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Evaluation of a central core MDR region as an optimum chromatin opening model

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ABSTRACT

Aim: To examine whether the sub-regions of the *HNRPA2B1 CBX3 UCOE* (A2UCOE) preserved UCOE functionality. This inquiry was driven by several reports that challenge the requirement of associated promoter activity for UCOE functionality.

Method: The 0.9kb central A2UCOE region, thought to form a potential MDR (methylation determining region), was placed upstream of the SFFV-eGFP cassette. These constructs were subsequently analyzed alongside the 1.5A2UCOE and 2.2A2UCOE to assess their ability to inhibit transcriptional repression.

Results: In this study, we investigated whether sub-regions of the *HNRPA2B1 CBX3 UCOE* (A2UCOE) retained UCOE activity. Lentiviral vectors were used to evaluate the stability and efficiency of eGFP expression in P19 and F9 embryonal carcinoma cells. LB medium was prepared, and recombinant plasmids were transformed into competent E. coli DH5α cells. HEK293T cells were cultured for lentiviral production and transduction experiments were performed. eGFP expression was analyzed by flow cytometry before and after differentiation of P19 and F9 cells. Statistical analysis was performed using Prism, with $p < 0.05$ considered significant.

Conclusions: This study demonstrates that the 0.9 kb core region of A2UCOE, containing the promoters of *HNRPA2B1* and *CBX3*, exhibits partial resistance to transgene silencing in both undifferentiated and differentiated P19 and F9 cells. The findings indicate that the size of the CpG-rich region is critical for maintaining open chromatin structure and ensuring full UCOE functionality. Our study highlights the potential of smaller A2UCOE subregions for gene therapy; however, further optimization is required to achieve full activity independent of promoter influence.

Keywords: UCOE, transgene silencing, lentiviral vectors, gene therapy.

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1. Introduction

Gene therapy aims to correct genetic mutations or modify cellular genetic processes by delivering therapeutic nucleic acids into living cells to treat genetic disorders. This methodology can be applied across a wide range of clinical pathologies [1]. Gene therapy vectors are primarily classified into two distinct modalities: viral-based and non-viral delivery systems. While viral vectors harness the innate ability of viruses to infiltrate cellular structures, non-viral approaches employ nucleic acid-pharmaceutical conjugates, including lipid-based nanoparticles such as liposomes [2,3]. Recent advancements in genetics have enabled the identification of viruses that are beneficial for gene therapy, leading to the increased application of

recombinant viral systems in both experimental and clinical settings [4,5].

Lentiviral vectors (LVs) and derived from HIV-1 are widely used RNA-based viral vectors. Their capacity to infiltrate both proliferating and quiescent cell populations renders them exceptionally advantageous for targeting specific tissues, including the central nervous system, hepatic structures, and stem cells with limited mitotic activity [6]. Lentiviral vectors possess a relatively large genomic capacity (~9 kb) and can be engineered to target specific cell types through pseudotyping with various viral glycoproteins [7]. However, recombinant viral vectors pose certain risks, including viral recombination or unintended integration into the host genome. This issue is particularly concerning in relation to wild-type HIV-1, as it may lead to uncontrolled gene expression or the activation of replication-competent viruses. To mitigate these risks, self-inactivating (SIN) lentiviral vectors have been developed, designed to reduce transcriptional disruption and mutagenesis [8].

Gene silencing remains a significant challenge in gene therapy. For instance, retroviral vectors frequently undergo transcriptional silencing following their integration into host cells [9,10]. This phenomenon occurs due to the methylation of CpG islands and histone modifications, which contribute to chromatin condensation, ultimately preventing stable transgene expression [11,12]. These challenges highlight the necessity for vectors that can prevent gene silencing and ensure consistent therapeutic protein production. To overcome this challenge, researchers have integrated universally active chromatin opening elements (UCOEs) into expression vectors. UCOEs prevent silencing by heterochromatin effect, ensuring stable gene expression in mammalian cells [13,14]. A prominent UCOE model involves the human HNRPA2B1-CBX3 locus (A2UCOE). This locus contains an unmethylated CpG island and encompasses two promoters with chromatin-opening properties. This configuration protects transgenes from silencing, even in heterochromatic regions such as centromeres [13,15].

The A2UCOE model operates with two key components: [1] an unmethylated CpG island and [2] bidirectional transcription from the HNRPA2B1 and CBX3 promoters. This system has exhibited reliable stability and reproducibility in both laboratory and organismal contexts, including murine hematopoietic stem cells (HSCs), when applied in LV-based vectors [16]. Notably, the A2UCOE enhances transgene expression by preventing silencing induced by DNA methylation.

Currently, the A2UCOE model is being evaluated with additional CpG islands and dual transcription promoters to identify new UCOE (Ubiquitous Chromatin Opening Element) candidates that could enhance gene therapy applications. As a result, the integration of UCOEs like A2UCOE into lentiviral vectors holds potential for improving the stability and efficacy of gene therapies by reducing silencing. Further research on the A2UCOE subregion fragments could pave the way for more effective and safer treatments by optimizing the therapeutic gene region in lentiviral vectors. A study by Lienert et al. revealed that specific subdomains within CpG islands, linked to gene promoters under developmental regulation, preserved precise DNA methylation patterns even when integrated into a transgenic construct [17]. Some of these subregions were absent from the promoter regions and were classified as methylation determining regions (MDRs). A computational genomics investigation pinpointed a segment within the A2UCOE constitutive gene locus containing distinct transcription factor interaction motifs, including

Sp1, CTCF, and USF. This analysis identified a central domain that could function as a multidomain regulatory element (MDR), encompassing the initial exons of both CBX3 and HNRPA2B1 and housing the associated promoters of both genes. To assess this hypothesis, two distinct configurations were designed. In these configurations, a 0.9 kilobase core segment of the A2UCOE, encompassing the putative MDR, was placed before the SFFV promoter in the context of the SEW lentiviral vector framework. This configuration aimed to evaluate the potential for expression stabilization (Figure 1; 0.9UCOE-F and 0.9UCOE-R).

The activity of UCOE in these vectors was assessed by introducing murine embryonal carcinoma cell lines, namely P19 and F9, which are widely recognized as reliable models for studying the stability of gene expression mediated by viral vectors [15,16). Subsequently, the consistency, effectiveness, and the intensity of enhanced green fluorescent protein (eGFP) expression was meticulously examined in the P19 and F9 cellular lineages, considering both their differentiated and undifferentiated conditions.

The aim of our study is to address the challenges in gene therapy applications by examining whether the HNRPA2B1 CBX3 UCOE (A2UCOE) subregions maintain the functionality of UCOEs. Additionally, we seek to identify smaller, functional UCOE units to overcome the limitations related to the carrying capacity of vectors used in gene transfer. Gene therapy aims to treat genetic diseases by transferring therapeutic nucleic acids into living cells to correct genetic mutations or alter cellular genetic processes [18].

The use of viral and non-viral vectors, especially RNA-based lentiviral vectors, has increased, playing a key role in genetic engineering and therapy. UCOEs are essential for stabilizing transgene expression. Integrating UCOEs into vectors is a common strategy to prevent gene silencing and ensure continuous therapeutic protein production. The A2UCOE model, containing the human HNRPA2B1-CBX3 locus, effectively maintains transgene expression sustainability by preventing silencing in heterochromatic regions through unmethylated CpG islands and bidirectional transcription [19,20].

In our study, examining the functionality of A2UCOE subregions is a critical step in developing more effective and safe treatment options in gene therapy applications. The subregions of this model aim to identify new UCOE candidates that could enhance the stability and efficiency of gene therapy vectors. Research by Lienert and colleagues has demonstrated that specific subregions within CpG islands associated with gene promoters remain functional when integrated into transgenic constructs, preserving DNA methylation. These findings have provided the foundation for experiments evaluating the effectiveness of A2UCOE subregions in our study. The analysis of A2UCOE subregions may provide smaller functional UCOE units that enhance vector carrying capacity, stabilize transgene expression, and prevent gene silencing, which could contribute to gene therapy strategies [21].

Random integration of transgenes into heterochromatic regions and the methylation of promoter DNA are critical factors that hinder gene expression, resulting in varied patterns of gene activation or repression [22,23]. Stable and high transgene expression is essential for clonal cell line production in biomanufacturing and therapeutic transgene expression in gene therapy. Thus, genetic regulators are needed to prevent gene silencing and maintain high expression levels. These elements can be classified into two

groups: those working with active chromatin remodeling mechanisms and those limiting the spread of heterochromatin marks to euchromatic regions.

Insulators, scaffold/matrix attachment regions (S/MARs), and balancing anti-silencing (STAR) elements are included, while the former encompasses locus control regions (LCRs) and widespread chromatin opening elements (UCOEs). LCRs and UCOEs are defined by their ability to ensure stable transgene expression that is independent of the integration site and proportional to the transgene copy number, even when integrated into heterochromatin [24,25]. LCRs, or locus control regions, are specialized regulatory elements that exhibit tissue-specific activity. They consist of several modular components, which are marked by their sensitivity to DNase I digestion and the presence of numerous transcription factor binding clusters [16,27]. In contrast, UCOEs function ubiquitously and do not consist of multiple DNase I hypersensitive regions, a characteristic of LCRs, and do not require surrounding a transgene at both the 5' and 3' ends for function, as is the case with insulators and S/MARs [28,29]. UCOEs represent a distinct class of genetic regulatory elements both structurally and functionally. They are commonly used in protein therapeutic biomanufacturing applications to accelerate the production of high-expressing recombinant cell clones while managing costs and resources. Furthermore, UCOEs hold significant potential in gene therapy by providing stable expression in somatic tissues and various stem cell types.

In our study, we examined the potential of the A2UCOE model to enhance the effectiveness of gene therapy strategies, with a particular focus on the functionality of various fragments within the A2UCOE subregions. Further optimization of these regions aims to increase the effectiveness of UCOEs in gene therapy applications. The use of additional CpG islands and bidirectional promoters to stabilize gene expression in the A2UCOE region may enhance gene therapy stability by counteracting the effects of DNA methylation. Certain subregions of the A2UCOE model can interact with transcription factors to create a strong regulatory effect. A more detailed investigation of these regions could further strengthen transgene expression within the A2UCOE model.

2. Materials and methods

In this study, the functionality of the sub-segments of the HNRPA2B1-CBX3 UCOE (A2UCOE) region was examined to determine whether they maintain UCOE activity. This exploration was driven by insights obtained from numerous studies examining the critical role of promoter activity. These studies underscore the necessity of promoter functionality in enabling the effective operation of ubiquitous chromatin-opening elements. The locations of the A2UCOE sub-segments analyzed are indicated in Figures 1 and 2. The UCOE activity of the vectors was tested through the transduction of P19 and F9 cell lines, which are our standard model used to assess the stability of viral vector expression [15,16]. Following this, the robustness, efficacy, and consistency of eGFP expression were evaluated across both differentiated and pluripotent cellular states.

To determine if the decrease in eGFP expression was due to the loss of vector-positive cells or lentiviral gene silencing, total genomic DNA was analyzed by real-time PCR. This measured vector genome copies per cell at various time points post-transduction. A monoclonal population with a single A2UCOE-eGFP integration, derived from transduced P19 and F9 cells via limiting dilution, served as a qPCR calibration standard for VCN

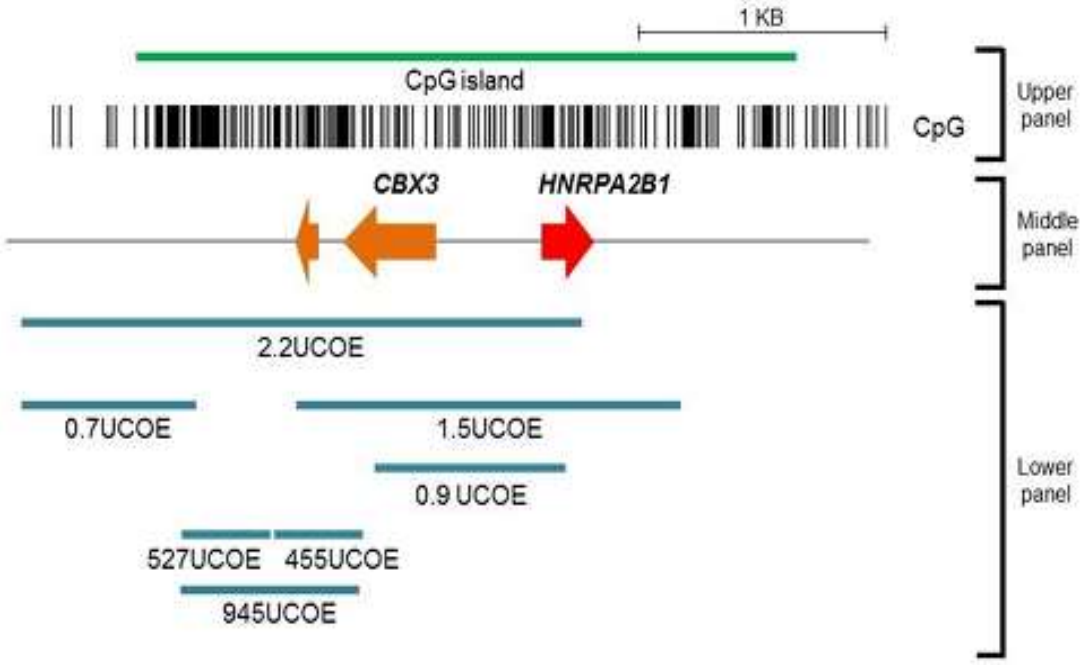


Figure 1. Positions of the subregions of the A2UCOE analyzed for UCOE activity.

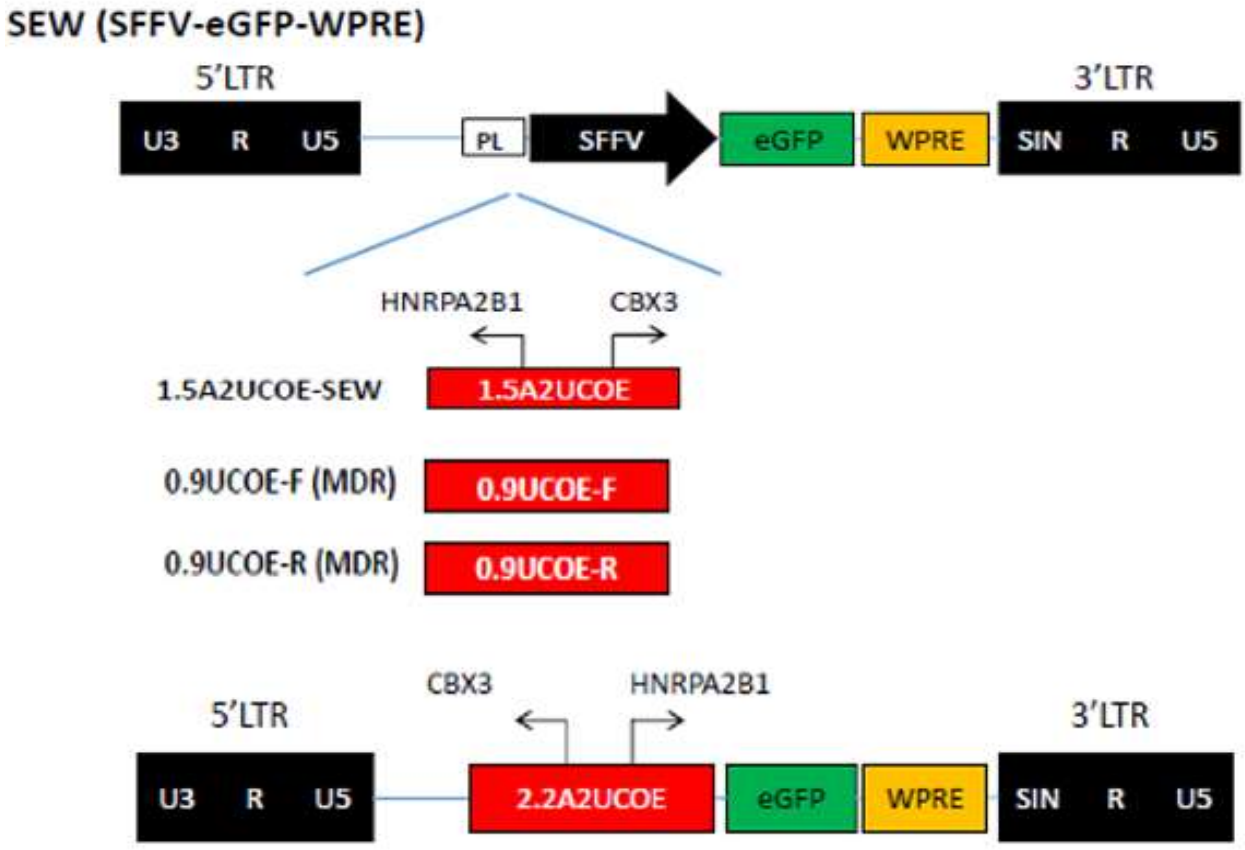


Figure 2. Depiction of the novel candidate ubiquitously acting chromatin opening element (UCOE) alongside the regulatory lentiviral vector constructs.

quantification. At least 2x10^5 fixed cells were analyzed for GFP fluorescence via flow cytometry. Viable cells were gated using size (forward scatter) and granularity (side scatter). GFP emission was measured at 525 nm (FL1) and 575 nm (FL2). Non-transduced controls set GFP positivity thresholds, enabling quantification of viable GFP+ cells. This was correlated with viral concentration to estimate transduction efficiency and calculate MOI. For accurate MOI, GFP+ percentages between 1% and 20% were ideal. Above 20% risked multiple integrations, while below 1% was excluded due to background noise.

2.1. Preparation of LB Medium: LB medium was prepared by dissolving 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1 liter of distilled water. The solution was autoclaved at 15 psi for 20 minutes, then cooled to 55°C, after which 50 µg/mL of ampicillin was added. The culture solution was preserved at a temperature of 4 degrees Celsius.

For LB agar plates, 20 g of agar was added to 1 liter of LB medium. Selective media were prepared by adding 100 µg/mL of ampicillin [30, 31].

2.2. Transformation of E. coli DH5α: The proficient *E. coli* DH5α cells (Life Technologies Ltd) were genetically modified in adherence to the guidelines provided by the supplier. Colonies containing plasmids were grown overnight at 37°C on plates containing 100 µg/mL of ampicillin, then cultured in LB medium with 100 µg/mL of ampicillin, either in small or large volumes. Plasmids were purified using Qiagen kits and eluted in TE buffer [32].

2.3. Cell Culture: HEK293T cells were cultured in DMEM medium enriched with 10% fetal bovine serum (FBS), Cells were cultured in a humidified incubator maintained at 37°C with 5% CO_2, in the presence of 1% L-glutamine and 10 µg/mL of penicillin/streptomycin. They were seeded into T162 culture flasks at a concentration of 2 × 10^7 cells per flask, and the cultures were allowed to reach 80–90% confluence before proceeding with transfection. Following the transfection procedure, the conditioned medium was harvested at intervals of 48 and 72 hours subsequent to transfection. To assess viral titers, a cellular suspension comprising 1–2 × 10^5 cells was seeded into 24-well plates and subsequently exposed to viral preparations serially diluted across a range of concentrations. The multiplicity of infection (MOI) was adjusted to values ranging from 1 to 10^{-5}. The detached cells were subjected to phosphate-buffered saline and tryple Red, after which the reaction was neutralized using a medium enriched with serum. For the purpose of flow cytometric analysis, the cellular samples were fixed utilizing a 4% paraformaldehyde-PBS solution and the specimens were maintained at 4°C until further examination and analysis could be conducted [33-36].

2.4. Cultivation of Mouse Embryonic Carcinoma Cells: P19 cells were cultivated in DMEM medium supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, and 10% fetal bovine serum (FBS). The cultures were maintained at 37°C in a humidified incubator. The incubator was set to contain 5% carbon dioxide (CO_2) and 10 µg/mL penicillin-streptomycin was added to the medium to prevent bacterial contamination. The induction of neuronal differentiation was commenced through the generation of embryoid bodies within a medium composed of DMEM, 5% fetal bovine serum (FBS), and 1 µM all-trans retinoic acid (RA). F9 cells were propagated in culture vessels coated with gelatin, utilizing a DMEM-based medium enriched with 10% FBS, 100 units per milliliter of penicillin, 100 micrograms per milliliter of streptomycin, and 2 millimolar L-glutamine. Differentiation of parietal endoderm

was induced using DMEM/F12 medium, 5% FBS, and 50 nM RA [37].

2.5. Reporter Gene Analysis: eGFP reporter constructs designed for the UCOE and A2UCOE in the LV system were transferred into P19 and F9 cells for analysis. The stability of gene expression was monitored both before and after the differentiation of these cells into neuroectodermal and endodermal cell types.

2.6. Statistical Evaluation: The dataset underwent rigorous statistical examination utilizing Prism 7, a sophisticated analytical tool, to ensure precise and methodical evaluation. The outcomes of flow cytometry pertaining to eGFP-expressing cells, in conjunction with the mean fluorescence intensity (MFI) and vector copy number (VCN) metrics within transduced cell cultures, were analyzed utilizing a Student's t-test. Statistical significance was ascribed to p-values falling below the threshold of 0.05 [38].

2.7. Lentiviral Constructs Used in the Study: In this study, the A2UCOE central region, which is 0.9 kilobases in length (illustrated in the lower section of Figure 1), has been identified as a methylation-determining region (MDR) [17]. This region is integrated into the self-Excising vector (SEW) in both the forward (F) and reverse (R) directions of the Spleen Focus-Forming Virus (SFFV) promoter (Figure 2).

Upper section: Depiction of CpG density distribution alongside an unmethylated CpG island. Central section: Positions of variant initial exons for the *CBX3* (indicated by amber arrows) and *HNRPA2B1* (marked by a crimson arrow) gene loci. Bottom section: Fragments of A2UCOE assessed for their enhancer capabilities. 2.2 UCOE: A positive regulatory element driven by the HNRPA2B1 promoter. 1.5 UCOE: A positive regulatory sequence positioned upstream of the SFFV promoter. 0.7 UCOE: The experimental "Daedalus" fragment under investigation. 0.9 UCOE: A core segment identified as a putative methylation-determinant region (MDR). 945/527/455 UCOE: Fragments derived from the CpG-dense region of A2UCOE. The capacity of these fragments to maintain stable expression of the SFFV promoter, relative to the 1.5 UCOE reference, will be evaluated.
The conventional self-inactivating (SIN) lentiviral construct, facilitating eGFP expression via the SFFV promoter and incorporating a WPRE component (SEW), was predominantly employed for the assembly of UCOE experimental vectors. The 1.5 kb HNRPA2B1-CBX3 UCOE (1.5A2UCOE), used as a positive control, was placed into a poly linker (PL) region located upstream of the SFFV promoter, generating the 1.5A2UCOE-SEW structure. The 2.2 kb HNRPA2B1-CBX3 UCOE (2.2A2UCOE) was combined with a GFP reporter gene driven by the HNRPA2B1 promoter to form the 2.2A2UCOE structure. Novel 0.9 kilobase methylation-sensitive regulatory (MSR) assay segments, designated as 0.9UCOE-F (forward primer) and 0.9UCOE-R (reverse primer), were strategically situated upstream of the SFFV enhancer within the SEW genomic locus. The abbreviation LTR denotes long terminal repeat sequences.

3. Results

3.1. Functional analysis of candidate 0.9UCOE-F and 0.9UCOE-R in undifferentiated P19 and F9 cells: The P19 and F9 cell lines were transduced with lentiviral vectors 0.9UCOE-F and 0.9UCOE-R, which encompass a putative multidrug resistance (MDR) locus, at multiplicity of infection (MOI) levels of 3 or 6 to achieve an approximate proportion of 40-60% enhanced green fluorescent protein (eGFP)-expressing cells (Figure 3). Following the transduction process, cellular samples, along with their corresponding control groups, were analyzed using flow

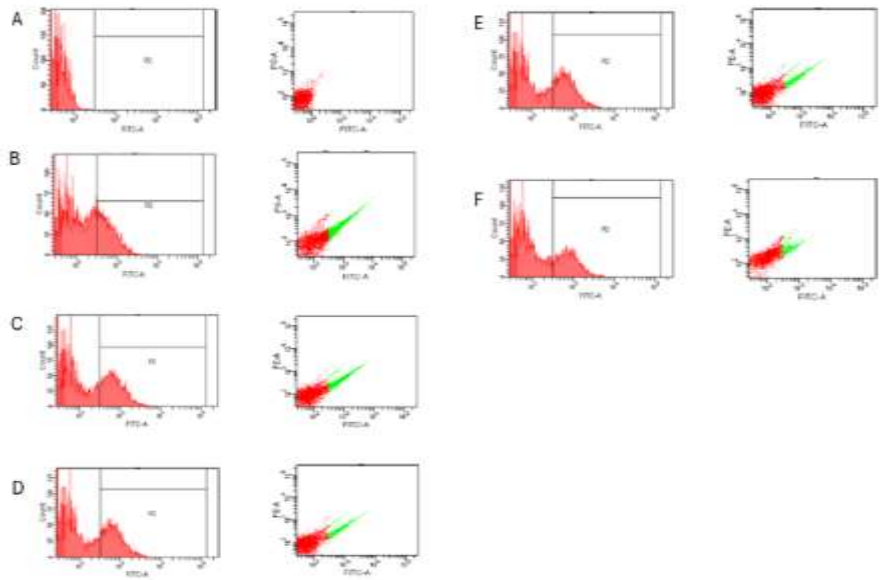
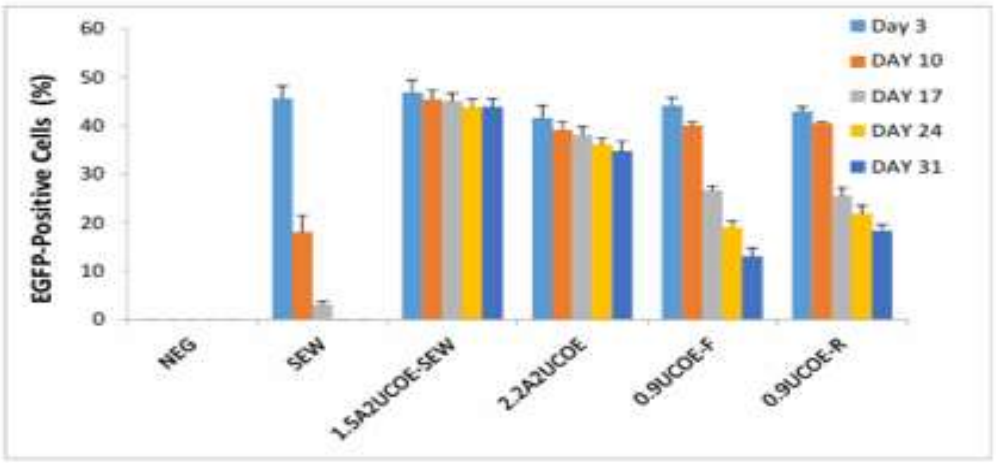


Figure 3A-D. Flow cytometric analyses depicting P19 and F9 cell populations, both transduced and non-transduced, preceding the induction of differentiation.

cytometry. This analysis aimed to determine the percentage of cells exhibiting eGFP expression and to measure the mean fluorescence intensity (MFI). The cellular cultures were systematically observed at seven-day intervals commencing from the third day through to the thirty-first day, as illustrated in Figures 4 and 5. Furthermore, DNA extraction was conducted to facilitate RT-qPCR analysis, aimed at evaluating the vector copy number within each individual cell. Figure 4.A presents the flow cytometric outcomes pertaining to eGFP-positive P19 cells. At the outset, the transduction efficacy across all vectors was comparable, with an estimated 45-60% of the cells exhibiting eGFP positivity. However, in cells infected with the SEW (SFFV-eGFP-WPRE) vector, eGFP expression significantly decreased from 45% to 2% within 17 days. In contrast, the 1.5A2UCOE-SEW and 2.2A2UCOE control groups maintained a stable percentage of eGFP-positive cells for up to 31 days. In cells infected with the 0.9UCOE-F and 0.9UCOE-R lentiviral vectors, eGFP expression showed a slower decline compared to SEW. While eGFP expression decreased by approximately 80% within two weeks in SEW-transduced cells, the reduction in cells infected with 0.9UCOE-F and 0.9UCOE-R vectors was only between 20-30%. The MFI values showed a similar trend (Figure 4.B); they remained stable in cells infected with 1.5A2UCOE-SEW, while no stability was observed in SEW and 0.9UCOE-F/R structures. Throughout the investigation, the mean vector copy number per cellular unit exhibited consistent stability (Figure 4.C). This finding corroborates that the attenuated eGFP expression linked to the SEW, 0.9UCOE-F, and 0.9UCOE-R lentiviral configurations did not arise from vector exhaustion but rather was a consequence of transcriptional silencing.

The cellular cohorts underwent genetic alteration through the application of recently devised UCOE constructs (0.9UCOE-F, 0.9UCOE-R, 455UCOE, 527UCOE, 945UCOE) in conjunction with analogous lentiviral delivery systems (SEW, 1.5A2UCOE-SEW, 2.2A2UCOE; Figure 2). Subsequent to transduction, flow cytometric evaluation was conducted after a 72-hour interval, with GFP-deficient cells illustrated in a crimson hue and

A)



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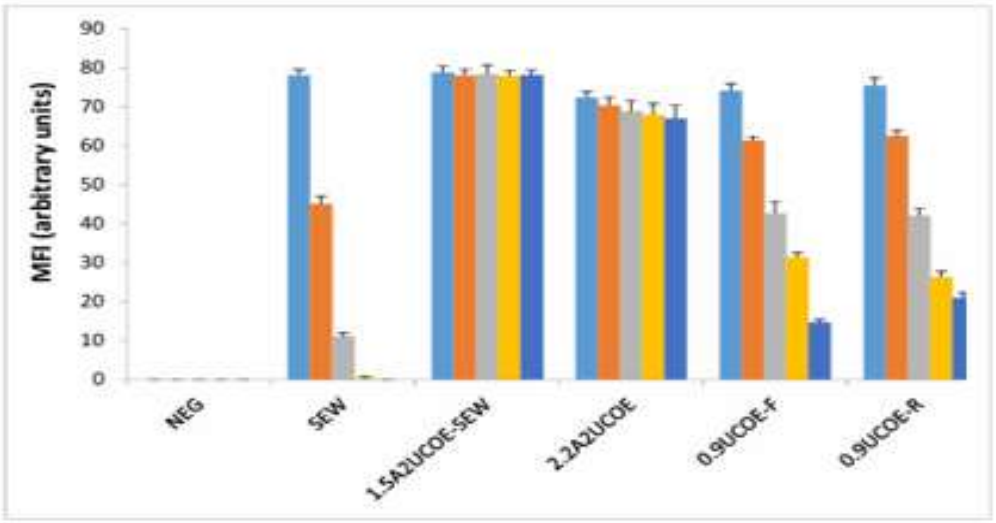

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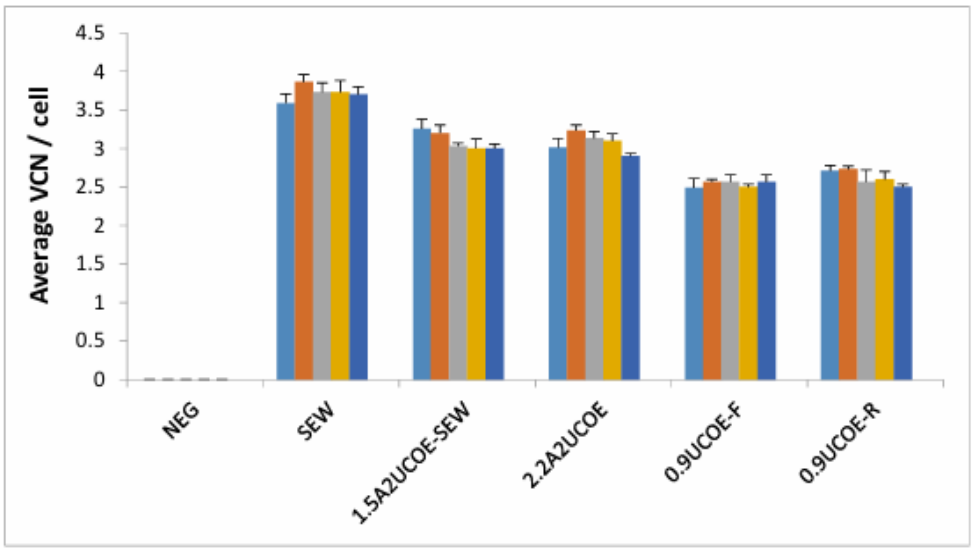

Figure 4A-C. The newly proposed candidate UCOEs, designated as 0.9UCOE-F and 0.9UCOE-R, exhibit a degree of resistance to transcriptional silencing in pluripotent P19 cells prior to differentiation.

GFP-expressing cells depicted in an emerald tone (A) Negative control — untreated cells. (B) Cells transduced with SEW. (C) Cells transduced with 1.5A2UCOE-SEW. (D) Cells transduced with 2.2A2UCOE. (E) Cells transduced with 0.9UCOE-F. (F) Cells transduced with 0.9UCOE-R.

P19 cell lines were genetically modified using lentiviral vectors (LVs) containing the innovative UCOE constructs, specifically 0.9UCOE-F and 0.9UCOE-R, alongside control vectors such as SEW, 1.5A2UCOE-SEW, and 2.2 kbA2UCOE (Figure 2). The evaluation of amplified green fluorescent protein (eGFP) manifestation, in conjunction with the measurement of average luminescence intensity (ALI) and genetic vector abundance per cellular unit (GVAC), was executed employing cytometric flow analysis and qRT-PCR methodologies. The results obtained from three independent infection experiments conducted for each vector, along with the negative control (NEG), were evaluated over the post-transduction period extending from day 3 to day 31. (A) Proportion of eGFP-expressing cells (Mean ± SEM, n=4; **p<0.01). (B) Median luminescence intensity (MLI) (Mean ± Standard Error of the Mean, n=4; **p<0.01). (C) Median genomic incorporation frequency per cellular entity (GIF) (Mean ± Standard Error of the Mean, n=4; **p<0.01).

Figure 5 illustrates the chronological fluctuations observed in the flow cytometric assessment of eGFP-expressing (eGFP+) cells within undifferentiated F9 cell populations transduced with lentiviral vectors (LVs) harboring the 0.9UCOE-F and 0.9UCOE-R constructs. The findings derived align harmoniously with the outcomes documented in P19 cellular models (Figure 4.A). Initially, the transduction efficacy of all vectors within the cellular milieu was relatively equivalent, ranging between 39% and 50% eGFP-positive cells. Nevertheless, the transcriptional activity mediated by the SEW (SFFV-eGFP-WPRE) construct demonstrated a pronounced decline, diminishing from 44% to a mere 3% across a span of 17 days. In contrast, the percentage of eGFP-expressing cells within specimens transduced with the 1.5A2UCOE-SEW and 2.2A2UCOE configurations demonstrated prolonged consistency across a span of 31 days (Figure 5.A). Gene expression transferred with 0.9UCOE-F and 0.9UCOE-R LVs showed a tendency to decrease over time, but this decline occurred much more slowly compared to SEW. The expression of eGFP in SEW-altered cells exhibited a significant decline of roughly 80% over a fortnight, whereas the diminution in eGFP-positive cells transduced with 0.9UCOE-F and 0.9UCOE-R lentiviral vectors was confined to a mere 5-10%. The median fluorescence intensity (MFI) measurements aligned closely with the observed eGFP expression outcomes (Figure 5.B). The MFI demonstrated consistent durability in cells transduced with the 1.5A2UCOE-SEW and 2.2A2UCOE configurations, whereas a marked reduction was observed under the SEW condition. Conversely, the recently evaluated UCOE variants demonstrated relative stability in MFI levels. Throughout the experiment, the vector copy number per cell did not vary under any condition (Figure 5.C).

Lentiviral vectors, encompassing 0.9UCOE-F, 0.9UCOE-R, SEW, 1.5A2UCOE-SEW, and 2.2A2UCOE, were utilized to transduce F9 cells (as illustrated in Figure 2). The percentage of cells exhibiting eGFP expression was assessed utilizing flow cytometric techniques, while the mean fluorescence intensity (MFI) and the vector copy number per cellular entity (VCN) were determined via RT-qPCR methodologies. Results obtained from three independent experimental replicates for each vector, in

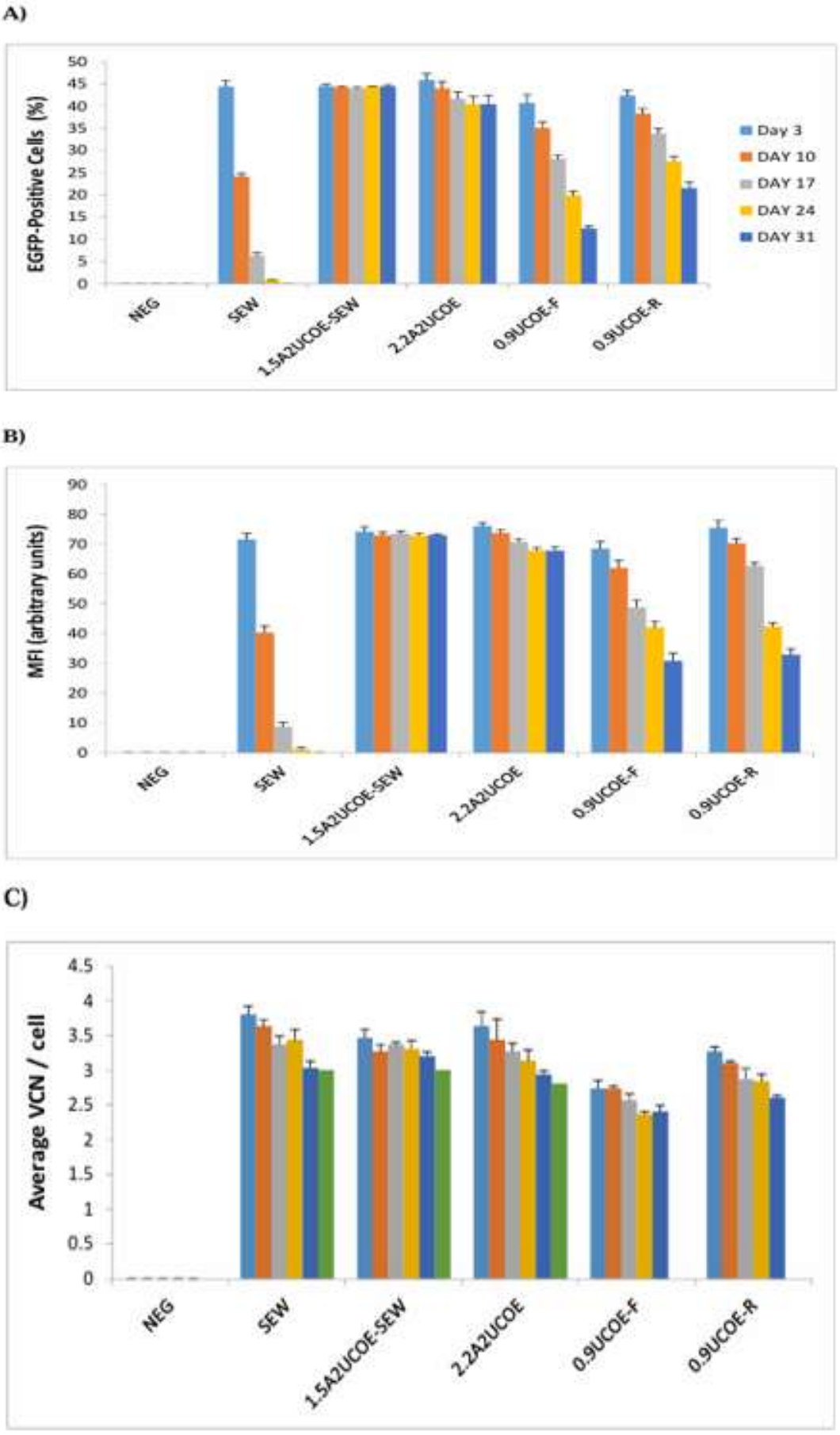

Figure 5A-C. The recently identified UCOE candidates, designated as 0.9UCOE-F and 0.9UCOE-R, demonstrate a moderate safeguarding influence against transcriptional repression in undifferentiated F9 cell

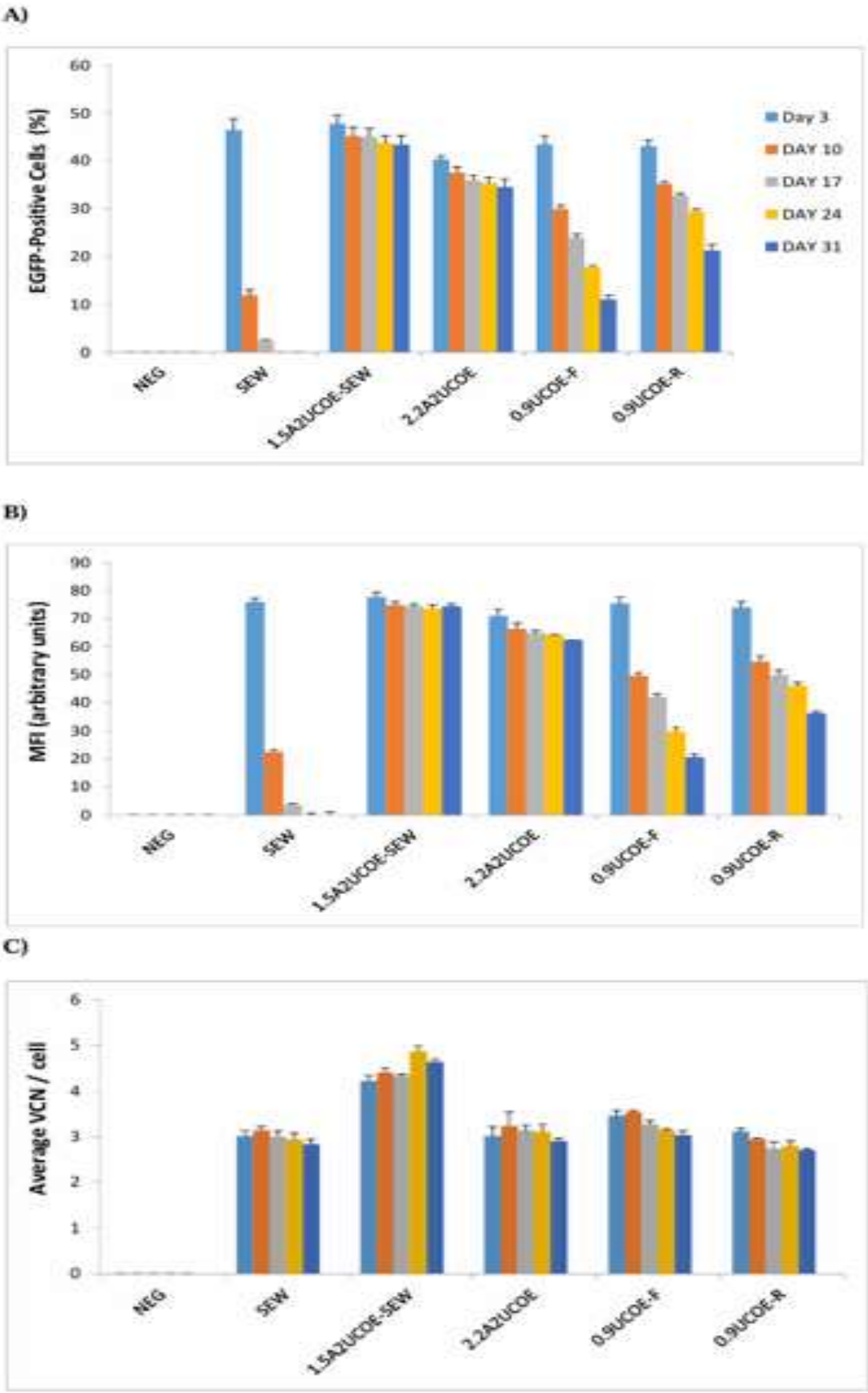

Figure 6A-C. The recently engineered 0.9UCOE-F and 0.9UCOE-R UCOEs demonstrate moderate protective impacts on alleviating gene expression suppression in differentiated P19 cells.

addition to the negative control (NEG), are presented over a chronological framework spanning from 3 to 31 days post-infection. (A) Percentage of cellular units demonstrating eGFP expression (Mean ± Standard Error of the Mean, n=4; $^{**}p<0.01$). (B) Median luminescent intensity (MLI) (Mean ± Standard Error of the Mean, n=4; $^{**}p<0.01$). (C) Median frequency of genomic integration per individual cell (GIF) (Mean ± Standard Error of the Mean, n=4; $^{**}p<0.01$).

3.2. Functional Assessment of 0.9UCOE-F and 0.9UCOE-R in P19 and F9 Cells Post-Differentiation: The 0.9UCOE-F and 0.9UCOE-R plasmids facilitated a partially balanced gene expression in undifferentiated P19 (Figure 4) and F9 (Figure 5) cell lines. Subsequently, the ability of these vectors to maintain stable gene expression throughout the differentiation process of P19 and F9 cells into neuroectodermal and endodermal lineages was thoroughly assessed. Lentiviral vector-modified P19 and F9 cells were initially cultured in a differentiation medium enriched with retinoic acid to induce the formation of embryoid bodies, followed by their differentiation into neuroectodermal and parietal endodermal lineages, respectively. Expression data obtained from triplicate samples of cells modified with SEW, 1.5A2UCOE-SEW, 2.2A2UCOE, 0.9UCOE-F, and 0.9UCOE-R LVs post-differentiation are presented in Figures 6 and 7. Our results demonstrate that the control LV vectors incorporating positive UCOE elements (1.5A2UCOE-SEW and 2.2A2UCOE) preserved the stability of gene expression following the differentiation of P19 (Figure 6.A) and F9 (Figure 7.A) cells. . In contrast, the newly developed 0.9UCOE-F and 0.9UCOE-R vectors showed a gradual decrease in eGFP expression over time, while the silencing control, the SEW vector, rapidly suppressed eGFP expression (Figures 6.A and 7.A).

P19 cell lines were infected with 0.9UCOE-F, 0.9UCOE-R, SEW, 1.5A2UCOE-SEW, and 2.2A2UCOE lentiviral vectors (LVs) (Figure 2). Flow cytometric analysis was used to evaluate the proportion of cells displaying eGFP expression and the median fluorescence intensity (MFI). Meanwhile, the mean vector copy number (VCN) per cell was determined using reverse transcription quantitative polymerase chain reaction (RT-Q-PCR). The results stem from three separate experiments carried out for each vector, incorporating a negative control group (NEG), and spanning a timeframe of 3 to 31 days following transduction. (A) Changes in the percentage of eGFP-positive cells over time (Mean ± Standard Error of the Mean, n=4; $^{**}p<0.01$). (B) MFI values corresponding to (A) (Mean ± Standard Error of the Mean, n=4; $^{**}p<0.01$). (C) Average VCN per cell in parallel with (A)/(B) (Mean ± Standard Error of the Mean, n=4; $^{**}p<0.01$).

F9 cell lines were transduced with 0.9UCOE-F, 0.9UCOE-R, and control lentiviral vectors (SEW, 1.5A2UCOE-SEW, and 2.2A2UCOE) as depicted in Figure 2. The proportion of eGFP-expressing cells, the average fluorescence intensity (AFI), and the vector copy number (VCN) per individual cell were assessed utilizing flow cytometry and quantitative real-time PCR (RT-qPCR) methods. . The results reflect the combined data from three independent experiments conducted over a 3 to 31-day period for each vector and the negative control (NEG). (A) Temporal variations in the proportion of eGFP-positive cells; (Mean ± Standard Error of the Mean (SEM), n=4; $^{**}p<0.01$). (B) Temporal alterations in the average fluorescence intensity (MFI); (Mean ± Standard Error of the Mean (SEM), n=4; $^{**}p<0.01$). (C) Temporal fluctuations in the average vector copy number (VCN) per cell; (Mean ± Standard Error of the Mean (SEM), n=4; $^{**}p<0.01$).

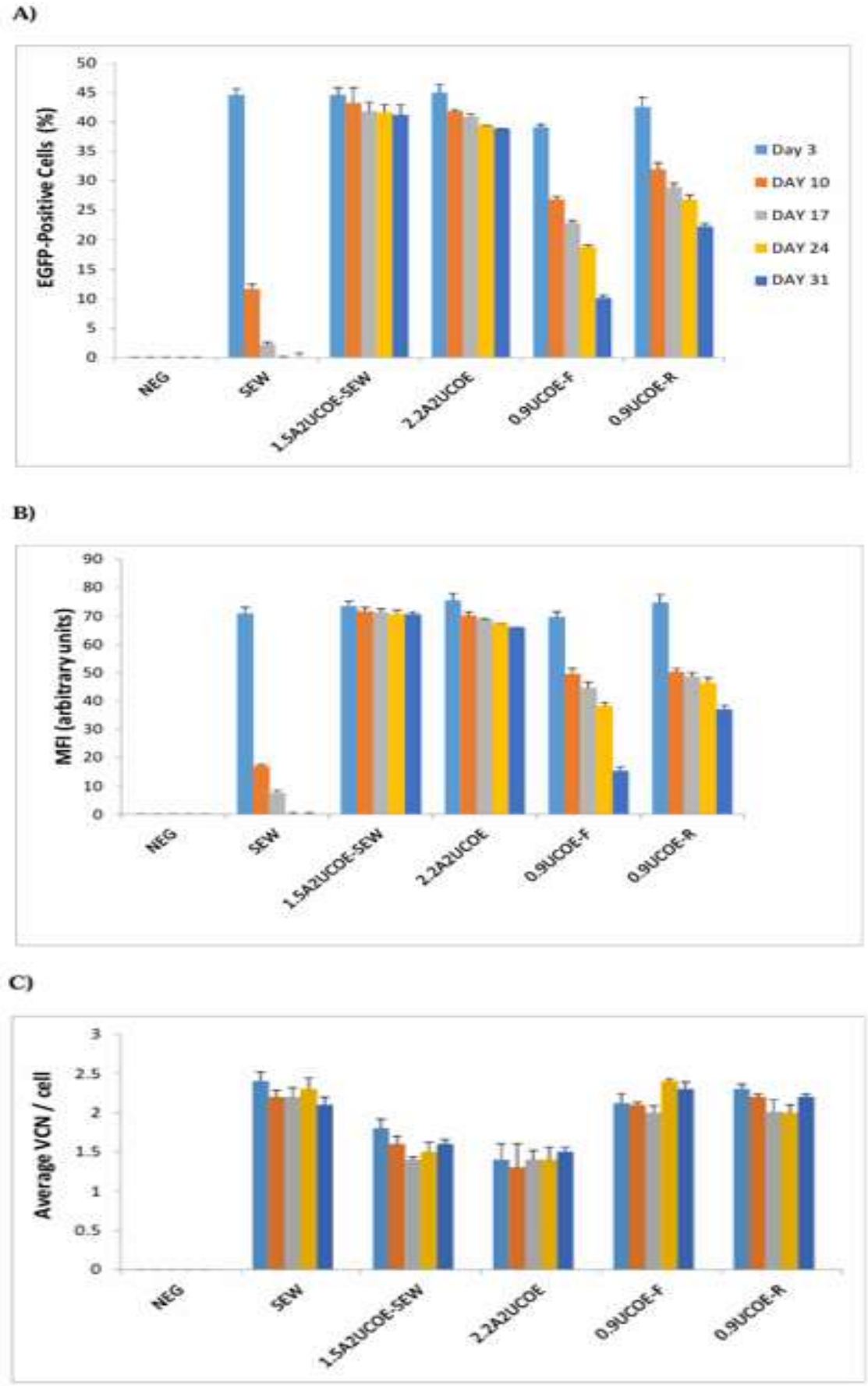

Figure 7. The recently engineered 0.9UCOE-F and 0.9UCOE-R UCOEs exhibit a partial shielding effect against gene suppression in differentiated F9 cells.

4. Discussion

Vector types, recognized for their integrative capabilities, are crucial in ensuring the stable uptake and expression of therapeutic genes, especially when targeted at expanding populations of stem cells. n the last decade and a half, clinical investigations performed ex vivo on hematopoietic stem cells (HSCs) have yielded encouraging outcomes with the application of these vectors. Particularly, conditions such as severe combined immunodeficiency (SCID), X-linked SCID (SCID-X1), and adenosine deaminase-deficient SCID (ADA-SCID) [17,39], in addition to Wiskott-Aldrich syndrome (WAS) and chronic granulomatous disease (CGD), have shown significant potential for therapeutic intervention [39,41], have been addressed through the genetic alteration of hematopoietic stem cells (HSCs) derived from patients, employing gammaretroviral delivery systems.

Recent investigations have highlighted the effective application of lentiviral vectors in the treatment of genetic myelinopathies, such as WAS [42]. X-linked adrenoleukodystrophy (X-ALD) [43] and metachromatic leukodystrophy (MLD) [44] are significant hereditary conditions. However, using these viral vectors, which integrate into the genome, introduces two main complications: the potential for insertional mutagenesis and the silencing of the introduced therapeutic gene through epigenetic mechanisms. Insertional mutagenesis, which induces oncogenesis through unintentional triggering of proto-oncogenes in host cells via gammaretroviral integration, was detected in five of twenty SCID-X1 individuals [17,31]. Furthermore, the repression of transgene expression due to DNA methylation in the promoter region contributed to the therapeutic failure in two CGD patients [40,41].

The principal aim of the experimental procedures undertaken during this stage was to examine whether distinct segments of the A2UCOE, originating from the HNRPA2B1-CBX3 locus in humans (Figure 1), exhibit particular characteristics and sustain UCOE activity. This investigation is informed by prior studies indicating that specific subdomains of CpG islands linked to genes regulated during development can retain proper DNA methylation patterns within a transgenic framework, even in the absence of a promoter [45]. These findings raise questions about the bidirectional divergent transcription model for UCOE functionality [46]. To pinpoint a methylation-sensitive region (MSR), potential UCOEs, 0.9UCOE-F and 0.9UCOE-R, were developed through bioinformatics analysis of the A2UCOE region. This design targets specific transcription factor binding sites, such as those for Sp1, CTCF, and USF. The 0.9-kb A2UCOE core segment, which includes the promoter and transcription initiation areas of the HNRPA2B1 and CBX3 genes, was linked to the SFFV promoter in the SEW vector. This construct showed partial resistance to suppression in both undifferentiated and differentiated P19 (Figures 4, 5) and F9 (Figures 6, 7) cells. The 0.9-kb A2UCOE core region can be regarded as a minimal functional regulatory element. However, it does not exhibit the same level of complete UCOE activity as the 1.5-kb A2UCOE, which spans a larger region within the HNRPA2B1 and CBX3 loci.

The disparity in UCOE functionality across the two central domains of A2UCOE may be ascribed to the extent of the CpG-rich region embedded within the 0.9-kb fragment. This length does not exceed a critical threshold, preventing the chromatin from remaining in an accessible configuration. As a result, the 0.9UCOE-F and 0.9UCOE-R subfragments from the A2UCOE region failed to demonstrate

complete UCOE activity. The configurations of 0.9UCOE-F and, more specifically, 0.9UCOE-R, which encompass the promoter sequences and transcription initiation zones of HNRPA2B1 and CBX3, conferred partial yet notable resistance to transgene silencing. In spite of conflicting outcomes, the targeted experiments performed did not reveal an A2UCOE subregion that maintains complete UCOE activity in the absence of promoter activity.

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