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Construction and Quantitative Evaluation of a Dual Specific Promoter System for Monitoring the Expression Status of *Stra8* and *c-kit* Genes

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Abstract Applications of genetic constructs with multiple promoters, which are fused with reporter genes and simultaneous monitoring of various events in cells, have gained special attention in recent years. Lentiviral vectors, with their distinctive characteristics, have been considered to monitor the developmental changes of cells in vitro. In this study, we constructed a novel lentiviral vector (FUM-M), containing two germ cell-specific promoters (*Stra8* and *c-kit*), fused with ZsGreen and DsRed2 reporter genes, and evaluated its efficiency in different cells following treatments with retinoic acid and DMSO. Several cell lines (P19, GC-1 spg and HEK293T) were transduced with this vector, and functional capabilities of the promoters were verified by flow cytometry and quantitative RT-PCR. Our results indicate that FUM-M shows dynamic behavior in the presence and absence of extrinsic factors. A correlation was also observed between the function of promoters, present in the lentiviral construct and the endogenous level of the *Stra8* and *c-kit* mRNAs in the cells. In conclusion, we recommend this strategy, which needs further optimization of the constructs, as a beneficial and practical way to screen chemical inducers involved in cellular differentiation toward germ-like cells.

Keywords Dual promoter · Lentiviral vector · Dynamic behavior · Stra8 · c-kit

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Introduction

Transcriptional regulation in eukaryotes is a strictly coordinated process. Although there are several types of cis- and trans-acting DNA sequence elements of this regulation, the most common elements are located on promoters [1–3], which can reflect the true biological activities. A simple and practical technique for detection of promoter activity in live cells is utilization of fluorescent proteins that can be detected via flow cytometry without any enzymatic pretreatments [4–7]. As a result of using these proteins, timescale monitoring of promoter activity would be easily possible, which is a way for tracking and measuring the properties of biological molecules in a short time [5, 8, 9].

Application of the vectors with dual promoters is a new strategy offering the opportunity for simultaneous study of inter-dependent events in a biological process [10–15]. On the other hand, the ability of lentiviral vectors to stably transduce both dividing and non-dividing cells has made them a unique implement for genetic modification of the animal models [14–23]. There are several studies in simultaneous application of different promoters to investigate cellular functions [11, 24, 25]. Among the synthetic promoters, bidirectional types are attractive and probably stronger replacements to the endogenous ones. However, promoter interference may damage the expression of adjacent transcription units placed in the same vector [26]. Application of insulators and terminator sequences for decreasing the position effect and placing both promoters in antisense orientation, relative to the viral LTRs, which result in avoiding the disruption of viral mRNA production by internal polyA sequences, are strategies to improve independent functions of the dual promoters [27–31].

In this study, we designed a lentiviral vector based on a dual promoter construct model [31], in order to track the simultaneous activity of two germ cell-specific promoters (*Stra8* and *c-kit*), monitored by expression analysis of their downstream reporter genes (ZsGreen and DsRed2, respectively). The *Stra8* gene encodes a cytoplasmic protein with a common role in embryonic ovary and adult testis. It is a premeiotic protein participating in transition to meiosis step in both sexes [32–36]. The stem cell factor receptor c-kit also participates in all stages of male germ cell development before (7.5-13 dpc) and after birth [37–39]. It plays a critical role in maintaining the ratio between self-renewal and differentiation of spermatogonial stem cells [40–42].

Here, we showed the activity of designed dual promoters with respect to expression of the reporter genes. Furthermore, these active promoters were induced by different inducing molecules (RA and DMSO) in order to evaluate the vector capabilities to track cell status.

Materials and Methods

Cell Culture

GC-1 spg (mouse spermatogonial type B cells; ATCC CRL-2053) obtained from Pasteur Institute, Iran, and HEK293T (human embryonic kidney; ATCC CRL-3216) obtained from Pasteur Institute, Iran, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Gibco, USA) and incubated at 37 °C in a humidified 5 % CO_2 atmosphere. P19 cells (mouse embryonal carcinoma) ATCC CRL-1825; obtained from Pasteur Institute, Iran, were cultured in α-MEM (Gibco, USA) supplemented with 10 % heat-inactivated FBS and incubated in the same conditions.

Plasmid Construction

The lentiviral backbone, used in this study for the construction of FUM-M dual promoter vector, was derived from FUW-OSKM (plasmid 20328, Addgene). As shown in Fig. 1, we designed and optimized a lentiviral vector carrying two independent transcriptional units that were spaced by a synthetic polyA, terminator (Tactb) and an insulator sequence (cHS4). A second insulator sequence (sMAR8) and terminator (TKpA) were added downstream of the second gene to relieve potential suppression of gene expression from the viral enhancer or surrounding genomic sequence after lentivirus integration, based on a model from Tian and Andreadis [31]. Two specific promoters in spermatogenesis process (*Stra8* and *c-kit*) were also employed. We examined the expression of ZsGreen gene as a reporter for *Stra8* promoter activity [43] and applied puromycin antibiotic to select the Stra8-positive cells. In addition to the *Stra8* promoter, we employed the DsRed2 gene as a second reporter that was expressed by *c-kit* promoter [44]. All sequences are located in antisense orientation relative to the viral LTRs. The sequence construct was made by Genscript Company (Piscataway, NJ, USA). pCDH-CMV-MCS-EF1-GFP-T2A-Puro lentiviral vector (system bioscience (SBI), USA) was also used as a control vector in these experiments.

Direct Transfection of the cells

P19 and HEK293T cells were directly transfected with FUM-M plasmid using the standard calcium phosphate precipitation method [45]. After 16 h, the culture medium was replaced with fresh DMEM, containing 10 % FBS.
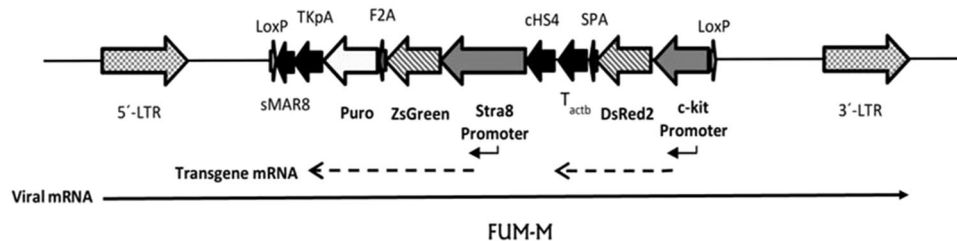
Fig. 1 Schematic representation of the designed lentiviral dual-promoter vector (FUM-M) with the following components: *sMAR8* synthetic MAR sequence as an insulator; *TKpA* thymidine kinase polyA from herpes simplex virus as a terminator; *Puro* Puromycin resistance gene; *F2A* 2A peptide; *cHS4* chicken hypersensitive site 4 as an insulator; *Tactb* a G-rich sequence from the extension of β-actin gene as a pause site; *SPA* synthetic polyA. ZsGreen and DsRed2 are reporter genes; Mouse *Stra8* and *c-kit* promoters are germ cell-specific promoters. All sequences are located in antisense orientation relative to the viral LTRs

Virus Production

HEK293T cells were transiently cotransfected with three plasmids using the standard calcium phosphate precipitation method [45]. Twenty-one micrograms of lentiviral vector, 21 µg of psPAX2 (a gift from Tronolab), as packaging vector, and 10 µg of pMD2.G (a gift from Tronolab), a vector encoding the G-protein of the vesicular stomatitis virus (VSV-G), were used for this purpose. After 16 h, the culture medium was replaced with medium containing 10 % FBS. Viral particles were harvested at 48, 72 and 96 h after transfection, filtered through 0.45-µm filter (Orange, Belgium), pelleted by ultracentrifugation (Beckman-Coulter ultracentrifuge XL-100K, USA) (at $28,000 \times g$, 4 °C for 1 h) and resuspended in fresh medium. Quantification of the vector stocks was performed using the RETRO-TEK HIV-1 p24 Antigen ELISA kit (Zepto Metrix Corporation, Buffalo, NY, USA).

Transduction Experiments

HEK293T, GC-1 spg and P19 cells were seeded in 6-well plates. On the day of infection, the media were changed with fresh ones containing the collected lentiviruses at an MOI: 20. 24 h after transduction, the culture media were changed and the cells were cultured for an additional 3–4 days. Puromycin (Life technologies, USA) selection was performed 4 days after transduction at 1 mg/ml for HEK293T and 2 mg/ml for GC-1 spg cell lines and maintained for 10–20 days. In order to test the functionality of the FUM-M vector, the transduced cells were treated with RA and DMSO. For RA treatment, HEK and GC-1 spg cells, transduced with FUM-M virus, were cultured for 48 h in a medium containing 10 µM RA (Sigma-Aldrich, USA), and For the DMSO, the GC-1 spg cells, transduced with FUM-M virus, were treated with 0.1 % of the DMSO (Merck, Germany) for 12 h.

Flow Cytometry

For flow cytometry analysis, the HEK293T, GC-1 spg and P19 cells were transduced with FUM-M lentiviruses. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then dissociated with 0.25 % trypsin-EDTA. After 5 min of centrifugation ($500 \times g$), the cells were washed twice in ice-cold PBS containing 2 % FBS, and then 500 µl paraformaldehyde (4 %) was added. After another wash with PBS, containing 2 % FBS, they were resuspended in 3–5 ml PBS and used for flow cytometry analysis. The proportion of the cells expressing plasmid-induced ZsGreen and DsRed2 fluorescence were examined using approximately 20,000 cells on a FACSCalibur flow cytometer (BD, USA) with reference to a baseline of the non-transduced HEK293T, GC-1 spg and P19 cells. Data were analyzed by flowing software 2.5.1 (University of Turku, Finland) program.

RNA Extraction, Reverse Transcription and Quantitative RT-PCR

Total RNA was extracted using Tripure reagent (Roche, Germany). DNA contamination was prevented by DNase I (Thermo Fisher Scientific, USA) treatment. Reverse transcription was performed using M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, USA) and quantitative RT-PCR reactions were performed by gene-specific primer (Microgene, Korea) (Table 1) sets and SYBR Green (Parstoos-Iran) with a Bio-Rad CFX-95 system (USA).

Statistical Analysis

Statistical analysis was performed using the SPSS 19.0 statistical package (SPSS, Chicago, IL). All data were expressed as mean ± s.d. and analyzed by t test. P values <0.05 or <0.01 were considered statistically significant.

Table 1 Gene-specific primer sets for quantitative RT-PCR

Primer sets	Forward primer (5′–3′)	Revers primer (5′–3′)
Human-stra8	CCTCAAAGTGGCAGGTTCTGAA	TCCTCTAAGCTGCTTGCATGC
Mouse-stra8	CGTGGCAAGTTTCCTGGACAAG	GGCTCTGGTTCCTGGTTTAATGG
Mouse-c-kit	AACCCTCAGCCTCAGCACATAG	TGAACACTCCAGAATCGTCAACTC
Human GAPDH	GACCACTTTGTCAAGCTCATTTCC	GTGAGGGTCTCTCTCTTCCTCTTGT
*Mouse β2*M	GTCGCTTCAGTCGTCAGCAT	TTTTCCCGTTCTTCAGCATTTGG
DsRed2	CGCCGACATCCCCGACTAC	CACGCCGATGAACTTCACCTTG

Fig. 2 Activity assesment of the dual-promoter vector (FUM-M), containing *Stra8* and *c-kit* promoters, in HEK293T and P19 cell. Micographs represent fluorescence images of HEK293T (**A**, **B**) and P19 (**C**, **D**) cells, transfected directly with FUM-M vector after 72 h. Notably that certain cells are double positive for the expression of *Stra8* and *c-kit* (*arrows*)
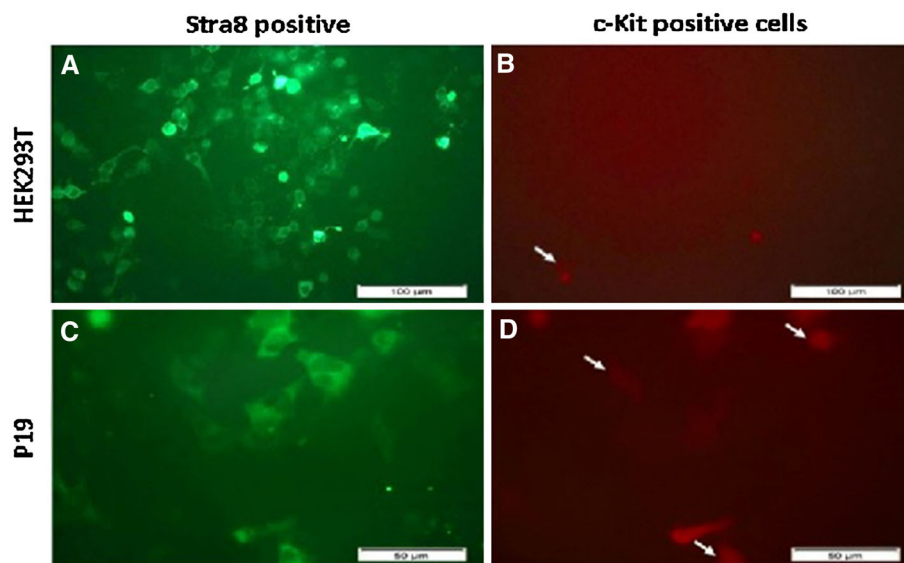
Results

Two Promoters are Simultaneously Active

To test whether the designed construct could support the simultaneous activity of the two promoters, HEK293T and P19 cell lines were transfected directly with FUM-M vector by calcium phosphate precipitation method. Fluorescent microscopic results indicated that the expression of ZsGreen and DsRed2 proteins were independently promoted by *Stra8* and *c-kit* promoters, respectively (Fig. 2).

Determination of the Activity of FUM-M Lentiviral Vector in Different Cell Lines

To determine the efficiency of the designed sequences in FUM-M lentiviral vector and investigate independent activity of both *Stra8* and *c-kit* promoters, the HEK293T, GC-1 spg and P19 cell lines were used. As a control of gene-transduction ability, we used a lentiviral vector containing EF1 constitutive promoter (pCDH-CMV-MCS-EF1-GFP-T2A-Puro) at an MOI: 20. As shown by fluorescence microscopy (Fig. 3A) and confirmed by flow cytometry (Fig. 3B), 96 h after transduction, percentages of the GFP-positive cells in the HEK293T and GC-1 spg cells were significantly higher than that in P19 cells, suggesting different transduction efficiency for this construct among different cell lines.

Next we transduced the HEK293T, GC-1 spg and P19 cell lines by FUM-M vector at the MOI: 20 similar to pCDH vector. As shown in Fig. 3C, D, fluorescent microscopic images and the flow cytometry results confirmed positive activity of the *Stra8* promoter in the HEK293T and GC-1 spg cell lines and non-detectable expression for the same promoter in the P19 cells. Flow cytometry results also showed a low expression rate for the *c-kit* promoter in the P19 cells (Fig. 3D) with little red fluorescence intensity (data not shown). Nevertheless, it did not demonstrate any DsRed2-positive cells in HEK293T and GC-1 spg cell lines 96 h after transduction (Fig. 3D). Overall, compared to the pCDH vector, with a constitutive promoter, the FUM-M lentiviral vector exhibited independent behaviors in *Stra8* and *c-kit* promoters.
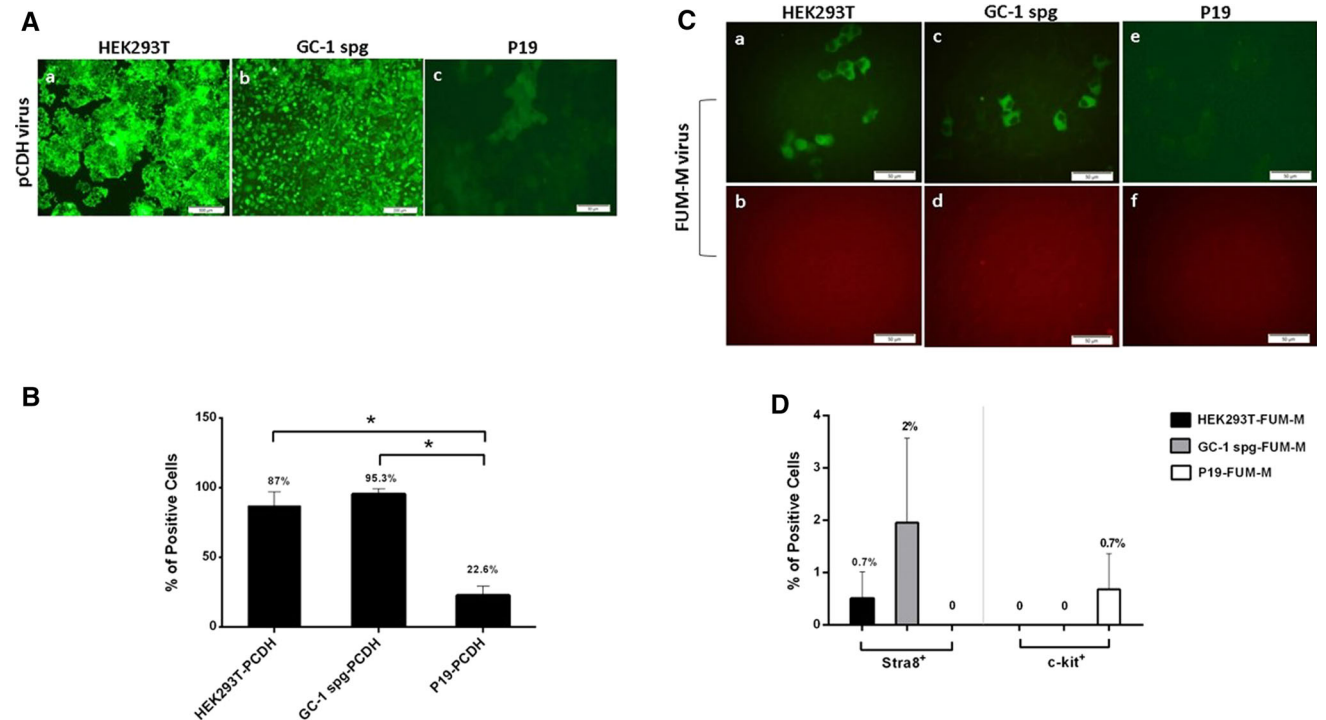
Fig. 3 Determination of the Transduction efficiency of FUM-M lentiviral vector in different cell lines. Panel **A** represents fluorescence images of HEK293T (*a*), GC-1 spg (*b*) and P19 (*c*) cell lines, transduced with pCDH-CMV-MCS-EF1-GFP-T2A-Puro virus (as a control virus) after 96 h. Panel **B** represents quantitative analysis of percentage of GFP-positive cells in HEK293T (87 %), GC-1 spg (93.3 %) and P19 (22.6 %) cell lines, transduced with pCDH-CMV-MCS-EF1-GFP-T2A-Puro virus after 96 h based on flow cytometry.

Panel **C** represents fluorescence images of Stra8 (*a*, *c*, *e*) and c-kit (*b*, *d*, *f*) positive cells, transduced with FUM-M virus after 96 h. Panel **D** represents quantitative analysis of the percentage of ZsGreen- and DsRed2-positive cells, transduced with FUM-M virus after 96 h based on flow cytometry. *Represents the significant difference as compared with control (*$P \leq 0.05$). The *error bars* indicate the standard deviations

FUM-M Lentiviral Vector Can be Used for Monitoring Stra8 Expression in Cells

To determine the stable transduction of FUM-M lentiviral vector, the HEK293T and GC-1 spg cells were treated by appropriate puromycin concentrations for about 3 weeks. As shown in Fig. 4A–C the expression of ZsGreen could be observed in 94 % of both HEK293T and GC-1 spg cells. In order to compare the gene expression pattern between the endogenous *Stra8* and the reporter gene and their possible coordination, the level of *Stra8* mRNA was determined by quantitative RT-PCR after puromycin selection (Fig. 4D, E). These results indicated that the percentage of green fluorescent cells was in accordance with the level of *Stra8* mRNA in both cell lines.

Dual Promoters are Active in Diverse Cellular Environments

To examine the dynamic behavior of FUM-M lentiviral vector in the presence of extrinsic factors, separate

experiments were designed. We selected HEK293T and GC-1 spg cells as models for human and mouse cells, according to our previous results. Since the percentages of Stra8 and c-kit-positive cells were low, to test the ability of the dual promoter activity for driving the simultaneous expression of two reporter genes, we selected Stra8-positive cells by appropriate puromycin concentrations.

Retinoic Acid Can Induce the Activity of Stra8 and c-kit Promoters

After transduction of the cells by FUM-M lentivirus and selecting the Stra8-positive cells by puromycin, they were treated by retinoic acid (RA) and the green fluorescence intensities were evaluated. As shown in Fig. 5A–C, infection of the HEK293T and GC-1 spg cells with RA significantly increased the expression level of ZsGreen in the cells.

The *c-kit* promoter activity in Stra8-positive cells was evaluated by measuring the percentage of c-kit-positive cells after induction by RA, where this percentage in HEK293T and GC-1 spg cells were significantly increased (Fig. 5D, E) after the treatment. However, DsRed2 intensity was not
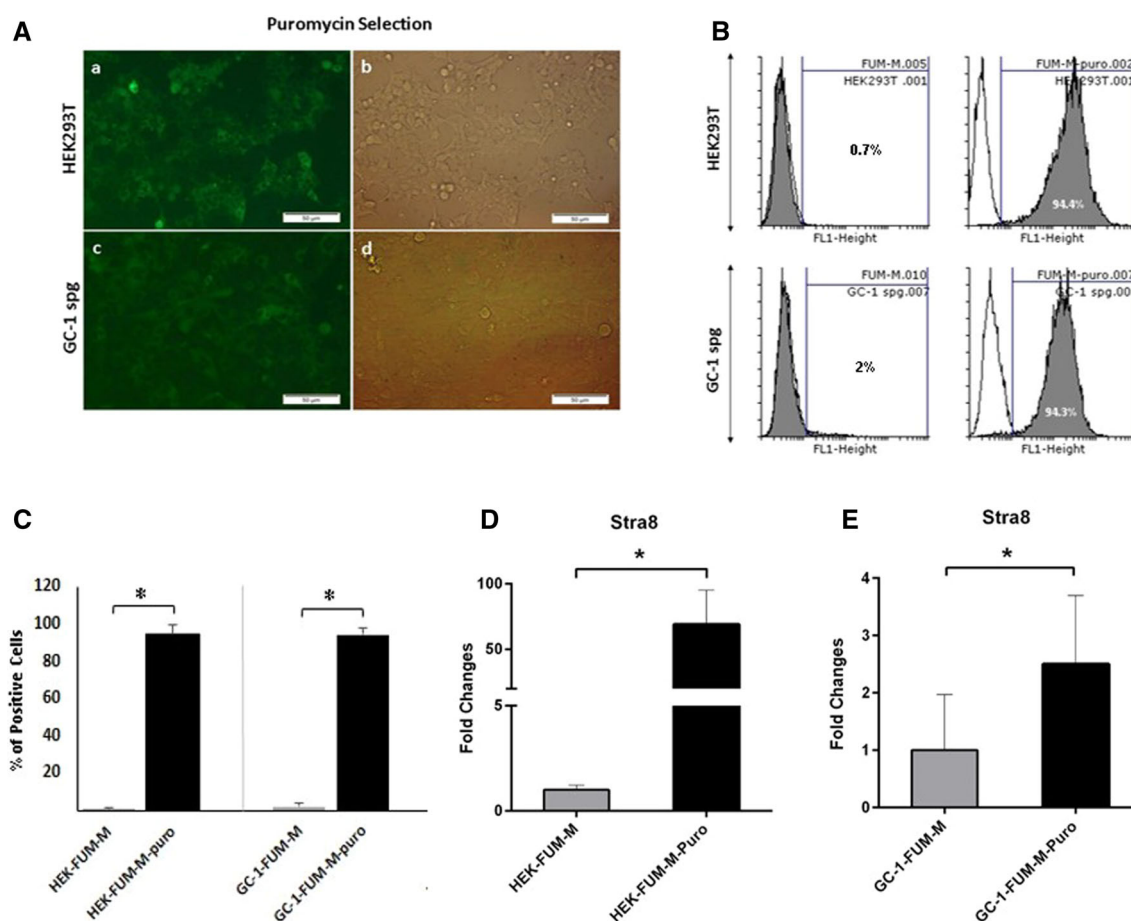
Fig. 4 FUM-M lentiviral vector function is in accordance with *Stra8* expression in the puromycin selcted cells. The panels represent: fluorescent (**A**a, c) and light microscopy (**A**b, d) analysis of transuced HEK293T and GC-1 apg cells; fluorescence histograms of ZsGreen-positive cells by flow cytometry analysis for about 3 weeks after puromycin selection (**B**); quantitative analysis of percentage of GFP-positive cells in HEK293T and GC-1 spg cells before and after puromycin selection based on flow cytometry (**C**); quantitative RT-PCR analysis of *Stra8* expression in HEK293T and GC-1 spg cells for about 3 weeks after puromycin selection (**D**, **E**). *Represents significant difference as compared with control (*$P \leq 0.05$). The *error bars* indicate the standard deviations

strong enough to be detected properly with fluorescence microscopy (data not shown). Therefore, to evaluate the DsRed2 expression under the *c-kit* promoter in mRNA level, we applied the quantitative RT-PCR. This experiment showed that the expression rate of DsRed2 increases significantly after RA treatment (Fig. 5F). These results are in line with the data from flow cytometry experiments implicating the efficient transcription of DsRed2.

Activity of Stra8 and c-kit Promoters are decreased in Response to DMSO Stimulation

In another attempt to investigate the FUM-M lentiviral vector activity, we treated GC-1 spg cells as a model for germ cells with DMSO. The Stra8-positive cells were selected by puromycine, and DMSO was added to the medium at a final concentration of 0.1 %. The behavior of FUM-M vector was analyzed by flow cytometry and quantitative RT-PCR, 12 h after treatment.

As shown in Fig. 6A, the activity of the *Stra8* promoter in response to DMSO was significantly decreased as compared with the untreated cells. These results indicated that DMSO could decrease the function of *Stra8* promoter in FUM-M lentiviral vector 12 h after treatment. Additionally, the reduction of *Stra8* mRNA level was also confirmed by quantitative RT-PCR (Fig. 6C).

Furthermore, to check the status of *c-kit* promoter activity in the Stra8-positive cells, the percentage of positive cells under the control of *c-kit* promoter was calculated. As shown in Fig. 6B, the percentage of c-kit-positive cells were significantly decreased 12 h after DMSO treatment. Quantitative RT-PCR results also indicated a significant decrease in *c-kit* mRNA level (Fig. 6C). Additionally, we checked the DsRed2 mRNA level after DMSO treatment in GC-1 spg cells. Our results confirmed that the expression of DsRed2 is decreased significantly after 12 h in presence of DMSO in accordance with flow cytometry results (Fig. 6D). These results suggest that
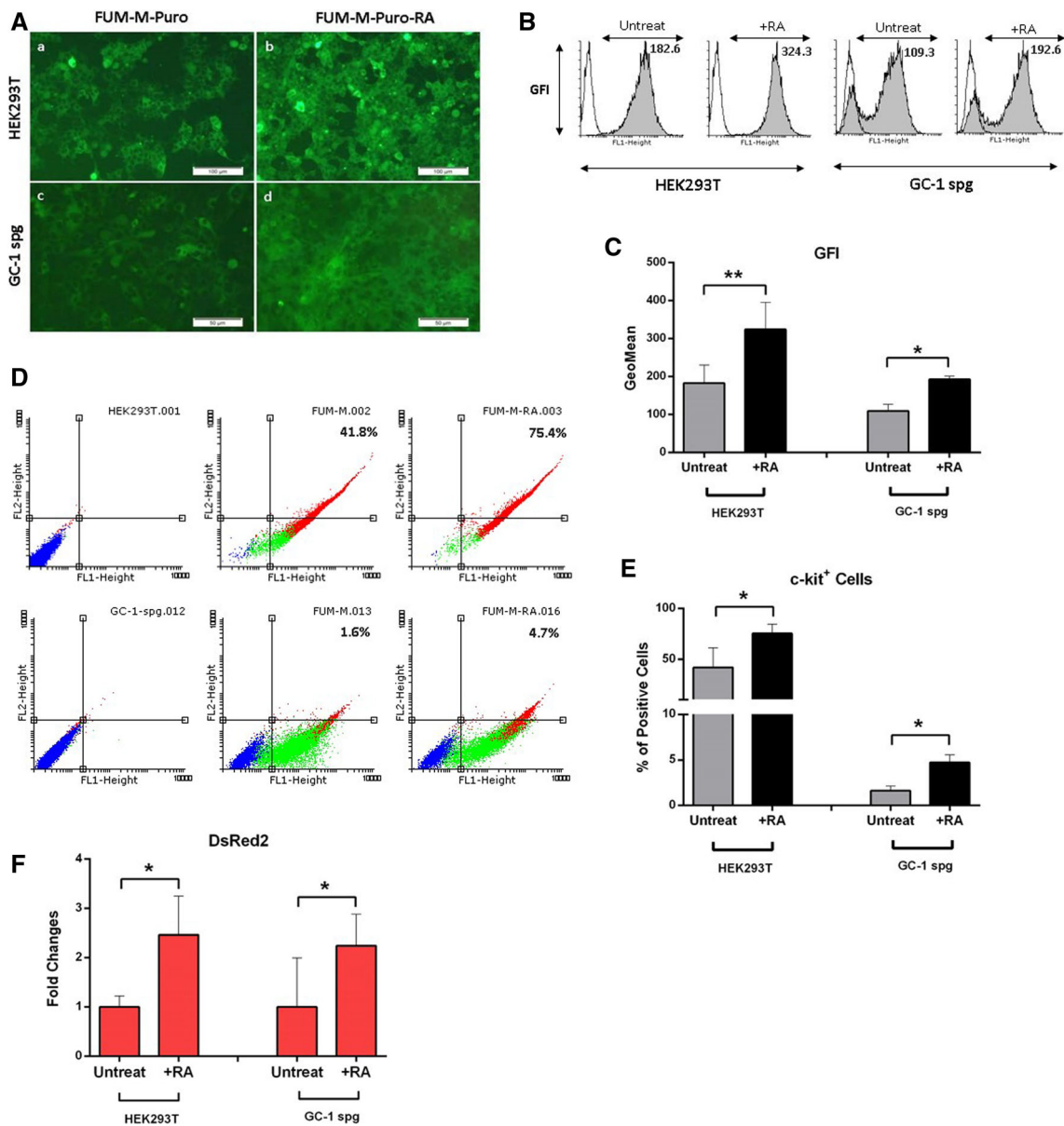
Fig. 5 Retinoic acid can significantly induce the activity of *Stra8* and *c-kit* promoters. The panels represent: fluorescence microscopic images of HEK293T (*a*, *b*) and GC-1 spg (*c*, *d*) cells transduced by FUM-M virus, which were selected by puromycin, after 48 h of RA treatment (**A**); fluorescence histograms of ZsGreen-positive cells by flow cytometry analysis after 48 h of RA treatment (HEK293T and GC-1 spg cells untransduced were as negative controls) (**B**); green fluorescence intensities (GFI) increased significantly 48 h after RA treatment in both cell lines (**C**); quantitative analysis of percentage of c-kit-positive cells in HEK293T and GC-1 spg 48 h after RA treatment (*Red* DsRed2, *Green* ZsGreen) (**D**, **E**); and quantitative RT-PCR analysis of DsRed2 expression 48 h after RA treatment in both cell lines (**F**). Untreat: cells transduced by FUM-M virus, which were selected by puromycin.*Significant difference as compared with control (*$*P \leq 0.05$, **$**P \leq 0.01$). The *error bars* indicate the standard deviations (Color figure online)

DMSO could negatively affect the promoter activity of the *Stra8* and *c-kit* promoters in FUM-M lentiviral vector.

Discussion

In the present study, we introduce a new strategy for timescale monitoring of activity of a dual promoter fused to reporter system for tracking the biological events in germ cell development. A novel lentiviral vector construct was made, based on an improved model from Tian and Andreadis [31]. The system described here has a number of advantages over the previously published system. First, using the dual specific promoters (*Stra8* and *c-kit*) for germ cells, with two reporter genes (ZsGreen and DsRed2), could report two independent events in the cells. They were

Fig. 6 DMSO reduces *Stra8* and *c-kit* promoters activity in FUM-M lentiviral vector. Panel **A** represents green fluorescence intensities (GFI) in GC-1 spg transduced by FUM-M virus, which selected by puromycin, after 12 h of DMSO treatment. Panel **B** represents percentage of c-kit-positive cells after 12 h of DMSO treatment. Panel **C** represents quantitative RT-PCR analysis of *Stra8* and *c-kit* expression after 12 h of DMSO treatment. Panel **D** represents quantitative RT-PCR analysis of DsRed2 expression after 12 h of DMSO treatment. Untreat: GC-1 spg cells transduced by FUM-M virus, which were selected by puromycin. *Represents significant difference as compared with control (*$P \leq 0.05$). The *error bars* indicate the standard deviations
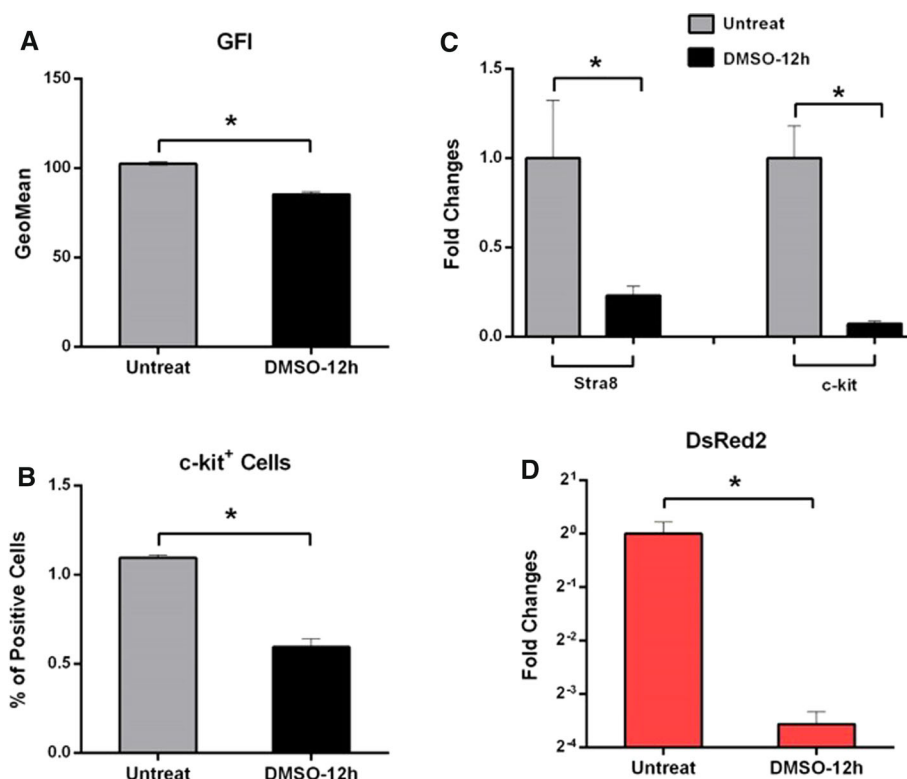
shown to be strictly regulated in response to different cellular environment. In addition, our system had an antibiotic (puromycin)-resistant gene fused to ZsGreen reporter gene. Application of 2A peptide, as a more reliable tool than the internal ribosomal entry site, leads to the expression of the resistant gene at equimolar level of ZsGreen [46]. This can provide an opportunity for using the system for selection of single cell clones that exhibit the *Stra8* promoter activity and checking the status of *c-kit* promoter, in the Stra8-positive cells in response to a particular treatment. Application of Two sets of insulators, cHs4 and SMAR8, and terminator sequences were included in the construct to overcome the possible position effects [31]. Besides both promoters were placed in antisense orientation relative to the viral LTR, to avoid the disruption of viral mRNA production by internal polyA, sequences were points to improve the designed independent dual promoters function [27–31]. The fact that we observed different levels of expressions for *Stra8* and *c-kit* promoters clearly shows that the insulator system is efficient.

In this study, we assayed the ability of dual promoter lentiviral vector (FUM-M) to drive the simultaneous expression of a pair of reporter genes in different cell lines. Although the flow cytometry analysis and fluorescent microscopy confirmed the functional capability of the promoters, the percentages of Stra8- and c-kit-positive cells were low. Thus, to monitor the dynamic behavior of FUM-M lentiviral vector in response to exogenous signals, we selected the Stra8-positive cells by an appropriate puromycin concentration. Antibiotic selection confirmed that the FUM-M construct was stably integrated into the genome, and its function is in accordance with the level of endogenous *Stra8* mRNA in the cells. In agreement with previous reports, flow cytometry results determined that RA could up-regulate the function of *Stra8* and *c-kit* promoters in FUM-M lentiviral vector in HEK293T and GC-1 spg cell lines [36, 47]. We also observed a down-regulation of *Stra8* and *c-kit* promoters in FUM-M lentiviral vector in response to DMSO according to quantitative RT-PCR [48–50]. However, DsRed2 intensity in all experiments was not strong enough to be detected with fluorescence microscopy, whereas both quantitative RT-PCR and also flow cytometric experiments confirmed sensitive response of the *c-kit* promoter in the presence of RA and DMSO.

Meanwhile, this experiment suffered from the fact that the red signals were not as strong as the green ones. This could be due to either inefficient maturation or folding of DsRed2 protein [51], low virus titer [52] or high specificity of the *c-kit* promoter [44]. This suggests that, in the system described here, DsRed2 gene cannot be an appropriate reporter to obtain true imaging and might be replaced with other reporter genes such as mCherry [51].

The potential of germ cells as a substitute for pluripotent stem cells can offer the opportunity to discover ambiguities in basic research [42, 53–55]. The activity of both *Stra8*

and *c-kit* promoters as germ cell markers [34, 37] has previously been reported using separate constructs [34, 37, 43, 44]. Our designed vector enjoys the advantage of having all the molecular components in a unique vector, to track the developmental events based on these two markers. This strategy would open new insights toward designing more complex vectors for monitoring the developmental events based on the promoter activity of the marker genes.

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