in transcriptional regulation. The presence of these motifs indicates that the corresponding gRNAs can enhance transcriptional efficiency by recruiting transcription factors to the target region. On the other hand, the effect of gRNA7, which includes a GC box but lacks a notable motif, is likely mediated through chromatin structure modulation or indirect mechanisms. When dCas9 activator complexes were tested, the highest activation of *PDX1* expression was achieved by the VPR system, resulting in a 19-fold increase. The VP64 activator also provided significant activation, although at a lower level (3.9-fold) compared to VPR. However, a combination of multiple gRNAs with the dCas9-p300 activator complex did not lead to a significant improvement in *PDX1* expression. This may be due to the more limited transcriptional activation capacity of p300 compared to VPR.

The concurrent activation of *PDX1* and *NGN3* genes resulted in the upregulation of pancreatic β-cell-related genes, suggesting that CRISPR-based gene activation could promote β-cell differentiation. Our findings indicate that the use of multiple gRNAs produces a synergistic effect in enhancing gene activation. The combination of VP64 for *PDX1* and VPR for *NGN3* provided the strongest gene expression, highlighting the critical role of activator selection in optimizing gene expression. Additionally, p300 was observed to be particularly effective in enhancing the expression of the *INS* gene, which is thought to be related to its role in histone acetylation and chromatin remodeling. These results support the idea that targeted epigenetic modifications can significantly improve the transcriptional environment in somatic cells. Furthermore, the upregulation of key β-cell markers such as *MAFA*, *NKX6.2*, *PAX4*, *GLUT2*, and *KIR6.2* indicates that CRISPR activation not only activates *PDX1* and *NGN3* genes but also triggers critical genetic programs essential for pancreatic β-cell development.

Immunohistochemistry analysis confirmed that the combination of dCas9-VPR and the target gRNAs successfully increased PDX1 and NGN3 protein expression in HEK293 cells, leading to a significant rise in insulin expression. These findings validate the specificity of cellular reprogramming and suggest that CRISPR/dCas9 activation could be an effective method for acquiring pancreatic β-cell features. However, functional evaluations of the β-cell-like cells are required. For these evaluations, it is recommended to validate the insulin production levels using Western blot analysis, assess cellular glucose sensitivity through static glucose stimulation tests, and analyze cellular response mechanisms such as calcium flux. Additionally, evaluating the phenotypic stability of the cells under long-term culture conditions will be critical to understanding the functional continuity of the generated β-cell-like cells.

Future studies should include validation of insulin production using Western blot analysis, assessment of glucose sensitivity, and investigation of long-term stability. Moreover, conducting similar experiments in other cell lines like INS-1, HP62, and HP74 will help ensure the broader biological relevance of these findings. The gRNAs identified in our study, along with the dCas9 activators we compared, make significant contributions to gene activation and cell differentiation studies. In conclusion, CRISPR/dCas9-based approaches hold great potential for cellular reprogramming and β-cell regeneration, and these methods could provide valuable insights for future biomedical applications.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study and in the Supplementary Material.

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