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RESEARCH ARTICLES

Testing of novel CpG chromatin fragments as UCOE candidates for improved gene therapy vectors

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ABSTRACT

Aim: The A2UCOE sequence, positioned between the HNRPA2B1 and CBX3 gene promoters in the human genome, supports durable and consistent expression of integrated transgenes, even within compact heterochromatin domains such as centromeres. This project focuses on evaluating the dual-component hypothesis of A2UCOE function by analyzing alternative DNA elements that possess CpG-rich content and divergent promoter features.

Method: To investigate expression stability, lentiviral vectors carrying eGFP reporter constructs driven by novel UCOE candidates and various A2UCOE subregions were introduced into P19 and F9 mouse embryonal carcinoma cells. Expression was tracked over time, both before and after lineage-specific differentiation toward neuroectoderm and endoderm. To examine the proposed bipartite model of UCOE function, we employed two types of CpG-rich, bidirectionally transcribed elements: the endogenous *SETD3–CCNK* housekeeping gene pair, and a synthetically arranged divergent configuration composed of *RPS11* and *HNRPA2B1* promoters.

Results: Placing these regulatory elements in either orientation upstream of the SFFV-eGFP reporter gene—known for its susceptibility to transcriptional silencing—conferred a noticeable, though incomplete, resistance to silencing when compared to the full activity of the reference 1.5A2UCOE-SFFV-eGFP construct. This partial protective effect was consistently observed in both P19 and F9 cell lines, prior to and following their differentiation. In conclusion, we successfully identified a naturally occurring (*SETD3–CCNK*) and synthetically engineered (*RPS11–HNRPA2B1*) pair of divergent promoters that exhibited measurable but incomplete UCOE-like activity relative to the established HNRPA2B1–CBX3 core element.

Conclusion: This study demonstrates that natural and synthetic divergent promoter pairs confer significant, though partial, resistance to transgene silencing. This finding directly supports the A2UCOE's dual-component hypothesis, confirming that CpG-rich bidirectional architecture is key for sustaining stable expression through differentiation and in challenging genomic contexts.

Keywords: chromatin remodelling, gene silencing, lentiviral vector, neuroectodermal and endodermal differentiation, UCOE

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INTRODUCTION

Gene therapy is an emerging method that is used to treat genetic diseases, both inherited and acquired. It is applied by inserting therapeutic nucleic acids into living cells. These nucleic acid molecules either correct faulty genes or alter how genes function (1). For the delivery of these nucleic acid molecules, there are mainly two types of systems: viral and non-6viral. Viral vectors operate by using viruses' natural ability to enter cells and non-viral delivery methods involve carriers like DNA-lipid complexes (e.g., liposomes) (2,3).

Recent advancements in molecular genetics have made it possible for researchers to develop virus-based delivery systems for both research and clinical use (4,5). These include lentiviral vectors (LVs), which are derived from HIV-1, and are significantly advantageous because they can efficiently transduce both proliferating and non-proliferating cells. This feature makes them favorable for treating tissues with low cell renewal activity, such as the central nervous system and some types of stem cells (6).

Lentiviral vectors are widely used because they can carry large genetic inserts (up to ~8 kb) and can be modified by altering their envelope proteins, which enables targeting of specific cell types (7). However, the use of these vectors comes with potential safety risks such as the risk of recombination or unwanted gene activation caused by random integration into the genome. To be able to reduce these risks, researchers have developed self-inactivating (SIN) systems. These minimize promoter interference and decrease the likelihood of insertional mutagenesis (8).

Challenges in gene silencing and transgene expression

A significant concern in gene therapy is maintaining stable and long-term expression of the introduced gene, referred to as a transgene. Retroviral vectors can integrate the transgene into the host genome, which is important for ensuring persistent expression (9,10). However, over time, this expression may decrease or stop altogether due to epigenetic silencing. This process of epigenetic silencing may be triggered by DNA methylation at CpG sites or it may be mediated by histone modifications which cause the chromatin to become more compact and make it harder for the molecular components involved in transcription to access the gene. As a result, the therapeutic effect may weaken or be lost (11,12).

To address this problem, vector systems have been optimized through the incorporation of ubiquitous chromatin-opening elements (UCOEs). These regulatory DNA sequences prevent heterochromatin-mediated silencing and promote stable and long-term gene expression in mammalian cells (13,14). A well-characterized example is A2UCOE, which is derived from the HNRPA2B1-CBX3 locus. This element encompasses two promoter regions and contains a naturally unmethylated CpG island, which helps secure an open chromatin structure and supports active transcription that includes transcriptionally repressive regions like centromeric DNA (13,15).

Functional role and applications of A2UCOE

There are two pathways for the A2UCOE element to function. The first one is a dual-promoter system (HNRPA2B1 and CBX3), and the second is a CpG island that shows natural resistance to DNA methylation (1,2). This unique structure supports an open chromatin configuration and enables consistent and long-term gene expression. It has proven to be a reliable regulatory element in lentiviral vector systems, which support stable transgene expression in both in vitro cell cultures and in vivo models, such as murine hematopoietic stem cells (16). Similarly, A2UCOE is widely used to overcome the issues of epigenetic silencing in gene therapy applications.

Recent studies have explored new CpG-rich DNA regions and other dual-promoter designs to develop improved versions of ubiquitous chromatin-opening elements (UCOEs). The goal of these efforts was to create next-generation vector systems that are more resistant to gene silencing and more effective in delivering therapeutic genes. In summary, incorporating regulatory elements such as A2UCOE into lentiviral vectors has been shown to optimize gene expression for stability, reliability, and reduced risk, which makes them more suitable for gene therapy applications.

MATERIALS AND METHODS

Luria-bertani (LB) medium preparation

To prepare LB broth, 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride were added to one liter of deionized water. The mixture was sterilized in an autoclave at 15 psi for 20 minutes and then cooled to about 55°C. To enable antibiotic selection, ampicillin was added at the final concentration of 50 µg/mL. To make LB agar plates, 20 g of agar was added per liter of LB broth. For selective growth, both plates and broths were supplemented with 100 µg/mL ampicillin.

Competent E. coli DH5α transformation

Chemically competent DH5α *Escherichia coli* cells (sourced from Life Technologies) were transformed following the supplier's instructions. Plasmid-containing bacterial colonies were cultured on ampicillin plates (100 µg/mL) for a duration of approximately 16 hours at 37°C. Selected bacterial colonies were subsequently cultivated in LB broth supplemented with 100 µg/mL ampicillin, using either small (5 mL) or larger volumes (200–500 mL), depending on the experimental scale. Plasmids were isolated using Qiagen kits and eluted in TE buffer.

Cell culture

HEK293T cells were maintained in high-glucose DMEM enriched with 10% bovine serum, L-glutamine (2 mM), and antibiotics (penicillin and streptomycin, 10 µg/mL), and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. A total of 2×10^7 cells were plated into T162 flasks and cultured under standard conditions until reaching approximately 80–90% cell density prior to transfection. Medium was collected 48 hours after transfection, replaced with fresh DMEM, and harvested again at 72 hours. For viral titration, $1–2 \times 10^5$ cells were plated individually into separate compartments of a 24-well tissue culture dish and transduced with serially diluted virus stocks, achieving a multiplicity of infection (MOI) ranging from 1 to 10^{-5} per µL. Detached cells were treated with PBS and Tryple Red reagent, and neutralized with serum-based medium. For flow cytometry, cells were fixed in 4% formaldehyde-PBS, shaken, and placed at 4°C in darkness until they were analyzed.

Mouse embryonic carcinoma cells culture

P19 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 2 mM glutamine, 1% non-essential amino acids, 10% FBS, and antibiotics (penicillin and streptomycin, 10 µg/mL), under standard incubation conditions (37°C, 5% CO_2). Neuronal differentiation was initiated by culturing embryoid bodies in suspension using DMEM supplemented with 5% FBS and a final concentration of 1 micromolar retinoic acid, applied at a cell density of 1×10^5 per milliliter. F9 cells were cultured in gelatin-coated flasks using DMEM supplemented with fetal bovine serum, antibiotics to prevent contamination, and L-glutamine to support cell metabolism. Differentiation toward extraembryonic endodermal lineage was induced by culturing cell aggregates in DMEM/F12 supplemented with 5% FBS and 50 nM retinoic acid at a density of 1×10^5 cells per milliliter.

Reporter gene analysis

Within the lentiviral system, UCOE and A2UCOE-driven eGFP reporter constructs were introduced into P19 and F9 cells, and the persistence of gene activity was assessed before differentiation as well as during their development into neural and endodermal cell types. Differentiated embryoid bodies were transferred to laminin-coated coverslips in 6-well plates. Immunofluorescence staining was performed post-differentiation to assess the expression of reporter genes.

A minimum of 2×10^5 preserved cells were examined for green fluorescent protein expression using a BD FACSymphony™ flow cytometer. Viable cells were identified by assessing their size and granularity through forward and side scatter measurements. Subsequently, GFP+ cells were distinguished by detecting fluorescence emission at 525 nm relative to 575 nm, with non-transduced cells serving as controls to accurately define positive gating. This approach estimates the proportion of live cells within the sample that are actively expressing GFP. The fraction of GFP+ cells was correlated with the amount of virus applied to each sample and extrapolated to determine the number of cells infected by the viral preparation, allowing for the calculation of the multiplicity of infection (MOI) based on mean fluorescence intensity.

For accuracy, values between 1% and 20% GFP-positive cells were selected. Higher percentages likely indicate multiple viral integrations per cell, which can lead to an underestimation of viral titre, while lower percentages may be unreliable due to background fluorescence interference.

Statistical analyses

Statistical analyses were conducted using Prism version 7. Statistical analysis of eGFP+ flow cytometry, MFI, and vector copy number estimation results of transduced cell cultures was performed using Student's t-test. A threshold of $p < 0.05$ was used to determine statistical significance.

RESULTS

The experimental approach aimed at testing the UCOE two-component model involved the use of unmethylated CpG islands and dual transcribed regions, including the SETD3-CCNK gene duo—commonly involved in basic cellular functions—and an engineered combination of RPS11 with HNRPA2B1 regulatory sequences. These sequences were connected in both directions to the silencing-prone SFFV-eGFP reporter to assess their potential for preventing gene expression repression (Figure 1). Although partial protection was noted when compared to the benchmark 1.5A2UCOE-SFFV-eGFP construct, the observed effectiveness remained modest, especially in P19 and F9 cells, throughout the undifferentiated as well as the differentiated conditions. Testing A2UCOE fragments (450–950 bp) positioned before the SFFV-eGFP sequence, regardless of promoter presence, did not preserve the complete UCOE activity. Sequences originating within the initial intronic region of *CBX3*, characterized by a high density of CpG sites but lacking promoter function, showed minimal ability to prevent gene silencing. However, a 0.9-kb subcore fragment of the 1.5-kb A2UCOE, encompassing the transcriptional start sites of CBX3 and HNRPA2B1, provided partial resistance to silencing. These findings suggest that both natural (SETD3-CCNK) and artificial (RPS11-HNRPA2B1) dual promoters exhibit partial UCOE activity, but their protective effects are weaker than the HNRPA2B1-CBX3 prototype.

To further explore the impact of these UCOEs, test vectors were generated using a lentiviral system engineered for reduced self-activation, incorporating the SFFV regulatory sequence to control eGFP expression, along with the WPRE element (SEW). The 1.5 kb 1.5A2UCOE element derived from the HNRPA2B1-CBX3 locus was cloned upstream of the SFFV promoter via a polylinker (PL), generating the 1.5A2UCOE-SEW construct. To broaden the panel of candidate UCOEs, the endogenous SETD3–CCNK locus and an engineered divergent promoter segment combining RPS11 with HNRPA2B1 were bidirectionally cloned into the PL vector, producing constructs named SET-CCN-SEW/CCN-SET-SEW and B1-RPS-SEW/RPS-B1-SEW (Figure 1).

A self-inactivating LV containing an SFFV promoter responsible for initiating eGFP production (SEW) was employed to generate test UCOE vectors that include the WPRE regulatory sequence. A 1.5 kb segment of the HNRPA2B1-CBX3 UCOE (1.5A2UCOE) was introduced before the SFFV promoter via a polylinker, generating the 1.5A2UCOE-SEW plasmid. Promoter regions derived from the human *SETD3–CCNK* locus and the engineered *RPS11–HNRPA2B1* sequence were cloned in both orientations within the PL vector, resulting in the creation of constructs named SET-CCN-SEW/CCN-SET-SEW and B1-RPS-SEW/RPS-B1-SEW. (LTR refers to the long terminal repeat).

The efficiency of lentiviral transduction in the P19 cell line (at an MOI of 3, see Figure 2) was evaluated by assessing cells via flow cytometry at 72 hours following transduction (Figure 3). Cells lacking GFP expression are indicated in red, whereas GFP-expressing cells appear in green. Panels represent the following groups: (A) non-transduced control; (B) cells transduced with SEW; (C) cells transduced with 1.5A2UCOE-SEW; (D) cells transduced with SET-CCN-SEW; (E) cells transduced with CCN-SET-SEW; (F) cells transduced with B1-RPS-SEW; and (G) cells transduced with RPS-B1-SEW.

P19 cells were subjected to transduction using candidate UCOE vectors (SET-CCN-SEW, CCN-SET-SEW, B1-RPS-SEW, RPS-B1-SEW) alongside control vectors as illustrated in Figure 1. The analysis included

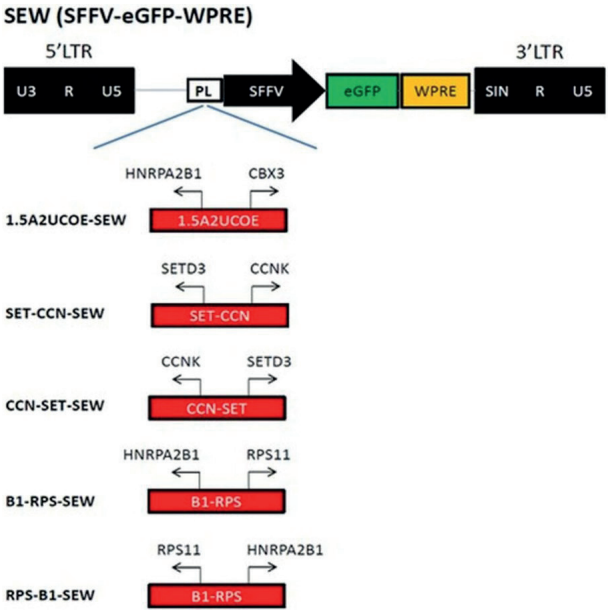

Figure 1. Representation of novel candidate UCOE and control lentiviral vectors.

the percentage of eGFP+ cells, their MFI, and the quantification of integrated vector sequences per cell. The data shown combine findings from three separate transduction experiments per vector and the NEG, collected over a period ranging from 3 to 52 days after transduction. (A) Percentage of eGFP+ cells tracked over the course of the experiment ($p<0.01$). (B) Changes in mean fluorescence intensity recorded across time points ($p<0.01$). (C) Average VCN per cell monitored longitudinally ($p<0.01$).

The transduction of F9 cells was performed using both experimental UCOE constructs (SET-CCN-SEW, CCN-SET-SEW, B1-RPS-SEW, RPS-B1-SEW) and control vectors (SEW and 1.5A2UCOE-SEW), as illustrated in Figure 1. Flow cytometry was used to assess eGFP+ cell percentage, MFI, and VCN. The results combine findings from three independent transduction experiments for each vector and NEG, tracked from day 3 to day 52 post-transduction. (A) Timeline of the percentage of eGFP+ cells (mean ± SEM, based on four samples; $^{**}p<0.01$). (B) Changes in mean fluorescence intensity over time (mean ± SEM, based on four samples; $p<0.01$). (C) Average vector copy number per cell throughout the study period (mean ± SEM), based on four samples; $p<0.01$).

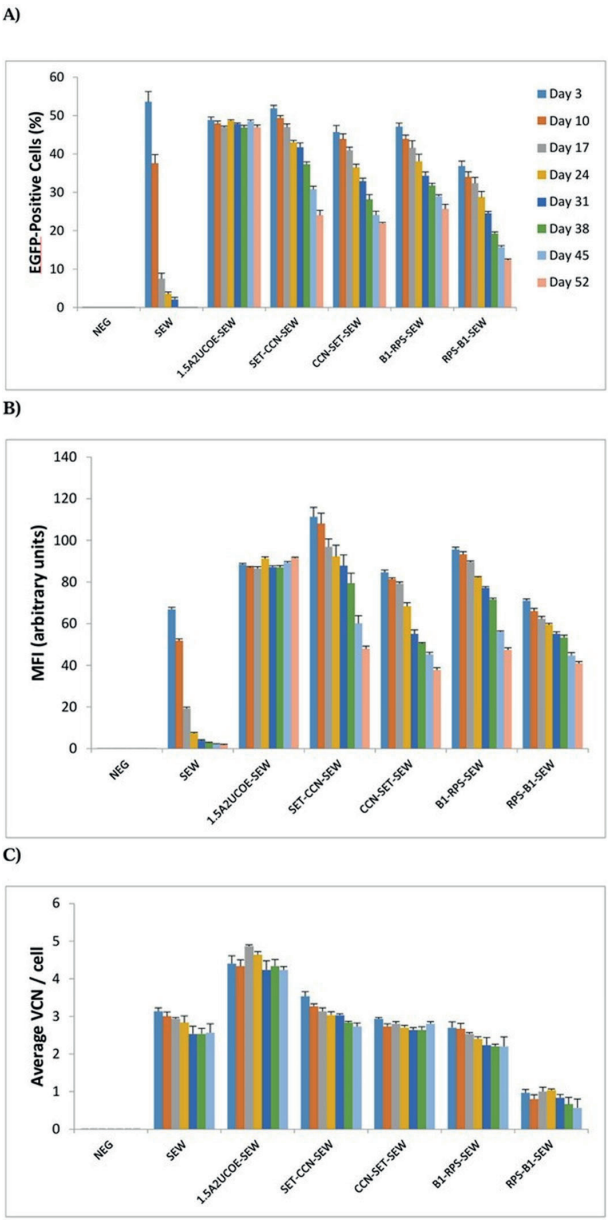

Figure 2. Novel candidate UCOEs provide only partial protection against silencing in undifferentiated P19 cells. (A) Percentage of eGFP+ cells tracked over the course of the experiment, (B) Changes in mean fluorescence intensity recorded across time points, (C) Average VCN per cell monitored longitudinally.

P19 cells underwent genetic transduction with potential UCOE vectors alongside control vectors (SEW and 1.5A2UCOE-SEW), as illustrated in Figure 1, and were induced to differentiate into the neuroectodermal lineage 72 hours following transduction. Flow

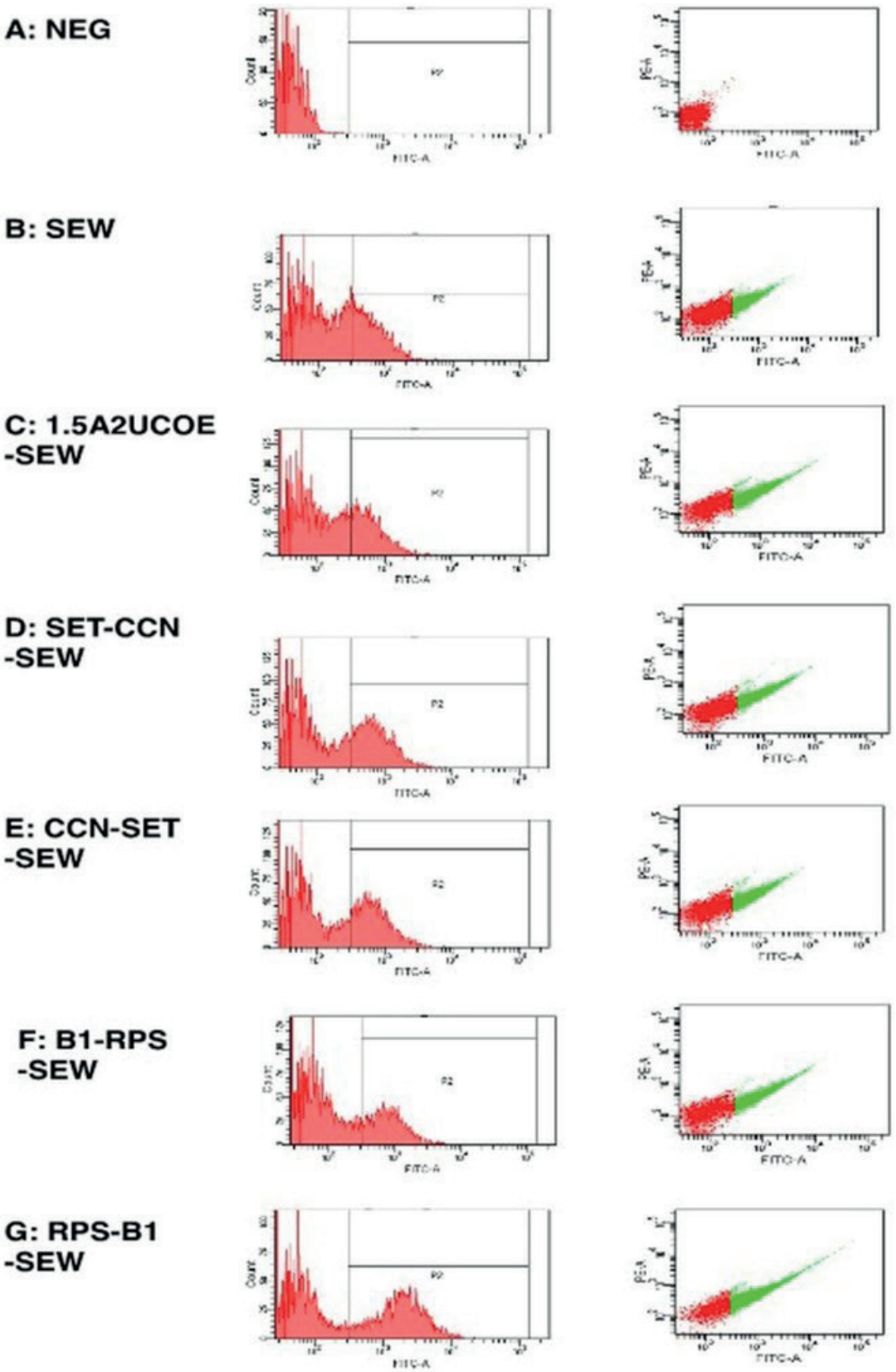

Figure 3. Untransduced and transduced P19 array flow cytometry plots.

cytometry was used to analyze eGFP+ cell percentage and MFI, while VCN was assessed using real-time qPCR at various time points post-differentiation. Data are presented as the aggregate of three separate transduction experiments conducted for each vector, including NEG, monitored from day 3 to day 45 after transduction. (A) eGFP+ cell percentage timeline; ($p<0.01$). (B) MFI timeline ($p<0.01$). (C) Mean VCN/cell timeline ($p<0.01$).

Selected UCOE vectors (SET-CCN-SEW, CCN-SET-SEW, B1-RPS-SEW, RPS-B1-SEW), along with control vectors (SEW and 1.5A2UCOE-SEW), were used to transduce F9 cells (Figure 1). Seventy-two hours after transduction, cells were stimulated to differentiate toward the endodermal lineage. Flow cytometry was performed to measure the percentage of eGFP+ cells and MFI, while VCN was assessed using real-time qPCR at multiple intervals. The data represent combined results from three independent transduction experiments per vector, including NEG, collected between day 3 and day 41 after transduction. (A) eGFP+ cell percentage timeline ($p<0.01$). (B) MFI timeline ($p<0.01$). (C) Mean VCN/cell timeline ($p<0.01$). The findings at 3 days post-transduction reflect the undifferentiated state of the cells.

To confirm the flow cytometry findings (Figures 2-6), P19 and F9 cells that had differentiated into neuroectodermal and endodermal lineages, respectively, were labeled with specific markers and examined using fluorescence-based immunostaining techniques (Figures 7, 8).

P19 and F9 cells were transduced with newly developed UCOE vectors alongside control vectors (Figure 1), and were induced to differentiate along the neuronal and endodermal lineages 3 days post-transduction. Cultures were then analyzed by flow cytometry according to eGFP fluorescence reference points. The results showed that novel UCOE candidates indicated partially stable activity after differentiation compared to the A2UCOE control vector (Figure 9, 10).

P19 cells were transduced with novel UCOE vectors (SET-CCN-SEW, CCN-SET-SEW, B1-RPS-SEW, RPS-B1-SEW) and control vectors (SEW, 1.5A2UCOE-SEW)

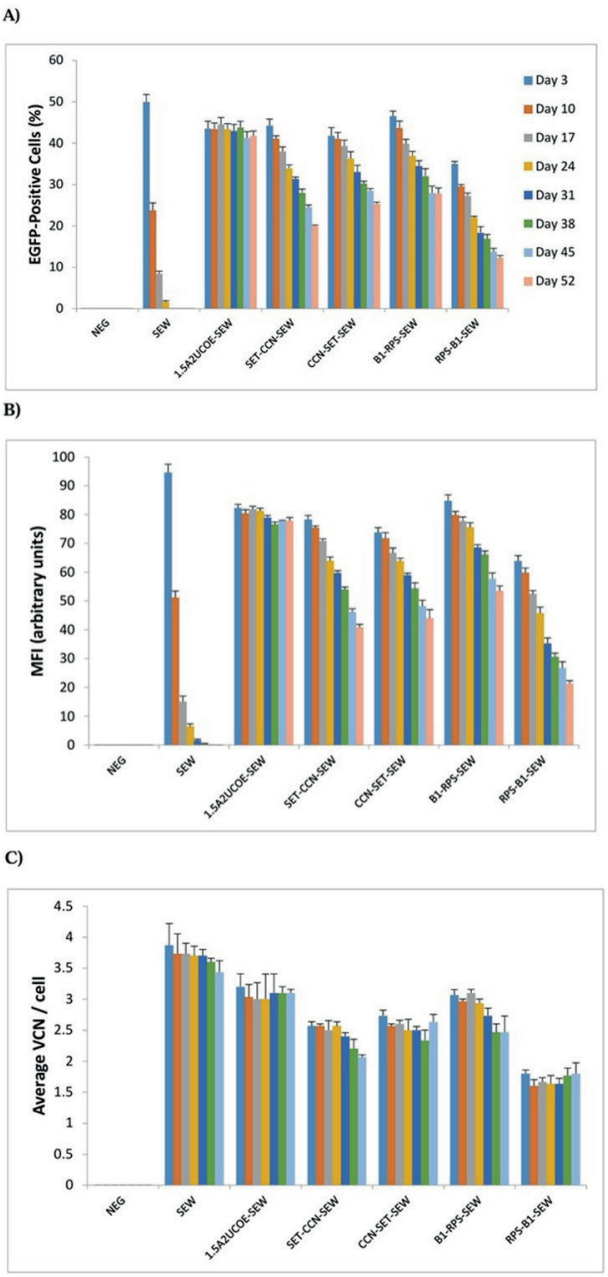

Figure 4. Novel candidate UCOEs provide only partial protection against silencing in undifferentiated F9 cells. (A) Timeline of the percentage of eGFP+ cells, (B) Changes in MFI over time, (C) Average VCN per cell throughout the study period.

(Figure 1). The cells were subsequently induced to differentiate toward the neural ectodermal pathway three days after vector delivery. Fluorescence

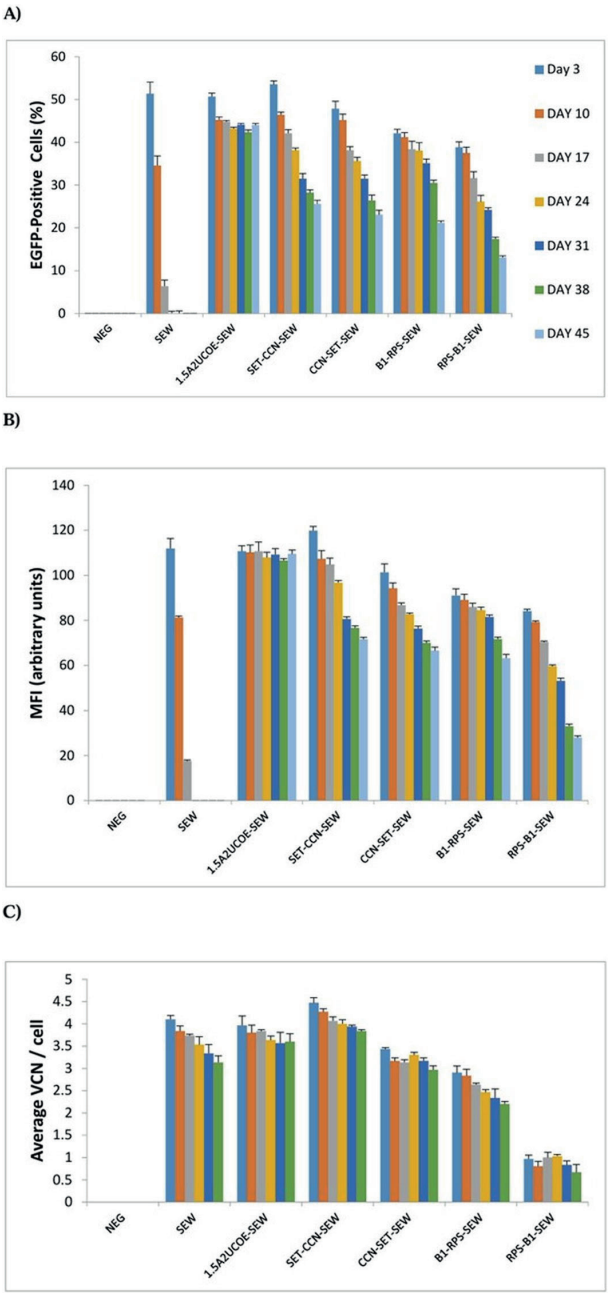

Figure 5. Novel candidate UCOEs provide only partial protection against silencing in differentiated P19 cells. (A) eGFP+ cell percentage timeline. (B) MFI timeline, (C) Mean VCN/cell timeline.

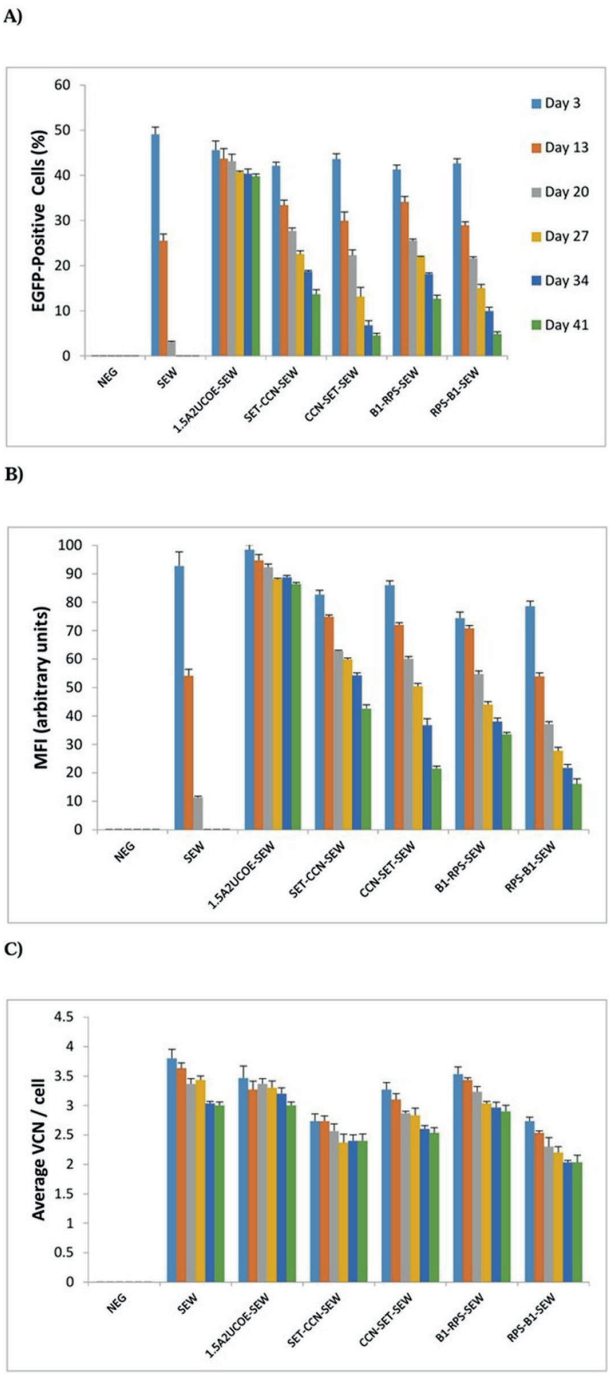

Figure 6. Novel candidate UCOEs provide only partial protection against silencing in differentiated F9 cells. (A) eGFP+ cell percentage timeline, (B) MFI timeline, (C) Mean VCN/cell timeline.

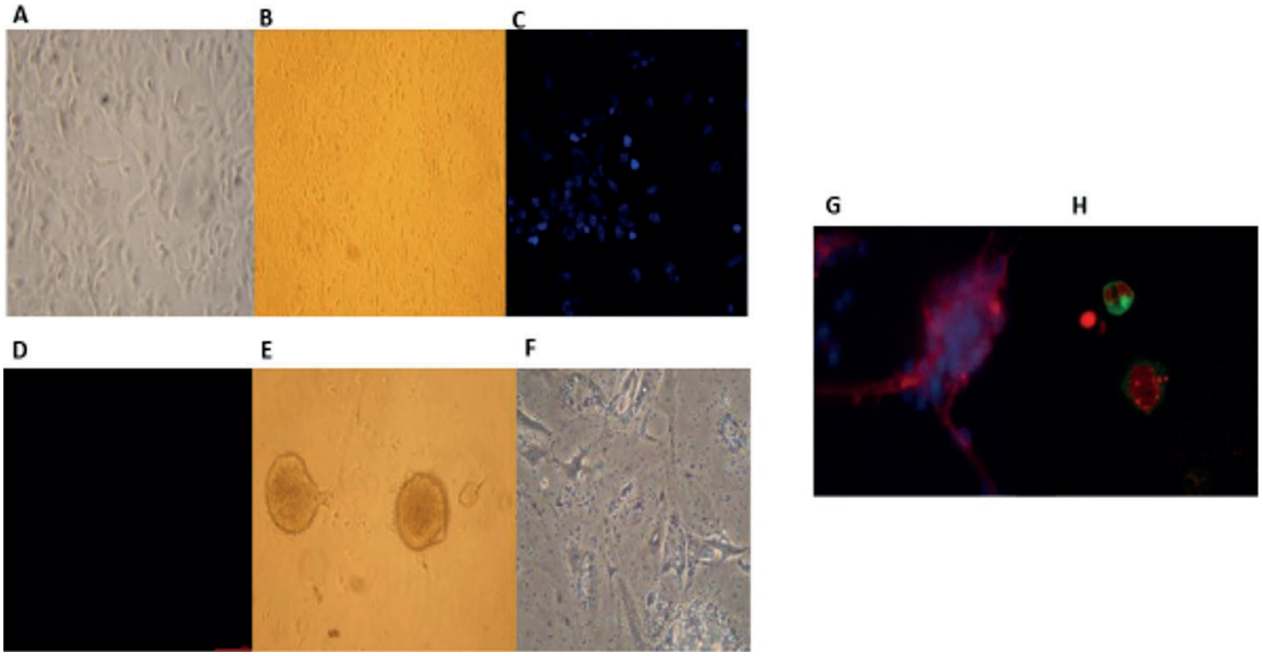

Figure 7. Microscopic Visualization of Undifferentiated and Differentiated P19 Cells

A–B: Phase-contrast microscopy images of undifferentiated and non-transduced P19 cells captured at 40× magnification.

C–D: Fluorescent labeling of the same cell population using DAPI (C) and anti-β-III tubulin (D), visualized at 40× and 100× magnifications, respectively. Cells exhibit positive nuclear staining with DAPI (blue), while showing no detectable expression of neuronal marker β-III tubulin (red) or GFP signal (green).

E–F: Formation of embryoid-like aggregates after 48 hours of culture in non-adherent conditions (E; 40× magnification). Differentiating cells were subsequently replated onto adherent surfaces and imaged after four days of neuroectodermal induction (F; 100× magnification).

G–H: Dual immunofluorescence analysis of P19 cells transduced with UCOE-eGFP vector following neuroectodermal differentiation. Panel G shows co-localization of DAPI (blue) and β-III tubulin (red), while Panel H displays β-III tubulin (red) alongside eGFP signal (green); both at 100× magnification.

microscopy was used at selected intervals to assess cells co-expressing eGFP and β-tubulin III. The findings are based on the pooled outcomes of three separate transduction experiments conducted for each vector and the negative control group, spanning a 3 to 24-day observation period. Analysis revealed statistically meaningful differences ($p < 0.01$)

F9 cells were transduced with novel UCOE vectors and control vectors (Figure 1), then induced to differentiate along the endodermal lineage 3 days post-transduction. Cells were analyzed by fluorescence microscopy at various time points, scoring for eGFP and anti-Oct3-4 double-positive cells. Data represent combined results from three independent transductions for each vector and the NEG over 3–24 days ($p<0.01$).

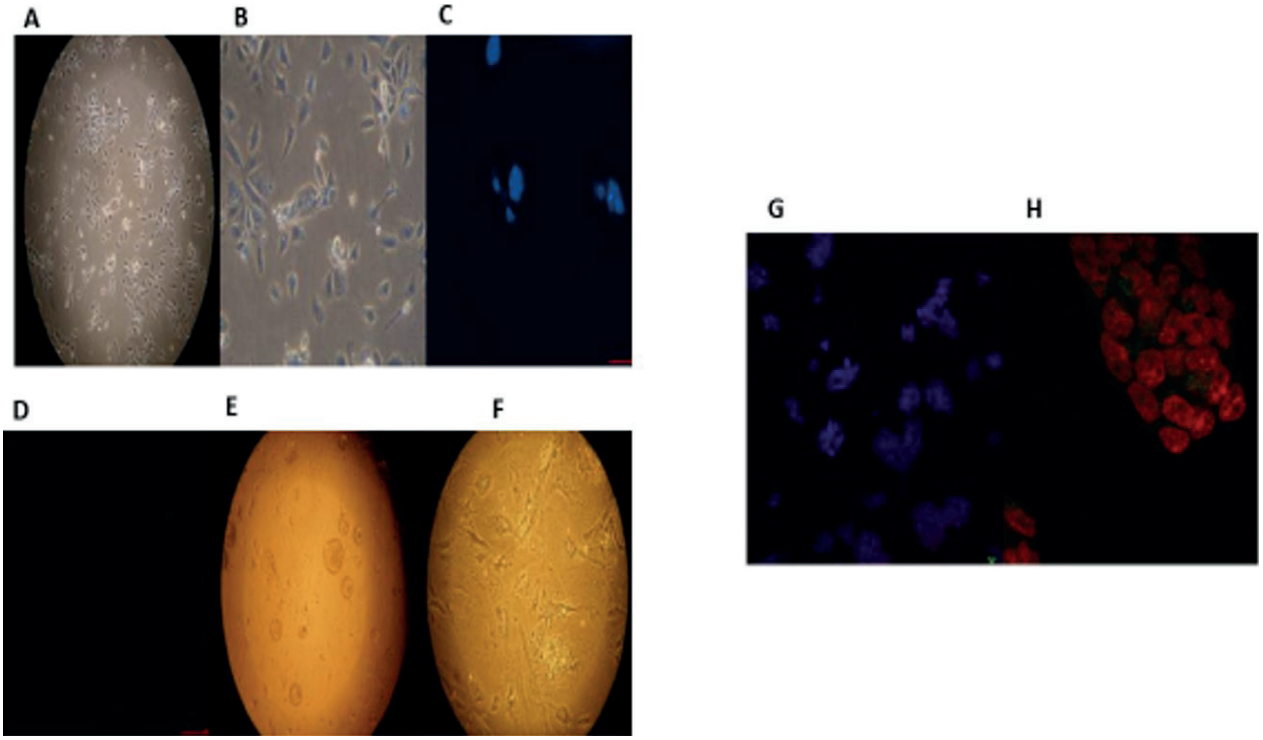

Figure 8. Morphological Examination of F9 Cells Prior to and Following Differentiation.

A-B: Phase contrast images illustrating the morphology of undifferentiated and non-transduced F9 cells at 20x (A) and 40x (B) magnifications.

C-D: Immunofluorescent labeling of undifferentiated F9 cells with nuclear stain DAPI (C) and anti-Oct3/4 antibody (D), both captured at 40x magnification. Cells exhibit DAPI-positive nuclei (blue), but no detectable Oct3/4 (red) or eGFP (green) signal.

E-F: Formation of embryoid bodies following 2-day suspension culture on non-adherent bacterial-grade dishes (E; 40x) and differentiated F9 cells after 5 days of endodermal induction, subsequently plated on adherent tissue culture surfaces (F; 100x).

G-H: Immunofluorescence analysis of F9 cells following endodermal differentiation and lentiviral transduction with UCOE-eGFP constructs. G: Nuclear (DAPI, blue) and Oct3/4 (red) staining shown at 20x magnification. H: Co-localization of Oct3/4 (red) and eGFP expression (green) visualized at 100x magnification.

DISCUSSION

This stage of the study focused on determining whether the newly designed UCOEs operate in accordance with the bidirectional transcriptional mechanism previously attributed to A2UCOE elements (17-19). Additionally, the experiments were designed to assess whether these UCOEs could ensure sustained gene expression when positioned adjacent to a non-native promoter, regardless of the DNA strand orientation in which they were inserted. The A2UCOE has been demonstrated to stabilize gene expression at linked promoters, both ubiquitous(16,18) and tissue-specific(20,21), generally requiring a specific alignment, especially when the CBX3 terminus lies adjacent to the linked promoter. The hypothesis of this study proposed that the two promoters within each chosen gene pair would exhibit comparable expression levels across various tissues, aiming to address the problem related to orientation dependency.

Integrating gammaretroviral and lentiviral vector classes remain the most effective approach to ensure consistent maintenance and expression of a therapeutic gene. This is particularly applicable when focusing on mitotically active stem cell populations. In fact, over the past 15 years, clinical trials employing an ex vivo strategy targeting HSCs with these vector types have consistently demonstrated successful outcomes (4). However, the use of these integrase classes involves two critical challenges that must always be considered.

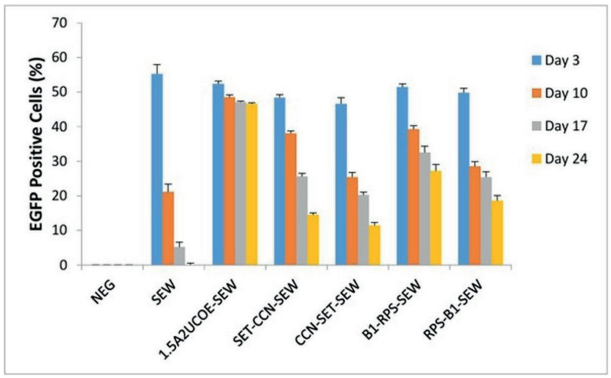

Figure 9. Novel candidate UCOEs provide only partial protection against silencing in differentiated P19 cells.

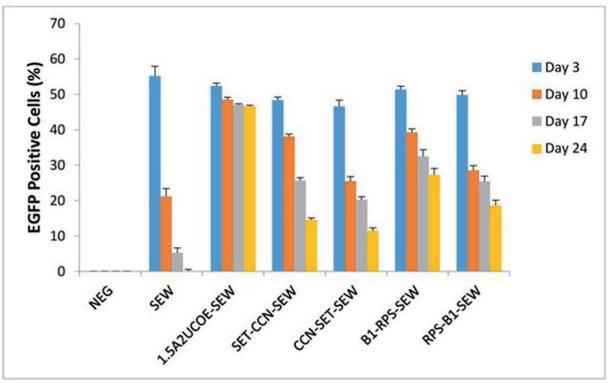

Figure 10. Novel candidate UCOEs provide only partial protection against silencing in differentiated F9 cells.

The first is insertional mutagenesis, and the second concerns therapeutic gene inactivation mediated by epigenetic mechanisms (3). Notably, gammaretroviral integration caused insertional mutagenesis in 5 out of 20 SCID-X1 patients, which led to unintended activation of host proto-oncogenes and subsequent oncogenesis (22). Additionally, therapeutic gene inactivation caused by methylation of promoter DNA eventually led to the loss of therapeutic efficacy in two individuals treated for CGD (23).

The findings were encouraging, as stable expression driven by the SFFV promoter was preserved in both configurations of the SETD3-CCNK element as well as the synthetic HNRPA2B1-RPS11 pair, independent of their orientation (Figures 3 and 10). This observation supports the hypothesis that orientation might not be as critical if both promoters exhibit similar expression profiles. The stability of the new candidate UCOE vectors and controls was evaluated in both undifferentiated and differentiated P19 and F9 cells (representing neuroectodermal and endodermal lineages, respectively). This study extends previous work by examining not only undifferentiated P19 cells but also differentiated cells, which have been less frequently studied in prior research (16,24).

The results showed that while both SETD3-CCNK and HNRPA2B1-RPS11 elements contributed to maintaining expression from the SFFV promoter, they were less effective than the positive control UCOE vector in preventing gene inactivation. Specifically, expression from the RPS-B1-SEW LV vector decreased the most rapidly, indicating less stability. In contrast, the positive control UCOE vector provided excellent, stable expression not only in undifferentiated P19 and F9 cells but also in differentiated neuroectodermal and endodermal lineages. These results confirm the robustness of the 1.5A2UCOE-SEW vector in preserving stable transgene expression during both cell types' differentiation, which aligns with findings from Zhang, Frost et al. (16).

The underlying mechanism enabling the A2UCOE to ensure both consistent and stable gene expression, independent of the transgene's integration site, involves two key elements: a prolonged CpG-free region resistant to methylation, alongside the inherent chromatin-opening properties of the HNRPA2B1 and CBX3 promoters (25,26). Consequently, the goal was to discover a compact yet fully active A2UCOE variant suitable for integration into lentiviral vectors, thus allowing for greater capacity to accommodate therapeutic genes. Previous studies have shown that positioning the core 1.5 kb or 1.2 kb A2UCOE sequence upstream of various heterologous promoters enhances expression stability in an orientation-dependent fashion. Specifically, this stability is achieved when the CBX3 end of the A2UCOE is adjacent to heterologous ubiquitous promoters such as SFFV (16) and EF1α (25), or the tissue-specific MRP8 promoter (27).

The orientational dependence observed in A2UCOE functionality has been attributed to the comparatively lower transcriptional strength of the CBX3 promoter relative to HNRPA2B1 (28). Consequently, the stronger activity of the HNRPA2B1 promoter in a divergent transcriptional arrangement is thought to create a more effective barrier, preventing the spread of repressive epigenetic modifications such as DNA methylation and histone changes to the transgene region, thereby maintaining gene expression (29). To evaluate this theory, our initial experimental series was designed with the goal of identifying UCOEs that possess a bidirectional transcriptional architecture and can operate effectively in either orientation when linked to heterologous promoters. We hypothesized that directional preference issues in UCOE-heterologous promoter combinations might be overcome if both promoters within selected gene pairs exhibited comparable expression levels and variability across different tissues.

In conclusion, while the new candidate UCOEs demonstrated some protective ability, they did not perform as well as the original A2UCOE, particularly in preventing silencing. Therefore, further investigation will focus on dissecting the 1.5A2UCOE structure to identify specific subregions critical to its function. These insights will guide future developments of more effective UCOE elements for gene therapy applications, aiming to enhance the stability and reproducibility of transgene expression.

CONCLUSION

This study supports the dual-component hypothesis of A2UCOE function, highlighting the key role of CpG-rich, bidirectional promoter architecture in maintaining stable transgene expression. Both the natural SETD3–CCNK and synthetic RPS11–HNRPA2B1 pairs exhibited partial UCOE-like activity, providing orientation-independent resistance to silencing in undifferentiated and differentiated P19 and F9 cells. However, the canonical 1.5A2UCOE element performed superiorly, indicating additional sequence-specific features are required for full potency. The HNRPA2B1–CBX3 promoter asymmetry likely enhances chromatin opening and transcriptional stability. These findings validate simplified UCOE-like elements and guide future research toward identifying minimal subregions for optimized, reliable therapeutic gene expression.

Ethical approval

In this study, ethical approval is not required.

Author contribution

Surgical and Medical Practices: ÖFA; Concept: ÖFA; Design: ÖFA, AOA; Data Collection or Processing: ÖFA; Analysis or Interpretation: AOA; Literature Search: ÖFA, AOA; Writing: ÖFA, AOA. All authors reviewed the results and approved the final version of the article.

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Conflict of interest

The authors declare that there is no conflict of interest.

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