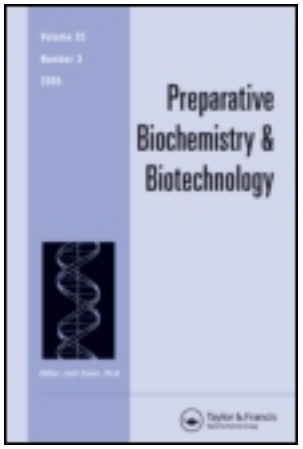

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A RAPID AND CONVENIENT METHOD TO ENHANCE TRANSGENIC EXPRESSION IN TARGET CELLS

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A RAPID AND CONVENIENT METHOD TO ENHANCE TRANSGENIC EXPRESSION IN TARGET CELLS

Chia-Hung Chen,[1] Yen-Ku Liu,[1] Yu-Ling Lin,[1] Huai-Yao Chuang,[2] Wei-Tung Hsu,[1] Yi-Han Chiu,[2] Tian-Lu Cheng,[3] and Kuang-Wen Liao[1,2]

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□ *Gene therapy provides a novel strategy and a new hope for patients with cancer. Unfortunately, the specifics of the delivery systems or the promoters have not achieved the specified efficacy so far, and the perfection of either system will be extremely difficult. In this study, we introduce a simple concept that a combination of a partially specific delivery system and a partially specific promoter activity may achieve a more specific effect on transgenic expression in target cells. The first section describes tumor-related transcription factors that were assayed in tumors or rapidly proliferating cells to determine their activities. The activities of nuclear factor (NF)-κB, CREB, and HIF-1 were higher, and three copies of each response element were used to construct a transcription factor-based synthetic promoter (TSP). The results showed that the expression of the TSP was active and partially specific to cell types. As described in the second section, the multifunctional peptide RGD-4C-HA was designed to absorb polyethyleneimine (PEI) molecules, and this complex was targeted to integrin αvβ3 on B16F10 cells. The results indicated that RGD-4C-HA could associate with PEI to mediate specific targeting in vitro. Finally, the combination of the PEI–peptide complex and TSP could enhance the specifically transgenic expression in B16F10 cells. This strategy has been proven to work in vitro and might potentially be used for specific gene therapy in vivo.*

Keywords integrin αvβ3, polyethyleneimine (PEI), RGD-4C-HA, transcription factor-based synthetic promoter (TSP)

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BACKGROUND

Mutations in oncogenes or tumor suppressors are thought to be critical factors in tumorigenesis. Thus, gene therapy is generally considered to be a useful treatment in the cancer therapy. There are several methods that provide benefits to patients with cancer, including the expression of tumor-suppressor genes in tumor cells, the ablation of an oncogene's function by RNA interference and ribozymes, and expression of a suicide gene that converts a harmless prodrug into a potent toxin in tumor cells.[1–4] The cancer gene therapy could be considered as having two parts: The delivery system and the regulation system depend on promoter activities.

The delivery systems consist of either a viral vector or a nonviral vector, and both have advantages and disadvantages.[5] In this study, we choose the nonviral vector polyethylenimine (PEI) due to its DNA capacity, safety, versatility, and ease of preparation. However, the use of PEI-derived gene delivery vehicles is still limited by a relatively low transfection efficiency and a short duration of gene expression.[6,7] Modifications of PEI are a potential way to improve its therapeutic efficiency.[8–10]

The specific expressions of therapeutic genes in different tumor cells are regulated by the promoter sequences prior to the transgenes. A highly specific and efficient transgenic expression in cancer cells is not yet available. Several promoters were reported to specifically regulate the expression of certain transgenes in different tumor cells,[11–13] and these promoters may be classified as cancer-specific promoters and tumor-related promoters. Nevertheless, most of these promoters were also expressed in nontumorigenic cells, and there have been difficulties in finding specifically tumor-expressed promoters.

Due to imperfections in both the delivery and regulation systems of cancer gene therapies, we proposed a simple concept: that the combination of a partially specific delivery system and a partially specific promoter activity may improve the efficacy of the expression of a specific gene in targeted cells. We chose the highly metastatic melanoma B16F10 cell line, which causes a high mortality rate in mice, as the targeted cell line. For improvement of the low transfection efficacy and low specificity, we modified PEI with the peptide RGD-4C-HA, which has the RGD-4C sequence that specifically binds to integrin $\alpha_v\beta_3$[14–16] on B16-F10 cells. On the other hand, we created a novel promoter based on the activities of transcription factors in B16F10 cells. The efficacy of a combination of the two modified systems was measured by use of the reporter gene hrGFP.

METHODS

Plasmids

pAP-1-hrGFP (containing seven copies of the AP-1 binding site), pCREB-hrGFP (containing four copies of the CREB binding site), pNF-κB-hrGFP (containing five copies of the NF-κB binding site), and pNFAT-hrGFP (containing four copies of the NFAT binding site) were purchased from Stratagene, Cedar Creek, TX. pAsRed2-N1 were purchased from Becton Dickinson, Moutain View, CA. pMZF-1-hrGFP (containing three copies of MZF-1 binding site), pCRII-hrGFP (containing seven copies of HIF-1 binding site), pAAV-MCS-hrGFP, and pARE-hrGFP were from our lab. All primers used in this study were purchased from MDBio, Taiwan, ROC.

Cell Lines

All cell lines used in this study were obtained from the BCRC (Food Industry and Development Institute, Taiwan) and cultured with Dulbecco's minimum essential medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) and 1% PSA (Biological Industries).

Construction of the Transcription Factor-Based Synthetic Promoter

The pD5-hrGFP was obtained by replacing the CMV promoter of pAAV-MCS-hrGFP with TSP. TSP was obtained by a direct ligation of insert1 and insert2. The insert1 and insert2 were obtained by primer annealing. The primers 5′ TSP1 (5′-CGC GTG GGA CTT TCC GCT GGG GAC TTT CCG CTG GGG ACT TTC CGC TGT GAC GTC AGA GAG-3′) and 3′ TSP2 (5′-TCA GCT CTC TGA CGT CAC AGC GGA AAG TCC CCA GCG GAA AGT CCC CAG CGG AAA GTC CCA-3′) were annealed by heating to 95°C for 5 min and then cooled to room temperature. The insert2 was obtained by annealing of 5′ TSP2 (5′- C**TG ACG TCA** GAG AGC **TGA CGT CA**G AGA GCT ACG TGT GTG TAC GTG TGT GTA CGT GAT-3′) and 3′ TSP1 (5′- CGA TCA CGT ACA CAC ACG TAC ACA CAC GTA GCT CTC **TGA CGT CA**G CTC TC**T GAC G** -3′) as described earlier. The primers contained three copies of the binding sites of NF-κB (underlined), CREB (bold), and HIF-1 (dotted). Each binding site was separated by at least a 4-nucleotides spacer according to the commercial design (Stratagene, Cedar Creek, TX). The 5′ end of insert1 and the 3′ end of insert2 were designed as a *MluI* and a *ClaI* protruding end,

respectfully. The insert1 and insert2 were phosphorylated by T4 polynucleotide kinase (NEB, Hitchin, UK) according to the manufacturer's protocol. The vector pAAV-MCS-hrGFP was double digested by *MluI* and *ClaI* (Fermentas, Burlington, Canada) and the CMV promoter was replaced by insert1 and insert2. The resulting clone, pD5-hrGFP, was obtained and sequenced.

Transcription Factor Activity Assay

There were 10^5 cells seeded in a 24-well plate and they were approximately 50% confluent at the time of transfection. Cells were transfected with the different plasmid DNAs respectively by polyethyleneimine.[17] Briefly, 1 μg of plasmid DNA and 6 μL of 5 μ*M* PEI (Aldrich, St. Louis, MO) were each diluted in 50 μL of 150 m*M* NaCl respectively and mixed. The PEI solution was added into DNA solution after 5 min and then mixed. After 20 min, the PEI-DNA mixture was gently added to each well and supplemented with 200 μL of Opti-MEM I Medium (Gibco, Grand Island, NY). After 18 hr of incubation, 700 μL of fresh growth medium was added into each well. After 48 hr, the cells were harvested and the hrGFP reporter gene expressions were measured by FACScan flow cytometry (BD, Moutain View, CA). In addition, the plasmid pAsRed2-N1, which has a transgene encoding the red fluorescent protein that is driven by the CMV promoter, was used as an internal control to normalize the transfection efficiency between each sample. In addition, ARE is a binding site of the prokaryotic transcription factor ampR and it was used as a negative control. Thus, the expression index of each transcription factor was calculated according to the following formula:

$$\text{Expression index} = \frac{\text{TFI of ARE} - \text{hrGFP/TFI of AsRed2 in ARE}}{\text{TFI of TFBS} - \text{hrGFP/TFI of AsRed2 in TFBS}} \times 100\%$$

where TFI is the total fluorescent intensity, and TFBS-hrGFP represented each plasmid containing the transcription factor binding sites.

RGD-4C-HA Cell-Binding Activity Assay

There were 2×10^5 cells suspended in staining buffer (1% bovine serum albumin [BSA], 0.05% NaN_3 in 1 × PBS) and incubated with 2 μ*M*, 200 n*M*, or 20 n*M* of RGD-4C-HA peptide (CDCRGDCFCGGGYPYDVPDYAGGGDD-DEC, MDBio, Taiwan, ROC) at 4°C for 1 hr. The cells that not incubate with RGD-4C-HA peptide served as a negative control. Then cells were subsequently washed and incubated with an anti-HA-FITC in staining buffer at

4°C for 1 hr. After 1 hr of incubation, the cells were washed and the surface immunofluorescence was measured by FACScan flow cytometer:

$$\text{RGD-4C-HA cell-binding activity} = \left(\frac{\text{total intensity of each sample}}{\text{total intensity of negative control}}\right) \times 100\%$$

PEI-RGD4CHA Complex Preparation

The PEI and RGD-4C-HA were incubated at room temperature for 30 min at a molar ratio of 1:1. The PEI-RGD4CHA complex was applied into a gel filtration column that was packed with Sephacryl S-200 (GE Healthcare, Chalfont St., UK), which followed the manufacturer's protocol for separating the un-absorbed products. The elution products were collected as 0.5 mL per fraction by fraction collector.

Ninhydrin Test

The PEI in the elution product of the PEI-RGD4CHA complex was identified by a ninhydrin test. One microliter of the elution products was dropped onto thin-layer chromatography (TLC) plates for 10 min and 1 μL of 15% ninhydrin solution (dissolved in methanol) was added. After 10 min of incubation, the TLC plate was dried and analyzed.

Dot Blotting

The PEI in the elution product of the PEI-RGD4CHA complex was identified by dot blotting. Ten microliters of the elution products was applied to a nitrocellulose (NC) membrane (Pall, Ann Arbor, MI) by a dot-blot machine (Bio-East, Taiwan). The NC membrane was blocked by 5% blocking buffer (5% skim milk in 1 × PBS buffer) at 37°C for 3 hr with shaking. The membrane was then washed with PBST (1 × PBS containing 0.05% Tween 20) 3 times at room temperature for 5 min. It was incubated with an anti-HA antibody (Roche, Basel, Switzerland) at room temperature for 1 hr with shaking. The NC paper was washed with PBST three times as already described and incubated with a polyclonal rabbit anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP; DakoCytomation, Denmark) for another 1 hr with shaking. Then the blots were washed, developed, and visualized by ECL detection according to the manufacturer's instructions (Pierce, USA).

PEI–Peptide Complex Transfection

The PEI and RGD-4C-HA were incubated at room temperature for 5 min at different molar ratios. After 5 min of incubation, the PEI–peptide complex was used as the native PEI. The transfection was performed as described earlier.

Statistical Analysis

The results were expressed as the mean $\pm$ SE. Each experiment was performed at least three times and duplicated in each group. All results were compared by a Student's *t*-test, and $p < 0.05$ was considered to be significant.

RESULTS

Screening for Activities of Several Tumor-Related Transcription Factors in B16-f10 and Other Cells

In order to construct a transcription factor-based synthetic promoter that is selectively expressed in tumors, particularly in B16F10 (mouse melanoma cells with high metastatic potential), we used several plasmids that contain tumor-related transcription factor binding sites, respectively, to screen for the activities of each transcription factor in B16F10 cells and HeLa cells (human cervical carcinoma cells). The respective transcription factor activity was represented by an expression index, as described in the Methods section. Among the transcription factors analyzed (nuclear factor [NF]- κB, CREB, HIF-1, AP-1, NFAT, and MZF-1), the activities of HIF-1 and NF-κB were higher than those of the other transcription factors in these cells. Besides, the expression indexes of CREB were higher than negative control (ampR) in all cell types, but the data were not significant under statistical analysis (Figure 1).

The Transcription Factor-Based Synthetic Promoter is Highly Expressed in B16-F10 and Hela Cells

According to the screening results, the activities of NF-κB and HIF-1 were relatively higher in these tumor or rapidly proliferating cells. In addition, CREB was considered to play an important role in cell transformation, including melanoma, and to contribute to tumor metastasis and invasion.[18] Thus, we assembled the binding sites of NF-κB, CREB, and HIF-1 (three copies of each binding site) to create a novel synthetic mini-promoter that is selective for highly metastatic tumors (Figure 2A).

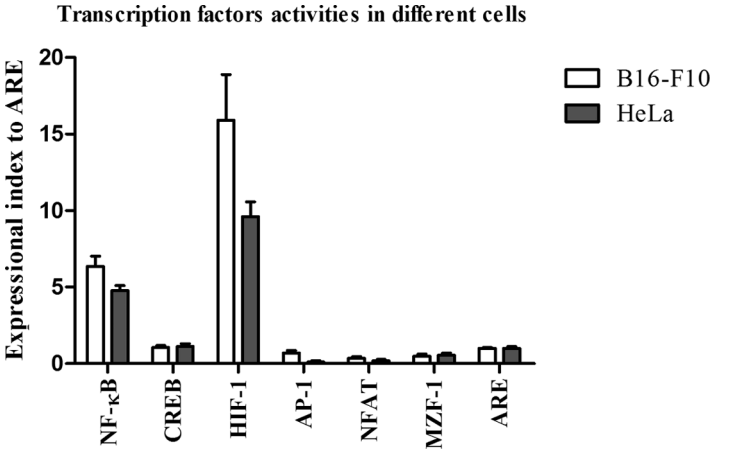

FIGURE 1 An investigation of the activities of several transcription factors in different cells. The activities of NF-κB, CREB, HIF-1, NFAT, and MZF-1 were analyzed in different cells by co-transfection with a reporter plasmid (pAsRed2-N1). The fluorescence intensity of 10^4 viable cells was determined by flow cytometry after 24 hr of transfection. The expression indexes represented the activities of the transcription factors. The results showed means ± SE of two independent experiments ($n = 4$).

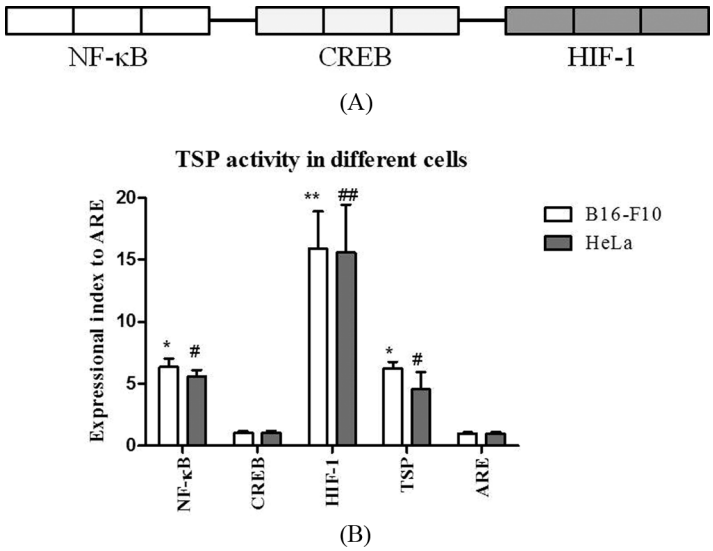

FIGURE 2 The schematic diagram and activities of the transcription factor-based synthetic promoter (TSP). (A) The novel mini promoter TSP was composed of three copies for the binding sites of NF-κB, CREB, and HIF-1. (B) The activities of NF-κB, CREB, HIF-1, and TSP were analyzed in different cells by co-transfection with a reporter plasmid (pAsRed2-N1). The fluorescence intensity of 10^4 viable cells was determined by flow cytometry after 24 hr of transfection. The expression indexes represented the activities of transcription factors. The results showed means ±SE of two independent experiments ($n = 4$). In B16F10 cells groups, asterisk indicates significant at $p < 0.05$ compared to ARE group, and double asterisk, significant at $p < 0.01$ compared to ARE group. In HeLa cells groups, # indicates significant at $p < 0.05$ compared to ARE group, and ## indicates significant at $p < 0.01$ compared to ARE group.

The CMV promoter in pAAV-MCS was replaced with the artificial promoter,[17] and the new plasmid was named pD5-hrGFP. In order to verify the efficiency of TSP, the transcription factor binding site-containing plasmids pD5-hrGFP, pCRE-hrGFP, pCRII-hrGFP, pNF-κB-hrGFP, and a control plasmid (pARE-hrGFP) were cotransfected with pAsRed2-N1 respectively into different cells. The results showed that the expression indexes of TSP were sixfold and fourfold higher than ampR (ARE) in B16-F10 and HeLa cells, respectively. The results suggested that TSP was indeed active in these tumor or rapidly proliferating cells. In addition, the expression indexes of HIF-1 were 15-fold and 15-fold higher than ampR (ARE) in B16F10 and HeLa cells, respectively. It was noticed that the HIF-1 retained the highest activity in two different cells, which meant the promoter could also potentially be utilized in cancer gene therapy (Figure 2B).

RGD-4C-HA is Selectively Bound to B16F10 Cells Compared to Hela Cells

To target to the highly metastatic B16F10 cells by two partially specific methods, we designed the multifunctional peptide RGD-4C-HA (Figure 3A) and investigated its targeting activity. Different concentrations of RGD-4C-HAs were tested on both types of cells to determine the binding activity. The results showed that the total fluorescent intensities increased significantly in B16F10 cells compared to the negative control for all concentrations, whereas the total fluorescent intensities had no differences compared to negative control in the HeLa cells (Figure 3B). This result revealed that the RGD-4C-HA peptide could selectively target B16F10, and that the targeting was more specific with higher concentrations (2 μM and 200 nM).

The RGD4CHA-PEI Complex Specifically Enhanced Reporter Gene Expression in B16F10 Compared to Hela Cells

To verify that our design for RGD-4C-HA could interact with positively charged residues and the primary amine group of PEI to form a new complex, we prepared and confirmed the components of this complex. The RGD4CHA-PEI complex was prepared by gel filtration as described in the Methods section. Then each fraction was examined to analyze PEI by a ninhydrin assay and RGD-4C-HA by a dot-blot analysis with an anti-HA antibody. Ninhydrin reacted to a primary or secondary amine group to produce a colored product (usually yellow to brown). Although the RGD-4C-HA peptide also has the primary amine group, the ninhydrin in this assay would not interact with RGD-4C-HA, probably because the concentrations

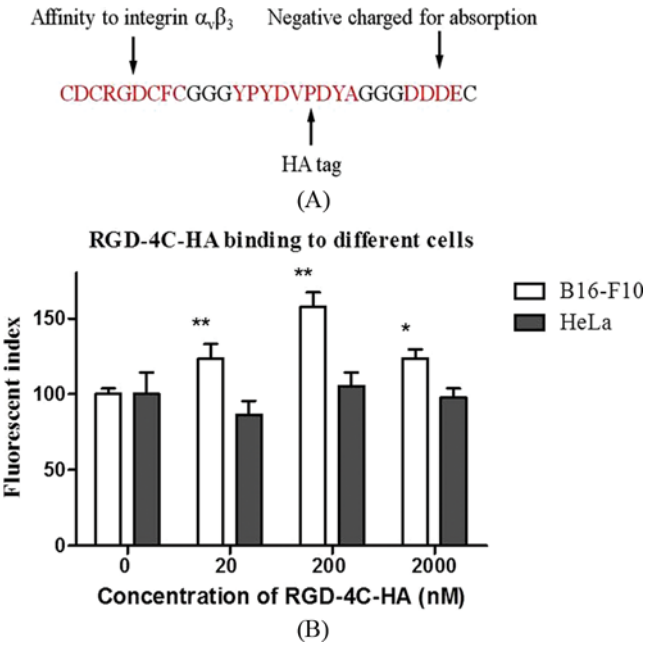

FIGURE 3 The schematic diagram and binding efficacy of RGD-4C-HA to different cells. (A) The RGD-4C-HA was a multifunctional peptide. The affinity region was the RGD-4C (CDCRGDCFC) sequence, which possessed a high affinity toward B16-F10. The identity region, the HA tag (YPYDVPDYA), acted as a spacer and an epitope that was used for detection. The absorption region (DDDE) contained four continuous amino acids with negatively charged residues. The two GGG sequences were spacers to separate the functional domains. The RGD-4C-HA with different concentrations were used to bind to the different cells, and the peptides on the cell surface were then detected by an anti-HA antibody that was conjugated to FITC. (B) The surface immunofluorescence of 10^4 viable cells was determined by flow cytometry. The result showed that the total fluorescent means in the different cells. The data shown were the means $\pm$ SE of two independent experiments ($n = 4$). *Indicates significant at $p < 0.05$ compared to control, and **Indicates significant at $p < 0.01$ compared to control (color figure available online).

of the amine groups in RGD-4C-HA were below the detection limits of ninhydrin. The elution product was positive for both the ninhydrin test and the dot immunoblotting (Figure 4), which revealed that the RGD-4C-HA peptide might interact with the PEI to form complexes by either electrostatic forces or covalent bonds.

The RGD4CHA-PEI Complex Selectively Enhanced TSP-Driven Gene Expression in B16F10 But Not in Hela Cells

To verify the efficacy and specificity of the RGD4CHA-PEI complex on reporter gene expression in the different cells; the plasmids containing TSP promoter were transfected into HeLa and B16F10 cells. Compared to

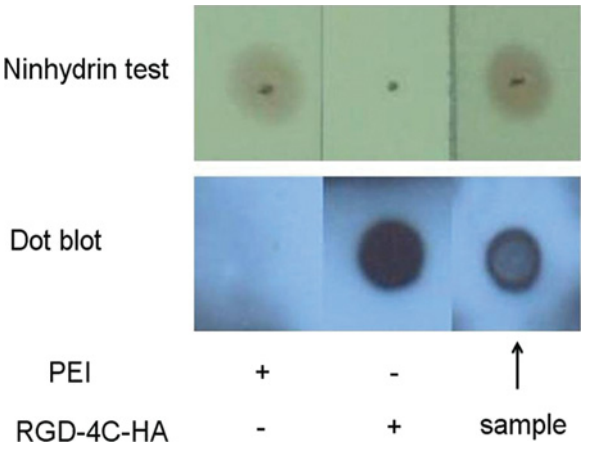

FIGURE 4 PEI and RGD-4C-HA absorption assay. The mixture of the PEI and RGD-4C-HA were purified by using a gel filtration column Sephacryl S-200 and the products were collected and tested after elution. The ninhydrin test was used to monitor the existence of PEI, and dot immunoblotting was used to determine the existence of RGD-4C-HA. PEI alone and RGD-4C-HA alone were used as control groups, and the elution product from a Sephacryl S-200 column (PEI–peptide complex) was used as the sample. The results shown are from one out of three independent experiments (color figure available online).

the PEI transfection, the RGD4CHA-PEI complex only selectively enhanced TSP-driven reporter gene expressions in B16F10 cells but not in HeLa cells (Figure 5). According to these results, the combination of a partially specific delivery system and a partially specific expression system could selectively express a specific gene in B16F10 cells.

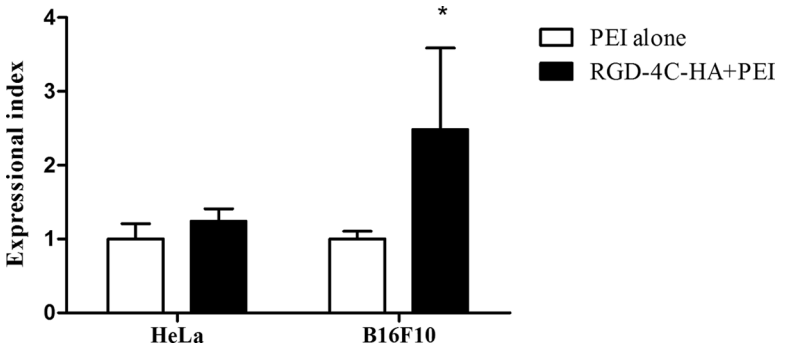

FIGURE 5 The gene expression of the plasmid in different cells. The transfection experiments were performed as already described. The fluorescence intensities of the transfectants were measured by flow cytometry, and the ratios of fluorescent expression were compared to the PEI transfectants. The plasmid pD5-hrGFP, which had a transgene encoding the green fluorescent protein that was driven by TSP, was used as a reporter gene. The results showed means ± SE of two independent experiments ($n = 4$). *Indicates significant at $p < 0.05$ compared to control.

DISCUSSION

In this study, we provided a rapid and convenient method to improve the targeting efficacy of a cancer gene therapy by a dual-targeting strategy. First, we constructed a novel mini-promoter TSP, which contained three copies of the NF-κB, CREB, and HIF-1 response elements (Figure 2A) that were selected from several tumor-associated transcription factors through a screening of their activities (Figure 1). TSP could overexpress a gene in B16F10 and HeLa cells similar to NF-κB mini-promoter; however, the HIF-1 mini-promoter showed a stronger effect than the TSP (Figure 2B). Notably, the copy numbers were different among these mini-promoters. The NF-κB mini-promoter contains four copies of the NF-κB response element, and the HIF-1 mini-promoter contains seven copies of the HIF-1 response element. The copy number of each response element on the mini-promoter may affect the activity of expression. For another part of our strategy, we easily constructed the peptide-PEI complexes (Figure 4) containing the modified peptides, RGD-4C-HA (Figure 3A), to target to B16F10 selectively (Figure 3B). The combination of a partially specific delivery system by RGD-4C-HA and a partially specific regulation by the TSP could achieve a specific enhancement in transgenic expression in B16-F10 cells (Figure 5).

The most concern for cancer therapies is to achieve the highest levels of specificity and efficacy. This was partly accomplished by targeting the transcription of therapeutic genes via specific response elements to malignant cells.[19,20] Many tumor-associated promoters have been applied for targeting gene therapies; for example, tyrosinase promoter expressed tissue-specifically in melanoma.[12] However, the activities of these promoters were too weak to have therapeutic effects, and combined or synthetic elements would be used.[20,21] The modified promoter has the advantages of being that more selective[22] and not being repressed at particular stages of tumor progression.[23] In addition to these benefits, the TSP mini-promoter in this study consisted of only 110 bps, which can be easily modified to improve specificity and efficacy by inserting other transcription factor response elements. Besides, the short transgenic genes can be easily manipulated for various viral and nonviral vectors for a range of applications.

The cationic PEI has recently emerged as a favorable candidate for nucleic acid (NA) delivery in vivo and in vitro because of its high effectiveness at a low cost.[24] The PEI transfection vesicle has advantages such being as easy to prepare, being relatively safe, and having large DNA capacity.[25] The selectivity of a cancer gene therapy that uses PEI as a DNA transfer vehicle could also be improved by modifying the PEI complexes with antibodies or ligands which bound to tumor-associated surface

molecules.[26–28] Similarly, PEI in combination with targeting peptides applies to encapsulate small interfering RNA (siRNA) and specifically and efficiently inhibits tumor growth.[29,30] In this study, we coupled PEI with the doubly cyclized peptide RGD4C, which has a higher binding affinity and specificity for the $\alpha_V\beta_3$ integrin than the linear peptide RGD.[16,31] It has been reported that $\alpha_V\beta_3$ integrin overexpressed in many tumor cells such as mouse melanoma B16F10 and plays important roles in tumor-induced angiogenesis and tumor metastasis,[32,33] which suggests that $\alpha_V\beta_3$ could be a good candidate for tumor targeting. It has been widely reported that RGD-PEI or RGD-PEG-PEI could be used for integrin-targeting gene transfer.[34,35] Considering the previous studies and our expectations, we first constructed RGD4CHA-PEI for the delivery system and then combined it with the TSP regulation system, which resulted in a selective gene expression in the targeted B16F10 cells.

CONCLUSIONS

Taken together, this study has provided a potential strategy to overcome traditional challenges of cancer gene therapy. It achieved a more selective transgenic expression in specific cells by a convenient dual targeting method, and it provided an easily modified platform for further applications in cancer gene therapy.

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