國立交通大學

生物科技系暨研究所

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建立一快速且便利的方式以增強轉殖基因在特定細胞中的表現

Development of a rapid and convenient method to enhance the transgenic expression in target cells

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摘要

基因治療對癌症病患提供了前所未有的治療策略及希望。不幸的是，無論是基因傳遞或啟動子系統迄今仍未達到專一性之療效，除此之外，任一系統的最佳化都極端困難。在本研究中我們希望介紹一個簡單的概念：亦即部分專一的基因傳遞系統以及部分專一的啟動子相結合，將可對標靶細胞達到更加專一性的表現。在第一部分，我們首先檢測與腫瘤相關的轉錄因子在腫瘤或快速生長細胞中的活性。接著利用表現量較高的轉錄因子(NF-κB，CREB 以及 HIF-1)之反應片段，取三倍體構築一轉錄因子相關之合成啟動子(Transcription factor-based synthetic promoter，TSP) 。實驗結果證實 TSP 在特定細胞中具有活性並有部分專一性。此外相對於 NF-κB 或 HIF-1 迷你啟動子，TSP 在抑制劑存在之下表現較佳的抵抗性。 在第二部分，多功能胜肽 RGD-4C-HA 可專一性結合至 B16-F10 細胞表面之 integrin $\alpha_v\beta_3$ 並且吸附至聚乙烯亞胺(Polyethyleneimine，PEI)。實驗結果顯示 RGD-4C-HA 能與聚乙烯亞胺形成複合物並且在 *in vitro* 實驗中引導專一性的指向。最後，聚乙烯亞胺及胜肽複合物與 TSP 的結合能夠使轉殖基因專

一性的表現在 B16-F10 細胞中。這種策略在 *in vitro* 實驗中已經證實為可行，並且在 *in vivo* 的專一性基因治療可能也具有潛力。

Development of a rapid and convenient method to enhance the transgenic expression in target cells

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ABSTRACT

Gene therapy provides a novel strategy and a new hope for the patients with cancer. Unfortunately, the specificity of the delivery systems or the promoters did not achieve the specific efficacy so far and the perfection of either system will be extremely difficult. In this study we had introduce a simple concept that the combination of partial specific delivery and partial specific promoter activity may achieve more specific effect for specific expression in target cells. In the first part, the tumor related transcription factors were assayed in tumor or rapid-proliferating cells to determine their activities. The activities of NF-κB, CREB, and HIF-1 were higher and three copies of each response elements were used to construct a transcription factor-based synthetic promoter (TSP). The results showed that the expression of TSP was truly active and partial specific to cell types. In addition, it was more resistant than NF-κB or HIF-1 mini-promoters at the presence of inhibitors. In the second part, the multi-functional peptide RGD-4C-HA was designed to specifically target integrin $\alpha_v\beta_3$ on B16-F10 cells and absorbed to polyethyleneimine (PEI) molecules. The results showed that RGD-4C-HA could associate with PEI to form complex and mediate specific targeting *in vitro*. Finally, the combination of PEI-peptide complex and TSP could enhance the specifically transgenic expression in B16-F10 cells. This strategy had been proven to work *in vitro* and might be also potential in specific gene therapy *in vivo*.

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不知不覺兩年的時間就過去了，回想起碩士班的時光，真是如夢似幻，從昨日無知的門外漢，到今天成為碩士班畢業生，歸根究柢，還是要感謝許多人的支持與幫助，父母親生我育我，教化之功自是不在話下，然而在學習的過程中，我的指導教授廖光文博士更是居功厥偉，承蒙他不嫌棄我這個應化出身對生物一無所知的稚子，本著教育理念在各方面都不吝給予指導，無論是學業或是待人接物，他告訴我如何能夠有效率的學習，是我碩士班的學業成績，一直名列前茅，甚至得到從未得過的書卷獎，重拾我對學習的信心，當研究遇到瓶頸時，也在在顯示其靈活的思維以及解決問題的頭腦，讓我獲益良多，了解科學的態度，學到許多做人做事的道理，如果沒有他，我不會學到這麼多。

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Abbreviations

AAV	Adeno-associated virus
AFP	α fetoprotein
ATF1	Activating transcription factor 1
CBP	CREB binding protein
CCSST	Clear-cell sarcomas of soft tissues
CEA	Carcinoembryonic antigen
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CREB	cAMP response-element binding protein
EWSR1	Ewing sarcoma gene
GCV	Ganciclovir
HER-2/neu	Human epidermal growth factor receptor 2/neu
HIF-1	Hypoxia-inducible factor-1
HREs	Hypoxia response elements
HSV-tk	Herpes simplex virus thymidine kinase
HTLV	Human T-cell leukemia virus
hTR	Human Telomerase RNA
hTRET	Human telomerase reverse transcriptase
IAPs	Inhibitor of apoptosis proteins
IKK	IκB kinase
IL-8	Interleukin-8
IPAS	Inhibitory PAS domain protein
kDa	Kilo dalton
NF-κB	Nuclear factor-kappaB
PEI	Polyethyleneimine
TFBS	Transcription factor binding sites
TLC	Thin layer chromatography
TSP	Transcription factor-based synthetic promoter
VEGF	Vascular endothelial growth factor

Chapter 1 Introduction

Gene therapy was defined as transferring genetic material into cells in order to cure a disease or improve the clinical status of a patient. It has been a promising tool in diseases caused by genetic defects such as severe combined immunodeficiency, cystic fibrosis, hemophilia, Parkinson's disease and Alzheimer's disease [1]. For example, cystic fibrosis (CF) is a lethal autosomal recessive disease caused by a mutation in the cystic fibrosis transmembrane regulator (CFTR) gene for a chloride ion channel expressed in epithelial cells of lung. The gene therapy for CF is to deliver CFTR cDNA to the epithelial cells that line the lumen of the conducting airways of the lung by inhaled aerosol, liposomes, or viruses [2-5]. However, there are still some limitations to impede successful outcome of gene therapy. The targeting of multiple therapeutic genes into the gene-defective cells may be required in order to develop an effective therapeutic strategy. In cancer gene therapy, the specific regulation of gene expression in tumor cells should also be improved.

1.1 Cancer gene therapy

Development of cancer has been suggested to occur through a series of molecular events in which multiple genetic abnormalities accumulate within cells

and has been called ‘‘multistep carcinogenesis.’’ Mutations in oncogenes may result in excessive activity or expression of the oncogene product. In contrast, tumor suppressor genes may be mutated or deleted resulting in decreased activity or expression of the tumor suppressor gene product. Either case could result in abnormal growth regulation resulting in the cancer phenotype. As above, gene therapy is generally considered as a useful tool in the treatment of cancer. There are several ways to provide the benefits to the patients with cancer including the expression of tumor-suppressor genes in tumor cells, ablation of oncogene function by RNA interference and ribozymes, and expression of a suicide gene that converts a harmless prodrug into a potent toxin in tumor cells [6-8]. Tumor suppressor genes such as *pRb* and *p53* play a critical role in the regulation of the cell cycle or promote apoptosis. Restoration of wild type *p53* gene expression using a retroviral *p53* vector inhibited cell growth and induced apoptosis in human lung cancer cells with mutated or deleted *p53* genes [9, 10]. Besides, the products of the *p16* tumor suppressor gene and a truncated *Rb* gene have been shown to suppress tumor growth in animal models [11, 12]. In ablation of oncogenes, adenovirus-mediated ribozyme targeting of HER-2/neu inhibited *in vivo* growth of breast cancer cells in a mouse model [13]. In similar way, intravenous tail injections of an Ad E1A construct in a mouse model inhibited the intratracheal growth of HER-2/neu overexpressing lung cancer cells [14]. In suicide gene therapy, the herpes simplex

virus thymidine kinase (*HSV-tk*) can specifically bind and phosphorylate nucleoside analogs such as acyclovir and ganciclovir (GCV), which blocks DNA synthesis and causes cell death. Human lung cancer cells have been shown to be selectively killed after transduction with retrovirus vectors carrying the *HSV-tk* gene and systemic administration of GCV [15]. As above, gene therapy provides a novel strategy and a new hope for the patients with cancer, although it is still not capable of completely eradicating malignant tumor cells in humans. The cancer gene therapy usually consists of two fields: the delivery system and the regulation system depend on promoter activities. In this study we focused on the non-viral delivery system and the promoter activities and they were discussed respectively in the following sections.

1.2 Delivery system for cancer gene therapy

The gene delivery systems can be divided into two categories: the viral vectors and non-viral vectors. Numerous viral or non-viral vectors for gene delivery to human body *in vivo* and *in vitro* have been developed. Whereas viral or non-viral vectors have certain advantages or limitations for themselves [16], they were both developing to reach available condition for the clinical treatment. In this study, we focused on the non-viral vectors as the gene delivery tool because of their safety, versatility and ease of preparation.

1.2.1 Viral vectors

Viruses are natural genetic material carriers that can efficiently introduce foreign DNA into specific cells. Viral vectors are available for clinical trials of gene therapy, such as almost 40% of all gene therapy clinical trials for cancer patients use adenoviral vector to deliver therapeutic genes [17]. Although recombinant adenoviral vectors have high titer advantage and are utilized for the clinical trials, their high immunogenecity for human impair their availabilities. Recently, adeno-associated virus (AAV) has been demonstrated that they are capable of inducing transgene expression in a broad range of tissues for a relatively long time without stimulation of a cell-mediated immune response [18]. However, the broad host tropism of AAV still remains a problem which impairs the availability in gene delivery. Moreover, the limited size of the genetic material through viral vectors is another limitation.

1.2.2 Non-viral methods

Investigators in non-viral vector development have introduced a variety of strategies to overcome barriers for gene delivery [19-21]. These include (a) polynucleotide degradation in the extracellular space, (b) internalization of the carrier, (c) intracellular trafficking from the endosome to the lysosome and the

escape of the polynucleotide from the endosome, (d) dissociation of polynucleotide from the carrier and (e) entry of the polynucleotide into the nucleus. Carrier molecules, such as polyethyleneimine (PEI), which can condense polynucleotides and provide protection against nucleases, is the major components of the delivery system. However, the positively charged DNA–cationic carrier complex tends to aggregate when injected into the blood and lacks tissue specificity. When entrapped in an acidic endosomal environment following endocytosis, components in the carrier that possess a proton sponge or endosomolytic activity will cause endosome rupture, thereby releasing the encapsulated polynucleotide. In this study, we used PEI as a gene delivery tool because of their ease of application.

1.2.2.1 Polyethyleneimine (PEI)

The cationic polymer polyethylenimine (PEI) has been widely used for non-viral transfection in vitro and in vivo and has an advantage over other polycations in that it combines strong DNA compaction capacity with an intrinsic endosomolytic activity. A large variety of different polymers and copolymers of linear, branched, and dendrimeric architecture, have been tested, in terms of their efficacy and suitability for in vitro transfection. It shows no morphology emerged as a general favorite [22]. The results from transfection experiments with PEI were impressive from the beginning. Depending on the linkage of the repeating

ethylenimine units, PEI occurs as branched or linear morphological isomers. Branched PEI derived vectors have been used to deliver oligonucleotides [23], plasmid DNA, and Epstein–Barr virus-based plasmid vectors [24] as well as RNA and intact ribozymes [25]. The efficacy of bPEI-derived vectors non-viral vectors and their cytotoxic effects depend to a remarkable extent on material characteristics like the molecular weight, the degree of branching, the cationic charge density and buffer capacity [26-28], polyplex properties, such as the DNA content, particle size and zeta potential and the experimental conditions like the polyplex concentration, the presence or absence of serum during transfection, the incubation time and the transfection model chosen for the gene delivery experiment. However, the use of PEI-derived gene delivery vehicles is still limited by a relatively low transfection efficiency and short duration of gene expression [29, 30]. The modification of PEI is a potential way to improve the therapeutic efficiency. Labeling of PEI/DNA complexes with receptor–ligand transferring could thereby enhance the gene expression in tumor cells, due to the efficient internalization of the transfecting complexes into the tumor cells via receptor-mediated endocytosis [31].

1.3 Promoters for cancer gene therapy

The specific expressions of therapeutic gene in different tumor cells are regulated by promoter sequences prior the transgenes. High specificity and efficacy

of transgene expression in cancer cells is not completely available until now. To address the purposes, the promoters of tumor associated antigen were used as tumor-specific promoters for gene therapy. Although the tumor-specific promoters are useful tools to accomplish specific expression in targeted tumor cells, low levels of gene expression is the chief defect of these tumor-specific promoters [32]. Several promoters were reported to specifically regulate certain expression of transgenes in different tumor cells [32-34] and these promoters may be classified as cancer specific promoters and tumor related promoters.

1.3.1 Cancer specific promoters

Cancer specific promoters are specific for the malignant process, such as telomerase related promoters. The activation of the telomerases activity is always considered as a critical step in cancer progression. The activities of telomerases exist in approximately 90% of human cancer cells, but are much lower or undetectable in normal somatic tissues [35-37]. Telomerase consists of an RNA component [human Telomerase RNA(hTR)] and a reverse transcriptase component [human telomerase reverse transcriptase (hTERT)] in human [38]. The hTR is not translated and remains as RNA and the hTERT functions as adding single-stranded telomere repeats into chromosome. Researchers had measured the expression levels of hTR and hTERT in a panel of 10 cell lines to demonstrate that the promoters of

hTR and hTERT are tumor-specific in tumor cells but not normal cells [39]. Moreover, there are cancer specific promoters oncofetally related with tissue specificity. Certain types of tumor often have genes overexpression of oncofetal origin that are silent in normal tissue. The most well-characterized promoters of these tumor-specific genes are the carcinoembryonic antigen (*CEA*) [40, 41] and α fetoprotein (*AFP*) [42, 43]. They are expressed in adenocarcinomas and hepatocellular carcinomas, respectively. These promoters have a potential in targeting a wide range of different tumor types and have been developed in cancer gene therapy.

1.3.2 Tumor related promoters

Tumor related promoters including tumor microenvironment-related promoters and tumor vasculature-related promoters. The former is responding to the tumor microenvironment and physiology such as hypoxia and glucose regulation. Many genes are transcriptionally upregulated in response to hypoxia which are mediated by the inducible transcription complex, hypoxia-inducible factor-1 (HIF-1). HIF-1 binds to hypoxia response elements (HREs) within these genes and activates the downstream gene expression. Therefore, HREs may be used to drive transgene expression specifically within tumor hypoxia areas. It is extremely important to target this population of cells since they are highly resistant

to other forms of treatment, such as radiotherapy and chemotherapy [44]. In addition to oxygen starvation, tumors can also be deprived of glucose that leads to the increased expression of genes involved in glucose metabolism. The promoters of these genes are also used to drive transgene expression specifically within a tumor [45, 46].

Another tumor related promoters are tumor vasculature-related promoters which are more active in the tumor vasculature than normal one. It has been reported that genes are upregulated in proliferating endothelium cells of tumor blood vessels [47]. The endothelial-specific kinase inserts domain receptor (KDR/flk-1) and E-selectin promoter have been indicated to enhance transgene expression in tumor endothelium [48]. Recently, it was demonstrated that the KDR/flk-1 promoter is not only endothelial cell-specific, but also actives in human ovarian cancer cell lines [49].

The use of cancer specific or tumor related promoters is promised to improve the safety of cancer gene therapy. However, the activities of these promoters are much weaker than the current benchmark CMV promoter [32]. The herapeutic efficacy might be limited when employing these kind of weak promoters.

1.4 Activities of NF-κB, HIF-1, and CREB in cancer progression and therapy

1.4.1 Transcription factor binding sites for expression

Eukaryotic transcriptional regulatory factors are conducted synergistically by multiple transcriptional regulatory factors [50]. These factors can bind to the promoter regions called transcription factor binding sites (TFBSs). TFBSs are usually short (about 5-15 base-pairs) and they are frequently degenerate sequence motifs [51]. The sequence degeneracy of TFBSs has been selected through evolution and is beneficial, because it confers different levels of activity upon different promoters, causing certain genes in specific cells to be transcribed at higher levels than other cells [51]. Although the sequences of TFBSs are degenerated, they still have consensus sequences, such as NF-κB element consensus sequence 5'-GGGPuNNPyPyCC-'3, which can be recognized by specific transcription factors. The orientations and functions of TFBSs are not absolutely correlated. The positions within a promoter can be varied in yeast, and in higher eukaryotes they can be placed upstream, downstream, or in the introns of the genes which they regulate. In addition, they can be placed close to or far away from regulated genes [51]. When one transcription factor interacts with other transcription factors and results in high levels of a transcriptional activation, it is called "synergism or synergistic effect". This phenomenon usually forms a ternary protein-protein-DNA complex which leads to altered DNA conformation and allowed other factors to bind on [52-54]. Interactions between two factors may be

direct or mediated by co-activators [40, 52]. For example, the coordination of c-Rel and ATF-1/CREB2 is mediated by p300/CREB-BP [53]. In some cases, two factors binding to DNA independently can still activate transcription synergistically [55-57]. A number of factors are known to bend the DNA structure and thus permit binding of other factors [58, 59]. For example, Fos and Jun can induce a corresponding alteration in the conformation of the DNA helix [59]. Furthermore, a variety of elements can contribute to promoter activity, but none is essential for all promoters [60]. Some transcription factors are specific in tissues and contributing to cell development [61]. Transcription factors play a major role in tumor progression. For example, NF-κB promotes cell cycle progression, regulates apoptosis, and facilitates cell adhesion [62]. Recently, many strategies have been used to enhance the potency of promoters needed to retain the tumor specificity in order to maintain potential therapeutic benefits. It is noticed that the transcription factors can recognize DNA sequence specifically and can be utilized in the promoter specificity. In next section, the roles and applications of NF-kB, HIF-1, and CREB in cancer progression and therapy will be discussed.

1.4.2 Nuclear factor-kappaB (NF-κB)

Nuclear factor-kappaB (NF-κB) is a common transcriptional factor that regulates many gene expressions. Many diseases are related to NF-κB, such as

cardiovascular diseases [63], muscular dystrophy [64], inflammatory diseases [65], and cancers [66]. In this section the relationship of NF-κB and cancers are discussed.

1.4.2.1 Biology of NF-κB

NF-κB was first found in B-lymphocytes [67] but NF-κB didn't only restrict to B-lymphocytes. For example, the stimulation of NF-κB by lipopolysaccharide [68] or phorbol ester was observed in a T cell line [53] and a non-lymphoid cell line [69] [70]. NF-κB belongs to the Rel family transcriptional factors, including Rel-A (also known as p65), Rel-B, c-rel, p50/p105 and p52/p100 [71]. The mature DNA-binding forms of p105 and p100 are shortened forms called p50 and p52, respectively. Unlike most transcriptional factors, proteins of this family reside in the cytoplasm and must translocate into the nucleus to work [72]. All NF-κB proteins contain a highly conserved Rel-homology domain (RHD) that is responsible for DNA binding, dimerization, nuclear translocation and interaction with the IκB proteins. The IκB proteins, including IκBα, β and ε, bind to NF-κB via ankyrin repeats and block its nuclear import and transcriptional activity [71]. Generally, NF-κB dimerization is the classical p50-p65 heterodimer which binds on the 5'-GGGANNYYCCC-3' consensus sequence [40] to regulate gene expression. NF-κB can regulate many gene expressions, such as cytokines/chemokines, cell

adhesion molecules, acute phase proteins, and cell-surface receptors, regulators of apoptosis and transcription factors.

1.4.2.2 NF-κB in cancer progression

The NF-κB family might act as tumorigenic transcription factors was first put forward upon the cloning of the p50/p105 subunit [73, 74] and analyzed its sequence. Sequence analysis revealed remarkable homology for over 300 amino acids at the amino-terminal end to the oncogene, v-rel. The v-rel is a potent transforming oncogene from the avian reticuloendotheliosis virus [75]. In many cancers, aberrant activation and nuclear localization of NF-κB is actually quite frequent but most often results from defects in the pathways regulating NF-κB [76, 77]. IκB kinase (IKK) can inhibit IκB resulting in enhancing NF-κB activation [76, 77]. Some oncogenesis are correlated with the levels of IkBα and IkBβ proteins and coincided with the activation of IKK that govern the destruction of IkB factors [78]. Other ways, the loss of negative feedback mechanisms, which inhibit the NF-κB response, can result in its aberrant activity. An example of this is the CYLD tumor suppressor gene, which is associated with a predisposition to familial cylindromatosis (tumors of skin appendages). Losses of CYLD can lead to NF-κB activation [79]. In addition, the microenvironment of a solid tumor frequently contains high levels of inflammatory cytokines and/or hypoxic conditions, which

both stimulate nuclear translocation of NF-κB [76, 77]. The constitutive activation of NF-κB also appears to have a role in cell proliferation. NF-κB prevent Hodgkin's lymphoma cells from undergoing apoptosis under stress conditions [80]. It was further shown that growth factors such as epithelial growth factor [81] and platelet-derived growth factor induce proliferation of tumor cells through activation of NF-κB [82]. NF-κB signaling was also shown to promote pheochromocytoma 12 (PC12) cells survivals by nerve growth factor ligand, TrkA [83]. Recently, research has indicated that NF-κB possesses the prosurvival and antiapoptotic functions [84]. Several gene products that negatively regulate apoptosis in tumor cells, including inhibitor of apoptosis proteins (IAPs) 1 and 2, X-linked IAP, cellular Fas-associated death domain-like interleukin-1β converting enzyme (FLICE)-like inhibitory protein (cFLIP), were shown to be controlled by NF-κB activation [84]. The production of angiogeneic factors, such as vascular endothelial growth factor (VEGF) and Interleukin-8 (IL-8) has been shown to be regulating by NF-κB activation. NF-κB expression was associated with VEGF expression and microvessel density in human colorectal cancer [85]. IL-8 also activate by NF-κB. Bombesin (BBS)-like peptide treated PC-3 cell stimulated an NF-κB-dependent migration of human umbilical vascular endothelial cells in vitro by activating VEGF and IL-8[86]. These findings suggest that increased expression of NF-κB contributes to tumor angiogenesis in cancer.

1.4.3 Hypoxia-inducible-factors (HIFs)

Cancer cells always have a higher growth rate whereas their expansion relies on nutrient supply. Oxygen limitation is central in controlling neovascularization, glucose metabolism, survival and tumour spread. Hypoxia occurs when available oxygen falls below 5%, triggering a complex cellular and systemic adaptation mediated primarily through transcription by hypoxia-inducible factors (HIFs). HIF-1α was first identified as a crucial regulator of erythropoietin expression inresponse to low oxygen [87]. HIF-2α and HIF-3α have also been described, with HIF-3α, also known as IPAS (inhibitory PAS domain protein), functioning as an inhibitor of transcription [88, 89].

1.4.3.1 Biology of HIFs

HIF was shown in vitro, in a variety of cell culture systems, to be activated at a cut-off point of about 5% oxygen (40 mmHg), and to progressively increase its activity with a decrease in oxygen gradient down to 0.2–0.1% oxygen (1.6–0.8 mmHg), close to anoxia. HIF belongs to the large family of basic-helix–loop–helix (bHLH) proteins and is a heterodimer of a constitutively expressed and stable HIF-1β subunit, and one of three oxygen-regulated HIF-α subunits (HIF-1α, HIF-2α or HIF-3α). HIF-1α and HIF-2α, complexed with the b-subunits ARNT and (more rarely) ARNT2, bind DNA at hypoxia response elements (HREs) [90, 91].

HIF subunits are continuously transcribed and translated, and their stability is regulated by oxygen availability. HIF activation is a multi-step process involving HIF-α stabilization, nuclear translocation, heterodimerization, transcriptional activation and interaction with other proteins [92, 93].

1.4.3.2 HIFs in cancer progression

HIF can induce a vast array of gene products controlling energy metabolism, neovascularization, survival, pHi and cell migration, and has become recognized as a strong promoter of tumor growth [94]. The chemokine receptor CXCR4, a major metastatic mediator, is upregulated by HIF [95]. In addition, metalloproteinases (MMPs) 2 and 9 are regulated by hypoxia [96]. Another key mediator of metastasis is lysyl oxidase which is also a HIF target strongly associated with hypoxia. Inhibition of the lysyl oxidase blocks *in vitro* migration and *in vivo* metastasis from subcutaneous xenografts or after tail vein injection [97]. HIF-1α is also associated with VEGF-C expression in invasive ductal carcinomas.

1.4.4 cAMP response-element binding protein (CREB)

cAMP response-element binding protein (CREB) has been found to mediate transcriptional responses to a variety of growth factor and stress signals. CREB regulate many gene expressions. Genome-wide studies put the number of putative

CREB target genes at about 5000, or nearly one-quarter of the human genome. CREB or related factors whose aberrant expression is often associated with certain cancers [98]. In this section, the relationship between CREB and cancer will be discussed.

1.4.4.1 Biology of CREB

CREB is a member of the CREB/ATF-1 (activating transcription factor 1)/CREM (CRE modulator) transcription factor family that mediates cyclic AMP (cAMP), growth factor-dependent, and calcium-dependent gene expression through the cAMP response element [99]. CREB is a 43-kDa basic/leucine zipper (bZIP) transcription factor that is expressed at the RNA level in most tissues. CREB binds to the consensus octanucleotide CRE element (5'-TGANNTCA-3') as a homodimer and heterodimers in conjunction with other members of the CREB/ATF superfamily of transcription factors [100]. In resting cells, CREB exists in the unphosphorylated state that is transcriptionally inactive but can still bind to DNA. Upon cell activation, CREB becomes phosphorylated, which induces its transcriptional activity by promoting its interaction with the 256-kDa co-activator protein CREB binding protein (CBP). CBP serves as a molecular bridge that allows CREB to recruit and stabilize the RNA polymerase II complex at the TATA box, leading to switch certain genes on or off.

1.4.4.2 CREB in cancer progression

A potential role for the CREB family in cellular transformation was first appreciated in clear-cell sarcomas of soft tissues (CCSST) [101]. CCSST is an unusual malignancy of adolescents and young adults that typically arises in the deep soft tissues of the lower extremities close to tendon, fascia, and aponeuroses [102]. CCSST is typified by a chromosomal t(12;22)(q13;q12) translocation resulting in a fusion between the Ewing sarcoma gene (EWSR1) and activating transcription factor 1 (ATF1) [63]. The EWS–ATF1 can enhance expression of numerous CREB target genes by functioning as a strong activator. Indeed, disrupting EWS–ATF1 activity appears sufficient to block cell proliferation and promote cell apoptosis [63, 103]. Virally encoded oncoproteins such as hepatitis B virus and human T-cell leukemia virus (HTLV-1) tax also influence CREB activity in their efforts to promote cellular transformation [104, 105]. Based on this evidence, CREB will appear to cooperate with other factors, either in the context of a fusion protein or as part of a complex with an oncoprotein, to induce transformation. But whether CREB alone is capable of promoting tumorigenesis remained unclear [98].

1.4.5 Transcription factors interaction in cancer progression

The activity of many inducible transcription factors, such as NF-κB, is regulated through their association with cellular co-activators [106] . Interaction with the co-activator CREB binding protein (CBP) appears to be necessary to optimize the transcriptional activity of NF-κB. The interaction of the p65 (Rel A) subunit of NF-κB with CBP involves the KIX region of CBP, which is the same region responsible for binding the transcriptionally active serine-133-phosphorylated form of CREB [107, 108]. In human germline (GL) Iγ1promoter, NF-κB interacts with CREB to enhance gene expression. The Human Iγ1 promoter has NF-κB binding sites and CREB sites; they are communicating with each other via direct or indirect interactions. When using EMSA to observe NF-κB and CREB, it was found that the co-activator p300 interacts with CREB and NF-κB [109].

1.5 Strategy

Specific expression of the therapeutic gene in target cells depends on the specific delivery or the specific promoter activity. Either one of the two systems can be improved to become completely specific therapy without side effects. However, the both systems do not achieve the specific efficacy so far and the perfection of either system is extremely difficult. In this study we introduced a simple concept that the combination of partial specific delivery and partial specific promoter activity may achieve more specificity for target cells (Figure 1). Besides,

this strategy can be done in a rapid and convenient fashion. The first part in our study is to rapidly create a novel promoter based on the activities of transcription factors. The transcription factors which are important in cancer progression will be roughly assayed in several tumor or rapid-proliferating cells. The response elements with higher activities in tumor cells will be processed to create a novel mini-promoter. This transcription factor-based synthetic promoter (TSP) which consists of several kinds of response elements might be flexible and partial specific in tumor cells. The second part is to enhance the delivery efficiency of PEI by a convenient method of peptide absorption. The multi-functional peptide RGD-4C-HA possesses the ability of specific targeting and can absorb to PEI. RGD-4C-HA contains RGD-4C sequence which was proved to specifically bind to integrin $\alpha_v\beta_3$ [110-112]. In addition RGD-4C-HA contains a negatively charged tail which can absorb to the positively charged PEI by electrostatic forces. This modification of PEI is rapid and convenient in laboratory compared to the complicated chemical coupling or modification of the functional groups. RGD-4C-HA should improve the delivery efficiency and specificity of PEI for integrin $\alpha_v\beta_3$ expessing cells such as B16-F10 cells. The partial specific promoter and the partial specific delivery system can be developed in a rapid and convenient method as described above. Finally, the combination of the two systems should achieve more specificity than either system alone.

Chapter 2 Materials & Methods

2.1 Materials

2.1.1 Primers

Table 1: Primers used in this study

Name	Primer Sequence (5'to 3')
5' TSP1	CGCGTGGGACTTTCCGCTGGGGACTTTCCGCTGGGGACTTTCCGCTG**TGACGTCA**GAGAG
3' TSP2	**TCA**GCTCTC**TGACGTCA**CAGCGGAAAGTCCCCAGCGGAAAGTCCCCAGCGGAAAGTCCCA
5' TSP2	C**TGACGTCA**GAGAGC**TGACGTCA**GAGAGCTACGTGTGTGTACGTGTGTGTACGTGAT
3' TSP1	CGATCACGTACACACACGTACACACACGTAGCTCTC**TGACGTCA**GCTCTC**TGACG**

The primers were purchased from commercial (MDBio, Taiwan, ROC, ROC). The binding sites of NF-kB (underlined), CREB (bold), and HIF-1 (dotted) were labeled.

2.1.2 Cell lines

Table 2: Cell lines used in this study

Cell line	Description	ATCC #
B16-F10	mouse melanoma cells	CRL-6475
Balb/3T3	mouse embryo fibroblast cells	CCL-163
HeLa	human cervical carcinoma cells	CCL-2

2.1.3 Plasmids

Table 3: Plasmids used in this study

Plasmid	Description	Source
pAAV-MCS	With multiple cloning site	Stratagene, Cedar Creek, TX
pAAV-MCS-hrGFP	With humanized renilla green fluorescent protein	From Dr. Liao's Lab
pAP-1-hrGFP	Containing 7 copies of AP-1 binding site	Stratagene, Cedar Creek, TX
pARE-hrGFP	AmpR assay plasmid	From Dr. Liao's Lab
pAsRed2-N1	With red fluorescent protein	Becton Dickinson, Moutain View, CA

pCRII-hrGFP	Containing 7 copies of HIF-1 binding site	From Dr. Liao's Lab
pCRE-hrGFP	Containing 4 copies of CREB binding site	Stratagene, Cedar Creek, TX
pD5-hrGFP	With synthetic promoter	From Dr. Liao's Lab
pNF-κB-hrGFP	Containing 5 copies of NF-κB binding site	Stratagene, Cedar Creek, TX
pNFAT-hrGFP	Containing 4 copies of NFAT binding site	Stratagene, Cedar Creek, TX
MZF-1-hrGFP	Containing 3 copies of MZF-1 binding site	From Dr. Liao's Lab

2.1.4 Chemicals, enzymes, and reagents

Table 4: Chemicals, enzymes, and reagents used in this study

Chemical	**Company**
100 bp DNA ladder	Protech, Taiwan, ROC
1kb DNA ladder	Protech, Taiwan,

Acetic acid	ROC Showa, Tokyo, Japan
Adenosine triphosphate (ATP)	Epicentre, Madison, WI
Agar	Amresco, Solon, Ohio
Agarose	MDBio, Taiwan, ROC
Albumin bovine Fraction V (BSA)	MP Biomedicals, Irvine, CA
Ampicillin	Amresco, Solon, Ohio
*Apa*I (restriction enzyme)	Promega, USA
*Bam*HI (restriction enzyme)	Fermentas, Burlington, Canada
Bsu15I (ClaI)	Fermentas, Burlington, Canada
Calcium chloride, dyhidrate	J.T.Baker,

	Phillipsburg, NJ
Coomssie Brilliant blue	Amresco, Solon, Ohio
Deoxy-nucleotide triphosphates (dNTP)	Promega, USA
Dimethyl sulfoxide (DMSO)	MP Biomedicals, Irvine, CA
Disodium hydrogen phosphate anhydrous (Na_2HPO_4)	Scharlau, Barcelona, Spain
Dulbecco's modified Eagle's medium (DMEM)	Sigma, St. Louis, MO
Ethanol	Sigma, St. Louis, MO
Ethidium bromide (EtBr)	Amresco, Solon, Ohio
Ethylenediaminetetraacetic acid (EDTA)	Tedia, Fairfield, OH
Fetal bovine serum	Biological Industries, Kibbutz Beit Haemek, Israel
Glycerol	Showa, Tokyo,

	Japan
Glycine	Amresco, Solon, Ohio
*Hind*III	Fermentas, Burlington, Canada
Hydroboric acid (H_3PO_4)	Riedel-de Haën, Seelze, Germany
Hydrochloric acid (HCl)	Scharlau, Barcelona, Spain
Isopropanol	C-Echo, Taiwan, ROC
Kanamycin	MDBio, Taiwan, ROC
Lipofectamine™ 2000	Invitrogen, Leek, The Netherlands
Luria Bertani (LB) agar	Amresco, Solon, Ohio
Luria Bertani (LB) broth	Scharlau, Barcelona, Spain

Methanol	C-Echo, Taiwan, ROC
*Mlu*I	Fermentas, Burlington, Canada
Ninhydrin	Sigma, St. Louis, MO
Penicillin-streptomycin amphotericin B (PSA)	Biological Industries, Kibbutz Beit Haemek, Israel
Pfu polymerase	MDBio, Taiwan, ROC
Polyethyleneimine	Aldrich, St. Louis, MO
Potassium acetate (KOAc)	Showa, Tokyo, Japan
Potassium chloride (KCl)	Showa, Tokyo, Japan
Propidium iodide (PI)	Sigma, St. Louis, MO

Sephacryl S-200	GE Healthcare, Chalfont St., UK
Sodium azide (NaN_3)	Showa, Tokyo, Japan
Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$)	Showa, Tokyo, Japan
Sodium chloride	Amresco, Solon, Ohio
Sodium hydrogen carbonate ($NaHCO_3$)	MP Biomedicals, Irvine, CA
Sodium hydroxide (NaOH)	Showa, Tokyo, Japan
T4 kinase buffer	Fermentas, Burlington, Canada
T4 ligase (2U)	Epicentre, Madison, WI
T4 ligase (10U)	Epicentre, Madison, WI
T4 ligation buffer	Epicentre, Madison,

	WI
T4 polynucleotide kinase	NEB, Hitchin, UK
Taq polymerase	BioKit, Taiwan, ROC
Taq DNA polymerase XL	Protech, Taiwan, ROC
Tris base	MDBio, Taiwan, ROC
Tris-HCl	MP Biomedicals, Irvine, CA
Trypan blue stain	Gibco, Grand Island, NY
Trypsin	Gibco, Grand Island, NY
Tryptone	CONDA, Spain
Tween 20	MP Biomedicals, Irvine, CA
*Xho*I (restriction enzyme)	Fermentas, Burlington, Canada

Yeast extract	Conda, Madrid, Spain

2.1.5 Antibodies

Table 5: Antibodies used in this study

Antibody	Description	Company
Anti-HA-fluorescein, high affinity (3F10)	Recognizing the HA peptide sequence [YPYDVPDYA]	Roche, Basel, Switzerland
Polyclonal rabbit anti-mouse IgG/HRP	Secondary antibody recognizing the mouse IgG	DakoCytomation, Glostrop, Denmark

2.1.6 Kits

Table 6: Kits used in this study

Kit	Company	Used in
Geneaid gel/PCR DNA fragments extraction kit	Geneaid, Taiwan, ROC	DNA extraction, clean-up
NucleoBond PC100	Macherey-Nagel,	DNA extraction

	Duran, Germany	
SuperSignal West Pico Chemiluminescent Substrate	Pierce, Rockford, IL	The substrate of HRP in dot blot

2.1.7 Buffers

Table 7: Buffers used in this study

Buffer	Description	Used in
1X PBS	137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH7.4	Cell culture
5% Blocking buffer	5%(w/v) non-fat powdered milk in 1X PBS buffer	Dot blot
50X TAE buffer	48.4 g Tris base, 0.5 M EDTA (pH8.0) 20 ml, 11.42 ml acetic acid. dd H_2O was added to 200 ml.	Gel electrophoresis
Buffer S1	50 mM Tris-HCl, 10mM EDTA, 100μg/ml RNase A, pH8.0	Midi preparation
Buffer S2	200 mM NaOH, 1% SDS	Midi preparation

Buffer S3	2.8 KAc, pH 5.1	Midi preparation
Buffer N2	100 mM Tris,15% ethanol, 900 mM KCl, 0.15% Triton X100, adjusted to pH 6.3 with H_3PO_4	Midi preparation
Buffer N3	100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH6.3 with H_3PO_4	Midi preparation
Buffer N5	100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH 8.5 with H_3PO_4	Midi preparation
EDTA-trypsin	2.5 g trypsin, 0.1 M EDTA (pH8.0) in 1L 1X PBS, pH7.4, 0.2 μm filtered	Cell culture
PBST	0.05% Tween 20 in 1X PBS	Dot blot
Solution I	50mM Tris-HCl, 10mM EDTA , 10mg/ml RNase A, pH=8.0	Mini preparation
Solution II	0.2M NaOH, 1%（w/v）SDS	Mini preparation
Solution III	2.8M potassium acetate, pH=5.1	Mini preparation
Staining buffer	1% BSA , 0.05% NaN_3 in 1X PBS	

Versene	0.2g EDTA in 1L 1X PBS	Cell culture

2.1.8 Media

Table 8: Media used in this study

Media	Description	Used in
DMEM growth medium	10% FBS, 1% PSA in Dulbecco's Modified Eagle's Medium	Cell culture
LB (Luria-Bertani) broth	1% tryptone, 0.5% yeast extract, 1% NaCl	Bacteria culture
LB (Luria-Bertani)/Ampicillin agar	1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50µg/ml ampicillin	Bacteria culture
LB (Luria-Bertani)/Ampicillin broth	1% tryptone, 0.5% yeast extract, 1% NaCl, 50µg/ml ampicillin	Bacteria culture
LB (Luria-Bertani)/Kanamycin agar	1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 30µg/ml kanamycin	Bacteria culture
LB	1% tryptone, 0.5% yeast extract, 1%	Bacteria

(Luria-Bertani)/Kanamycin broth	NaCl, 30μg/ml kanamycin	culture
Opti-MEM I	Medium without serum	Cell culture
SOB broth	2% tryptone, 0.5% yeast extract, 0.05% NaCl, %0.0186 KCl, 10mM $MgCl_2$	Bacteria culture

2.1.9 Equipment

Table 9: Equipment used in this study

Equipment	**Company**
–20°C low temperature refrigerator	Frigidaire, Pittsburgh, PA
–80°C low temperature refrigerator	Nuaire, Caerphilly UK
4°C refrigerator	MINI KINGCON, Taiwan, ROC
Biophotometer DPU-414	Eppendorf, Hamburg, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany

Centrifuge 5804 R	Eppendorf, Hamburg, Germany
DNA electrophoresis unit Gel Mate 2000	Toyobo, Japan
Dot-blot machine	Bio-East, Taiwan, ROC
Econo column 737-0722	Bio-Rad, Taiwan, ROC
Flow cytometer, FACScan	Becton Dickinson, Moutain View, CA
Heating plate	Firstek, Taiwan, ROC
Inverted research microscope, IX71	Olympus, Tokyo, Japan
Biological safety cabinet, Forma Class II, A2	Thermo, USA
Lead blocker	Okamoto, Fukuyama, Japan
Microscope, CX31	Olympus, Tokyo, Japan
Orbital Shaking incubator OS1500R	TKS
pH meter SP701	Suntex, Taiwan, ROC

Thermal cycler	Eppendorf, Hamburg, Germany
Uni-photo gel image system	EZ lab, Taiwan, ROC
Water bath	Firstek, Taiwan, ROC

2.2 Methods

2.2.1 Construction of transcription factor-based synthetic promoter (TSP)

The pD5-hrGFP was obtained by replacing the CMV promoter of pAAV-MCS-hrGFP with TSP. Briefly, the vector pAAV-MCS-hrGFP was double digested by *MluI* and *ClaI* (Fermentas, Burlington, Canada) to eliminate the CMV promoter. TSP was obtained by 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 heated to 95°C for 5 minutes and then cooled down 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 above. The primers contained three copies of the binding sites of NF-kB (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 was designed as a *MluI* protruding end and the 3' end of insert2 was designed as a *ClaI* protruding end. The inserts were phosphorylated by T4 polynucleotide kinase (NEB, Hitchin, UK) according to the manufacturer's protocol. The vector and inserts (insert1 and insert2) were then ligated with a molar ratio 1:5:5 or 1:10:10 at 16°C for 16 hours. After 16 hours incubation, the ligation products were transformed into DH5α competent cells by heat shock method. The colonies were picked and checked by restriction enzyme digestion. The correct clone pD5-hrGFP was then obtained and sequenced.

2.2.1.1 Restriction enzyme digestion

The restriction enzyme digestion of DNA was performed following the manufacturer's protocol (Fermentas, Burlington, Canada). Generally, 1 μg DNA was digested with 5 unit of restriction enzyme in a 10 μl volume reaction at 37°C overnight.

2.2.1.2 DNA extraction

After digestion by restriction enzyme, the DNA was cleaned up by Geneaid gel/PCR DNA fragments extraction kit (Geneaid, Taiwan, ROC) following the manufacturer's protocol.

Briefly, the digestion product was spun at 13,000 rpm for 30 seconds in the spin column. The filtrate in the collection tube was discarded. 700 µl Washing buffer (Geneaid, Taiwan, ROC) was added and the solution was spun at 13,000 rpm for 1 minute. This step was repeated twice. The filtrate was discarded by centrifugation at 13,000 rpm for 3 minutes to remove residual trace of ethanol. The column was additionally incubated at 65°C for 5 minutes to evaporate ethanol. The DNA was eluted by 30 µl ddH_2O in a new tube and stored at -20°C.

2.2.1.3 Ligation

The ligation reaction was performed following the manufacturer's protocol (Epicentre, Madison, WI). Briefly, 500 µg vector was used in a 10 µl volume reaction with 1mM ATP. The molar ratio of the vector and the inserts (insert1 and insert2) was 1:5:5 or 1:10:10. The mixture was then incubated at 16°C for 16 hours.

2.2.2 Transformation of *E. coli*

2.2.2.1 Preparation of competent cells for heat shock

Single colony of *E. coli* was inoculated in 3 ml of LB broth and grew for 12 hours at 37°C with agitation until the OD_{600} was between 0.35~0.45 (about 12 hours). 1 ml of the overnight culture was transferred into 100 ml LB broth and was then incubated at 37 °C with agitation until the OD_{600} was between 0.35~0.45. The cells were havested by centrifugation at 4100 rpm for 10 minutes and then re-suspended in 30 ml ice-cold 0.1M $CaCl_2$. The cells were pelleted by centrifugation at 4100 rpm for 10 minutes. The pellet was re-suspended in 2 ml 0.1M $CaCl_2$ containing 10% glycerol. The cells were dispensed at 100 µl per tube and stored at -80°C.

2.2.2.2 Transformation of competent cell by heat shock method

Stored competent cells were thawed on ice. 1 ng DNA was mixed with 100 µl competent cells and was then stored on ice for 30 minutes. The mixture was incubated in a preheated 42°C heating block for 90 seconds and quickly placed on ice for 2 minutes. Then 250 µl of LB broth was added to the cells. The culture was incubated at 37°C with shaking for 50 minutes. 100 µl of the culture was plated on the LB agar plate with 50µg/ml ampicillin or 30µg/ml kanamycin. The plate was incubated at 37°C for 16 hours later.

2.2.3 Plasmid DNA extraction

2.2.3.1 Minipreparation method

A single colony of *E. coli* was inoculated in 3 ml of LB broth (with antibiotics) and allowed to grow overnight at 37°C with agitation. 1 ml culture was recovered by centrifugation at 13,000 rpm for 1 minute and then re-suspended in 200µl ice-cold Solution I buffer in a new tube. 250 µl Solution II buffer was added and mixed gently. After 3 minutes, 250 µl Solution III buffer was added to the mixture and mixed gently until a homogeneous suspension containing an off-white flocculate was formed. The mixture was incubated on ice for 5 minutes and then spun at 13,000 rpm for 5 minutes at 4°C. The supernatant was transferred to a fresh tube. Equal volume of phenol: chloroform (700 µl) was added. The organic and aqueous phases were mixed by vortex and then the emulsion was centrifuged at 13,000 rpm for 3 minutes at 4°C. The aqueous upper layer was transferred to a fresh tube. Nucleic acids from the supernatant were precipitate by adding 0.7 volumes of isopropanol at room temperature. The solution was mixed completely and incubated for 2 minutes at room temperature. The precipitated DNA was collected by centrifugation at 13,000 rpm for 20 minutes at 4°C. The supernatant was removed by gentle aspiration. 1 ml of 70% ethanol was added to the pellet and the DNA was recovered by centrifugation at 13,000rpm for 5 minutes at 4°C. The supernatant was removed by gentle aspiration and the tube was incubated opened at room temperature to evaporate ethanol (7 minutes). The DNA was dissolved in 50

μl ddH_2O and vortexed gently for few seconds. The products were stored at -20°C.

2.2.3.2 Midipreparation method

The midipreparation was performed by NucleoBond PC 100 kit (Macherey-Nagel, Duran, Germany) following the manufacturer's protocol. Briefly, a single colony of *E. coli* was inoculated in 100 ml of LB broth (with antibiotics) and grew overnight at 37°C with agitation. The cells were recovered by centrifugation at 8,000 rpm for 15 minutes at 4°C. The pellet was collected, and 4 ml buffer S1 (Macherey-Nagel, Duran, Germany) was added to dispense the pellet. Then 4 ml buffer S2 (Macherey-Nagel, Duran, Germany) was added to the suspension**.** The lysate was mixed gently and incubated at room temperature for 3 minutes (no more than 5 minutes). The pre-cooled 4 ml buffer S3 (Macherey-Nagel, Duran, Germany) was then added to the solution and mixed gently until a homogeneous suspension containing an off-white flocculate was formed. The mixture was incubated on ice for 5 minutes and then spun at 13,000 rpm for 25 minutes at 4°C. The supernatant was loaded onto the NucleoBond AX 100 Midi column which was equilibrated with 2.5 ml buffer N2 (Macherey-Nagel, Duran, Germany). The flow-through was emptied by gravity flow and discarded. 10 ml buffer N3 (Macherey-Nagel, Duran, Germany) was added to wash the column twice. The DNA was eluted by 5 ml buffer N5 (Macherey-Nagel, Duran, Germany).

Then 3.5 ml isopropanol was added to precipitate the DNA. The mixture was incubated on ice for 10 minutes and recovered by centrifugation at 13,000 rpm for 30 minutes at 4°C. 6 ml 70% ethanol was added to the pellet and the solution was spun at 13,000 rpm for 5 minutes. Finally, the pellet was dissolved in appropriate amount of ddH_2O and stored at -20°C.

2.2.4 Cell culture

All cells were cultured following the ATCC's instructions. Generally, cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% PSA (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were incubated in tissue culture incubator with 5% CO_2 at 37°C. All cells were subcultured to ensure that the confluency was no more than 80%.

2.2.4.1 Procedures of subculture

All cells were passaged following the ATCC's instructions. Generally, the culture medium was removed and discarded. The cell layer was briefly rinsed with 1X PBS to remove all traces of serum that contains trypsin inhibitor. 3 ml Trypsin-EDTA solution was added to the flask for about 5 minutes until the cell layer was dispersed. The cells were centrifuged at 1,200rpm for 5 minutes at 4°C

and were re-suspended by 2 ml growth medium. Appropriate aliquots of the cell suspension were added to a new culture vessel. Cells were incubated in tissue culture incubator with 5% CO_2 at 37°C.

2.3 Transcription factors and TSP activity assay

The transcription factors assay were performed to measure the activities of several transcription factors and TSP in different cells. Briefly, the assay plasmids (pAP-1-hrGFP, pCRII-hrGFP, pCRE-hrGFP, pD5-hrGFP, pNF-κB-hrGFP, pNFAT-hrGFP , MZF-1-hrGFP) and a control plasmid (pARE-hrGFP) were co-transfected with a reporter plasmid (pAsRed2-N1) into B16-F10, Balb/3T3, and HeLa cells. The assay plasmids pAP-1-hrGFP, pCRII-hrGFP, pCRE-hrGFP, pNF-κB-hrGFP, pNFAT-hrGFP, and MZF-1-hrGFP each contained 7, 7, 4, 5, 4, and 3 copies of the responding binding site respectively. ARE was a binding site of a prokaryotic transcription factor ampR and it was used as a negative control group. In addition, the reporter plasmid (pAsRed2-N1) with a transgene encoding the red fluorescent protein driven by CMV promoter was used to normalize the transfectant efficiency between each sample. 24 hours after transfection, the gene expressions were measured by FACScan flow cytometry (Becton Dickinson, Moutain View, CA).

2.3.1 Transfection of mammalian cells

2.3.1.1 Seeding cells

The culture medium was removed and discarded. The cell layer was briefly rinsed with 1X PBS and then 3 ml Trypsin-EDTA solution was added to the flask for about 5 minutes until the cell layer was dispersed. The pellet was recovered by centrifugation at 1,200rpm for 5 minutes at 4°C. The supernatant was discarded. Cells were re-suspended by 2 ml growth medium. Certain amount of cells was stained by trypan blue and calculated by a bright-line chamber (Marienfeld, Germany). Appropriate cells were plated in 6-well or 24-well plate and incubated in tissue culture incubator with 5% CO_2 at 37°C.

2.3.1.2 Polyethyleneimine transfection

10^5 cells were plated in 24-well plate to be approximately 50% confluent at the time of transfection. Cells were transfected with different plasmid DNA by polyethyleneimine (PEI). Briefly, 1 μg plasmid DNA and 6 μl of 5μM PEI (Aldrich, St. Louis, MO) were each diluted into 50 μl of 150mM NaCl and vortexed. The PEI solution was added into DNA solution after 5 minutes **(**Notice: not the reverse order**)**, and then vortexed. After 20 minutes, the cells were rinsed and supplemented with 200 μl Opti-MEM I Medium (Gibco, Grand Island, NY). The PEI-DNA

mixture was gently added to each well. After 18 hours incubation, 700 μl fresh growth medium were added into each well. After 24~48 hours, the gene expressions were measured by FACScan flow cytometry (Becton Dickinson, Moutain View, CA).

2.3.1.3 Lipofectamine™ 2000 transfection

2×10^5 cells were plated in 24-well plate to be approximately 80% confluent at the time of transfection. Cells were transfected with different plasmid DNA by Lipofectamine™ 2000 (Invitrogen, Leek, The Netherlands). The transfection procedure was performed according to the manufacturer's protocol. Briefly, 3 μg DNA was diluted in 250 μl Opti-MEM I Medium (Gibco, Grand Island, NY) and mixed gently. 10 μl Lipofectamine™ 2000 was gently mixed with 250 μl Opti-MEM I medium and incubated for 5 minutes at room temperature. The diluted DNA was combined with the diluted Lipofectamine™ 2000 for 20 minutes at room temperature. The medium in the cells were discarded and cells were gently washed with Opti-MEM I medium twice. The DNA- Lipofectamine™ 2000 mixture was added into each well gently. 500 μl Opti-MEM I medium was added into each well gently and the cells were incubated at 37°C in a CO_2 incubator for 12 hours. 2 ml of growth medium (DMEM or RPMI) was added into each well at 6 hours after

transfection. After 24 hours, the gene expressions were measured by FACScan flow cytometry (Becton Dickinson, Moutain View, CA).

2.3.2 Measurement of reporter gene expression by flow cytometry

After 24 hours transfection, cells were harvested to measure the gene expression. Briefly, the medium was discarded and each well was rinsed with 1 ml PBS. 1ml versene or trypsin was then added, and the cells were incubated at 37°C for 5 minutes. 1 ml growth medium was added into each well and the cells were recovered by centrifugation at 1,500rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended by 1ml staining buffer in FACS tube. The reporter gene expression was measured by FACScan flow cytometry (Becton Dickinson, Moutain View, CA). Fluorescence intensities were analyzed with CELLQUEST software (Becton Dickinson).

2.4 RGD-4C-HA binding assay

The culture medium was removed and discarded. The cell layer was briefly rinsed with 1X PBS to remove all traces of serum that contains trypsin inhibitor. 5 ml versene was added to the flask (Notice: the use of trypsin might digest the ligands on cell membrane). After centrifugation to remove versene and the

measurement for the cell number, 2×10^5 cells were suspended in staining buffer (1% BSA, 0.05% NaN_3 in 1X PBS) containing different concentrations (2μM, 200nM or 20nM) of RGD-4C-HA peptide (CDCRGDCFCGGGYPYDVPDYAGGGDDDEC which was purchased from MDBio, Taiwan, ROC) at 4°C for 1 hour. After 1 hour incubation, cells were washed, suspended in staining buffer with anti-HA-FITC and incubated at 4°C for 1 hour. After 1 hour incubation, cells were washed and the surface immunofluorescence was measured by FACScan flow cytometer (Becton Dickinson, Moutain View, CA). Fluorescence intensities were analyzed with CELLQUEST software (Becton Dickinson, Moutain View, CA).

2.5 The absorption of RGD-4C-HA to PEI assay

2.5.1 Separating PEI-peptide complex with un-absorbed PEI and RGD-4C-HA by gel filtration column S-200

The PEI and RGD-4C-HA were incubated at room temperature for 30 minutes at a molar ratio 1:1. The PEI-peptide complex was applied into a gel filtration column which was packed with Sephacryl S-200 (GE Healthcare, Chalfont St., UK) following the manufacturer's protocol to separate the un-absorbed products. Briefly, the S-200 gel was first equilibrated to room temperature, and gently shaken to make slurry. The homogeneous suspension was poured into an empty glass column.

The column was packed following two steps:

STEP 1: The column was packed at 0.5 ml/min for 2 hours.

STEP 2: Increased the flow rate to 0.9 ml/min for 1 hour.

The elution products were collected 0.5 ml per fraction by fraction collector.

2.5.2 Ninhydrin test

The elution products were put onto a thin layer chromatography (TLC) plate. The 15% ninhydrin solution (solved in methanol) was then put onto the TLC plate. After 10 minutes incubation, the TLC plate was pictured.

2.5.3 Dot-blotting

The elution products were applied onto the nitrocellulose (NC) paper (Pall, USA) which was rinsed with 1X PBS buffer on a dot-blot machine (Bio-East, Taiwan). Samples were gently for 10 minutes. The NC paper was blocked by 5% of skin milk at 4°C overnight or 37°C for 3 hours with shaking. The paper was then washed with 1X PBS containing 0.05% Tween 20 three times at room temperature for 5 minutes. The anti-HA antibody (Roche, Basel, Switzerland) were diluted 200X in 5% blocking buffer (5% skin milk in 1X PBS buffer) and applied onto the NC paper gently at room temperature for 1 hour with shaking. The mixture was then discarded. The NC paper was washed with 0.05% PBST three times at room

temperature for 5 minutes. The 2nd antibody conjugated with HRP (DakoCytomation, Denmark) was diluted 100X in 5% blocking buffer and applied on the NC paper in dark for 1 hour with shaking. After 1 hour incubation, the NC paper was washed as above and the substrate was applied onto the NC paper for 10 minutes in dark. The NC paper was covered in the lead blocker (Okamoto, Japan) with the film for several minutes depended on the intensity of the signals. Then, the film was developed in the developer for 1 minute. The film was washed in water and then stained in the fixer for 1 minute.

2.6 PEI-peptide complex transfection

The enhancement of transgenic expression *in vitro* by RGD-4C-HA was performed by PEI-peptide complex transfection. The PEI and RGD-4C-HA were incubated at room temperature for 5 minutes at different molar ratios. After 5 minutes incubation, the PEI-peptide complex was used as the native PEI. The transfection was done as described above.

2.7 Statistical analysis

Results were expressed as mean ± SE. Statistical significance of differences between mean values was estimated using the Student's *t*-test (Microsoft Excel). $p < 0.05$ was considered significant.

Chapter 3 Results

3.1 Establishment of the transcription factor-based mini-promoter (TSP) system

3.1.1 Screening of the activities of several transcription factors in different cells

Previous literatures have indicated that the activities of transcription factors are different in different cell types. In order to character the activities of the transcription factors in tumor cells, the plasmids pAP-1-hrGFP, pCRE-hrGFP, pCRII-hrGFP, pNF-κB-hrGFP, pNFAT-hrGFP, MZF-1-hrGFP, and a control plasmid (pARE-hrGFP) were respectively co-transfected with a reporter plasmid (pAsRed2-N1) into different cells. ARE was a binding site of a prokaryotic transcription factor ampR and it was used as a negative control group. In addition, the reporter plasmid (pAsRed2-N1) with a transgene encoding the red fluorescent protein driven by CMV promoter was used to normalize the transfectant efficiency between each sample. B16-F10 cells (mouse melanoma cells with high metastatic potential), Balb/3T3 cells (mouse immortalized fibroblast cell with high proliferating activity) and HeLa cells (human cervical carcinoma cells) were assayed their certain activities of transcription factors as described above. The

expression index of each transcription factor was calculated according to the following formula:

$$\text{Expression Index} = \frac{\text{TFI of TFBS-hrGFP / TFI of AsRed2 in TFBS}}{\text{TFI of ARE-hrGFP/TFI of AsRed2 in ARE}}$$

TFI = Total fluorescence intensity

The results showed that the expression indexes of HIF-1 and NF-κB were higher than other transcription factors in all cells. NF-κB activities were 6-fold, 12-fold, and 4-fold higher than ampR (ARE) in B16-F10, Balb/3T3, and HeLa cells, respectively. The expression indexes of HIF-1 were 15-fold, 43-fold, and 9-fold higher than ampR (ARE) in B16-F10, Balb/3T3, and HeLa cells, respectively. Except of HIF-1 and NF-κB, the expression indexes of CREB were higher than ampR in all cell types, but the data were not significant after statistical calculation (Figure 2).

3.1.2 Construction of the transcription factor-based synthetic promoter (TSP)

The activities of NF-κB and HIF-1 were found that they were relatively higher in these tumor or rapid-proliferating cells. In addition to CREB, it was considered as the important role in cell transformation such as myeloid leukemia and

contributing to tumor metastasis and invasion. Based on above, the binding sites of NF-κB, CREB, and HIF-1 were assembled to create a novel synthetic mini-promoter. The CMV promoter of pAAV-MCS-hrGFP was replaced by the transcription factor-based synthetic promoter (TSP) and the new plasmid (pD5-hrGFP) was verified by restriction enzyme digestion (Figure 3).

3.1.3 Transcription factor-based mini-promoter activity in different cells

The pD5-hrGFP contained a novel mini-promoter TSP with 9 transcription factor binding sites of 3 NF-κB response elements, 3 CREB elements and 3 HIF-1 elements. In order to verify the efficiency of this promoter, the plasmids pD5-hrGFP, pCRE-hrGFP, pCRII-hrGFP, pNF-κB-hrGFP, and a control plasmid (pARE-hrGFP) were co-transfected with a reporter plasmid (pAsRed2-N1) into different cells. ARE was a binding site of a prokaryotic transcription factor ampR and it was used as a negative control group. In addition, the reporter plasmid (pAsRed2-N1) with a transgene encoding the red fluorescent protein driven by CMV promoter was used to normalize the transfectant efficiency between each sample. The expression index of each transcription factors was calculated according to the following formula:

$$\text{Expression Index} = \frac{\text{TFI of TFBS-hrGFP / TFI of AsRed2 in TFBS}}{\text{TFI of ARE-hrGFP/TFI of AsRed2 in ARE}}$$

TFI = Total fluorescence intensity

The results showed that the expression indexes of TSP were 6-fold, 36-fold, and 4-fold higher than ampR (ARE) in B16-F10, Balb/3T3, and HeLa cells respectively. The results indicated that TSP was truly active in these tumor or rapid-proliferating cells. In addition, the expression indexes of HIF-1 were 15-fold, 54-fold, and 15-fold higher than ampR (ARE) in B16-F10, Balb/3T3, and HeLa cells respectively. It was noticed that the HIF-1 remained the highest activity in all three cells which meant the promoter might be also utilized in cancer gene therapy (Figure 4).

3.1.4 Inhibition effect of TSP in HeLa cells

We had proven that TSP was active in several types of cell including tumor cells in previous study. The activity of TSP was apparently related to the activities of the transcription factors: NF-κB, CREB, and HIF-1. Generally, the activity of some transcription factor might be reduced under several physiological conditions. For example, NF-κB inhibitors were occasionally utilized in the treatment of cancer. In addition, tumor progression was largely depended on angiogenesis. When the

angiogenesis in tumor was completed, the activity of HIF-1 would reduce because of the sufficient oxygen supply. Since NF-κB and HIF-1 responsive element were partial components of TSP, the activity of TSP might be lost in these circumstances. In order to verify whether the activity of TSP was affected under such circumstance, several compounds were used to inhibit the activities of certain transcription factors. The experiment was performed as described above. The samples were treated with the corresponding inhibitors after 24 hours transfection. After drug treatment for 16 hours, the gene expression was determined by FACScan flow cytometer (Becton Dickinson, Moutain View, CA). The results indicated that the activity of NF-κB was inhibited by 25μM hydroquinone and the activity of the promoter was reduced to 83.7%. Similarly, the activity of HIF-1 was inhibited by D609 (50μg/ml) and the activity of the promoter was reduced to 69.07%. However, the activities of TSP were not significantly lowered than control group at the presence of inhibitors (Figure 5).

3.2 Design of RGD-4C-HA and the functional regions

Many peptides had been identified by phage display to react strongly to certain receptors or molecules. The RGD-4C (CDCRGDCFC) peptide was discovered to specifically bind to integrin $\alpha_v\beta_3$ expressed on the surface of B16-F10 cells. The binding activity of RGD-4C peptide was utilized to improve the DNA delivery

efficiency of PEI to certain cells. For the purpose, this peptide must contain several additional functional domains. The peptide RGD-4C-HA (CDCRGDCFCGGGYPYDVPDYAGGGDDDEC which was purchased from MDBio, Taiwan, ROC) was designed as a multi-functional peptide. The RGD-4C (CDCRGDCFC, underlined) sequence is the targeting region to direct the molecule binding to integrin $\alpha_v\beta_3$ on B16-F10 cell surface. The HA tag (YPYDVPDYA, bolded) was designed to act as a spacer to separate from the absoption domain and as an epitope for antibody detection. The absorption region (DDDE, dotted) was designed for absorption with PEI. The four continuous amino acids (DDDE) sequence contained negatively charged residues would absorb to the positively charged PEI by electrostatic forces. The final amino acid cysteine with the sulfyl group can be used to couple with the primary amine group of PEI. The two GGG sequences were spacers to separate the functional domains (Figure 6).

3.3 The binding affinity of RGD-4C-HA

The multi-functional peptide RGD-4C-HA was determined whether the other functional regions interfered with the activity of RGD-4C to abolish the targeting activity. The RGD-4C-HAs with different concentrations were mixed with target cells to determine the binding activity. The results revealed that the total fluorescence intensities increased significantly for B16-F10 cells compared to

negative control under all concentrations. However, the total fluorescence intensities were no differences compared to negative control in Balb/3T3 or HeLa cells (Figure 7a). Generally, the total fluorescence intensity was obtained by the events time the fluorescence mean. The events represented the binding percentage of RGD-4C-HA to the population and the fluorescence mean represented the binding strength on a single cell. When we focused on the two parameters respectively, it was found that the binding percentages of RGD-4C-HA increased significantly in Balb/3T3 cells under 2μM and 200nM. The binding percentages increased from 45.30% (NC) to 47.61% and 49.28% under 2μM and 200nM RGD-4C-HA in Balb/3T3 cells. It was noticed that the binding percentages increased from 44.53% (NC) to 53.84%, 59.13%, and 52.53% under 2μM, 200nM, and 20nM RGD-4C-HA in B16-F10 cells at the same time. The binding percentages were no differences compared to negative control (Figure 7b). In other way, the total fluorescence mean didn't increase in Balb/3T3 and HeLa cells as in B16-F10 cells (Figure 7c). These results indicated that RGD-4C-HA bound to B16-F10 and Balb/3T3 cells significantly since the binding percentages increased, but it was shown that the binding percentages were apparently larger in B16-F10 than in Balb/3T3 cells. However, the differences of the total fluorescence mean showed that there were more ligands on a single cell in B16-F10 rather than in Balb/3T3 cells.

3.4 The absorption of RGD-4C-HA to PEI

RGD-4C-HA peptide could bind to the surface of B16-F10 cells that represented the targeting region still remained the activity as in its native RGD-4C form. Moreover, the absorption region was determined whether it had the activity to absorb to PEI. The PEI and RGD-4C-HA were incubated at room temperature for 30 minutes at a molar ratio 6:1. After incubation, the PEI-peptide mixture was separated by a gel filtration column Sephacryl S-200 (GE Healthcare, Chalfont St., UK) and the elution products were collected to test whether the PEI could form complex with RGD-4C-HA. The ninhydrin test was used to identify the existence of PEI (Figure 8a) and the dot immunoblotting was used to determine the existence of RGD-4C-HA by anti-HA antibody (Figure 8b). Ninhydrin reacted to primary or secondary amine group to give a colored product (usually yellow to brown). Although the RGD-4C-HA also has the amine group, the ninhydrin test in this experiment would not be false positive because the concentration of the amine groups in RGD-4C-HA was far below the sensitivity range of ninhydrin. The elution product was double positive in ninhydrin test and dot immunoblotting that revealed the negatively charged absorption region might act with the positively charged PEI to form complex by electrostatic forces.

3.5 The enhancement of transgenic expression by PEI-peptide complex

The binding affinity to B16-F10 and the absorption ability to PEI were proved in the previous study. In this section, the PEI-peptide complex was used as a modified transfection reagent to determine whether it could increase the level of transgenic expression or not. The plasmid pAAV-MCS-hrGFP with a transgene encoding the green fluorescent protein driven by CMV promoter was used as reporter gene. The results showed that the levels of expression were 2.8-fold, 2.3-fold, and 4.8-fold higher than PEI alone in B16-F10, Balb/3T3, and HeLa cells respectively at 10μM RGD-4C-HA (Figure 9). When the concentrations of RGD-4C-HA were decreased, the levels of expression were also reduced for B16-F10 cells. However, the levels of expression were reduced to 63% and 71% than PEI alone in HeLa cells at 1μM and 100nM RGD-4C-HA respectively. The results indicated that RGD-4C-HA combined with PEI increased the expression of transgene in B16-F10 cells and the effects on variant cells were different.

3.6 The enhancement of transgenic expression by PEI-peptide complex (HIF-1)

The PEI-peptide complex was proved to increase the transgene expression in

B16-F10 cells under all concentrations of RGD-4C-HA. However, the transgene expression also increased in Balb/3T3 and HeLa cells under 10μM RGD-4C-HA. The previous study indicated that the increase was only partial specific for B16-F10 cells under some conditions. In previous study, the HIF-1 had been shown to have higher activity in tumor or rapid-proliferating cells (Figure 2 and figure 4). It might be a potential mini-promoter for cancer gene therapy. Therefore, the pCRII-hrGFP which contained 7 copies of HIF-1 responding site was used to determine whether it had the activity of specific expression for B16-F10 cells. The results indicated that the levels of expression were 2.7-fold, 4.6-fold, and 4.4-fold higher than PEI alone for B16-F10, Balb/3T3, and HeLa cells respectively under 10μM RGD-4C-HA (Figure 10). When the concentration of RGD-4C-HA reduced, the levels of expression were 1.3-fold and 1.5-fold than PEI alone in B16-F10 under 100nM and 10nM RGD-4C-HA respectively. However, the levels of expression were no differences than PEI alone in Balb/3T3 and HeLa cells under other RGD-4C-HA concentrations. The results indicated that RGD-4C-HA combined with PEI increased the transgene expression under HIF-1 mini-promoter in B16-F10. However, the transgene expression still increased for all cell types without specificity under 10μM RGD-4C-HA.

3.7 Enhancement of transgenic expression by PEI-peptide complex combined with TSP in B16-F10 cells

The previous study had proved that the PEI-peptide complex could enhance the tansgene expression especially in B16-F10 cells in a partial specific fashion. The combination of HIF-1 mini-promoter with PEI-peptide complex failed to achieve specific expression for target cells. It was shown that TSP had higher activity in tumor or rapid-proliferating cells in previous study. In this section, the pD5-hrGFP which contained TSP was combined with the PEI-peptide complex to determine whether it could achieve specific therapy in B16-F10. The transfection experiments were performed as described above. The results indicated that the levels of expression were 6.7-fold, 2.4-fold, and 1.7-fold higher than PEI alone for B16-F10 cells at 10μM, 100nM, and 10nM RGD-4C-HA respectively (Figure 11). Surprisingly, the levels of expression were almost no differences than PEI alone for Balb/3T3 and HeLa cells under the same condition. The level of expression even reduced to 41% than PEI alone in HeLa cells at 1μM. The results indicated that TSP combined with RGD-4C-HA could achieve specific enhancement of transgenic expression for B16-F10.

Chapter 4 Discussion

The measurements of several transcription factors associated tumorigenecity were rapid and convenient in laboratory. Since the activities of transcription factors varied in different types of tumor cells, the response elements of TSP can be varied according to the transcription factor profiles in target cell. Moreover, the activities of various transcription factors in target cells can be obtained by high-through-put screening systems or reference searching. The information for transcription factors should be helpful in the designation of different TSP.

The novel mini-promoter TSP contained three copies of NF-κB, CREB, and HIF-1 response elements and was active in tumor and rapid-proliferating cells such as B16-F10, Balb/3T3, and HeLa cells. In this study, the activity of NF- B mini-promoter was similar to TSP whereas the HIF-1 mini-promoter was higher than TSP (Figure 4). However, the copy numbers were different among these mini-promoters. The NF-κB mini-promoter contains four copies of NF-κB response elements, CREB mini-promoter contains 5 copies of CREB response elements, and the HIF-1 mini-promoter contains 7 copies of HIF-1 response elements. The copy number of each response element on the mini-promoter may affect the activity of expression. In addition, the spacers which separate different response elements also have influences on the activity of whole promoter.

The activity of TSP was obviously related to the activities of the selected transcription factors: NF-κB, CREB, and HIF-1. In the inhibition experiment, it was shown the activities of TSP were more resistant to inhibitors and were not significantly lowered than control group at the presence of inhibitors (Figure 5). TSP consists of three kinds of response elements and it may result in when the activity of one transcription factor is reduced under certain physiological conditions, the others are still active and maintain the promoter activity. The resistance to such inhibitors may be beneficial for the therapy combined with other therapy such as chemotherapy or certain micro-environment in tumor such as highly angiogenic condition.

TSP mini-promoter consists of only 110 bp which can be modified to improve the activity regulation of expression such as insertion of other transcription factor response element or enhancer. Moreover, the small size of TSP can also be utilized for gene therapy by viral delivery systems. The viral vectors usually use CMV promoter that is always larger than 1kb, thus it may have limitations to result in the fail of package of viral particle containing the large size of therapeutic gene.

RGD-4C-HA was designed as a multi-functional peptide to bind to integrin $\alpha_v\beta_3$ and to absorb to PEI. The RGD-4C (CDCRGDCFC) sequence of RGD-4C-HA could bind to integrin $\alpha_v\beta_3$ expressing cells (B16-F10) as in its native form (Figure 7). Besides, the absorption domain of RGD-4C-HA could bind to PEI

(Figure 8). These results indicate that the functional domains could act without interfering to each other.

In the binding assay of RGD-4C-HA to different cells, RGD-4C-HA strongly bound to B16-F10 rather than others and the total fluorescence intensity was 1.5-fold higher than negative control group under 200nM RGD-4C-HA. Focus on the binding percentage of RGD-4C-HA for Balb/3T3 cells, it was increased significantly but the increase was far lower than for B16-F10 (Figure 7b). However, the total fluorescence mean was no significant differences between negative control group and Balb/3T3 group (Figure 7c). These results may mean that Balb/3T3 cells express few integrin $\alpha_v\beta_3$ on the surface but the level should be below the high sensitivity range.

It was noticed that the total fluorescence intensity was highest under 200nM RGD-4C-HA rather than 2μM. This phenomenon may result from the formation of the inter- or intra-molecular disulfide bond between RGD-4C-HA. There are five cysteines in the RGD-4C-HA sequence and the rate of spontaneous formation of disulfide bond may probably increase at high concentration. The cysteines can form certain disulfide bond to fold proper or improper structures for ligation to integrin $\alpha_v\beta_3$. It was reported that the affinity of RGD-4C to integrin $\alpha_v\beta_3$ is seriouly affected by its correctly secondary structures. Thus RGD-4C-HA may have improper secondary structures to impede its affinity at high concentration.

The PEI-peptide complex can be produced in an easy mixture without complicated chemical coupling reactions. The PEI-peptide complex can enhance the transgene expression in target cells and reduce it in other cells under certain conditions (Figure 9). It was showed that the levels of expression were higher than PEI alone in B16-F10 cells since the RGD-4C-HA can preferentially bind to B16-F10. At the same time, the levels of expression reduced to 63% and 71% than PEI alone in HeLa cells at 1μM and 100nM RGD-4C-HA respectively. The RGD-4C-HA absorbed to PEI and acted as a targeting molecule which may impede the delivery efficiency to non-target cells. However, when the concentrations of RGD-4C-HA were raised to 10μM, the levels of expression also increased in Balb/3T3 and HeLa cells. It would be possible that the toxicity of positively charged PEI was reduced by high concentration of the negatively charged RGD-4C-HA. Therefore the efficiencies of transfection were raised for all cells without specificity. The results of HIF-1 mini-promoter were similar with CMV promoter as above description (Figure 10).

Surprisingly, the combination of RGD-4C-HA and TSP could achieve specific enhancement for transgenic expression in B16-F10 cells (Figure 11). The reasons might be complicated. There are several lines of possibilities to explain these results. First, TSP was more active in B16-F10 than HeLa cells according to previous results and RGD-4C-HA specifically binds to B16-F10 rather than HeLa

cells (Figure 4 and 7). The simultaneity may dramatically increase the specificity of transfection for B16-F10. Besides, RGD-4C-HA interact with more integrins on B16F10 cells to trigger the phosphorylation of endogenous CREB [113] that enhance the expression of transgene via the activity of TSP.

Generally, the perfect specificities of delivery and promoter systems are difficult to achieve so far. In this study we had introduced a simple concept that the combination of partial specific delivery and partial specific promoter activity, it could achieve more specificity for target cells in a rapid and convenient fashion. Besides, the modifications of TSP and RGD-4C-HA should further improve the therapeutic efficacy and will be easier and more applicable than the improvement of either delivery or promoter alone. The strategy of TSP can also apply to the individual therapy by a high-through-put screening for transcription factors. These results may provide a potential way to cancer gene therapy.

Figures

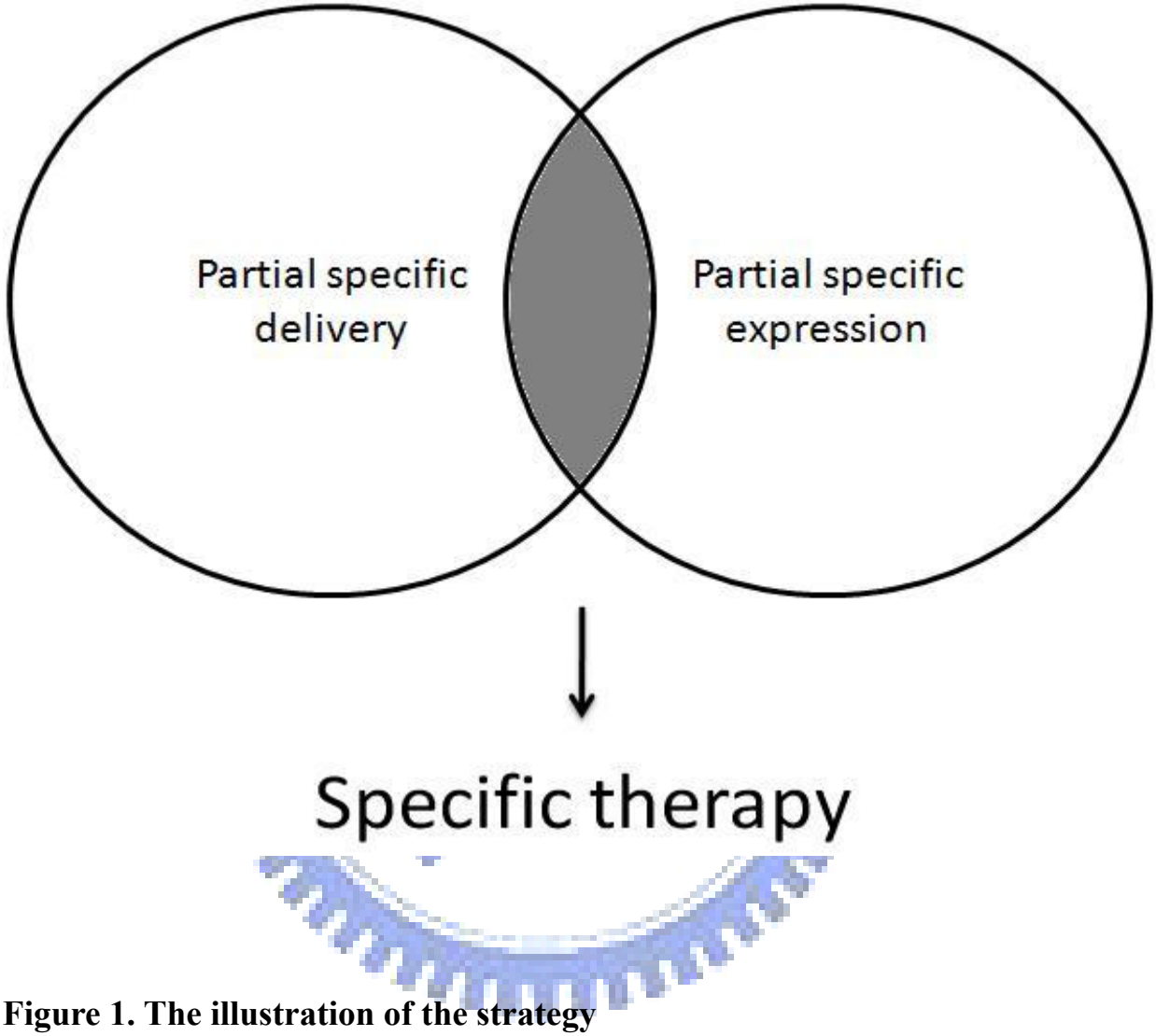


Figure 1. The illustration of the strategy

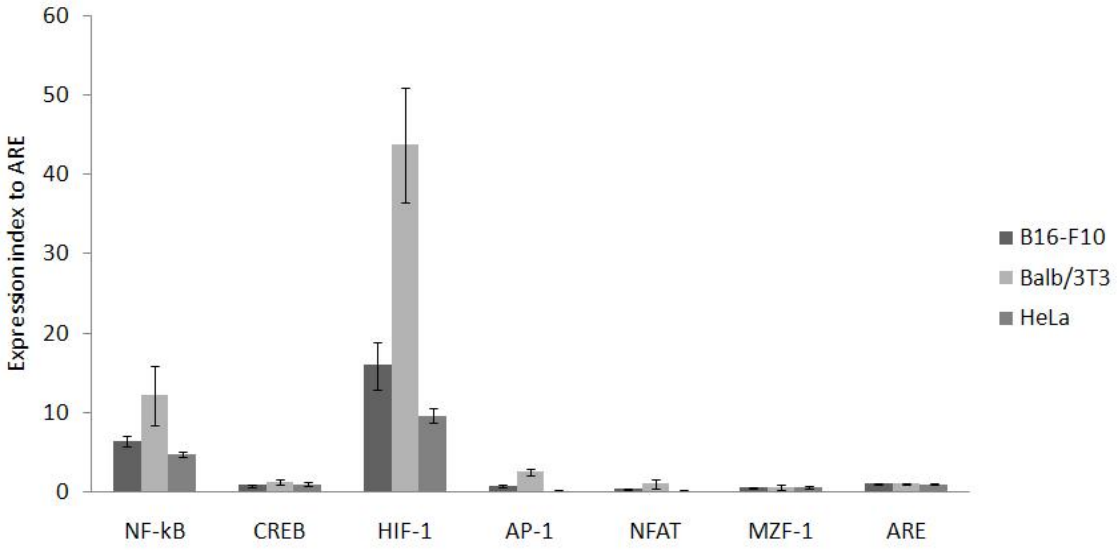


Figure 2. The activities of several transcription factors in different cells

The activities of NF-κB, CREB, HIF-1, AP-1, NFAT, and MZF-1 were assayed 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 hours transfection. The expression indexes represented the activities of transcription factors. The results shown are means ± SE of two independent experiments (n=4).

(a) (b)

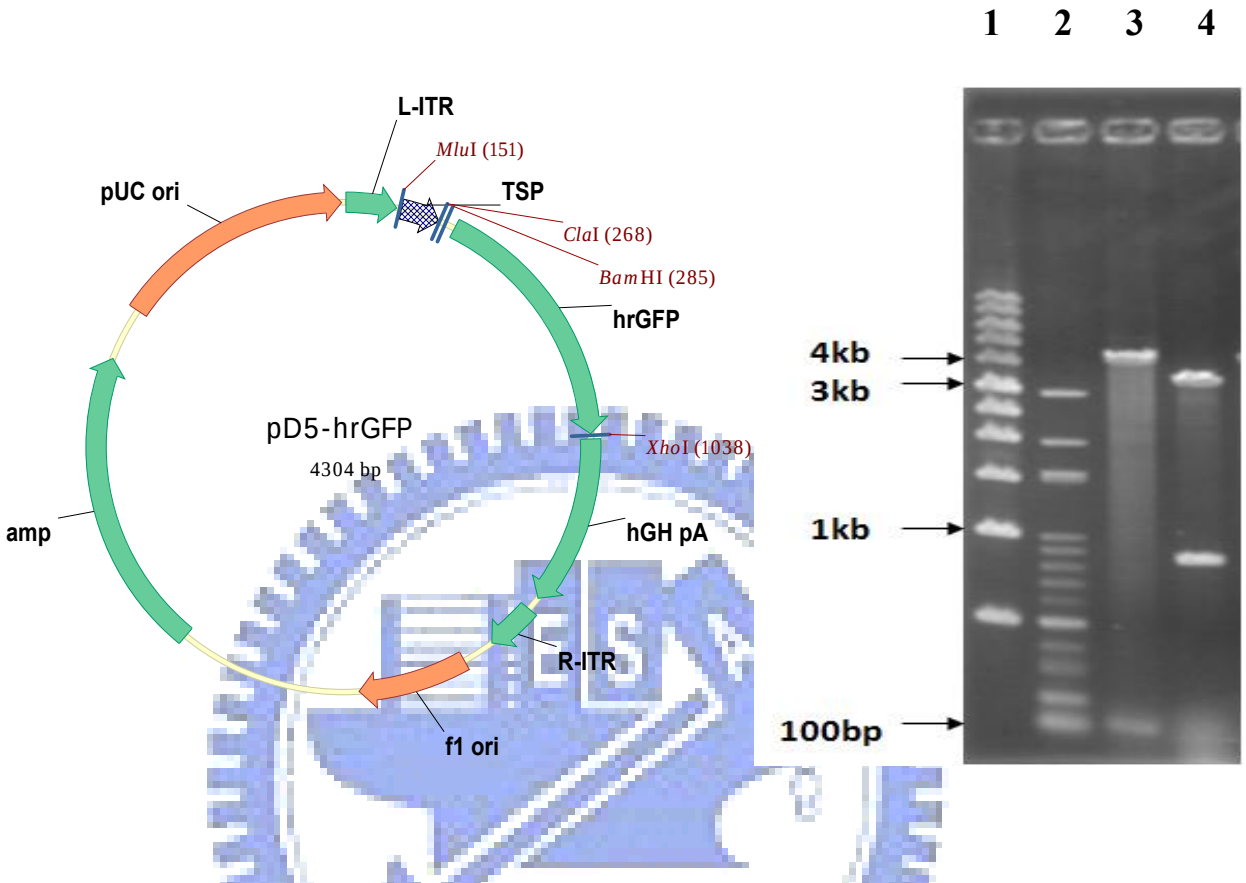


Figure 3. Construction of transcription factor-based synthetic promoter (TSP)
(a) The map of pD5-hrGFP. It was constructed by replacing the CMV promoter of pAAV-MCS-hrGFP with TSP.
(b) Restriction enzyme digestion check
Lane 1: 1kb DNA marker
Lane 2: 100bp DNA marker
Lane 3: MluI & BamHI digestion products of pD5-hrGFP as 4170, 134bp
Lane 4: XhoI & MluI digestion products of pD5-hrGFP as 3417, 887bp

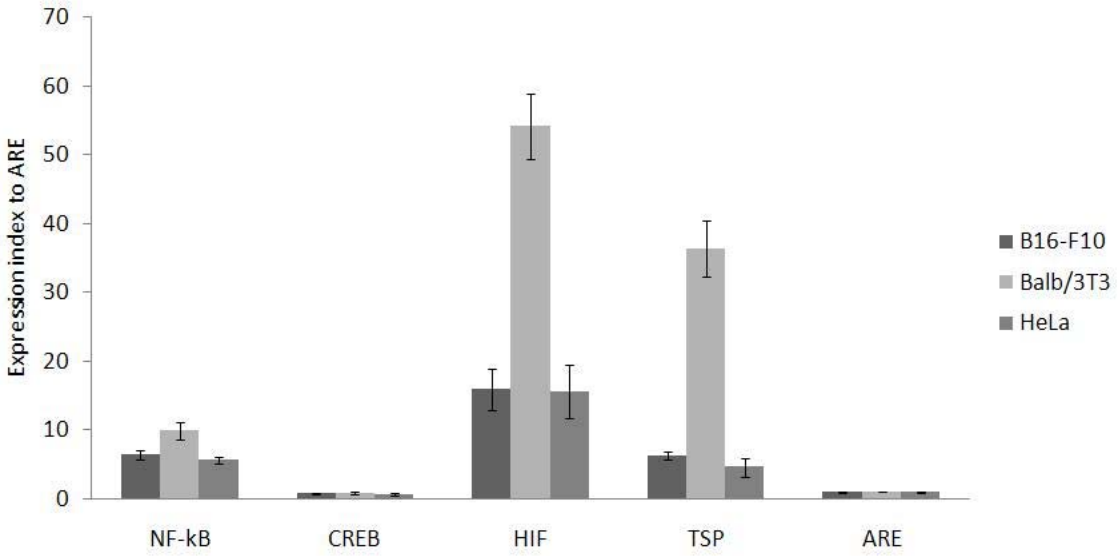


Figure 4. Activities of the transcription factor-based synthetic promoter (TSP) in different cells

The novel mini-promoter TSP contained three copies of the binding sites of NF-κB, CREB and HIF-1. The activities of NF-κB, CREB, HIF-1, and TSP were assayed 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 hours transfection. The results shown are means ± SE of two independent experiments (n=4).

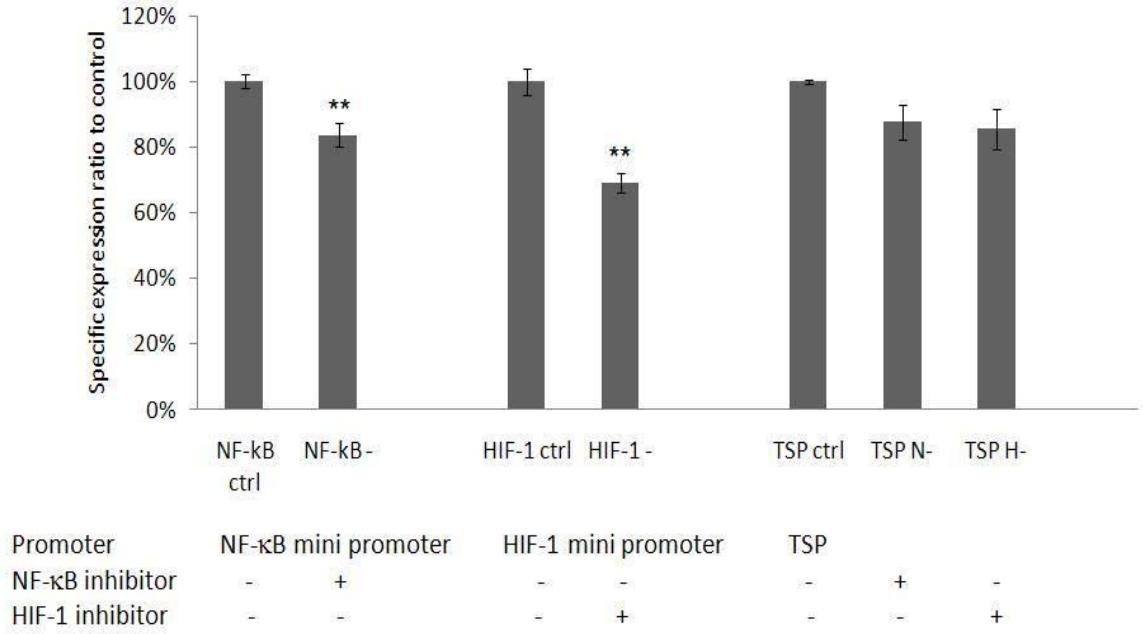


Figure 5. The effect of inhibiting transcription factors to TSP in HeLa cells

NF-κB, HIF-1, and TSP mini-promoters were transfected into HeLa cells to assay the activities when the corresponding transcription factors decreased. Samples were treated with corresponding inhibitors after 24 hours transfection. The fluorescence intensity of 10^4 viable cells was determined by flow cytometry after 16 hours drug treatment with NF-kB inhibitor (25μM hydroquinone) or HIF-1 inhibitor (50μg/ml D609). The results shown are means ± SE of two independent experiments (n=4). ** p<0.01 compared to corresponding control group.

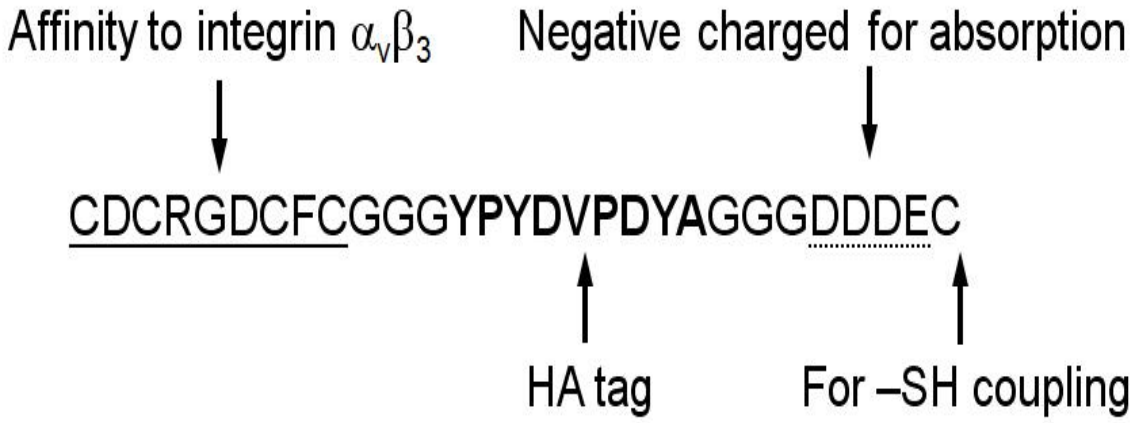
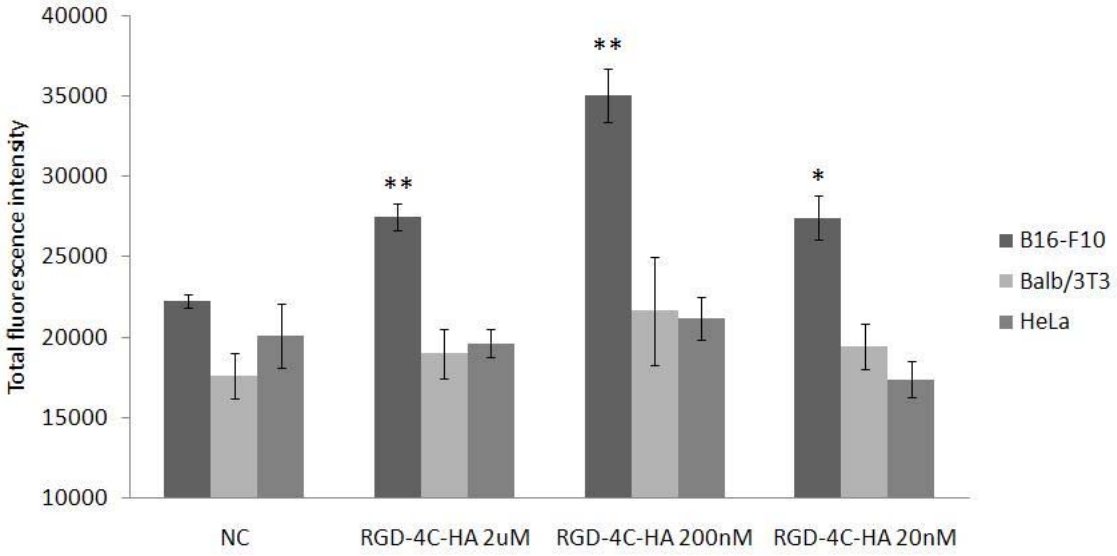
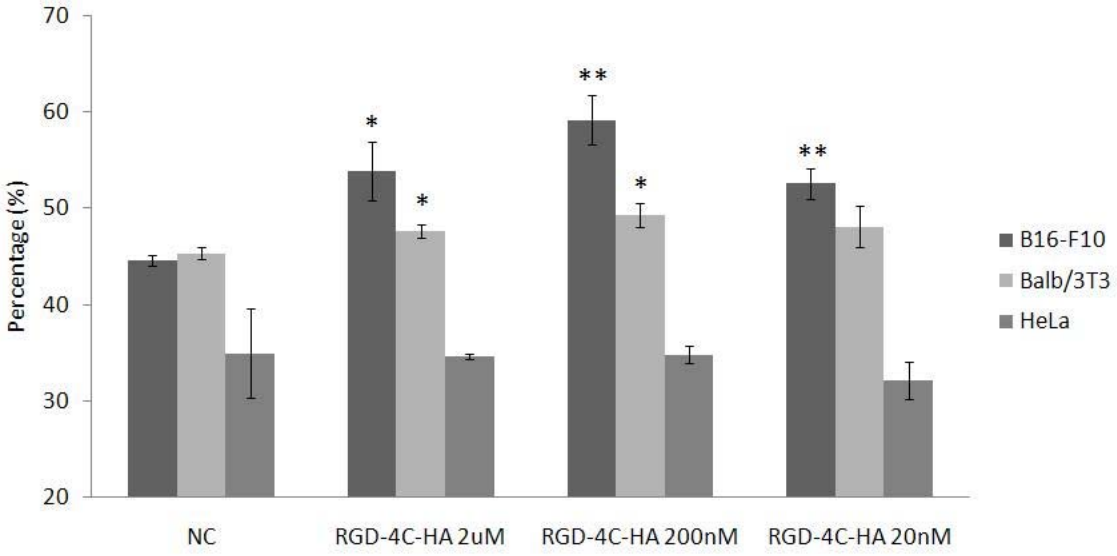


Figure 6. Design and illustration of the multi-functional peptide RGD-4C-HA
The RGD-4C-HA was a multi-functional peptide. The affinity region was the RGD-4C (CDCRGDCFC, underlined) sequence which possessed high affinity to B16-F10. The identity region was a HA tag (YPYDVPDYA, bolded) which acted as a spacer and an epitope for detection. The absorption region (DDDE, dotted) contained four continuous amino acids with negatively charged residues. The final amino acid cysteine which contained a sulfyl group was used to coupling with the primary amine group of PEI. The two GGG sequences were spacers to separate the functional domains.

(a)


40000
35000
30000
25000
20000
15000
10000
Total fluorescence intensity
**
**
*
NC
RGD-4C-HA 2uM
RGD-4C-HA 200nM
RGD-4C-HA 20nM
B16-F10
Balb/3T3
HeLa

(b)


70
60
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30
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Percentage (%)
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NC
RGD-4C-HA 2uM
RGD-4C-HA 200nM
RGD-4C-HA 20nM
B16-F10
Balb/3T3
HeLa

(c)

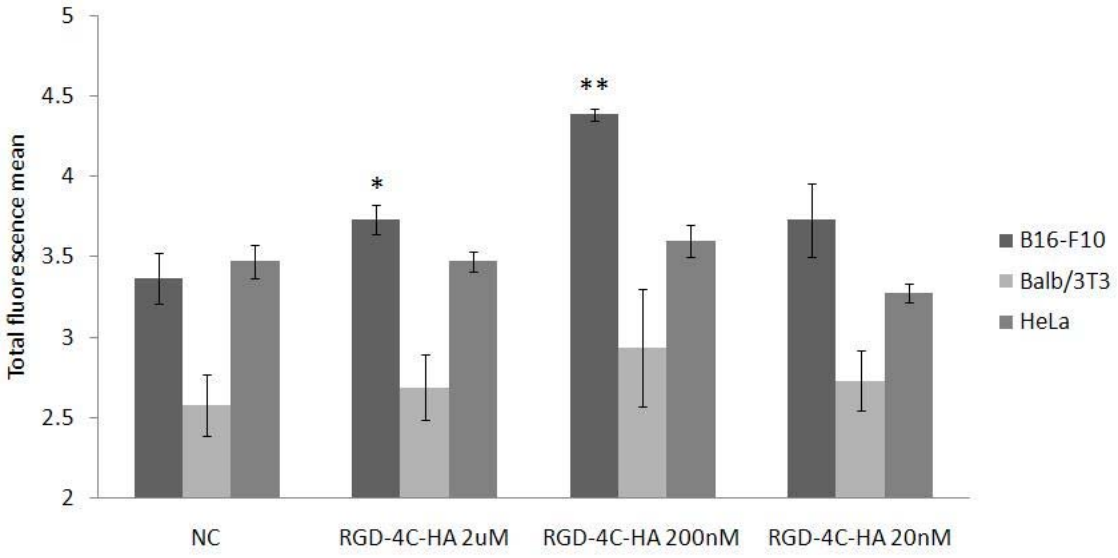

Figure 7. The binding efficacy of RGD-4C-HA to different cells

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 anti-HA antibody conjugated FITC. The surface immunofluorescence of 10^4 viable cells was determined by flow cytometry. The results were shown as (a) the total fluorescence intensities (= the event number × the fluorescence mean), (b) the binding percentages (= the percentage of overexpression) or (c) the total fluorescence mean in different cells. The data shown are means ± SE of two independent experiments (n=4). * p<0.05 compared to control, ** p<0.01 compared to control.

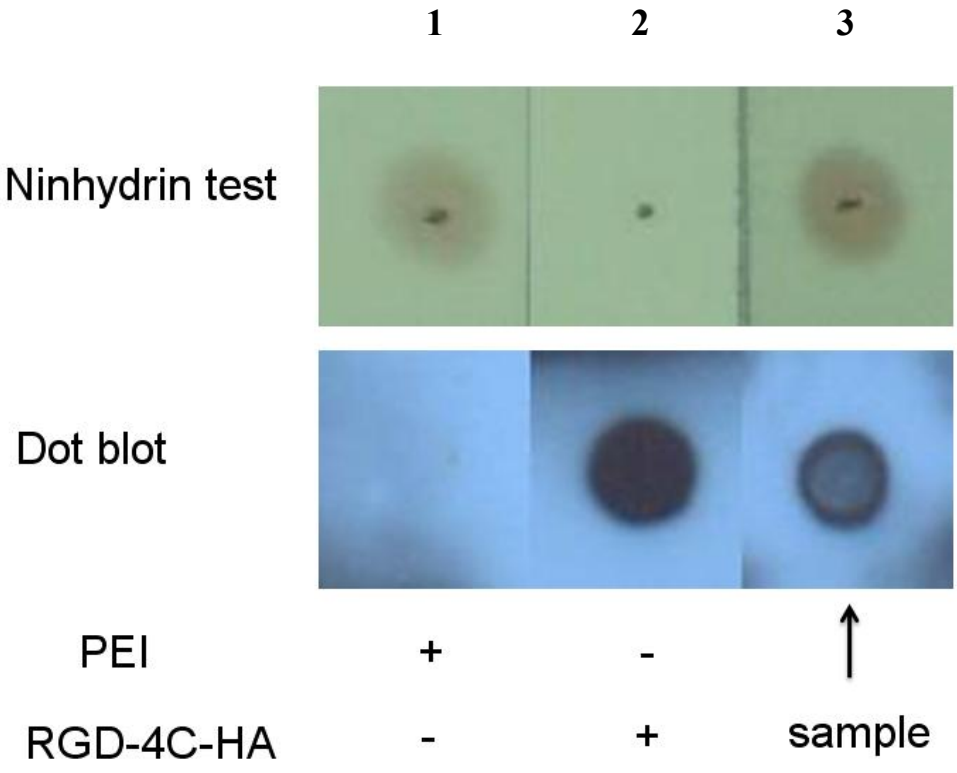

Figure 8. PEI and RGD-4C-HA absorption assay

The mixture of the PEI and RGD-4C-HA were purified by 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 the dot immunoblotting was used to determine the existence of RGD-4C-HA. PEI alone and RGD-4C-HA alone were used as control group, and the elution product from Sephacryl S-200 column (PEI-peptide complex) was used as sample. The results shown are one of three independent experiments.

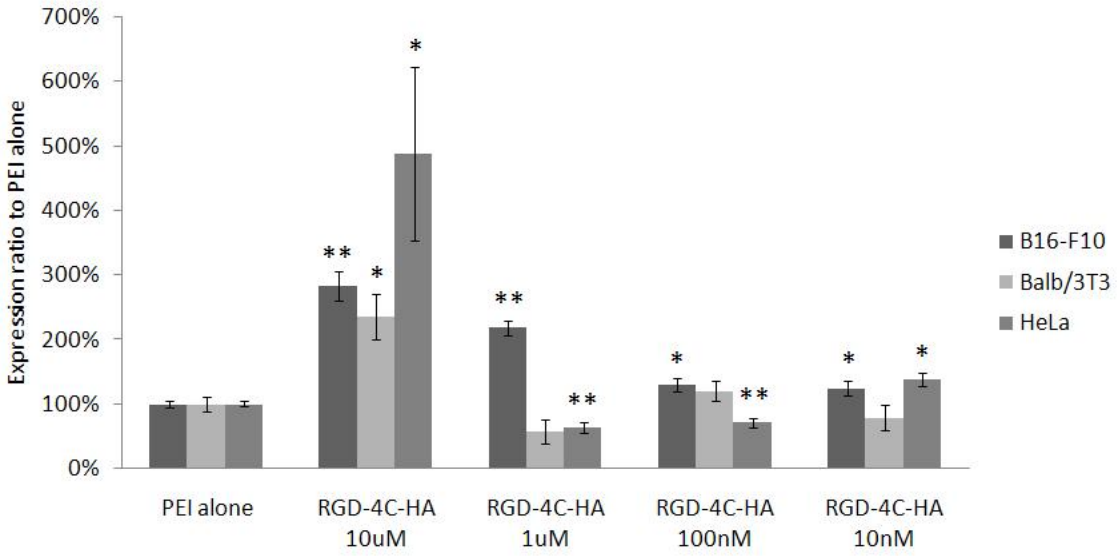

Figure 9. The enhancement of transgenic expression by PEI-peptide complex (pAAV-MCS-hrGFP)

The transfection experiments were performed as described above. The plasmid pAAV-MCS-hrGFP was mixed with PEI-peptide complex to transfect into cells. After transfection for 24 hours, the fluorescence intensities of transfectants were measured and the ratios of fluorescent expression comparing to PEI-transfectants were showed. The results shown are means ± SE of two independent experiments (n=4). * $p<0.05$ compared to control, ** $p<0.01$ compared to control.

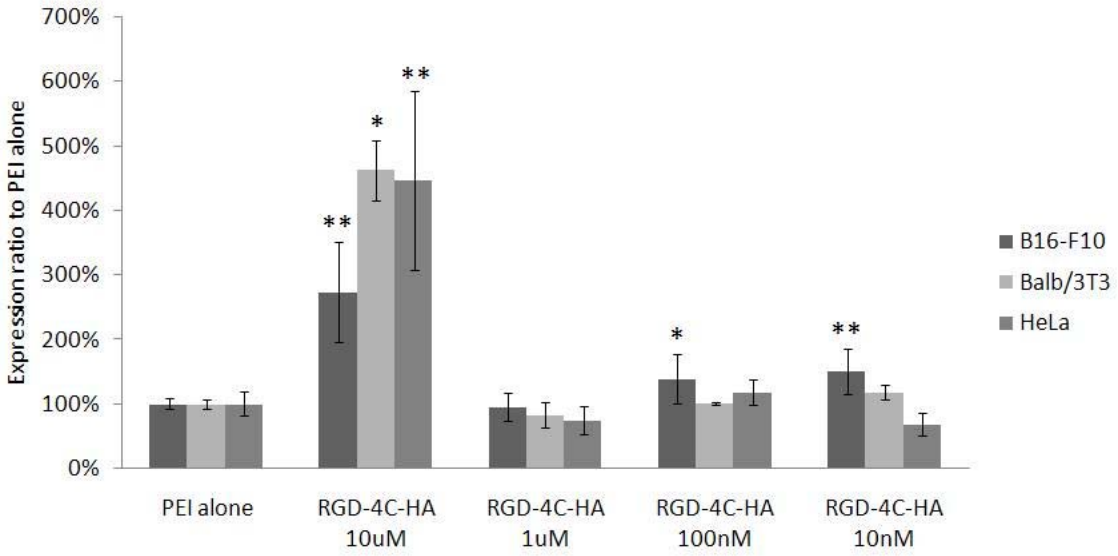

Figure 10. The enhancement of transgenic expression by PEI-peptide complex (pCRII-hrGFP)

The plasmid pCRII-hrGFP with a transgene encoding the green fluorescent protein driven by HIF-1 mini-promoter (7 copies of HIF-1 responding site) was used as reporter gene. The fluorescence intensities of 10^4 viable cells was determined by flow cytometry after 24 hours transfection. The ratios of fluorescent expression comparing to PEI-transfectants were showed. The results shown are means ± SE of two independent experiments (n=4). * $p<0.05$ compared to control, ** $p<0.01$ compared to control.

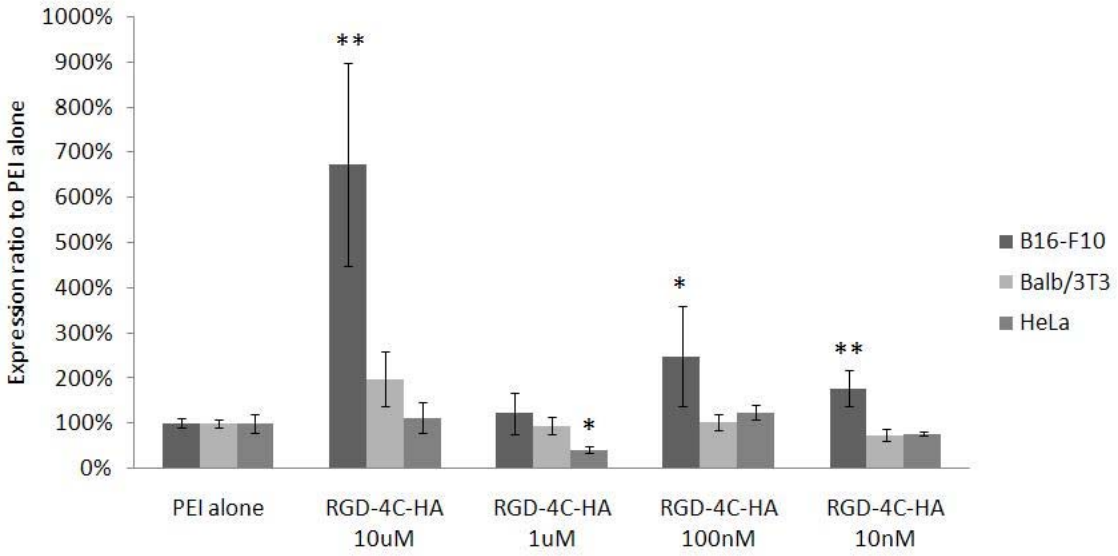

Figure 11. Enhancement of transgenic expression by PEI-peptide complex combined with TSP in B16-F10 cells

The plasmid pD5-hrGFP with a transgene encoding the green fluorescent protein driven by TSP was used as reporter gene. The fluorescence intensities of 10^4 viable cells were determined by flow cytometry after 24 hours transfection. The ratios of fluorescent expression comparing to PEI-transfectants were showed. The results shown are means ± SE of two independent experiments (n=4). * $p<0.05$ compared to control, ** $p<0.01$ compared to control.

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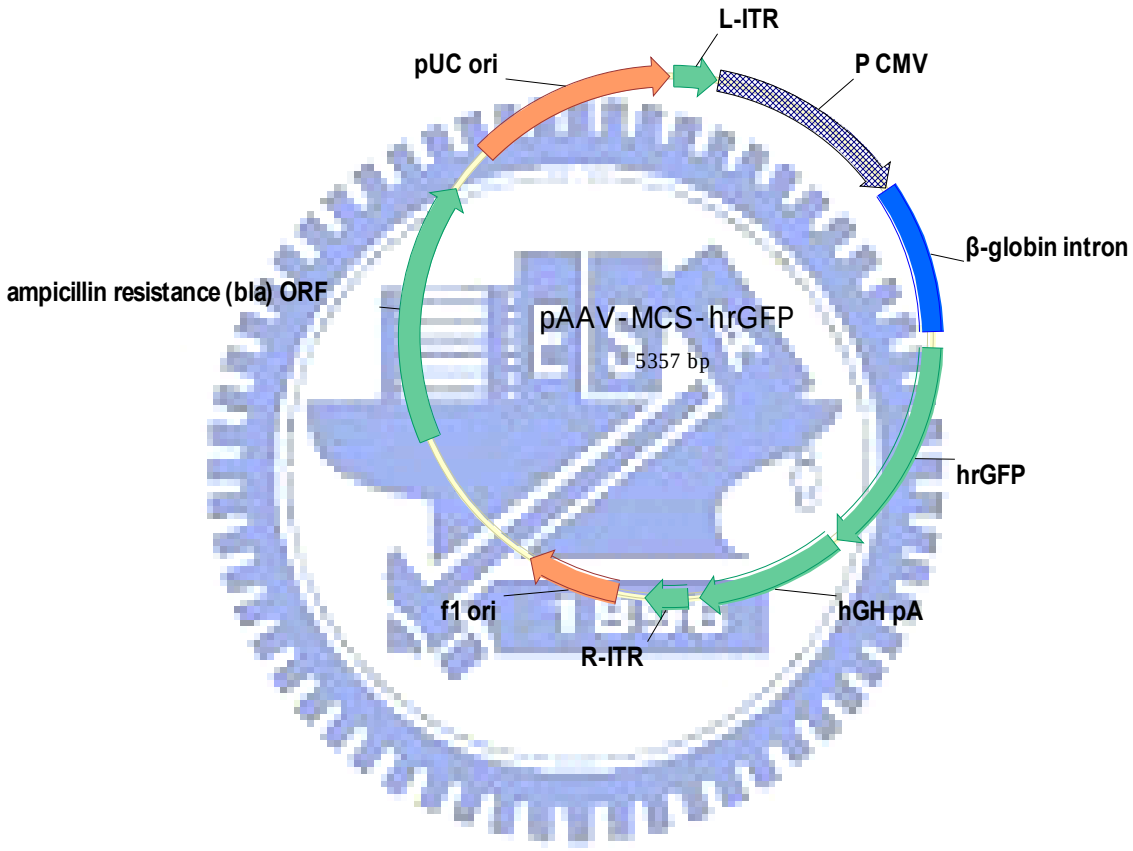
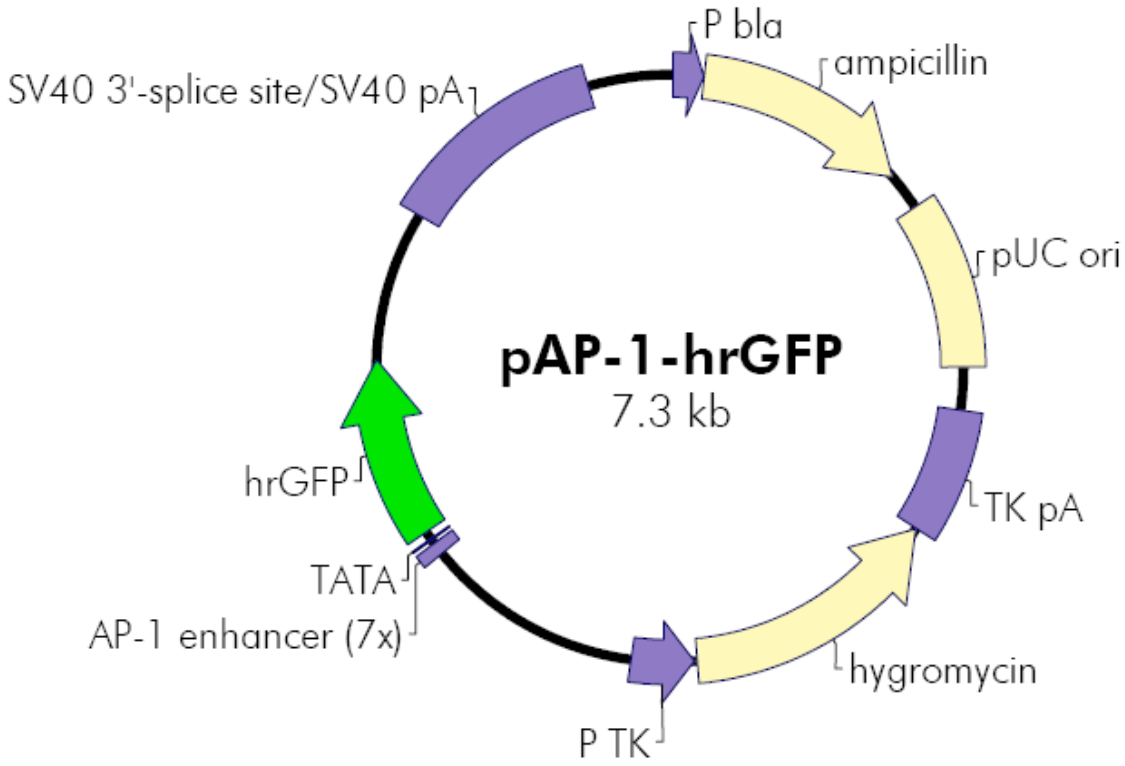
Appendices

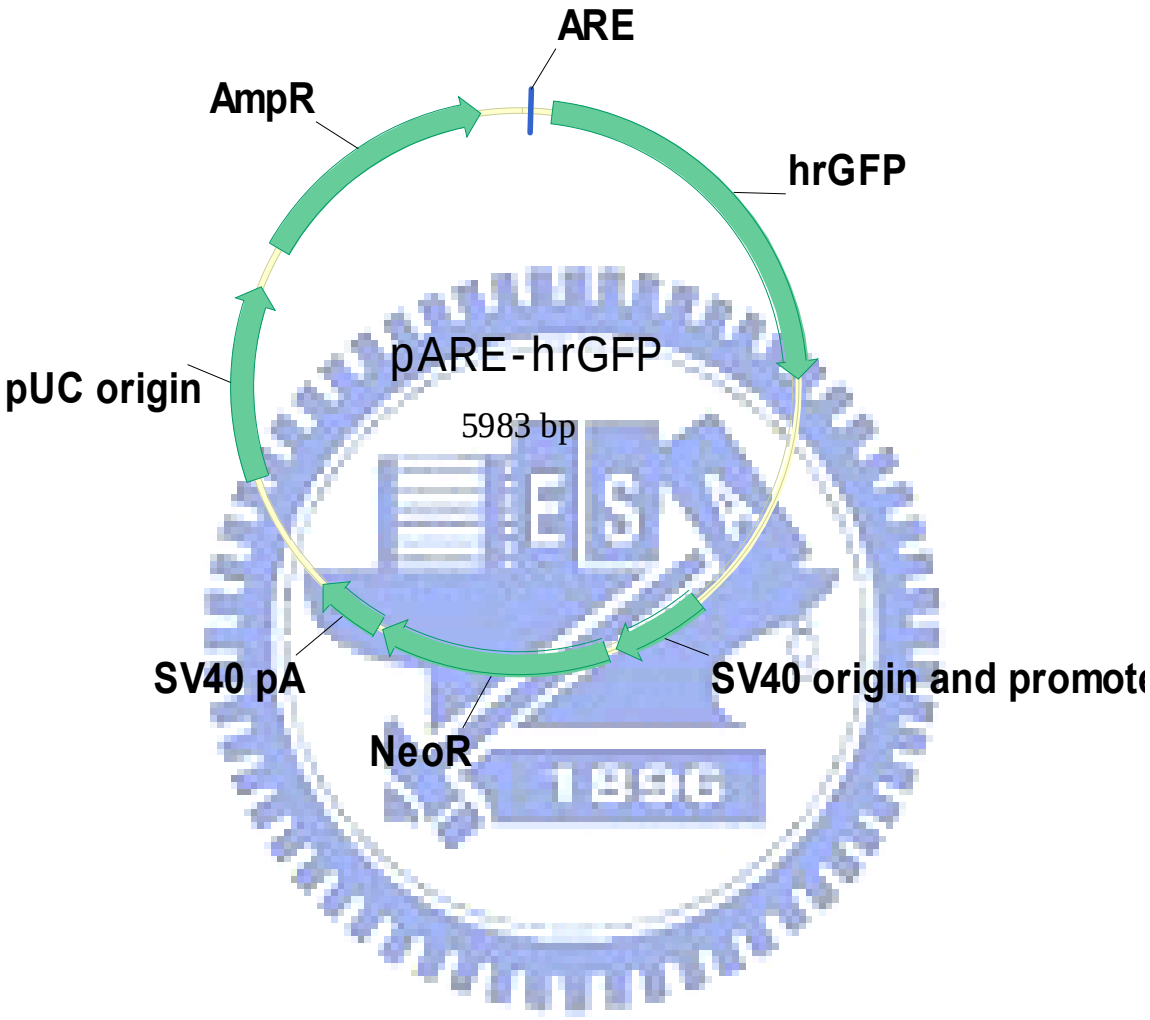
A1. The map of pAAV-MCS-hrGFP



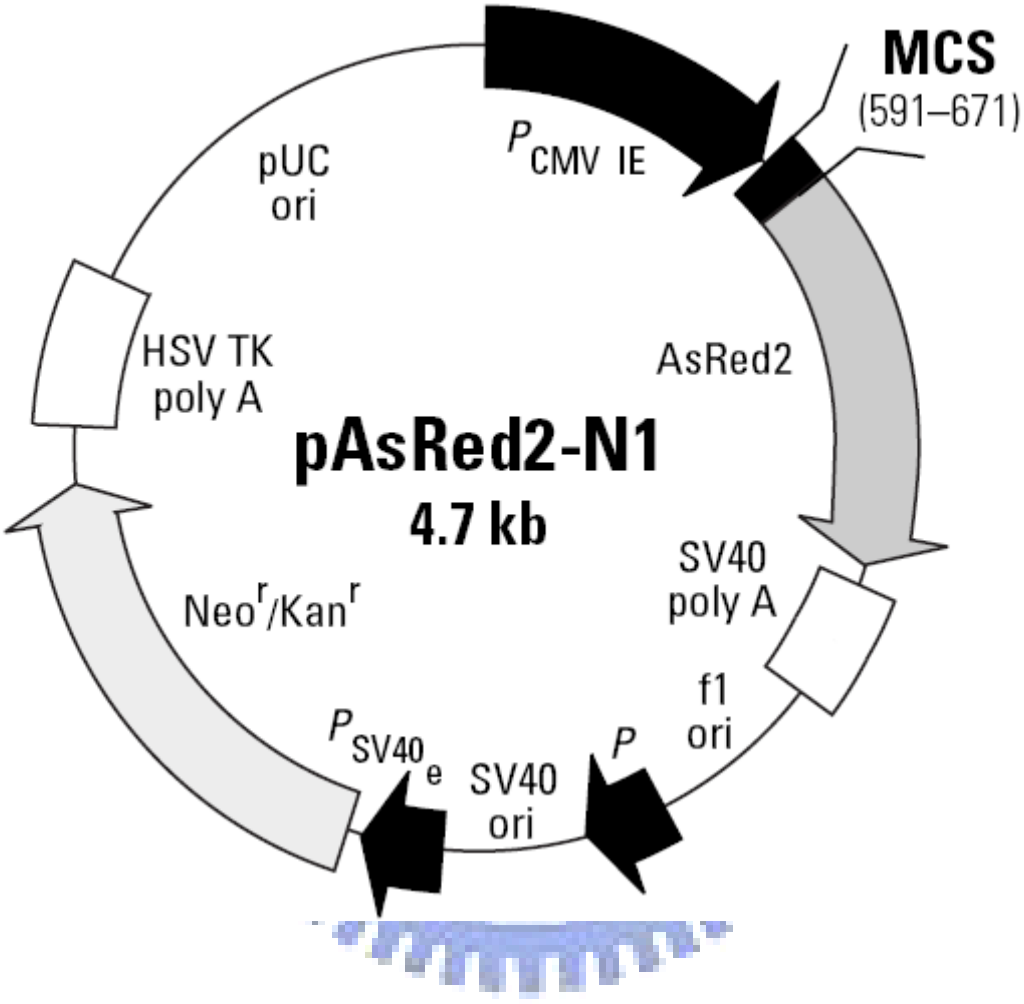
A2. The map of pAP-1-hrGFP



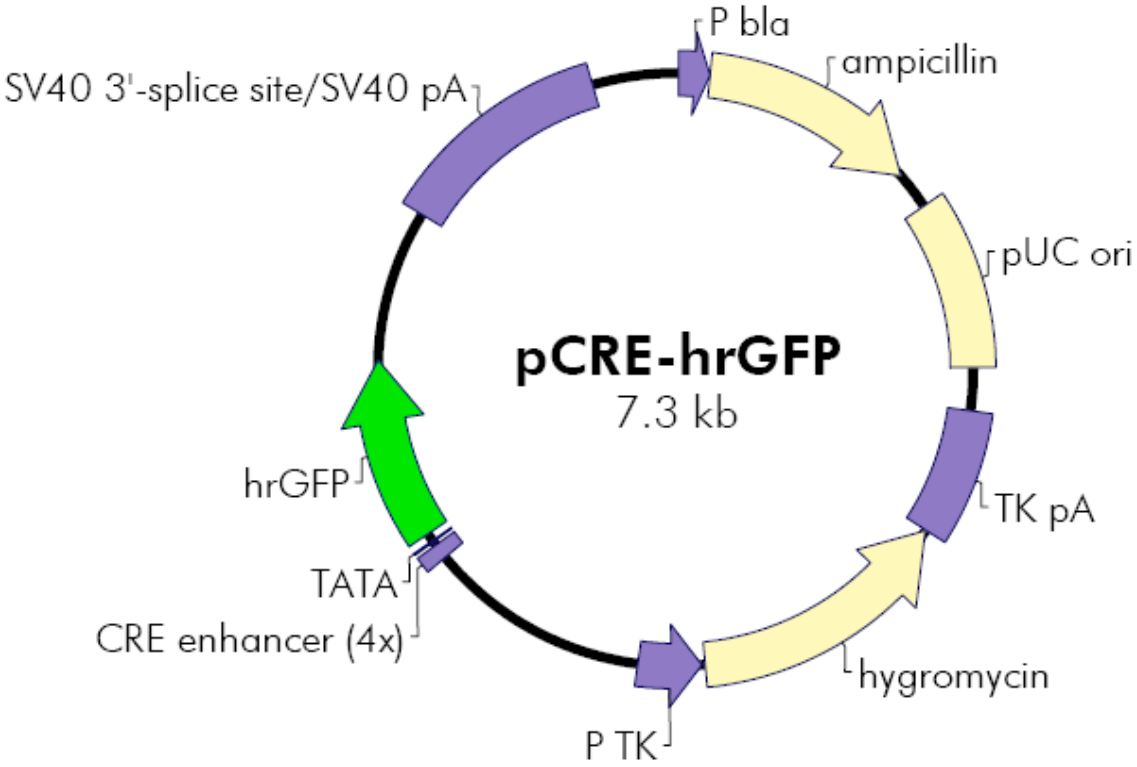
A3. The map of pARE-hrGFP



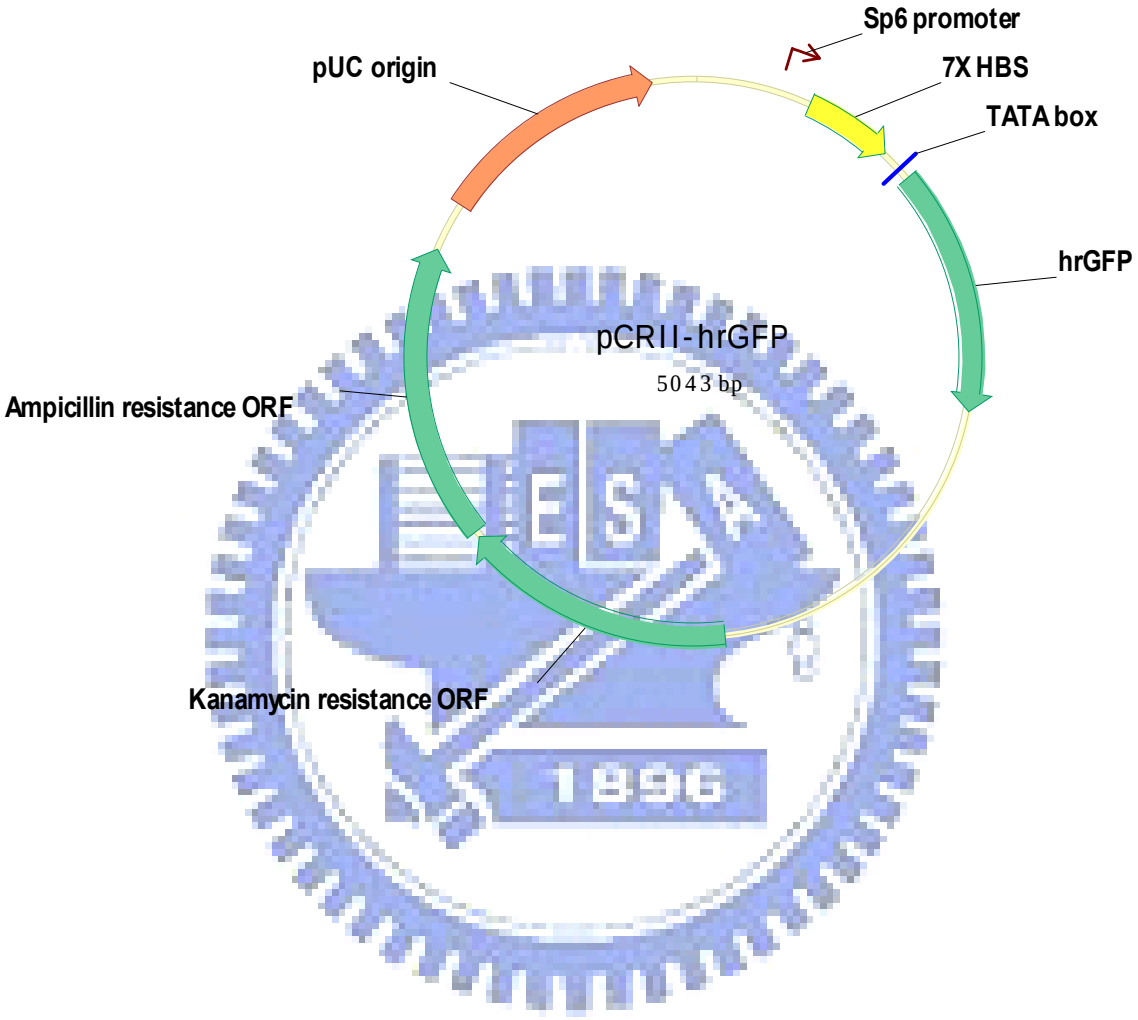
A4. The map of pAsRed2-N1



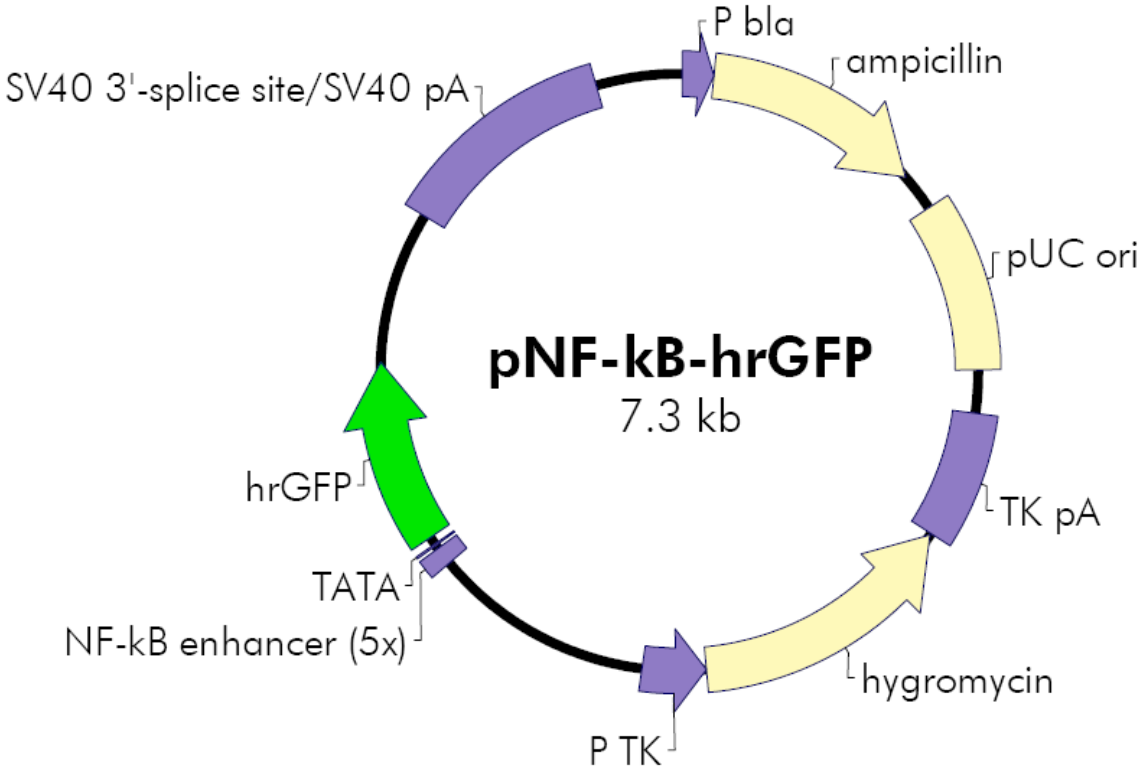
A5. The map of pCRE-hrGFP



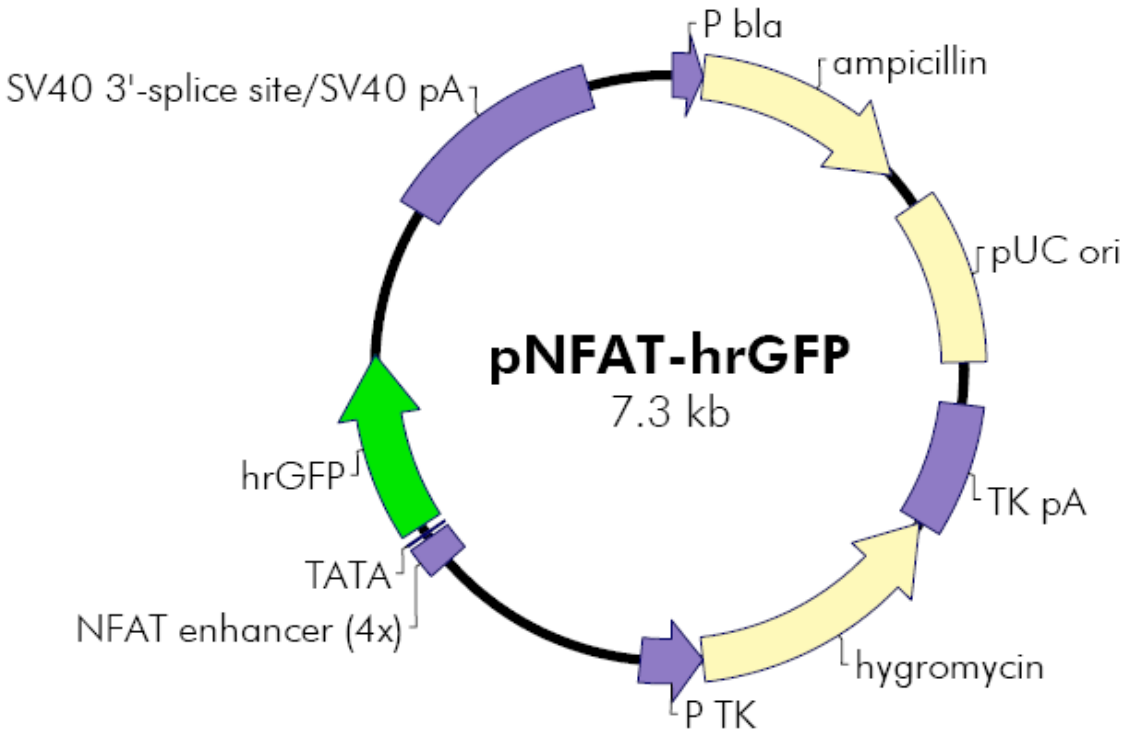
A6. The map of pCRII-hrGFP



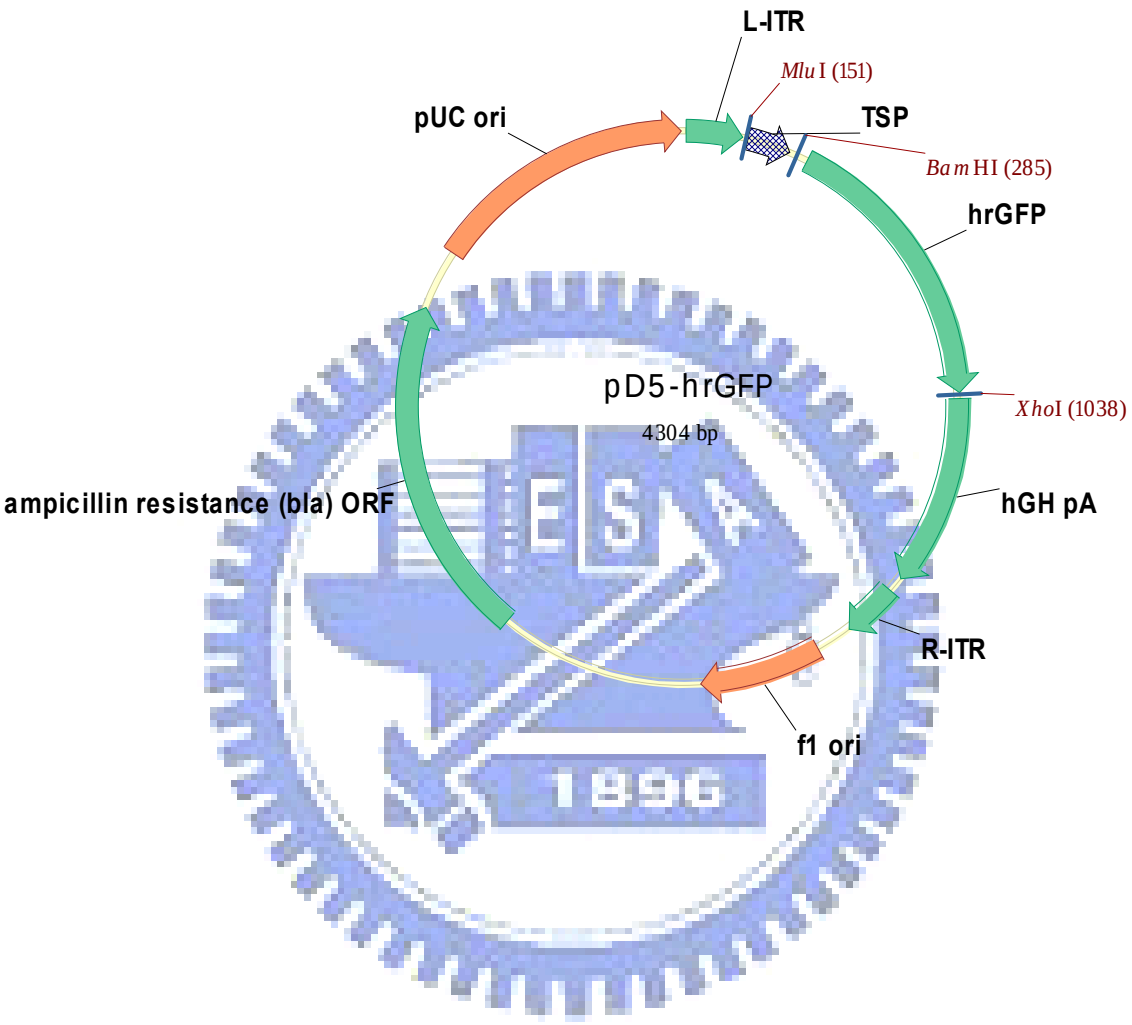
A7. The map of pNF-κB-hrGFP



A8. The map of pNFAT-hrGFP



A9. The map of pD5-hrGFP



A10. Sequence of pD5-hrGFP

pD5-hrGFP 4304bp

left inverted terminal repeat 1–141

TSP mini-promoter 156–265

hrGFP ORF 314-1034

hGH polyA 1049-1527

right inverted terminal repeat 1567–1707

f1 origin 1799-2105

mpicillin resistance (bla) ORF 2624-3481

pUC origin 3632-4299

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   1 CCTGCAGGCA GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC CCGGGCAAAG
  51 CCCGGGCGTC GGGCGACCTT TGGTCGCCCG GCCTCAGTGA GCGAGCGAGC
 101 GCGCAGAGAG GGAGTGGCCA ACTCCATCAC TAGGGGTTCC TGCGGCCGCA
 151 CGCGTGGGAC TTTCCGCTGG GGACTTTCCG CTGGGGACTT TCCGCTGTGA
 201 CGTCAGAGAG CTGACGTCAG AGAGCTGACG TCAGAGAGCT ACGTGTGTGT
 251 ACGTGTGTGT ACGTGATCGA TTGAATTCCC CGGGGATCCC CGGGTACCGA
 301 GCTCGAATTC ACCATGGTGA GCAAGCAGAT CCTGAAGAAC ACCCGCCTGC
 351 AGGAGATCAT GAGCTTCAAC GTGAACCTGG AGGGCGTGGT GAACAACCAC
 401 GTGTTCACCA TGGAGGGCTG CGGCAAGGGC AACATCCTGT TCGGCAACCA
```

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501 ACATCCTGAG CCCCGCCTTC CAGATCGGCA ACCGCACCTT CACCAAGTAC

551 CCCGAGGACA TCAGCGACTT CTTCATCCAG AGCTTCCCCG CCGGCTTCGT

601 GTACGAGCGC ACCCTGCGCT ACGAGGACGG CGGCCTGGTG GAGATCCGCA

651 GCGACATCAA CCTGATCGAG GAGATGTTCG TGTACCGCGT GGAGTACAAG

701 GGCCGCAACT TCCCCAACGA CGGCCCCGTG ATGAAGAAGA CCATCACCGG

751 CCTGCAGCCC AGCTTCGAGG TGGTGTACAT GAACGACGGC GTGCTGGTGG

801 GCCAGGTGAT CCTGGTGTAC CGCCTGAACA GCGGCAAGTT CTACAGCTGC

851 CACATGCGCA CCCTGATGAA GAGCAAGGGC GTGGTGTAGG ACTTCCCCGA

901 GTACCACTTC ATCCAGCACC GCCTGGAGAA GACCTACGTG GAGGACGGCG

951 GCTTCGTGGA GCAGCATGAG ACCGCCATCG CCCAGCTGAC CAGCCTGGGC

1001 AAGCCCCTGG GCAGCCTGCA CGAGTGGGTG TAATAGCTCG AGAGATCTAC

1051 GGGTGGCATC CCTGTGACCC CTCCCCAGTG CCTCTCCTGG CCCTGGAAGT

1101 TGCCACTCCA GTGCCCACCA GCCTTGTCCT AATAAAATTA AGTTGCATCA

1151 TTTTGTCTGA CTAGGTGTCC TTCTATAATA TTATGGGGTG GAGGGGGGTG

1201 GTATGGAGCA AGGGGCAAGT TGGGAAGACA ACCTGTAGGG CCTGCGGGGT

1251 CTATTGGGAA CCAAGCTGGA GTGCAGTGGC ACAATCTTGG CTCACTGCAA

1301 TCTCCGCCTC CTGGGTTCAA GCGATTCTCC TGCCTCAGCC TCCCGAGTTG

1351 TTGGGATTCC AGGCATGCAT GACCAGGCTC AGCTAATTTT TGTTTTTTTG

1401 GTAGAGACGG GGTTTCACCA TATTGGCCAG GCTGGTCTCC AACTCCTAAT

1451 CTCAGGTGAT CTACCCACCT TGGCCTCCCA AATTGCTGGG ATTACAGGCG

1501 TGAACCACTG CTCCCTTCCC TGTCCTTCTG ATTTTGTAGG TAACCACGTG

1551 CGGACCGAGC GGCCGCAGGA ACCCCTAGTG ATGGAGTTGG CCACTCCCTC

1601 TCTGCGCGCT CGCTCGCTCA CTGAGGCCGG GCGACCAAAG GTCGCCCGAC

1651 GCCCGGGCTT TGCCCGGGCG GCCTCAGTGA GCGAGCGAGC GCGCAGCTGC

1701 CTGCAGGGGC GCCTGATGCG GTATTTTCTC CTTACGCATC TGTGCGGTAT

1751 TTCACACCGC ATACGTCAAA GCAACCATAG TACGCGCCCT GTAGCGGCGC

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2251 CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGCCC CGACACCCGC

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3001 CATGAGTGAT AACACTGCGG CCAACTTACT TCTGACAACG ATCGGAGGAC

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3151 TGACACCACG ATGCCTGTAG CAATGGCAAC AACGTTGCGC AAACTATTAA

3201 CTGGCGAACT ACTTACTCTA GCTTCCCGGC AACAATTAAT AGACTGGATG

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