

Daedalus fragment from the CBX3 ubiquitous chromatin opening element as an optimum UCOE candidate

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ABSTRACT

Aim: The A2UCOE, a well-characterized chromatin-modifying element originating from a constitutively expressed human gene locus, promotes consistent and durable gene activity, even when foreign DNA sequences are inserted into densely packed heterochromatin regions. The aim of this study is to examine the Daedalus fragment in A2UCOE to identify key sub-regions responsible for its prominent chromatin opening activity. This would enable the discovery of a minimal yet fully functional element, allowing for more efficient utilization of vector capacity to accommodate therapeutic transgenes when incorporated into lentiviral vectors (LVs).

Method: To assess the chromatin-opening activity of the Daedalus sub-region within the A2UCOE, lentiviral vectors carrying eGFP reporter constructs were generated and transduced into murine embryonal carcinoma cell lines (P19 and F9). Transgene expression stability was assessed across both pluripotent cells and those differentiated with retinoic acid. HEK293T cells were used for lentiviral packaging. Plasmid propagation was performed in *E. coli* DH5α, and standard molecular cloning techniques were applied. Flow cytometry was used to measure eGFP expression levels, and significance was assessed via Student's t-test ($p < 0.05$).

Results: Flow cytometry and RT-qPCR analyses revealed that the positive control vector (1.5A2UCOE-SEW) maintained stable eGFP expression in both differentiated states of P19 and F9 cells, whereas the UCOE vectors named Daedalus-F and Daedalus-R were unable to prevent transgene silencing. In undifferentiated cultures, eGFP expression from these vectors decreased by 40–50% within two weeks, with a similar decline observed in differentiated cells. The average vector copy numbers remained stable, indicating transcriptional silencing rather than vector loss.

Conclusion: The Daedalus sub-region of A2UCOE alone could not prevent transgene silencing in pluripotent or differentiated cells, indicating additional A2UCOE elements are needed for stable long-term expression.

Keywords: chromatin structure modulation, daedalus vector system, lentiviral gene delivery, neuroectodermal and endodermal differentiation, transcriptional silencing, ubiquitous chromatin opening element

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INTRODUCTION

Gene therapy involves introducing therapeutic nucleic acids into living cells to correct genetic mutations or to alter cellular functions for medical treatment (1). These nucleic acids can be delivered using either viral or non-viral vectors. While non-viral systems, such as DNA-drug complexes like liposomes, are used, viral vectors remain the more efficient option due to their natural infection mechanisms (2,3). Advances in the field have led to the development of recombinant viral systems that are now widely applied in both research and clinical settings (4,5).

Lentiviral vectors (LVs), derived from HIV-1, are a prominent type of RNA-based viral vectors. Their capacity to effectively deliver genetic material into both proliferating and quiescent cells renders them ideal for targeting a range of tissues, including the brain and stem cells (6). Additionally, LVs have a relatively large carrying capacity (~8 kb) and can be modified to target specific cell types by changing the viral envelope glycoproteins (7). Despite their advantages, recombinant viral vectors pose certain biosafety concerns, such as the risk of insertional mutagenesis or activation of replication-competent viruses, especially in the context of HIV-1. To address this, self-inactivating (SIN) lentiviral vectors have been designed, reducing the likelihood of transcriptional interference and off-target gene activation (8). One major challenge in gene therapy is the epigenetic silencing of integrated vectors, often caused by methylation of CpG islands and chromatin condensation (9,10). Such silencing impedes stable transgene expression, particularly in retroviral vectors (11,12). To mitigate this, researchers have incorporated elements known as ubiquitous chromatin opening elements (UCOE) into vectors. UCOEs help resist silencing by maintaining an open chromatin structure and, ensuring sustained gene expression (13,14).

A widely studied UCOE is derived from the HNRPA2B1-CBX3 locus, which includes a methylation-resistant CpG island with dual divergent promoters. This element, known as A2UCOE, has been shown to support stable gene expression even in heterochromatic regions (13,15). It functions via two main components: an unmethylated CpG island and

bidirectional transcription from the native promoters. A2UCOE's performance in lentiviral contexts, including in hematopoietic stem cells, highlights its utility in gene therapy (16).

Currently, researchers are exploring whether shorter sub-regions of A2UCOE can preserve its anti-silencing capabilities while providing more space for therapeutic genes within lentiviral vectors. The aim was to discover compact and effective UCOE variants for future applications in stable gene therapy.

The primary goal of this study was to enhance the stability of transgene expression while minimizing gene silencing during lentiviral gene therapy. Lentiviral vectors are particularly advantageous because they can efficiently transduce both dividing and non-dividing cells, making them suitable for targeting complex tissues such as the liver and brain. To combat epigenetic silencing, elements like UCOEs—especially A2UCOE—are incorporated into these vectors to preserve an open chromatin configuration. Ongoing research is investigating novel CpG-rich segments and smaller A2UCOE fragments that may improve functionality while optimizing the vector capacity. These advancements have the potential to make gene delivery safer and more effective.

MATERIALS AND METHODS

This study aimed to determine whether a specific sub-region of the A2UCOE, known as the 'Daedalus fragment', retains its chromatin-opening functionality. This was based on emerging questions regarding whether UCOE efficacy depends on intrinsic promoter features. The exact genomic locations of the tested sub-fragments are illustrated in Figures 1 and 2.

To assess the activity of the vector constructs, embryonic carcinoma cell lines originating from mice, namely P19 and F9, were used. These cell lines are well-established models for evaluating the expression stability of viral vectors (15,16). To evaluate the reliability and strength of expression mediated by the various vector designs, eGFP production was tracked in cells maintained in both their pluripotent state and following differentiation.

Luria-bertani (LB) medium preparation

To prepare Luria-Bertani (LB) medium, 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl were dissolved in one liter of deionized water. The solution was autoclaved at 121°C (15 psi) for 20 minutes to ensure sterility. Once cooled to approximately 55°C, ampicillin was incorporated to a final concentration of 50 µg/mL. The medium was subsequently stored at 4°C until needed. For solid media, 20 g of agar was added per liter of LB prior to sterilization. Selective LB broth or agar was prepared by supplementing the medium with ampicillin at a concentration of 100 µg/mL.

Competent E. coli DH5α Transformation

Chemically competent DH5α E. coli cells, obtained from Life Technologies, were transformed following the standard procedure provided by the supplier. Plasmid-containing colonies were cultured on ampicillin plates (100 µg/mL) overnight at 37°C and then grown in LB medium with ampicillin (100 µg/mL) in small (5 mL) or large (200–500 mL) cultures. Plasmids were extracted using Qiagen kits and eluted in TE buffer.

Cell culture

HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), and penicillin-streptomycin (10 µg/mL each) at 37°C in a humidified incubator containing 5% CO₂. Cell cultures were initiated by plating 2×10^7 cells into T162 flasks and maintained until they reached approximately 80–90% confluence prior to transfection. The culture supernatant was collected 48 hours after transfection, the medium was refreshed with DMEM, and a second harvest was performed at the 72-hour mark. Culture medium was collected 48 hours post-transfection, replaced with fresh DMEM, and harvested again at 72 hours. For viral titration, $1\text{--}2 \times 10^5$ cells were seeded in the wells of a 24-well culture plate, followed by exposure to gradually diluted viral suspensions, resulting in a multiplicity of infection (MOI) ranging from 1 to 10^{-5} per µL. Detached cells were treated with PBS and Tryple Red reagent, then neutralized with serum-based medium. For flow cytometry, cells were fixed with 4% formaldehyde-PBS, shaken, and kept in darkness at 4°C until they were ready for analysis.

Mouse embryonic carcinoma cells culture

P19 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 2 millimolar L-glutamine and supplemented with 1% non-essential amino acids. The culture conditions included the addition of 10 µg/mL penicillin-streptomycin, and incubation at 37°C within a 5% CO₂ atmosphere. Neuronal differentiation was initiated by forming embryoid bodies in non-adherent dishes containing DMEM supplemented with 5% FBS and retinoic acid at a concentration of 1 micromolar. The culture of F9 cells was carried out in flasks coated with gelatin, employing DMEM enriched with 10% FBS, alongside antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL), plus 2 mM L-glutamine. The formation of embryoid bodies in DMEM/F12 supplemented with 5% FBS and 50 nM RA triggered the cells to differentiate toward a parietal endoderm lineage.

Reporter gene analysis

The UCOE and A2UCOE eGFP reporter constructs within the LV system were assessed by transducing P19 and F9 cells. The stability of gene expression was then monitored both prior to and following differentiation into neuroectodermal and endodermal lineages.

At least 2×10^5 fixed cells were analyzed for GFP expression using flow cytometry (BD FACSymphony™). During analysis, only viable cells were included by gating based on cell size and granularity, determined respectively through forward scatter and side scatter measurements. GFP+ cells were identified by detecting fluorescence emission at 525 nm (FL1 channel) compared to 575 nm (FL2 channel). Untransduced cells served as negative controls to set the gating threshold for positive GFP signals. The resulting percentage of GFP-positive cells represents the fraction of viable cells within the sample that expressed GFP. Subsequently, the proportion of GFP-positive cells for each sample was analyzed in relation to the amount of virus administered. By scaling this relationship, the quantity of cells infected with a specified volume of viral preparation could be estimated, allowing for the calculation of the multiplicity of infection (MOI) based on mean fluorescence intensity. For this calculation, values ranging from 1% to 20% were selected. Samples

exhibiting more than 20% GFP-positive cells likely contained multiple integration events per cell, which could lead to an underestimation of the viral titer. Conversely, samples with less than 1% GFP expression were considered unreliable, as detectable signals at such low levels are prone to interference from background noise.

Explanation of the chemicals and devices used in the study: Thermo Fisher Scientific, Gibco, Qiagen, Sigmaaldrich (MERK), Bio-Rad, BD FACSymphony

Statistical analyses

Statistical evaluations were conducted using Prism version 7 software. To assess the eGFP-positive populations from flow cytometry, as well as mean fluorescence intensity (MFI) and vector copy number (VCN) measurements in transduced cell cultures, Student’s t-test was employed. Results with p-values below 0.05 were considered statistically significant.

In our cell culture study, the cell line used was obtained commercially. Therefore, ethical approval was not required for our study.

Lentiviral vectors used in this study

The experimental approach aimed at testing the UCOE two-component model involved the use of 750bp length Daedalus fragments. To test their effectiveness in preventing suppression of gene activity, these sequences were inserted in both forward and reverse directions into the silencing-prone SFFV-eGFP vector. Approximate locations and plasmid schematics for the Daedalus fragments derived from A2UCOE sub-regions examined in this study are presented in Figures 1 and 2.

RESULTS

Evaluation of the functional properties of Daedalus-F and Daedalus-R lentiviral vectors containing candidate UCOEs in undifferentiated P19 and F9 cell lines

The generated lentiviral vectors (LVs) were utilized to transduce P19 and F9 cells at infection ratios (MOI) of 3 and 6, aiming for an initial eGFP+ cell proportion between 40 and 60% across all pools. Cells

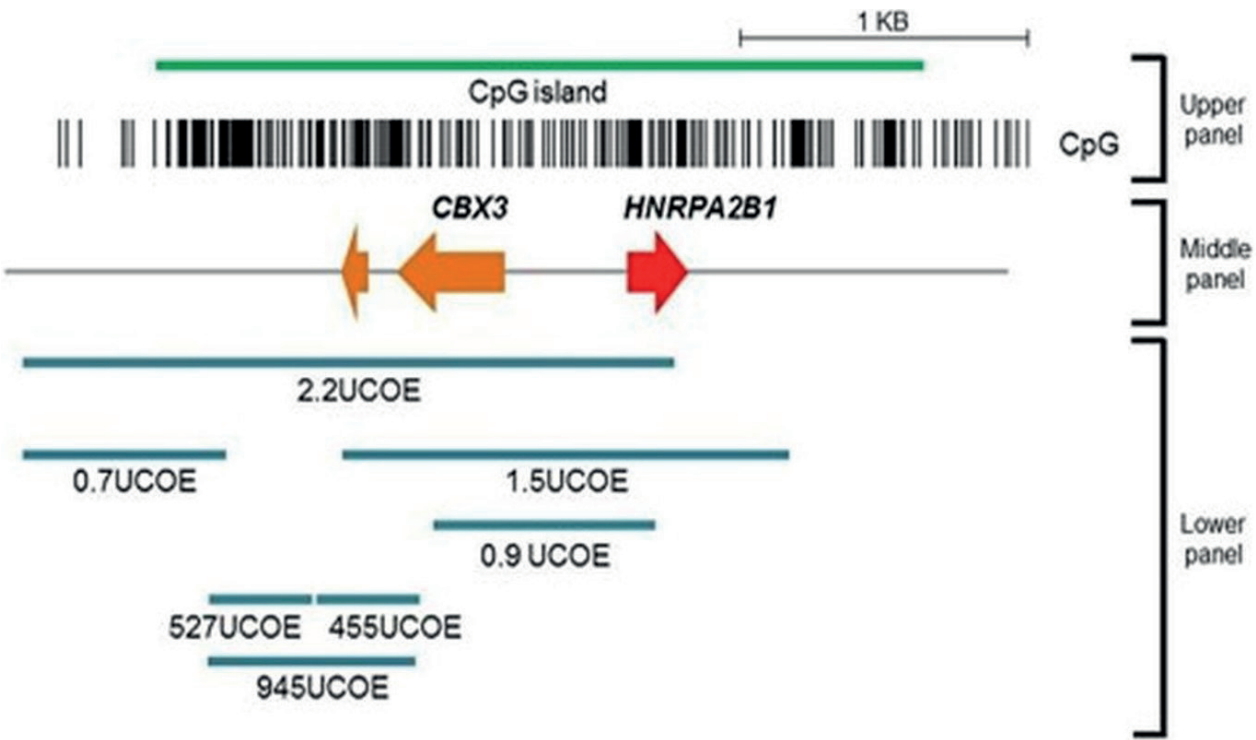


Figure 1. The positions of A2UCOE region sub-fragments analyzed for UCOE activity.

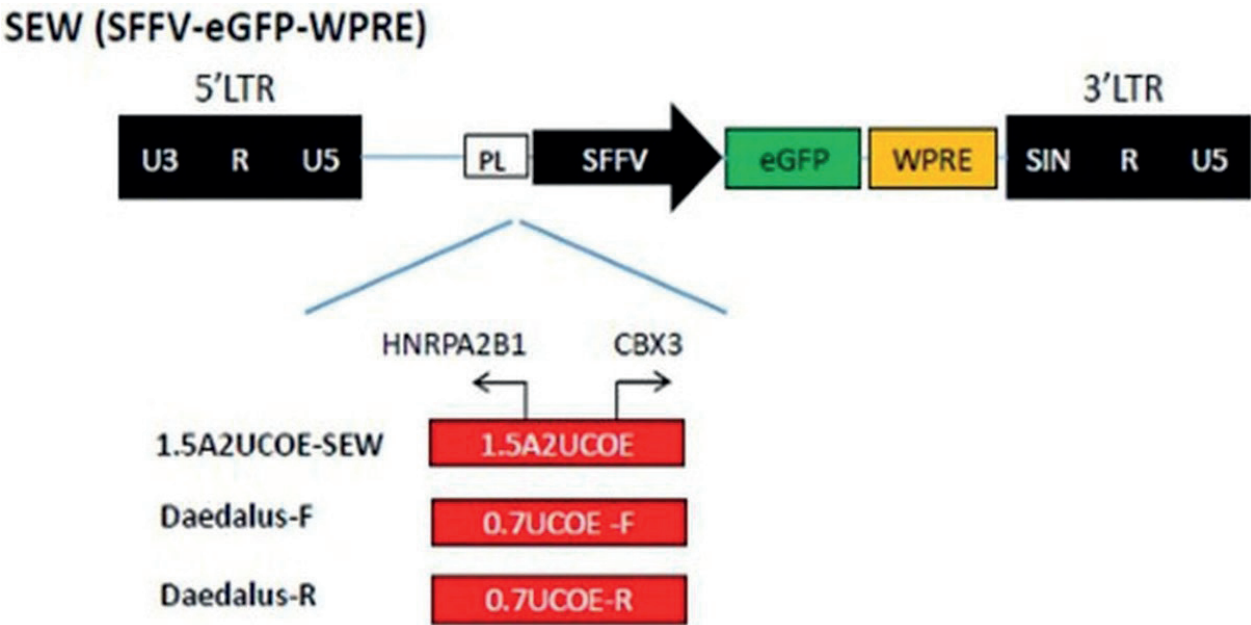


Figure 2. Illustration of novel candidate UCOE and control lentiviral vectors.

that underwent transduction, along with negative controls, were maintained in culture and periodically assessed using flow cytometric analysis to quantify the percentage of cells expressing eGFP, as well as their mean fluorescence intensity during ongoing culture following a single vector transduction. The first three days after transduction, cell samples were collected once a week up to 45 days (see Figure 3) to analyze eGFP expression via flow cytometry. At designated time points, DNA was isolated for RT-qPCR to quantify the average number of vector copies present in each cell. Flow cytometry analysis results in Figure 4A illustrate the proportion of eGFP+ cells. At the start, all vectors demonstrated comparable transduction efficiencies, ranging between 45% and 60% eGFP+ cells; however, the expression driven by SEW (SFFV-eGFPWPRE) sharply decreased from 46% to just 3% of positive cells over a period of 17 days. Conversely, the percentage of eGFP+ cells within the 1.5A2UCOE-SEW group showed consistent stability throughout the 45-day cultivation period. Therefore, while the proportion of eGFP+ cells was notably greater in 1.5A2UCOE-SEW transduced samples compared to alternative vectors, the reduction in expression observed in both Daedalus-F and Daedalus-R mirrored the pattern seen with SEW. SEW transduced cells saw an almost 80%

reduction in eGFP expression after two weeks. Except for 1.5A2UCOE-SEW, the remaining vectors, including both Daedalus-F and Daedalus-R, demonstrated a 40–50% decline in eGFP+ cell populations within the corresponding time interval. The MFI across all vectors reflected the percentage of eGFP+ cells (Figure 4B). MFI remained stable in 1.5A2UCOE-SEW transduced cells but fluctuated in SEW and the new Daedalus-F and Daedalus-R constructs.

Figure 5A presents the flow cytometry analysis showing the proportion of eGFP-expressing cells measured at successive time points following transduction in undifferentiated F9 cells. These findings are consistent with those observed in P19 cells (Figure 4A). Initial transduction efficiencies were comparable across all vectors, ranging from 39% to 50% eGFP+ cells. However, expression driven by the SEW vector declined sharply, dropping from 47% to just 7% within 17 days. Conversely, cells genetically transduced with the 1.5A2UCOE-SEW vector sustained a stable eGFP-positive population of approximately 45% throughout the 38-day culture period. The candidate UCOE constructs, Daedalus-F and Daedalus-R, showed a reduction in gene activity comparable to the decrease observed with the SEW vector. Specifically, eGFP

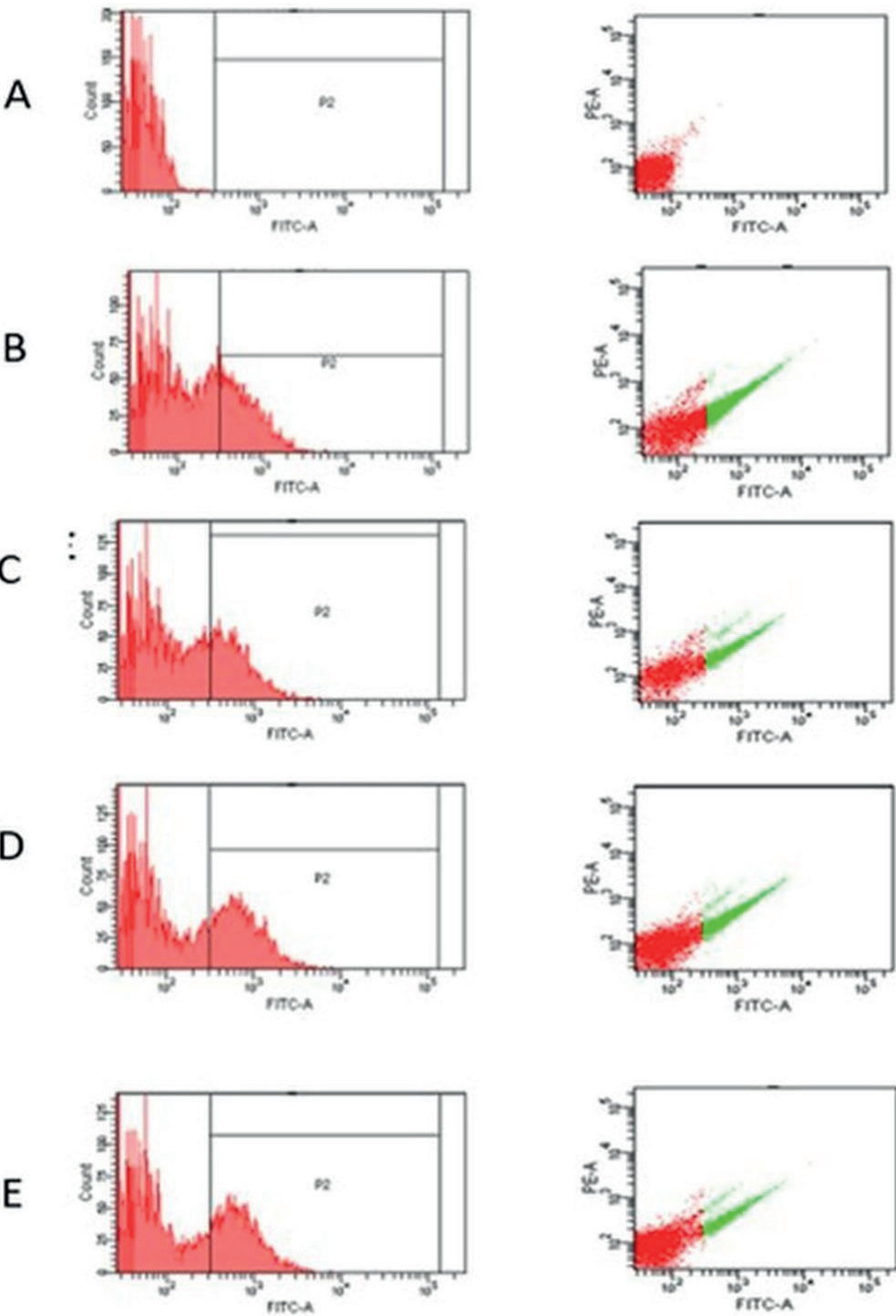


Figure 3. Flow cytometry plots of untransduced and transduced P19 and F9 cells prior to differentiation.*

* Flow cytometry analysis was performed 3 days post-transduction, showing GFP-negative cells in red and GFP-positive cells in green. (A) Negative control—untransduced cells. (B) SEW transduced cells. (C) 1.5A2UCOE-SEW transduced cells. (D) Daedalus-F transduced cells. (E) Daedalus-R transduced cells.

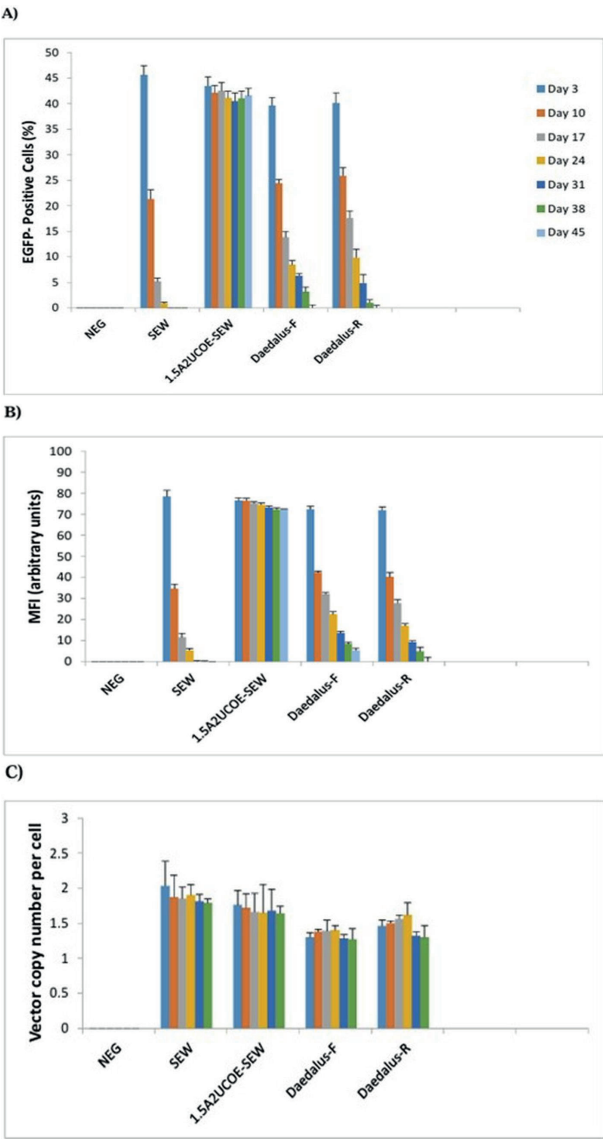


Figure 4. Novel candidate Daedalus-F and Daedalus-R UCOEs offer only partial protection against silencing in undifferentiated P19 cells. *

* Cells were analyzed by flow cytometry to detect the percentage of eGFP reporter gene expressing (eGFP+) cells, mean fluorescence intensity (MFI) and by RT-qPCR for average VCN per cell. The data show combined results from three independent transductions for each vector, plus negative control (NEG), over a period of 3 to 45 days post transduction. (A) Time course of percentage eGFP positive cells; (Mean + SEM, n=4; **p<0.01). (B) As in (A) but showing MFI; (Mean + SEM, n=4; **p<0.01). (C) As in (A)/(B) but average VCN/cell; (Mean + SEM, n=4; **p<0.01).

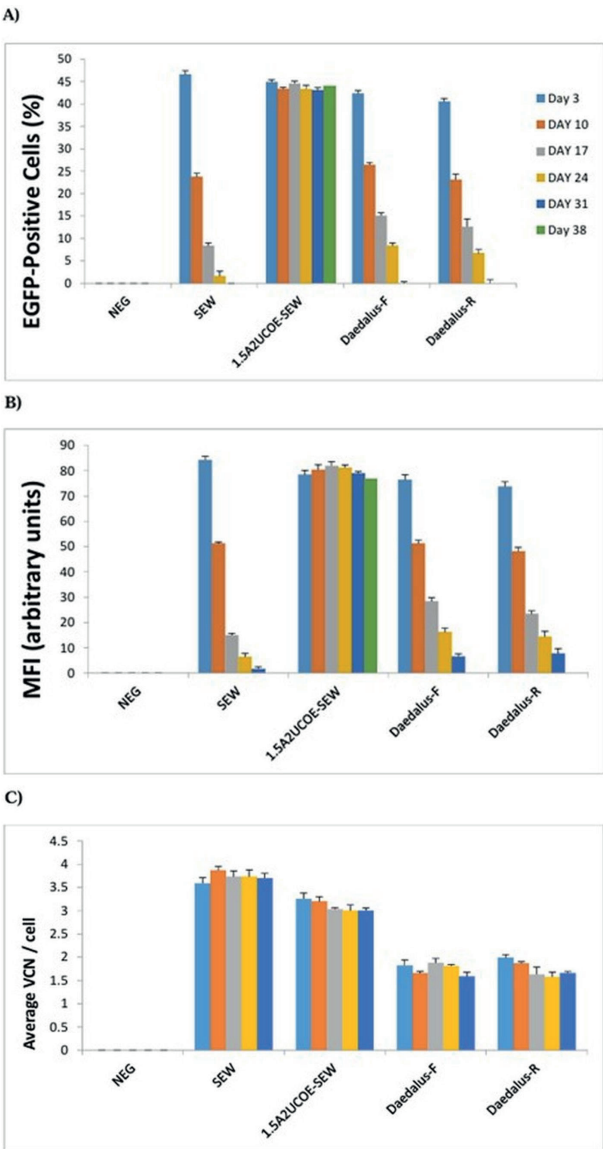


Figure 5. Novel candidate UCOE Daedalus-F and Daedalus-R offer only partial protection against silencing in undifferentiated F9 cells. *

* Cells were analyzed by flow cytometry to detect the percentage of eGFP reporter gene expressing (eGFP+) cells and MFI, and to quantify the average VCN per cell by real-time qPCR. The data show combined results from three independent transductions for each vector, plus negative control (NEG), over a period of 3 to 38 days post-transduction. (A) Time course of percentage eGFP positive cells; (Mean + SEM, n=4; **p<0.01). (B) As in (A) but showing MFI; (Mean + SEM, n=4; **p<0.01). (C) As in (A)/(B) but average VCN/cell; (Mean + SEM, n=4; **p<0.01).

levels in cells treated with SEW dropped by nearly 80% fourteen days after gene delivery. Apart from the 1.5A2UCOE-SEW group, the reduction in eGFP+ cells for Daedalus-F and Daedalus-R vectors ranged between 40% and 50% over the same timeframe. The MFI measurements across all vectors reflected the trends seen in eGFP expression (Figure 5B), showing stability in cells transduced with 1.5A2UCOE-SEW, instability in those with SEW alone, and partial stability in cells treated with the new UCOE vectors. Throughout the entire culture duration, the mean number of vector copies per individual cell in the P19 (Figure 4C) and F9 (Figure 5C) transduction experiments remained stable, suggesting that the reduction in eGFP-positive cells observed with the SEW, Daedalus-F, and Daedalus-R vectors was due to transcriptional silencing of the integrated sequences rather than a loss of vector presence.

Assessment of the functional performance of Daedalus-F and Daedalus-R candidate UCOEs in P19 and F9 cells following differentiation

The newly developed UCOE vectors, Daedalus-F and Daedalus-R, failed to sustain consistent gene expression in undifferentiated P19 (Figure 4) and F9 (Figure 5) cell lines. Subsequently, we evaluated their effectiveness in maintaining expression stability throughout the differentiation process of P19 and F9 cells into neuroectodermal and endodermal lineages, respectively. Cells transduced with the respective lentiviral vectors were cultured in differentiation medium supplemented with retinoic acid, on non-adherent plastic surfaces to promote the formation of embryoid bodies, a critical step for their subsequent differentiation into neuronal and endodermal lineages. The cells differentiated into a mixture of neuroectodermal and parietal endoderm cell types, with fibroblast-like cells being the most abundant during the early stages—2 to 3 days for P19 cells and 5 to 6 days for F9 cells. Neurons and endoderm cells were also detected in the cultures. To enrich the population for non-dividing cells, 10 μ M cytosine arabinoside was added, which selectively eliminated dividing cells and allowed only neurons and endoderm cells to survive.

The optimal duration for embryoid body formation and differentiation into neuroectodermal and endodermal lineages was 2 to 3 days for P19 cells and 5 to 6 days for F9 cells. Following the differentiation process, Figures 6 and 7 display the flow cytometric profiles of entire cell populations derived from triplicate cultures transduced with the SEW, 1.5A2UCOE-SEW, Daedalus-F, and Daedalus-R lentiviral constructs. The data indicate that the lentiviral vector incorporating the 1.5 kb A2UCOE sequence (1.5A2UCOE-SEW), used as a positive control, maintained consistent transgene expression following the differentiation of P19 and F9 cells into neuronal and endodermal lineages, respectively (Figures 6A and 7A). In contrast, the newly developed UCOE vectors, Daedalus-F and Daedalus-R, exhibited a rapid decline in eGFP expression. The suppression pattern mirrored that of the SEW vector used as a silencing control, wherein eGFP expression was rapidly diminished, as illustrated in Figures 6A and 7A. As seen in undifferentiated cells (Figures 4B and 5B), the MFI values measured during the differentiation process of both P19 and F9 cells displayed a pattern consistent with the eGFP+ cell data (Figures 6B and 7B). The mean VCN remained consistent over the entire experimental timeline (see Figures 6C and 7C). Comparable results were observed in both pluripotent and differentiated forms of F9 and P19 cells following transduction with the lentiviral constructs employed in this study. It is noteworthy that differentiating cells exhibited a greater loss of eGFP expression after being transduced with lentiviral particles carrying SEW and newly characterized UCOE elements, as seen in Figures 6A and 7A. This decline was more pronounced compared to undifferentiated cells, illustrated in Figures 4A and 5A. Additionally, because Daedalus-F and Daedalus-R vectors failed to sustain stable expression in differentiated P19 and F9 cells, immunofluorescence analysis for neuroectodermal and endodermal markers was not performed.

Given that neither of the Daedalus constructs exhibited stable expression, we decided against performing immunofluorescence staining on the differentiated cells.

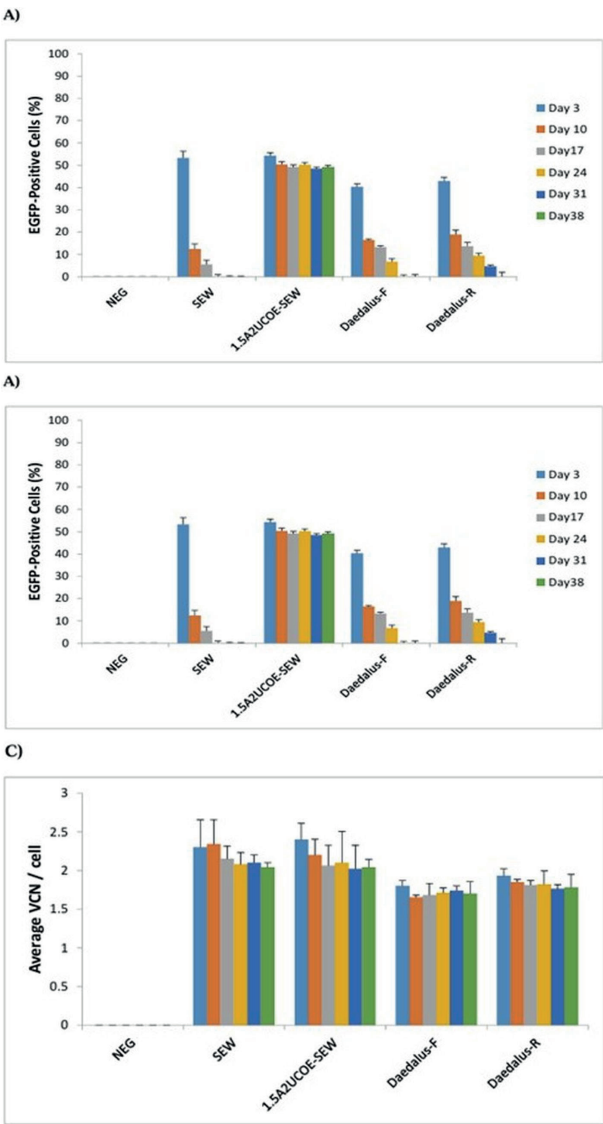


Figure 6. Novel candidate UCOEs Daedalus-F and Daedalus-R offer only partial protection against silencing in differentiated P19 cells. *

* Cells were analyzed by flow cytometry to detect the percentage of eGFP reporter gene expressing (eGFP+) cells and MFI, and to quantify the average VCN per cell by real-time qPCR. The data show combined results from three independent transductions for each vector, plus negative control (NEG), over a period of 3 to 38 days post-transduction. (A) Time course of percentage eGFP positive cells; (Mean + SEM, n=4; **p<0.01). (B) As in (A) but showing MFI; (Mean + SEM, n=4; **p<0.01). (C) As in (A)/(B) but average VCN/cell; (Mean + SEM, n=4; **p<0.01).

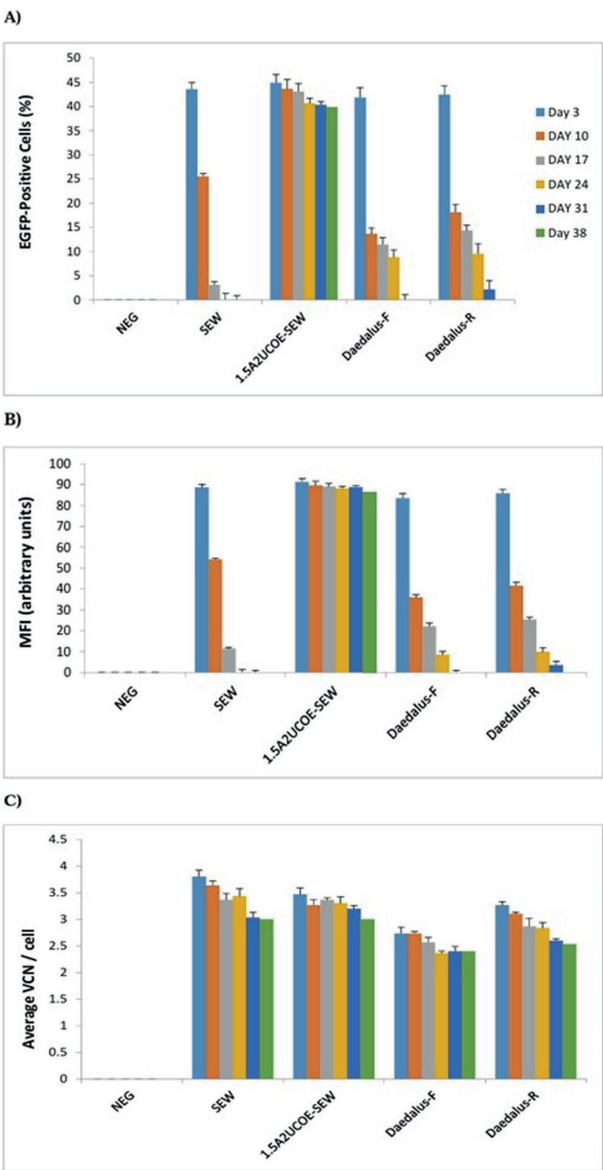


Figure 7. Novel candidate UCOEs Daedalus-F and Daedalus-R offer only partial protection against silencing in differentiated F9 cells. *

* Cells were analyzed by flow cytometry to detect the percentage of eGFP reporter gene expressing (eGFP+) cells and MFI, and to quantify the average VCN per cell by real-time qPCR. The data show combined results from three independent transductions for each vector, plus negative control (NEG), over a period of 3 to 38 days post-transduction. (A) Time course of percentage eGFP-positive cells; (Mean + SEM, n=4; **p<0.01). (B) As in (A) but showing MFI; (Mean + SEM, n=4; **p<0.01). (C) As in (A)/(B) but average VCN/cell; (Mean + SEM, n=4; **p<0.01).

DISCUSSION

The gammaretroviral and lentiviral vector classes, known for their integration capability, continue to be the most reliable tools for ensuring stable incorporation and expression of therapeutic genes. This is particularly evident in the context of stem cell subsets undergoing active proliferation. Over the course of the last fifteen years, clinical trials employing these vector systems in *ex vivo* applications targeting hematopoietic stem cells (HSCs) have yielded promising results. Genetic modification of patients' hematopoietic stem cells (HSCs) using gammaretroviral vectors has shown therapeutic success in various primary immunodeficiencies, including severe combined immunodeficiency subtypes such as SCID-X1 and SCID-ADA (17,18), as well as in disorders like Wiskott-Aldrich syndrome and chronic granulomatous disease (19,20). More recently, lentiviral vectors have demonstrated success in treating inherited demyelinating disorders, including X-ALD (21) and MLD (22), in addition to WAS (23). Despite these advancements, two critical issues continue to arise in the context of using integrating viral vectors and require ongoing attention. A major limitation is the potential for insertion-related genomic alterations; another is the suppression of therapeutic gene activity over time through epigenetic mechanisms (1). Insertional mutagenesis resulting from gammaretroviral integration, which inadvertently triggered the activation of proto-oncogenes within the host genome and contributed to malignant transformation, was detected in five patients out of a cohort of twenty undergoing treatment for SCID-X1 (17,18). Meanwhile, epigenetic silencing triggered by promoter methylation resulted in a loss of sustained therapeutic benefit in two CGD patients, despite an initially positive response (19,20).

The main goal of this study phase was to evaluate whether particular sub-regions within the A2UCOE, which originates from the human HNRPA2B1-CBX3 locus (see Figures 1 and 2), retain the characteristic UCOE activity. Earlier investigations have indicated that a 0.7 kb fragment within the first intron of CBX3 preserves UCOE functionality when fused to an exogenous CMV promoter (24), and that this segment can sustain appropriate DNA methylation patterns in the context of transgene expression (25). These

results prompt reconsideration of the mechanism by which UCOEs operate through bidirectional divergent transcription (26). To directly evaluate whether a 0.7 kb segment derived from the first intron of CBX3 (see Figure 2) possesses UCOE functionality, lentiviral constructs named Daedalus-F and Daedalus-R (Figures 1 and 2) were engineered. Our findings from experiments involving P19 and F9 cells, both before and after their differentiation into neural ectoderm and endoderm cell types, distinctly demonstrates that this sequence fails to promote expression stability when associated with an SFFV promoter (Figures 4-7). The difference observed between our findings and earlier studies likely stems from the fact that previous investigations reporting UCOE activity of the 0.7 kb intronic region within CBX3 were conducted using transductions that yielded approximately 8 to 10 lentiviral vector copies per cell (24). Under these conditions, even a single active copy of the integrated vector within a cell can generate detectable GFP reporter gene expression. This may misleadingly suggest that the transgene remains functionally stable. However, a notable reduction of approximately 55% in MFI was observed at four weeks after transduction with the 0.7UCOE-GFP ("Daedalus") lentiviral vector. This decline highlights considerable vector silencing, despite the continued presence of GFP+ cells (see Figure 4) (24). Our findings provide strong evidence that the expression of the 0.7UCOE (Daedalus) vector undergoes silencing. This is supported by experiments specifically designed to generate cells containing a low average vector copy number per cell, between 1 and 4 (Figures 4C, 7C). Furthermore, the P19 and F9 embryonal carcinoma cell lines used in our study may serve as a more sensitive and reliable model for evaluating functional gene silencing compared to the CHO cells employed in previous research (24,15,16).

CONCLUSION

In summary, our findings demonstrate that the 0.7 kb Daedalus sub-region of A2UCOE is insufficient to maintain stable transgene expression in both pluripotent and differentiated P19 and F9 cells. Despite stable vector integration, significant transcriptional silencing occurred, particularly at low vector copy numbers. These results indicate that additional regulatory sequences within A2UCOE are necessary

to sustain long-term and robust transgene expression. Identifying a minimal yet fully functional UCOE element remains crucial for optimizing lentiviral vector design and enhancing the efficiency of therapeutic transgene delivery.

Ethical approval

In this study, ethical approval is not required.

Author contribution

Surgical and Medical Practices: ÖFA; Concept: ÖFA; Design: ÖMA, 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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