# TESTING A NON-VIRAL VECTOR FOR ITS LONG TERM STABILITY AND REPLICATION ABILITY

By

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To my mom

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LIST OF ABBREVIATIONS

| | |
|---|---|
| 6-MPH4 | 6-Methyl-5,6,7,8-tetrahydropterine |
| AAT | α1 antityrpsin |
| AAV | Adeno-associated Virus |
| Bcl-2 | B-cell lymphoma 2 apoptotic regulator |
| $BH_4$ | Tetrahydrobiopterin |
| Bp | Basepair |
| BPV | Bovine papilloma virus |
| cDNA | Complementary DNA |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CHO | Chinese hamster ovary |
| CMV | Cytomegalovirus |
| CpG sites | Cytosine guanine rich sites |
| CsCl | Cesium Chloride |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dubelco's Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| DOPE | 1,2-Dioleoyl-sn-Glycero-3-phophoethanolamine |
| DOTAP | Dioleoyl-trimethylammonium propane |
| Dpm | Decay per minute |
| Dpt | Days post-transfection |
| EBNA1 | Epstein-Barr encoded nuclear antigen |
| EBV | Epstein –Barr virus |
| FACS | Fluorescent activated cell sorting |

| | |
|---|---|
| FBS | Fetal bovine serum |
| GFP | Green Fluorescent Protein |
| HBS | Hepes Buffered Saline |
| hCA2 | Human carbonic anhydrase 2 |
| HTV | Hydrodynamic tail vein injections |
| ITR | Inverted Terminal Repeat |
| Kb | Kilobase |
| KSHV | Kaposi Sarcoma-associated Herpesvirus |
| LANA | Latency Associated Nuclear Antigen |
| NAb | Neutrilizing antibody |
| NAD(H) | Nicotineamide Adenine dinucleotide |
| ORMOSIL | Organically modified silica nanoparticle |
| PAH | Phenylalanine hydroxylase |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PKU | Phenylketonuria |
| Rb | Retinoblastoma |
| RFP | Red fluorescent protein |
| SAF-A | Scaffold Attachment Factor A |
| SCID-X1 | X-linked Severe combined immunodeficiency syndrome |
| siRNA | Small interfering RNA |
| SLK | An endothelial cell line |
| SMAR | Scaffold/Matrix Attachment Region |

| | |
|---|---|
| SMGT | Sperm mediated Gene Transfer |
| SV40 | Simian virus 40 |
| TR | Terminal Repeat |
| UTMD | Ultrasound targeted microbubble destruction |
| VEGF | Vascular endothelial growth factor |

Abstract of Thesis Presented to the Graduate School
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An ideal non-viral gene therapy vector should provide long term transgene expression in dividing and non-dividing cells without being dependent on integration since it was shown that integration can cause potential mutations. Stable expression without integration can be achieved by using vectors which persist episomally and replicate upon cell division. Thus far, Scaffold/Matrix Attachment Region (SMAR)-based vectors have attracted attention for their replication abilities in the presence of an initial selection. Here, we tested the long-term maintenance of SMAR vectors in 293 cells in terms of reporter Green Fluorescent Protein (GFP) expression without applying an initial selection pressure. The rate of GFP expression loss over time was similar for SMAR and non-SMAR control vectors suggesting that initial selection pressure is necessary for long-term SMAR-based vector replication.

We also designed and tested a novel vector which combines a SMAR element with the minimal replicator (Latency Associated Nuclear Antigen and Terminal Repeats) of Kaposi Sarcoma-Associated Herpesvirus (KSHV). This vector was designed with a goal of increasing episomal maintenance and partition efficiency. The novel vector was also unable to provide long term GFP expression and GFP positive cells displayed a

dying cell phenotype. The underlying reason for rapid loss of GFP positive cells was hypothesized to be the competition between the two different episomal maintenance factors, SMAR and LANA-TR, for binding to different nuclear locations. To address that possibility, a fluorescence-tagged KSHV replicator protein was utilized to identify the presence of disordered nuclear structures in the SMAR/LANA-TR vector transfected cells. It was found that the novel vector causes multi-nucleation after several cell divisions. In summary, it was found that the SMAR element is insufficient for long term maintenance without initial selection and the initial attempts of constructing a LANA-SMAR combination vector did not yield a vector suitable for gene therapy purposes. Vectors derived from episomally stable viruses are still a promising area of inquiry, but further development will be necessary.

# CHAPTER 1
# INTRODUCTION

## The Importance of Non-viral Vectors

Gene therapy has become a promising option for treatment of genetically inherited and acquired diseases. Our understanding of possible mechanisms for gene introduction has progressed at a rapid pace. One of the main hurdles in gene therapy is the requirement for vectors which can efficiently deliver transgenes to the target cell, transit into the nucleus, and express the gene without otherwise altering the cell. Historically, the first recombinant viral vectors such as Retroviruses, Adenoviruses, and Adeno-associated viruses (AAV) were used as natural carriers in preclinical and clinical trials. Another approach is to use non-viral vectors in which transgene carrying DNA can be delivered either in a naked form or complexed with lipids or nanoparticles.

Viral vectors are quite promising, but there are problems associated with the safety, large scale manufacture, and complex manipulation steps required for viral vector construction.[1] In 2001, a clinical gene therapy trial for X-linked severe combined immunodeficiency, known as SCID-X1, was conducted in France and nine out of ten patients were corrected for the disease with *ex vivo* retrovirus mediated gene therapy. However, three years later it was reported that two of the ten patients developed leukemia due to uncontrolled integration events. [2] Additionally, in AAV mediated gene therapy studies, it has been reported that pre-existing neutralizing antibodies (NAb) against AAV capsid in human population may restrict the clinical use of AAV serotypes.[3] When GFP-encoding AAV8 was intravenously injected into macaques, liver transduction was diminished depending on the pre-existing NAb levels. [4] The large scale production of viral vectors poses a problem since those vectors should be

replication defective for safety purposes. Recombinant Adenovirus vectors which lack most viral sequences can be generated when all the required factors are supplied *in trans*. However, helper virus contamination and absence of stable cell lines expressing viral factors makes the production harder to scale up for clinical applications.[5] For such reasons, non-viral vectors are becoming prominent alternative tools for gene delivery. First of all, they are cheaper for large scale production. Manipulation and construction of non-viral vectors are easier when compared to Adenovirus-based vectors. It is also possible to target specific cell types via modification of lipid or nanoparticles. As an example, addition of galactose and lactose moieties onto the cationic lipids showed increased hepatic uptake of systemically injected DNA-lipid complexes.[6]

**Construction and Production of the Non-viral Vectors**

Non-viral gene therapy vectors depend on plasmid biology. Plasmids encoding the therapeutic protein have bacterial sequences such as the replication origin for amplification in bacteria and a drug resistance gene for selection. The therapeutic protein expression cassette should include following elements: a promoter region which can be designed for tissue specific expression, a 5' untranslated region including an intron for efficient processing of mRNA, an open reading frame, and a 3' untranslated region containing a polyadenylation signal.[7] The highest level of transgene expression is achieved when the plasmid DNA is in supercoiled form.[8] The protocols for bench scale plasmid amplifications are not suitable for large scale production of supercoiled DNA. Therefore, a combination of anion-exchange and size exclusion chromatography should be used for pharmaceutical DNA manufacturing.[9] Depending on the bacterial backbone, 500 L of fermentation can yield up to 20 grams of DNA. [10]

## Delivery of the Plasmid DNA

### Physical Delivery Methods

The naked DNA can yield gene expression when injected either locally or systemically. It was first reported that intramuscular injection of a reporter gene yielded gene expression in muscle cells.[7] One example of the clinical trials for intramuscular DNA injection is of Phase II trials for fibroblast growth factor 1(FGF-1)-encoding plasmid which was locally injected to the patients suffering from stomach ulcer. Even though there was no significant difference for ulcer healing, the risk of amputation was decreased significantly.[11] Systemic injection of naked DNA might not be suitable for clinical trials; however, it is a quite common method for preclinical studies in animal models. In this method plasmid DNA is administered via tail vein of mice or rats with a large volume of vehicle in a short time period (10 seconds) in order to form a hydrodynamic pressure. The high pressure allows DNA molecules access to tissues mainly liver. As soon as 30 minutes after injection fluorescently labeled DNA starts to accumulate in the liver. It was also shown that nucleic accumulation of DNA depends on plasmid size.[12, 13]

Gene transfer to the target organs can also be achieved by using sonication. A method called Ultrasound targeted microbubble destruction (UTMD) was conducted to deliver a reporter gene to mouse salivary glands and the VEGF gene to transplanted islets in mouse in two different studies.[14, 15] Basically, the method is based on mixing the naked plasmid DNA with activated microbubble solution which results in encapsulation of plasmid DNA in microbubbles. After systemic injection without a hydrodynamic pressure, the sound waves targeted to a specific site disrupt the microbubbles allowing plasmid DNA enter into cells. The initial gene expression was lower than Adenovirus-

based control vectors. However, gene expression with UTMD sustained constant levels while Adenovirus mediated gene expression dropped significantly.[15] This method is useful for local gene delivery, because it is noninvasive and neither of the studies reported inflammation in the target tissue. However, it should be noted that targeted sound waves might also destruct the cell membranes which results in death of cells taking up the plasmids.

**DNA Complex Formulations**

Besides to the physical methods discussed above, the most common method for non-viral gene delivery is to formulate lipid or nanoparticle complexes with DNA. Plasmids are large hydrophilic molecules which have negative surface charges due to the phosphate backbone of DNA. Therefore, cationic lipids are commonly used for both condensation and neutralization of DNA molecules for *in vivo* and *in vitro* gene transfers. There are a number of important factors which have to be considered carefully in designing a lipid- based DNA carrier: toxicity of lipid composition, nucleic acid dispersion characteristics of the lipid, and large scale manufacture.[16] Two commercially available lipids are used for liposome formations. DOTAP, dioleoyl-trimethylammonium propane, interacts with the negatively charged DNA molecule as the cationic lipid and DOPE or cholesterol is used as the helper lipid to provide the vector with better intracellular trafficking efficiency. [7]

The particle size should be between 50 to 200 nm for liposome and nanoparticle complexes. Particles smaller than 10 nm are cleared by the reticuloendothelium system while particles bigger than 200 nm are readily taken up by phagocytic cells. Therefore, it is a common practice for systemic administrations to cover particles with polyethylene glycol (PEG) in order to mask particles from immune recognition and protect from renal

clearance.[17, 18] While successful in animals, a Phase I trial for systemic delivery of liposome siRNA complexes was terminated due to its "potential immune stimulation to interfere with further dose escalation". [19] Additionally, cationic lipids were also used for transferring the cystic fibrosis transmembrane conductance regulator gene through the nasal route of cystic fibrosis patients. The study resulted in partial correction of chloride secretion; however, patients developed fever which was thought to be a side effect of innate immune system stimulation.[20] Again, further development is needed.

Another class of synthetic DNA condensing agents is nanoparticles. Many nanoparticles such as poly-L lysine, polyvinylpyrolidone, polyethylimine has been studied extensively in preclinical studies.[21] Among many polymer formulations, a recently developed silica-based nanoparticle is discussed in the context of this thesis. Silica nanoparticles can be modified with amino groups to condense DNA electrostatically. It was shown that nanoparticles protect DNA from enzymatic digestions *in vitro*.[22] Organically modified silica nanoparticles, so called ORMOSILs, can be easily synthesized in desired sizes and the surface of the particles can be covered with PEG to mask immunogenic properties. With the addition of hepatocyte-targeting ligands onto the PEG chain, the ORMOSIL nanoparticles can be targeted to the liver.[23] Even though there are few studies evaluating the efficacy of ORMOSIL nanoparticles, one study showed that local administration of pGFP plasmid condensed with ORMOSIL to mouse brain yielded a better expression profile than a herpesvirus vector control.[24] Additionally, ORMOSILs are studied for their drug delivery features in the Tan Laboratory in the Chemistry Department here at University of Florida. For future studies, these

nanoparticles can be modified with desired features such as targeting and can be utilized in non-viral gene delivery research.

## The Plasmid Design

As described above, non-viral gene therapy vectors are based on plasmids. An ideal gene therapy vector should provide efficient transgene expression for a long period of time without causing any cytotoxicity.[25] In Figure1-1, the minimal plasmid requirements are depicted mainly as two elements: bacterial backbone and eukaryotic expression cassette.

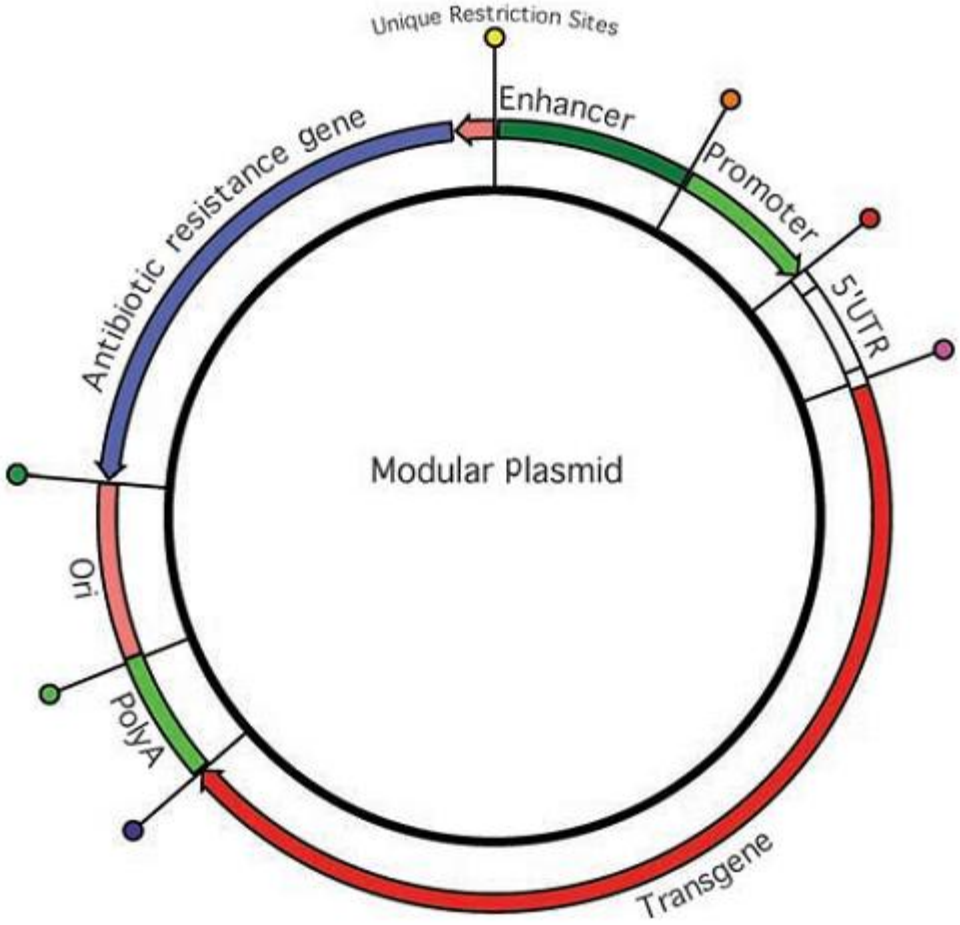


Figure 1-1. The minimal requirements of non-viral gene therapy vector. Plasmid encodes for the therapeutic gene for eukaryotic expression and antibiotics resistance gene for bacterial selection. Transgene expression cassette may also contain an intron at 5' for efficient mRNA processing.[25]

**Promoter Selection**

For efficient transgene expression, one of the most important elements is the promoter. It has been known that certain viral promoters such as CMV immediate early promoter may get silenced due to DNA methylation. In order to circumvent the promoter silencing problem, tissue specific promoters can be used for successful sustained transgene expression. Even with the viral gene delivery, using tissue specific promoters can enhance long term gene expression. Studies showed that CMV promoter driven expression dropped around ten thousand fold after nine months.[26] Hybrid promoters are also promising for sustained expression. As an example, CMV immediate early enhancer is fused to chicken β actin promoter. This hybrid promoter provided a robust long term transgene expression in several studies.[27, 28]

**CpG (Cytosine-Guanine Rich Islands) Free Plasmids**

Throughout the course of evolution cells have evolved protection mechanisms against infectious agents such as viruses. Upon entering into the cell plasmid DNA is recognized by the cell which may stimulate an immune response. It has been hypothesized that unmethylated CpG islands in the plasmid cause this innate immune response. Reduction of CpG islands by codon optimization in non-viral vectors containing CpG free CFTR cDNA resulted in a robust transgene expression without eliciting an inflammatory response. [29]

In order to maximize the therapeutic effect of gene for a long period of time, it is important to reduce host inflammatory response. In addition to codon optimization to remove CpG islands in the entire plasmid, another strategy to circumvent this problem is to eliminate bacterial sequences from the plasmid leaving only the expression cassette. Minicircle production procedure utilizes a site specific recombinase-mediated

recombination. Either Cre- recombinase or Flp-recombinase were used in an inducible way to produce minicircles.[30, 31] Basically, the transgene expression cassette is flanked by loxP sequences for Cre recombinase or frt sequences (Flp target sites) for Flp recombinase. Upon the transient induction of recombinases in the bacterial strain in which plasmids are amplified, the parental plasmid will give rise to two supercoiled DNA molecules: the miniplasmid which is composed of the bacterial selection marker and the bacterial replication origin, and the minicircle which contains the expression cassette and a site specific recombination sequence. A restriction enzyme which only cuts in the bacterial backbone is used to digest the miniplasmid. The undigested minicircle is then purified with a combination of CsCl density gradient purification and column chromatography.[30] Currently, a commercial company based in Germany, The Plasmid Factory, specializes on large scale minicircle production for non-viral gene therapy studies. An additional advantage of using minicircles for gene therapy is the small size of minicircles. First the molar amount of therapeutic DNA is increased since the bacterial backbone is removed. Second, smaller DNA molecules are taken up more efficiently and accumulate in the nucleus faster than larger molecules. [12, 13]

**Inclusion of SMAR (Scaffold/Matrix Attachment Regions)**

Upon transfection, plasmids stay in the nucleus temporarily. Since the integration frequency is quite low, the plasmids get diluted through each cell division or are degraded. By using a selection pressure, plasmids can be forced to integrate into host cell genome to produce cell lines which stably expresses the transfected gene. One strategy to overcome dilution of plasmid is the inclusion of Scaffold/Martix Attachment Regions, SMAR, into the plasmid.25 SMARs are AT rich regions which organizes the chromatin and chromosome structures. Initially, depending on the DNA protein

extraction methods for chromosome scaffold or nuclear matrix proteins, they were given the names SARs and MARs, respectively. SMARs are localized to every 50 to 200kb region in the human genome and form loops for higher chromatin organization.32 In a study done by Bode et al, an 800 bp region from upstream of the human β interferon gene was tested for its ability to unwind and bind to the scaffold proteins in in vitro assays. Therefore SMARs were first acknowledged for their characteristic similarity to eukaryotic origin of replications. 33

In 1999, a German group developed a novel vector based on SMAR elements which replicates episomally as an independent extrachromosomal entity. [34] In this study, SV40 large T-antigen coding region was replaced by a 2kb SMAR element in order to replace virally encoded protein requirements for long term gene expression. The resulting plasmid, called pEPI, was transfected into CHO cells and following a 2-week initial selection, stably GFP expressing colonies were obtained. The extrachromosomal and genomic DNA was extracted 8 weeks after initial selection. It was shown that inclusion of SMAR provided the vector with episomal maintenance and replication ability.[34]

After the development of the first generation SMAR including vectors, many studies were conducted to further investigate the characteristics of these vectors.[35-37] Even though SMAR-containing vectors have promising characteristics for using in non-viral gene therapy, the literature is somewhat contradictory on the issue.[30, 37, 38] This previous work will be discussed in the second chapter.

# CHAPTER 2
# EPISOMALLY REPLICATING NON-VIRAL VECTORS

## Targeted Integration Versus Episomal Maintenance

Since retrovirus-mediated gene therapy depends on random integration and AAV-mediated gene therapy is still far away from controlled integration into the chromosome 19 site, some researchers have investigated targeted integration with non-viral vectors.[39] In one example, a bacteriophage-derived integrase system, phiBT1, was used to integrate the murine phenylalanine hydroxylase gene to correct phenylketonuria. Naturally, the integration occurs in a unidirectional way into the attachment sites in bacteria. The idea was to utilize pseudo attachment sites naturally found in the mammalian genome. The group claimed that hyperphenylalaninemia was corrected after 10 weekly administrations of non-viral vectors. Additionally, they could not detect any random integration with PCR.[40] However, this paper was retracted three years later after re-examination of data.[41] So far, we are lacking safe site-specific integration systems without any random insertion risks.

The ideal non-viral gene therapy vector should be persistent without integration since random integration can cause suppression of tumor suppressor genes or activation of oncogenes. Episomal vectors are advantageous in this respect, however, in dividing cells, plasmids get lost due to the inability of replication. Certain viral replication factors such as SV40 large T-antigen and the EBNA1 protein of EBV were used for epsisomally replicating plasmids. However, these viral factors often lead to the transformation of cells.[42]

For those reasons, it was apparent that a non-transforming and non-integrating alternative for episomal replication has to be developed. As briefly discussed in the first chapter, SMAR elements may fulfill the criteria desired for non-viral gene therapy.

**Previous Work Done with SMAR Element Containing Vectors**

When the SMAR element-containing vectors were first introduced by the Lipps group, one of the conclusions was that SV40 origin of replication was required for long term maintenance of the vector. [34] In another paper by the same group, this requirement was further investigated. They constructed vectors which lacked SV40 origin of replication and found that only the SMAR element was sufficient for long term episomal maintenance in CHO cells.[36] Additionally, they also investigated the minimal SMAR element sequence requirements. A 155 bp synthetic oligo was identified which possessed unwinding and SAF-A (Scaffold Attachment Factor A) protein binding characteristics. This minimal SMAR module was tested for function in plasmids. While plasmids with dimeric 155 bp SMAR oligos resulted in random integration, inclusion of four copies of the synthetic SMAR oligos was sufficient for episomal maintenance and replication. One of the important aspects in designing the vector is the location of the SMAR element. It is known that mammalian replication origins are closely associated with transcriptionally active sites.[42] Therefore, the SMAR element has to be in a transcriptionally active state. Northern blot results showed that transcription is continued until a cryptic polyadenylation site within the SMAR element. Insertion of a polyadenylation site between the transcription unit and SMAR resulted in the loss of episomal replication ability.[36] This data showed that SMAR element has to be transcribed in order to convey episomal maintenance.

Another interesting paper showed that transgenic pigs can be produced with SMAR-based vectors.[35] A method, called Sperm Mediated Gene Transfer (SMGT), was used to deliver a GFP-encoding SMAR-based vector to the pig embryo. After organogenesis was completed, 18 fetuses were collected. 12 out 18 fetuses were PCR positive for almost every tissue examined. For further confirmation, extrachromosomal DNA was extracted for Southern analysis which detected the SMAR-based vector in different tissues without any integration.[35] Even though the results showed that SMAR element inclusion strategy was very successful for transgenic animal production, other papers do not support these results.[37, 38] The SMGT method used for gene delivery might have played a role in long term maintenance of the vectors.

The first *in vivo* experiment to examine the persistence of SMAR-based vectors was performed by Argyros *et. al.*[37] They hypothesized that CMV promoter driven reporter gene expression would be subjected to the silencing due to the methylation. Therefore, they used a liver specific promoter, the α1 antityrpsin (AAT) promoter. The luciferase reporter gene was advantageous for studying the long term expression since luciferase expression can be detected by in situ bioluminescent imaging without sacrificing the animals. 24 hours following hydrodynamic injections, all tested vectors mediated a high level of luciferase expression. A week after administration the expression levels from control plasmids, which lacked SMAR element or driven by CMV promoter with SMAR element, dropped ten thousand fold, whereas the plasmid which had the liver specific promoter and SMAR element persistently expressed luciferase for up to six months. In order to test the replication ability of the plasmids they conducted partial hepatectomy on mice which resulted in the decrease of luciferase expression for

every plasmid. A replication dependent assay was then conducted to check whether plasmids were replicating or luciferase expression was due to the plasmids administered. Both experiments showed that inclusion of a SMAR element was not sufficient for vectors to replicate. Another issue that they investigated was the methylation state of the promoter regions in plasmids. CpG-rich regions of four different vectors were analyzed for the methylation state by bisulfite PCR. Only the plasmid which has both AAT promoter and SMAR element was not methylated, but all the other controls were methylated up to 80% at the CpG sites. [37]

The same group later on developed an *in vivo* selection strategy to force SMAR-containing plasmids to replicate in liver.[38] The selection would provide transfected cells with a survival advantage compared to non-transfected cells. The selection strategy was basically dependent on expression of Bcl-2, an apoptosis inhibitor, from the SMAR containing plasmid. The plasmid was designed in a way which allows the expression of Bcl-2 and luciferase from the bicistronic transcription unit. The liver specific AAT promoter drove the transcription of Bcl-2 and luciferase which were separated by an internal ribosomal entry site. After the delivery of plasmids via hydrodynamic injections, mice were treated with the apoptosis inducing antibody Jo2 twice a week. 3 months after injections, luciferase expression was 13 times higher in the mice injected with the vector containing liver specific promoter AAT, selection gene Bcl-2 and SMAR element compared to the non-SMAR vector. The vector copy number per cell was also found to be ten times higher for SMAR-containing vector than non-SMAR vector. These results showed that Bcl-2 expression was providing a selective advantage to the transfected cells. In order to determine the replication ability of the plasmid, a replication dependent

assay was conducted with extrachromosomal DNA extracts. For the first time, it was shown that SMAR-containing plasmids replicated *in vivo* when a selection pressure was applied.[38] In terms of understanding the *in vivo* characteristics of SMAR elements, this study was elegantly designed; however, the *in vivo* selection strategy did not seem to be an applicable method for gene therapy.

As briefly discussed earlier, removal of bacterial sequences is an important issue in reducing the host immune response. Therefore, by two different groups, minicircle production strategy was applied to SMAR containing vectors.[30, 31] Broll *et. al*. developed a GFP encoding SMAR minicircle by an inducible Flp recombinase system.[31] After long term passaging of minicircle transfected CHO cells, PCR analysis showed that a subpopulation of cells had a reduced SMAR element with a size of 700 bp which happened probably due to an internal recombination event within the SMAR element. In the context of minimal SMAR sequence requirements this result was not entirely surprising since a former study showed that four adjacent repeats of 155 bp unwinding elements in SMAR was sufficient for long term maintenance.[36] The spontaneously size reduced SMAR element was PCR amplified to construct another minicircle, named M18. Both M18 clone and original minicircle were compared for their long term GFP expression and DNA duplex destabilization profiles. The M18 clone showed a robust GFP expression which stayed stable over time whereas the original minicircle followed a slight decrease in the GFP signal intensity. After 21 weeks, minicircle also showed a size reduction comparable to the M18 clone which suggested the recombination might be a repeated event for the SMAR element. Additionally, it was observed that full length SMAR-including vectors showed random integration. [31]

The first SMAR containing minicircle experiments were conducted *in vivo* by the same group which showed SMAR-based luciferase vector replication following an *in vivo* selection.[30] They utilized an inducible Cre recombinase system to produce minicircle DNA which includes the AAT promoter driven luciferase gene linked to a 2kb SMAR element. The *in vitro* experiments were done in U251 cells, a human glioma cell line. Even after sub-culturing the minicircle transfected cells for 60 days, Southern blots showed that minicircles stayed intact as replicating episomal entities. Subsequently, AAT-Luciferase-SMAR minicircle and controls were injected into mice via tail vein injection. SMAR-containing minicircle's expression levels were higher than any other vector tested until the end of the experiment. Interestingly, even the expression from non-SMAR minicircle DNA was an order of magnitude higher than the non-SMAR plasmid control. These results indicate that removal of bacterial backbone gives better transgene expression; therefore, it might be essential to consider a minicircle strategy when designing a gene therapy vector. Southern blots from the liver extrachromosomal DNA extracts showed that every vector administered remained as episomes and genomic integration was not detected.[30] The last two papers discussed here showed that minicircle provide better transgene expression *in vivo* and in vitro. However, their conclusion about random integration events of full length SMAR containing minicircles seems somewhat contradictory. It is explainable that integration might not occur frequently *in vivo* since the regeneration of hepatocytes is slow but for highly dividing cells, the integration frequency should be clearly determined.

**Viral Element Based Episomally Replicating Vectors**

The first developed episomally replicating vectors were based on viral factors for replication and partition. Simian virus 40-derived vectors were the very first developed

episomally replicating vectors only requiring the large T-antigen as a *trans* factor in order to replicate. Even though SV40-based vectors are useful for transient transfections, the vector replication is not in synchrony with cell cycle which leads to the excessively high vector copy number per cell and eventual cell death.[43] Another example is Bovine papilloma virus-derived vectors which require two viral proteins. When compared to the SV40-based vectors, the copy number is lower; however, BPV-based vectors cause cellular transformation.[44] These vectors were not suitable for gene therapy applications because they lack mechanisms such as control of vector copy number per cell, efficient partition, and faithful segregation to daughter cells.

A good candidate vector which can fulfill the aforementioned criteria was developed based on Epstein-Barr virus elements. EBV can latently infect B- cells *in vivo* and in vitro. During latency, the viral genome is duplicated once per cell cycle and distributed to daughter cells by “hitchhiking” onto the chromosomes. This step requires two viral elements; one of which is the viral *cis*-acting oriP sequence and the other is trans-acting EBNA 1 (Epstein Barr encoded Nuclear Antigen 1) protein. In vector systems containing both elements, EBNA 1 protein ensures the plasmid retention and replication through binding to oriP sequence.[45] EBNA-1 and oriP-containing plasmids were tested in 293 cells and human embryonic stem cells for their long term episomal maintenance. Both studies showed that inclusion of EBNA-1 and oriP increased plasmid retention over time and enabled plasmid replication.[46, 47] Given the successful episomal maintenance properties of EBNA-1-containing plasmids, the safety issues for the oncogenic properties of EBNA-1 protein is still an ongoing debate. EBNA-1-containing plasmids are commercially available for transient expression from the episomal state;

however, there are no *in vivo* studies evaluating the episomal maintenance of EBNA-1 containing vectors to the author's knowledge.

**KSHV Minimal Replicator as an Alternative**

Kaposi's sarcoma-associated herpesvirus (KSHV) belongs to the gammaherpesvirus subfamily and establishes long term latency in lymphocytes. Similar to its close relative EBV, viral genomes are maintained episomally in latently infected cells. The minimal cis- and trans-acting requirements for viral DNA replication and maintenance during latency are the terminal repeats (TR) and Latency Associated Nuclear Antigen (LANA).48 LANA binds to the highly GC-rich 801 bp TRs in KSHV genome and recruits cellular replication machinery. By tethering the viral episomes to the mitotic chromosomes, LANA also ensures the faithful segregation of viral genome to the daughter cells. 49 The attachment of LANA to interphase chromatin and mitotic chromosome is through protein-protein interactions. Figure 2-1 depicts the model for episomal maintenance and segregation by LANA and EBNA-1 proteins.

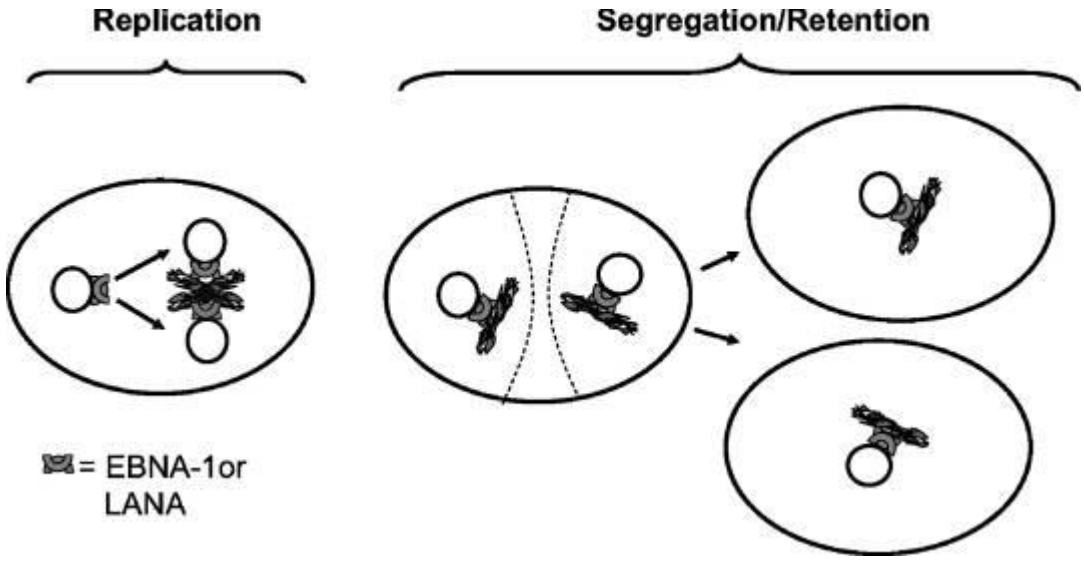


Figure 2-1. A model for LANA and EBNA-1-mediated episomal maintenance and segregation. LANA or EBNA-1 tethers the plasmid to mitotic chromosomes and interphase chromatin through protein-protein interactions. [50]

LANA has been shown to bind tumor suppressors such as p53 and Rb; however, no experimental evidence supports that LANA can induce transformation in primary cells.[50] Thus, it may be useful to include LANA and TR in plasmids in order to provide the gene therapy vectors with the ability for episomal maintenance and synchronous replication with cell cycle.

**Designing a Novel Vector Which Contains LANA Combined to SMAR Element**

One of the major limitations in utilizing non-viral vectors is the inefficient episomal maintenance through cell divisions. As the literature indicated, in the absence of a selective advantage, SMAR-including vectors cannot be maintained episomally in *in vitro* and *in vivo* experimental systems. Utilization of viral components for episomal replication also poses problems because the vector copy number cannot be controlled in SV40-like systems and EBV-based replicators are thought to have oncogenic properties. It is for these reasons, we hypothesized that combining the SMAR element and the minimal replicator of KSHV, LANA, would result in a non-integrating episomal vector which efficiently replicates in synchrony with the cell cycle and is segregated into daughter cells faithfully.

# CHAPTER 3
# EXPERIMENTAL APPROACH

## Specific Aims

**Specific Aim 1:** Determine if a SMAR element can efficiently maintain long term GFP expression without integration in the absence or presence of selection pressure in 293 cells.

**Specific Aim 2:** Test a novel GFP expressing episomal vector, which combines the minimal replicator of KSHV and SMAR elements together, for long term maintenance in 293 cells.

**Specific Aim 3:** Test the hypothesis that the SMAR element and LANA compete for binding to the different nuclear compartments which leads to genomic instability because it was found that transfection of cells with 2TR-LANA-GFP-SMAR vectors is detrimental to the cells.

We first aimed to verify that SMAR element's effect on long term stability for the plasmids. The plasmid loss rate was evaluated in the absence of selection pressure. It was expected that the presence of SMAR would increase plasmid retention. In a parallel set of experiments, stably GFP expressing cells were obtained upon application of selection pressure. Then, the novel vector, p2TR-LANA-GFP-SMAR, was tested in cell culture. We expected to see stable GFP expression in the absence of selection. Since it was repeatedly found that the co-presence of SMAR and 2TR-LANA on the same plasmid was not a viable system, we hypothesized that the nuclear structure could be damaged due to competitive binding. This hypothesis was tested by co-transfecting cells with the novel vector and LANA-RFP fusion protein encoding plasmid.

## Materials and Methods

### Molecular Cloning Protocols

Manufacturer's recommendations were followed for the enzymes and components used to construct vectors. The restriction, ligation and other enzymes were from New England BioLabs unless otherwise indicated.

### Construction of 2TR-LANA-GFP-SMAR vector

2TR-LANA-GFP-containing vector was obtained from Dr. Rebecca Skalsky (Renne Laboratory). This vector is based on pCRII (Invitrogen) and cloning strategy was previously described.[51] A GFP-SMAR containing vector was previously obtained from H.J. Lipps.[34] For better GFP expression, a Eco RI Bgl II SMAR fragment was inserted into pEGFP-C1 (Clontech) by Dr. Jianhong Hu. A 2kb SMAR element from pEGFP-SMAR was PCR amplified by Easy A High Fidelity Cloning Enzyme (Agilent Technologies). The primers had a Nhe I recognition site and 4 additional nucleotides on the 5' (Table 3-1). The PCR products were then digested with Nhe I and ligated into a unique Nhe I site in the 2 TR-LANA-GFP vector with T4 ligase (NEB). Half of the ligation reaction (5 µl) was used for transformation of E. coli DH5α strain. The colonies were analyzed with restriction enzyme analysis and one of the colonies which had the insert in the same orientation as pEGFP-SMAR was also confirmed by sequencing with four set of primers (Table 3-2) and this plasmid, named 2TR-LANA-GFP-SMAR, was used for further experiments.

Table 3-1. Primers used for SMAR element amplification were purchased from IDT. NheI recognition sites are in bold font.

| Primers | Sequence |
|---|---|
| Forward | 5' CCTA**GCTAGC**CACATGGCATGGATGAACTA 3' |
| Reverse | 5' TTGA**GCTAGC**CGTCGACTGCAGAATTCTAT 3' |

Table 3-2. Sequencing primers. The SMAR insert is located in between 7558 bp and 9599 bp.

| Primers | Sequence | Start site |
|---|---|---|
| 1 | 5’ GTCAGGCCATAGAATGACAG 3’ | 8291 |
| 2* | 5’ GATCTCATTCAGAATGCCAC 3’ | 8784 |
| 3 | 5’ GGGAACCCATATGTCATACC 3’ | 8059 |
| 4* | 5’ CAGCTATGGATAGTTAGGCG 3’ | 9012 |
| 5* | 5’ AACCACTACCTGAGCACCCA 3’ | 7406 |
| 6* | 5’ AGGTACCGAGCTCTGCAGAA 3’ | 9667 |
| 7* | 5’ CACATGGCATGGATGAACTA 3’ | 7558 |
| 8* | 5’ CGTCGACTGCAGAATTCTAT 3’ | 9599 |

(*) indicates that primers are designed for the complementary strand

**Construction of mPAH encoding vectors with and without SMAR element.**

In order to construct mPAH-containing vectors, a two-step cloning strategy was chosen. First, two intermediate recipient vectors were constructed based on pEGFP-C1 and pEGFP-SMAR. Two complementary oligonucleotides were designed to include one Bgl II and one Not I site in the middle and those sites are flanked with Pci I and Bgl II compatible ends (Table 3-3). Then those oligonucleotides were annealed, phosphorylated at 5’ ends, and ligated into Pci I and Bgl II digested pEGFP-C1 and pEGFP-SMAR backbones. The resulting intermediate recipient vectors were analyzed for newly introduced Not I cutting site. The cloning strategy of CB-mPAH vector was previously described by C.E. Charron.[27] The Bgl II- Not I fragment which includes the CMV enhancer/ chicken B actin promoter driven mPAH expression cassette inserted into the recipient vectors. The resulting vectors called CB-mPAH-C1 and CB-mPAH-SMAR were confirmed for the orientation of insert by restriction enzyme analysis.

Table 3-3. The oligonucleotides for cloning mPAH encoding vectors. Bgl II and Not I cutting sites are in bold font and 5' compatible ends are underlined.

| Strand | Sequence |
|---|---|
| Sense | 5' CATGTTT**AGATCT**CATTA**GGCGGCCGC**TTG 3' |
| Antisense | 5' GATCCAA**GCGGCCGC**CTAATG**AGATCT**AAA 3' |

**Cell Culture Protocols and Transfections**

HEK-293 cells were cultured in Dubelco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum and 1% Penicillin –Streptomycin (Cellgro). Cells were passaged twice a week with 0.25% trypsin. Passages were performed at one to four dilutions for long term GFP expression experiments and one to eight for cell line maintenance. For selection of transfected cells, G418 Sulfate (Cellgro) was used with 400µg/ml concentration for initial selection and 200µg/ml for the maintenance of stably transfected cells.

**Calcium Phosphate transfections**

Calcium phosphate transfections were mainly used for Phenylalanine Hydroxylase activity assays. The protocol was adapted from Current Protocols in Molecular Biology.[52] 293 cells were seeded on six well plates with $5x10^5$ cells per well to obtain 60% confluency at time of transfection. In general, 5µg DNA per well was diluted in 90µl water and mixed with 10µl $CaCl_2$. This solution was mixed with 100µl of HBS (Hepes Buffered Saline) while swirling the tubes gently. After 10 minute incubation at room temperature, 200µl solution was added dropwise to wells. Cells are harvested three days after transfection.

**Transfections with Mirus TransIT293 transfection reagent**

For the experiments done with 293 cells, $6x10^4$ cells were seeded into 24 well plates to reach 50% confluency at the time of transfection. For Hep2 and SLK cells, $3x10^4$ cells were seeded into 24 well plates per well in order to reach 50% confluency at the time of transfection. In general, 0.5µg DNA was used per well according to the manufacturer's recommendations. 50µl of serum free DMEM was mixed with TransIT293 reagent at a concentration of 3µl per µg DNA used and incubated for 15 minutes at room temperature. Then 0.5 µg of DNA was added to the reaction tube and incubated again for another 20 minutes at room temperature. This solution was added dropwise to the wells. GFP expression was analyzed16 to 24 hours later.

**Fluorescence-Activated Cell Sorting**

The transfected cells were analyzed for GFP expression. 293 cells were prepared for FAC sorting 48-72 hours after transfection. Cells were collected by removing medium, washing with PBS twice, trypsinizing and re-suspending in DMEM. Cells were centrifuged at 1100 rpm and re-suspended in PBS was containing 2% FBS and diluted to a concentration of $1\text{-}2x10^6$ cells per ml. Cell sorting was done by the Flow Cytometry Core in ICBR. The sort gate was set to sort 90% of GFP expressing cells in order to eliminate transfectants which had low copy number of plasmids. After sorting, cells were washed with PBS twice and seeded onto 6 or 12 well plates in 20% FBS- including DMEM. 16 hours later medium was changed with 10% FBS-including DMEM.

**Southern Blot Analysis**

For DNA extractions, one 10 cm dish of confluent 293 cells was used for each stably GFP expressing cell line. Extrachromosomal DNA was extracted with a modified HIRT protocol. $2x10^7$ cells from 10 cm dish was trypsinized and washed with PBS twice.

The collected cells were lysed with 700 µl of lysis buffer (10mM Tris,10mM EDTA and 0.6% SDS). Chromosomal DNA was precipitated with 0.83M NaCl at $4^0$C overnight. Supernatant was collected after 30 minutes centrifugation at 14,000 rpm. The extrachromosomal DNA was extracted by phenol-chloroform extraction and precipitated by ethanol and dissolved in 20µl of RNase A containing water. Half of the extract was linearized by a single cutter, Nhe I or Bgl II and the other half was double digested with the single cutter and Dpn I overnight. Digestion reactions were run on 0.8% agarose gels and transferred to nylon membranes (BioRad). The probes were prepared with 10ng of either the entire pEGFP plasmid or Nhe I –Bgl II fragment which contains the the ~800 bp GFP coding sequence by using random prime labeling system (Amersham) and purified with Quickspin columns(Amersham). The blots were hybridized in Church buffer at $65^0$C overnight, washed twice for 2 hours and then exposed to phosphoimaging screen (Molecular Dynamics). The screen was analyzed with ImageQuant.

Genomic DNA was extracted from one 10 cm dish of 293 cells which were transfected and selected with G418 sulfate. Cells were washed with PBS twice. To extract total genomic DNA, 1ml of DNAzol (Molecular Research Center) was directly applied onto the plate. The genomic DNA was precipitated by addition of 1ml 100% ethanol, washed twice with 70% ethanol and dissolved in 8mM NaOH. DNA concentration was determined by NanoDrop 1000 (Thermo Scientific). 20µg of genomic DNA was digested with either Bgl II or Nhe I in a final volume of 75 µl. In general for each µg of DNA, 2 or more units of enzyme was used. The digestion reactions were either phenol-chlorofom extracted and EtOH precipitated or only EtOH precipitated.

Genomic fragments were separated on 0.8% agarose gels and subjected to Southern blot analysis as described in the paragraph above. For genomic blots, an internal human carbonic anhydrase 2 (hCA2) cDNA containing plasmid was used as a probe for quantification purposes. 10ng of entire hCA2 plasmid was used for probe preparation as described above.

**Plasmid Rescue Analysis**

For plasmid rescue experiments, extrachromosomal DNA was extracted as described above. The entire volume of HIRT extract was used for transformation of either DH5α or supercompetent cells (Stratagene). The transformation reactions were plated on 30μg/ml Kanamycin-containing LB plates and incubated overnight. The genomic DNA isolation kit can also isolate episomal DNA, therefore; 5 μg of genomic DNA was used for transformation as described for HIRT extracts.

**Localization of Proteins by Fluorescence Microscopy**

A plasmid expressing LANA C-terminal-RFP fusion protein, DsRed-LANA (Renne Lab), was used for determination of LANA binding distribution. DsRed-LANA plasmid does not contain a TR element and can only express the fusion protein. 293, SLK and Hep2 cells were grown on autoclaved cover slips which were placed in wells of a 24 well plate. To improve adherence of 293 cells, the coverslips were covered with poly-lysine before cell seeding. Then, each well was co-transfected with 0.5μg of 2TR-LANA-GFP-SMAR and 0.5μg of DsRed-LANA. As controls 2TR-LANA-GFP and DsRed-LANA co-transfections were also performed. Cells were washed and fixed with 0.4% paraformaldehyde for 15 minute at room temperature and then permeabilized with 0.2% Triton X-100 for 10 minutes. Vectashield mounting medium (VectorLabs) was used for

DAPI staining in slide preparation. The slides were stored at $4^0$C and analyzed with fluorescence microscopy (Leica EL6000).

**Hydrodynamic Tail Vein Injections**

20 heterozygous BTBR male mice derived from crossing wild type mice with $Pah^{enu2}$ were injected with 25µg plasmid DNA via tail vein. 25µg DNA was dissolved in 2 ml Ringers solution and the entire 2 ml volume was injected into tail veins in ~8-10 seconds. 10 mice received pEGFP plasmid and other ten mice were injected with pEGFP-SMAR plasmid. At four different time points which corresponds to day 3, 10, 15 and 30, mice were sacrificed and four liver lobes were harvested and fixed in formaldehyde. Fixed liver tissue was cryo-sectioned and GFP expression was analyzed with microscopy. Procedures were performed by Dr. W. Zeile.

**PAH Activity Assay**

PAH activity assay is based on the oxidation of NADH to NAD in the synthesis of $BH_4$ cofactor (Figure 3-1). This assay measures the phenylalanine-linked oxidation of NADH at 340nm. Cells were seeded on a 6-well plate and transfected with 10µg plasmid DNA per well. CB-mPAH, pmPAH-C1, or pmPAH-SMAR were transfected as triplicate. To check the transfection efficiency, 1µg pEGFP was transfected as an internal control. If the transfections worked, cells were washed and 3 wells which received the same plasmid were pooled together in homogenization buffer in 1ml (1M KCL and 0.5µl of β mercaptoethanol). The lysate was homogenized in a glass homogenizer and centrifuged for 10 min at 14,000 rpm. 100 µl of the supernatant was mixed with 0.10 M potassium phosphate buffer (pH 6.8) 0.25 U catalase, 0.10 mU DHPR, 0.04 mM 6-Methyl-5,6,7,8-tetrahydropterine (6-MPH4), 0.2 mM NADH, and 1mM Phe in 1 ml reaction tubes. Each reaction was also set up without Phe as controls.

The reduction of NADH was read for every five minutes for 30 minutes by spectrophotometry. The protein concentration was measured with Bradford assays. 10µl of each sample was mixed with 790µl water and 200µl Biorad Protein Assay (Biorad) kit. After 5 minutes incubation, absorbance was read at 500nm. Protein concentration was determined by BSA standards according to the manufacturer's manual.

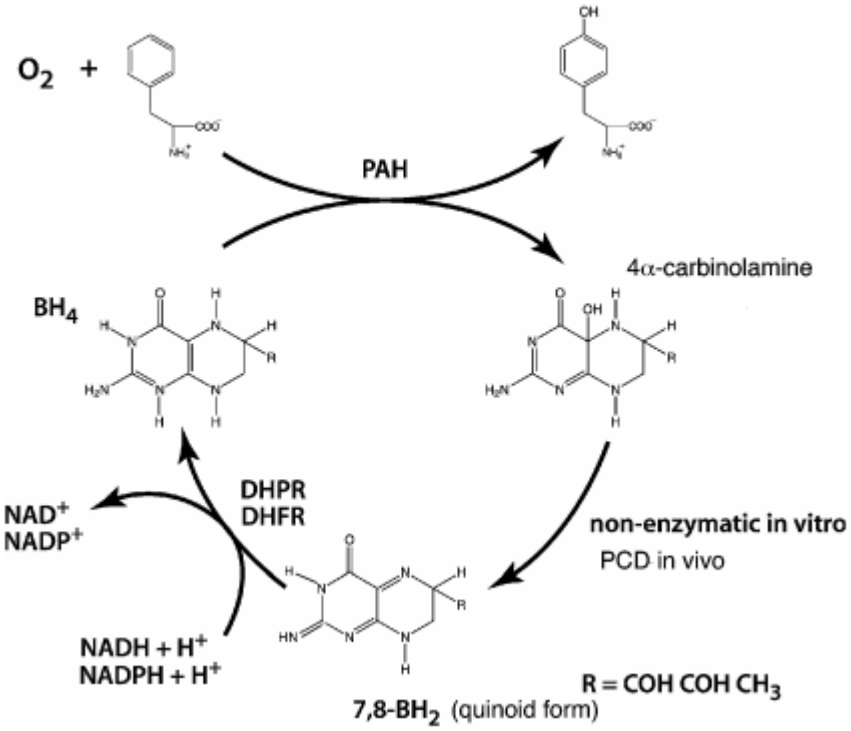


Figure 3-1. Phenylalanine tyrosine conversion requires BH4. The assay measures the oxidation of NADH to NAD.

# CHAPTER 4
# RESULTS

## Vector Construction

Initially, a blunt end ligation strategy was used to construct the p2TR-LANA-GFP-SMAR vector. The 3.4 kb GFP-SMAR fragment from pEGFP-SMAR was ligated into a 2TR-LANA vector backbone and transformed to DH5α strain of competent E. coli cells. Unfortunately, every colony tested with restriction enzyme analysis revealed unexpected bands on the gel. This unexpected result was probably due to the fact that both insert and 2TR-LANA vector had CMV promoters for GFP and LANA expression, respectively. It was thought that unusual bands might have resulted from homologous recombination. In order to make the cloning step easier, a PCR based approach was selected. As described in the Materials and Methods, 2TR-LANA- GFP harbors an Nhe I cutting site in between the GFP stop codon and polyadenylation signal. This was an ideal site because the SMAR element has to be actively transcribed to fulfill its long term effects. The 2kb SMAR element from pEGFP-SMAR vector was amplified with primers which have flanking Nhe I recognition sites and this fragment was cloned into Nhe I cutting site of 2TR-LANA-GFP. Figure 4-1 shows schematic representation of cloning strategy of 2TR-LANA-GFP-SMAR vector.

CB-mPAH expression cassette containing vectors were constructed in a two-step procedure. The intermediate plasmids were described in materials and methods. Briefly, for pEGFP and pEGFP-SMAR vectors, CMV promoter and GFP coding region was replaced by a 30 nucleotide double stranded oligonucleotide which had restriction enzyme sites for insertion of mPAH cassette in the correct orientation. The parental mPAH encoding plasmid was previously used for AAV vector production. Therefore, the

plasmid also contains ITRs (Inverted Terminal Repeat) flanking CB-mPAH expression cassette. 3.4 kb mPAH coding region with the CMV enhancer and chicken β-actin promoter was inserted into the intermediate plasmids (Figure 4-2). It is important to note that the resulting plasmids are free of ITR and the SMAR element was inserted in between the stop codon and polyadenylation signal.

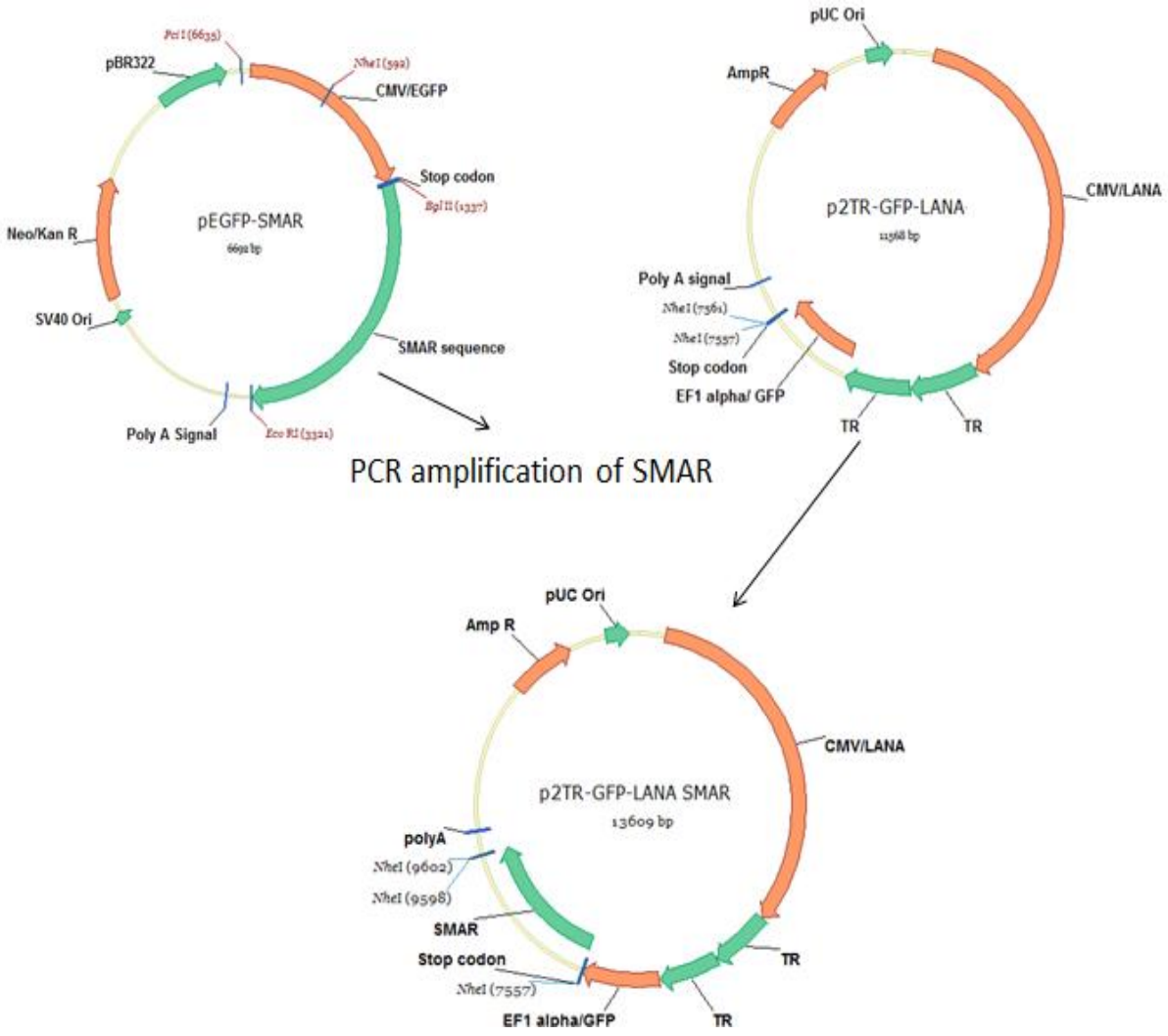


Figure 4-1. Cloning strategy of 2TR-LANA-GFP-SMAR. SMAR element was amplified from pEGFP-SMAR plasmid and inserted into Nhe I site of 2TR-LANA-GFP vector. Important features and restriction sites are indicated.

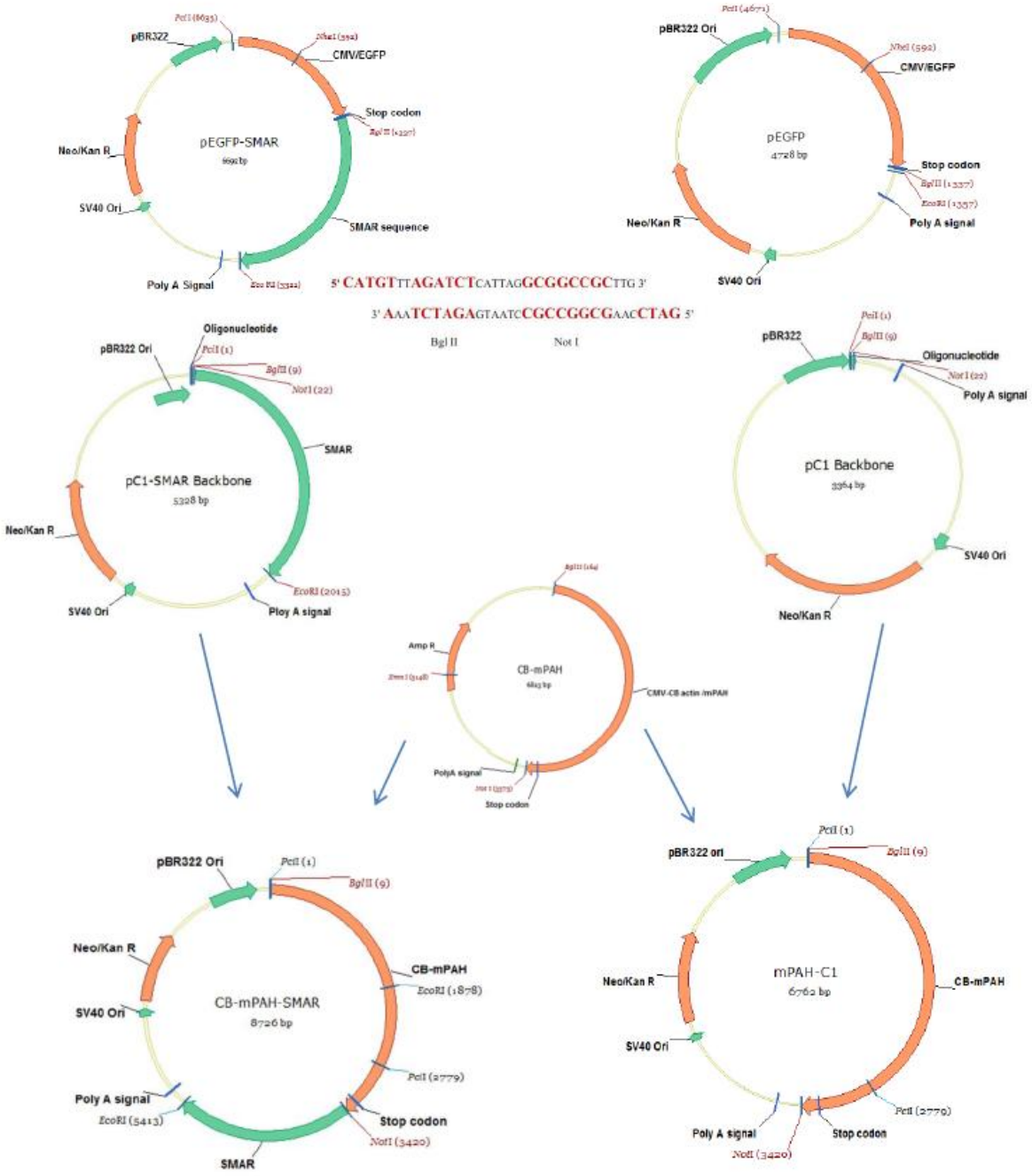


Figure 4-2. Cloning strategy of mPAH encoding vectors. Two oligonucleotides were hybridized to each other to harbor Bgl II and Not I cutting sites and flanking 5' sticky ends. First double stranded oligonucleotide was inserted in place of GFP expression cassette to construct the intermediate vectors. Then Bgl II-Not I mPAH expression cassette was inserted into corresponding cutting sites.

**Evaluation of the SMAR Element on the Long Term Stability of GFP Expression**

In the absence of selection pressure, pEGFP-SMAR and pEGFP transfected cells were monitored for GFP expression under microscopy for 9 passages. In order to compare SMAR–containing vector to non-SMAR control, cells were passaged at one to four dilution. It is important to note that initially, there were more GFP expressing cells on the plate transfected with pGFP plasmid. This indicates that smaller plasmids get into the nucleus more efficiently and higher copy number of the plasmid is present in equal amounts of DNA for smaller plasmids. Figure 4-3 shows the loss of GFP expression in both conditions over time. After nine passages, GFP expression was lost in both cells transfected with either pEGFP or pEGFP-SMAR. Evaluation of GFP expression by fluorescent microscopy through eight cell passages indicated that SMAR element-containing vector did not provide long term GFP expression when compared to non-SMAR control.

An internal drawback of this experiment was that transfection efficiency was higher with the smaller pEGFP plasmid. We wanted to test the effect of the SMAR element on the rate of GFP expression loss. To examine whether the SMAR element provides even a slight benefit to stability of GFP expression, we sorted GFP expressing cells by FACS to start with 100% GFP positive cells. As expected, FACS analysis showed that pEGFP transfected cells were 50.5% GFP positive whereas pEGFP-SMAR transfected cells were 40.5% GFP positive. Sorted cells were cultured and examined for loss of GFP expression rate through 16 cell passages. No difference in the rate of GFP expression loss was detected after comparing the pictures taken at every passage for both pEGFP and pEGFP-SMAR transfected cells (Figure 4-4).

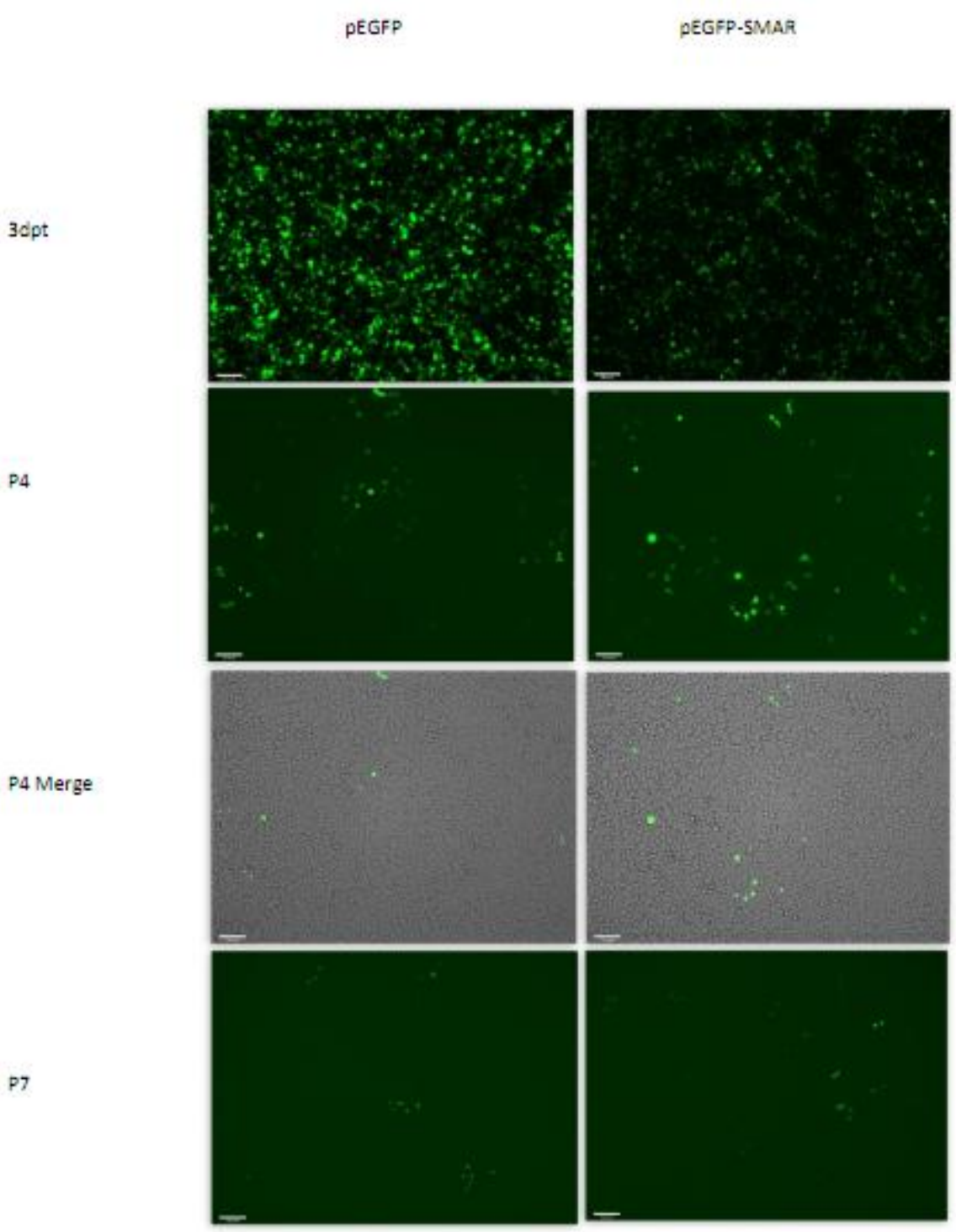


Figure 4-3. Loss of GFP expressions in the absence of selection pressure from both SMAR and non SMAR plasmids. Merge is the overlay image of GFP and Brightfield images.(dpt= days posttransfection, p= passage number)

Initially, the loss of GFP expression occurred quickly. However, in the later passages the loss rate of GFP expression reduced. Considering the fact that cell passages were performed at 1 to 4 dilutions and 12 passages were performed through the end of this experiment, it is implied that the GFP positive cells seen on the pictures taken at passage 12 most likely indicates integration events. At passage 16, cells were frozen and stored in liquid nitrogen for further experiments.

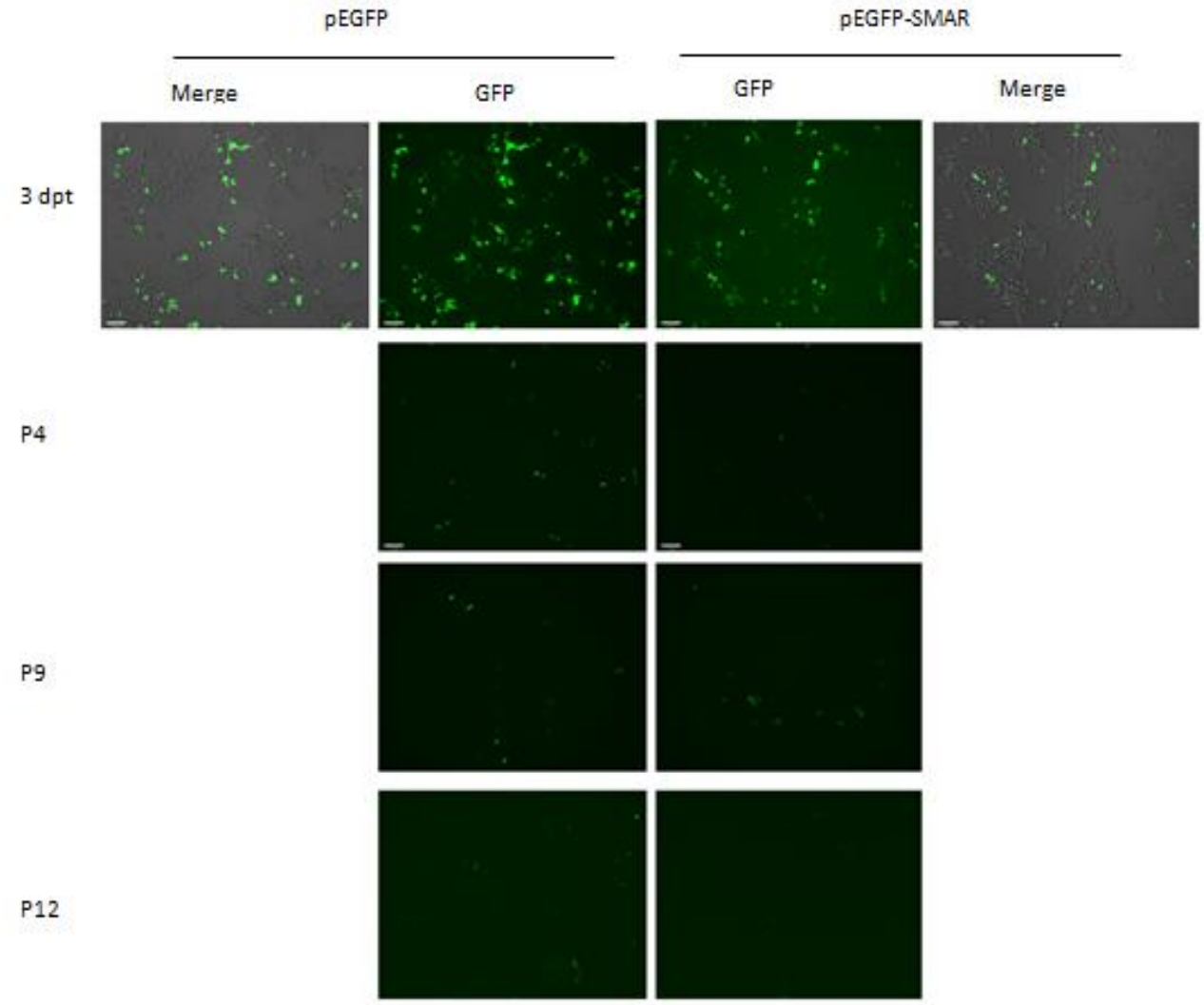


Figure 4-4. Loss of GFP expression over time after FACS. Cells were transfected and FACS sorted 3dpt. Cells were passaged 12 times without selection anitbiotics

In order to examine the long term stability of SMAR plasmids in the presence of antibiotic selection, 293 cells were transfected with pEGFP and PEGFP-SMAR plasmids. After 10 days of G418 selection, both pEGFP and pEGFP-SMAR transfected cells resulted in stable GFP expression. Figure 4-5 shows that GFP expression was stable in 293 cells even at passage 16 after removing G418 selection. At this level we assumed that expression from SMAR plasmids was due to the episomal maintenance and replication whereas non-SMAR control plasmids must have integrated into genome. To test this hypothesis, we have performed Southern blot experiments with genomic and extrachromosomal DNA extracted from stably GFP expressing 293 cells. Additionally, Southern analysis was performed on FACS sorted cells.

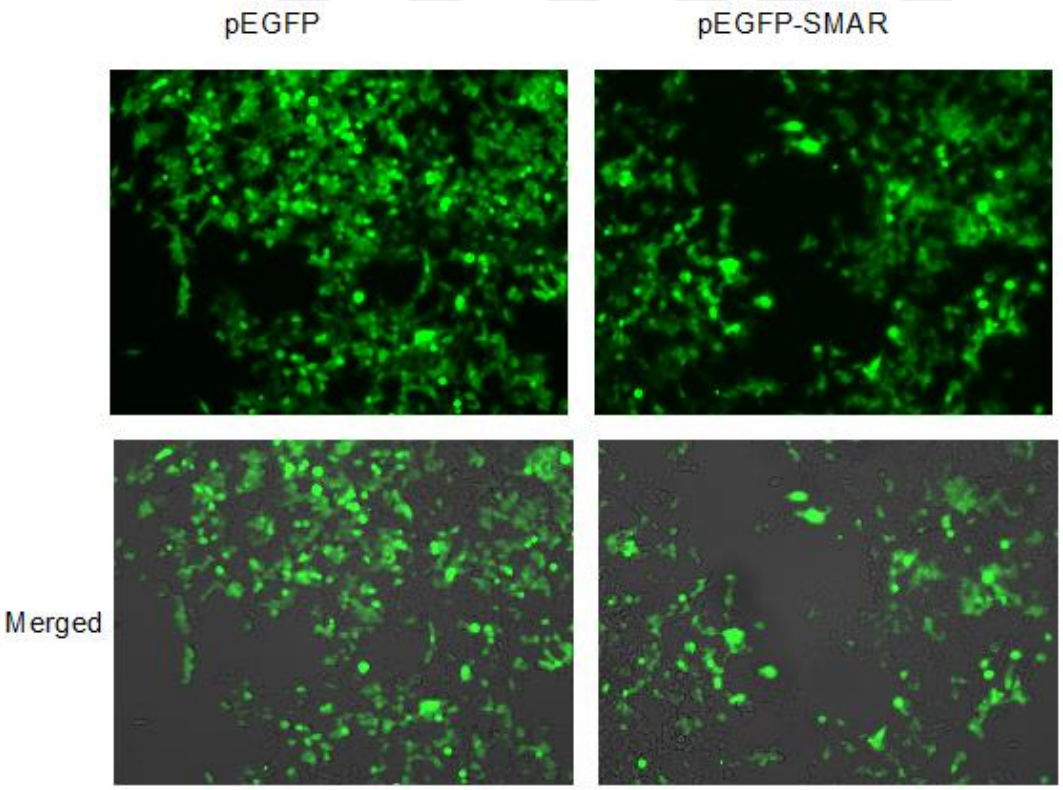


Figure 4-5. GFP expression at passage 16 after G418 selection. Cells were transfected and selected for G418 for ten days. Further passages were performed in the absence of G418.Lower panel shows the bright-field overlays of upper panel.

Previously it was shown that in the presence of an initial selection pressure, SMAR plasmids can be maintained episomally over long time in Chinese hamster ovary cells and HeLa cell lines. Replication dependent assays also showed that maintenance is due to episomal replication. [34, 36] We wanted to test whether long term GFP expression in pEGFP-SMAR transfected and G418 selected 293 cells is a result of episomal replication or random integration. Therefore, half of the HIRT extracts was subject to a restriction enzyme which linearizes the plasmid such as Nhe I or Bgl II and the other half was subjected to the single cutter and Dpn I followed by Southern blots anlysis. This assay depends on the fact that Dpn I cuts only bacterial methylation pattern. If the plasmids were replicated at least twice in 293 cells, they would lose the bacterial methylation and would be seen on the blots in linearized forms. Unfortunately, no signal was detected on Southern blots performed with HIRT extracts (Figure 4-6 A, B, C). In order to increase the amount of DNA loaded, the total HIRT extracts from 10cm dishes were only subjected to single cutters in other trials. However, the Southern blots were unable to detect the plasmids from HIRT extracts. An additional control, which tests the HIRT extraction protocol, was also included. Basically, cells were transfected and at day three, HIRT extraction was performed. This control yielded a strong signal but HIRT extracts of long term GFP expressing cells was again undetected (Figure 4-6 C).

Then, we decided to perform Southern analysis on genomic DNA to check the integration status of the plasmids. 20μg of genomic DNA was digested with one of the following enzymes: EcoRI, Bgl II or Nhe I. Unfortunately again, Southern blots did not produce any signal for genomic DNA extracted from pEGFP and pEGFP-SMAR transfected cells.

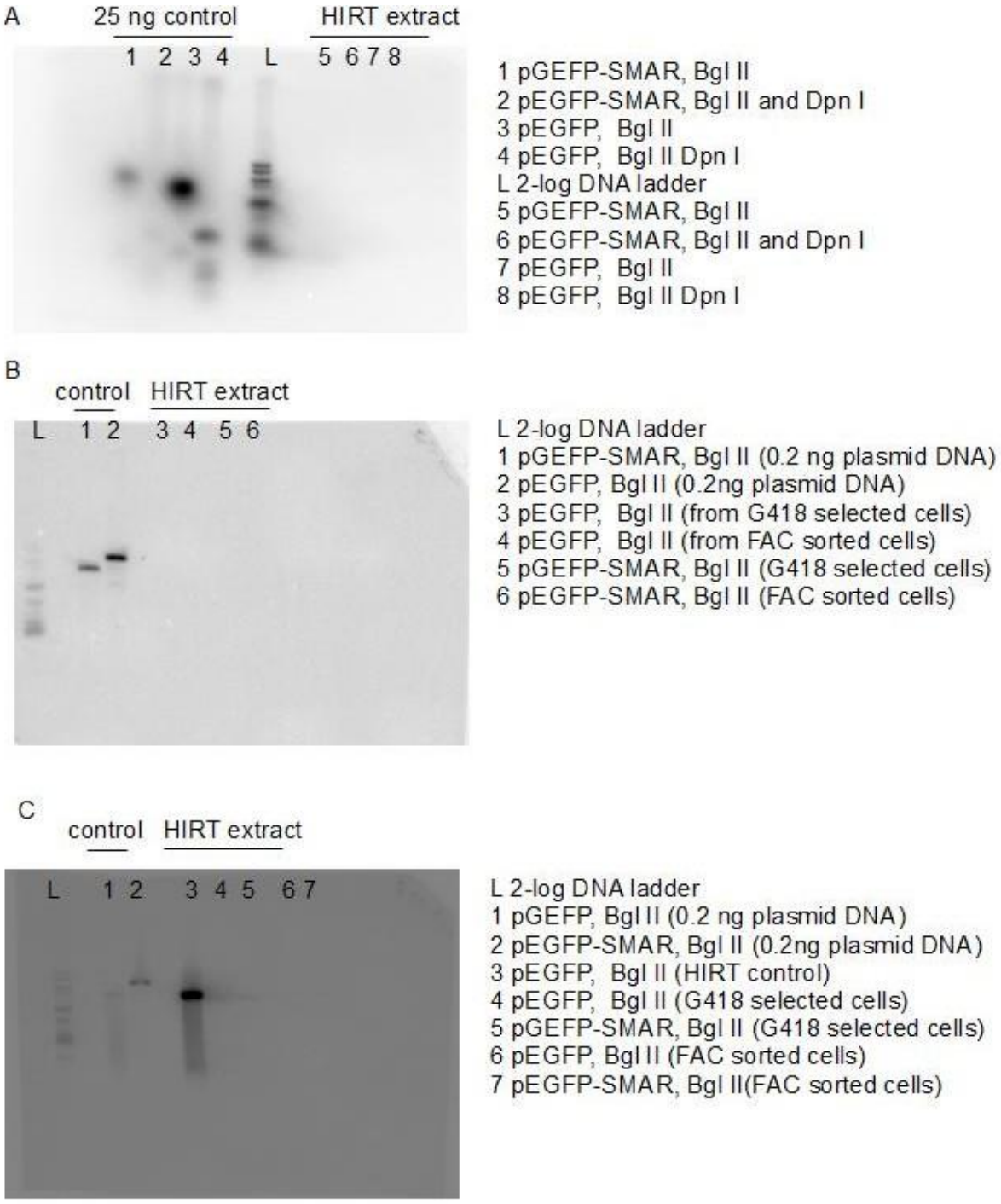


Figure 4-6. Southern analysis for extrachromosomal DNA. A) Methylation dependent replication assay. 25ng plasmid DNA was used as control. Probes were prepared from the entire pEGFP plasmid template. B) Linearized HIRT extracts from G418 selected cells and FAC sorted cells. Probes were prepared for EGFP coding region(700 bp) C) HIRT protocol control from transiently transfected cells. Probes were prepared for EGP coding region.

As an internal control, the genomic blots were also hybridized with a single copy gene, human carbonic anhydrase 2 cDNA probe. The rationale behind using the internal control was that if we could detect a single copy gene in the genome and we could not detect pEGFP and pEGFP-SMAR, we would conclude that the plasmid is maintained as an episome. Due to the low specific activity of probes (≤2x$10^8$ dpm/µg), the internal control human carbonic anhydrase was not detected on genomic blots (Figure 4-7). In the figure, it can be seen that probes prepared from EGFP region were able to detect as low as 0.2 ng control plasmids; however, probes prepared from hCA2 hardly detected the 0.2 ng hCA2 control plasmid.

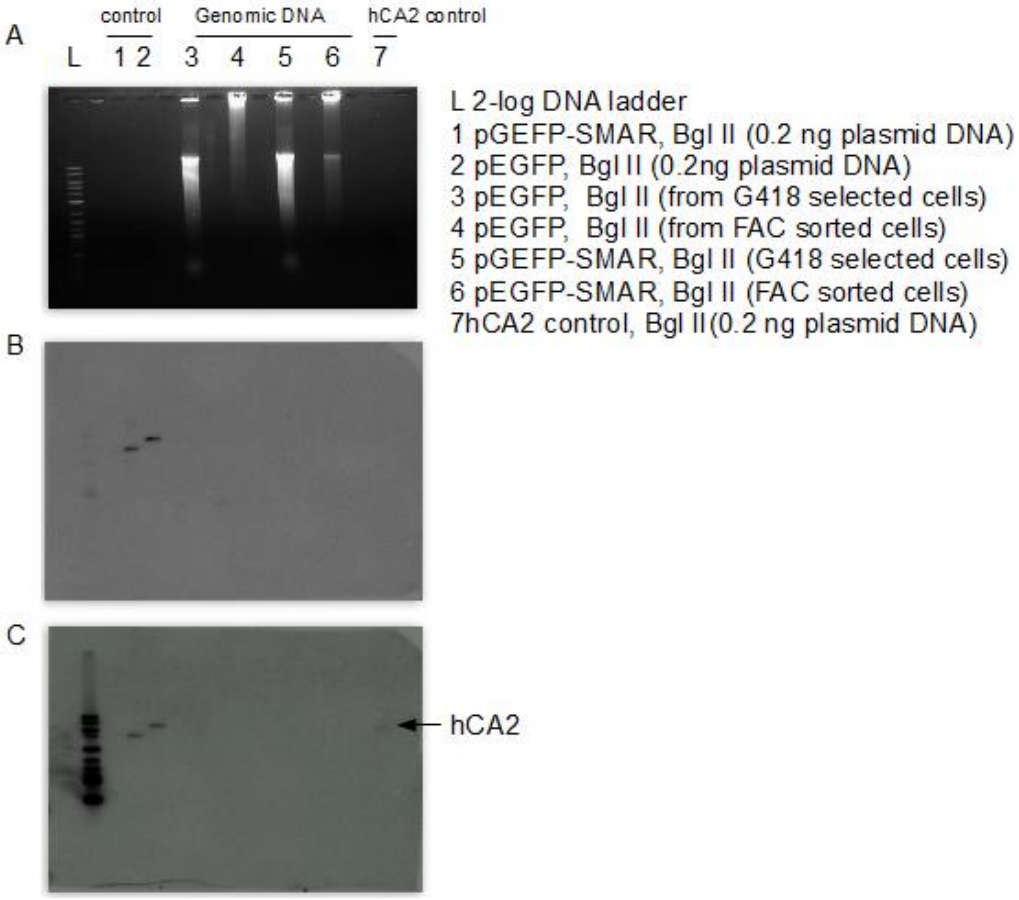


Figure 4-7. Southern blots for genomic DNA. A) 20 µg of Genomic DNA digested with Bgl II. B) Blot was hybridized with EGFP probe. C) Same blot was hybridized with hCA2 probe without stripping off the EGFP probe.

Plasmid rescue assay is an alternative way of confirming episomal maintenance of plasmids. Plasmids are extracted by HIRT protocol and used for transformation of competent E. coli cells. This assay is based on episomal plasmids remain intact and therefore, transformation of the plasmid into a competent cell yields the original plasmid. We wanted to check whether the plasmids were episomal by this assay. Transformation of HIRT extracts from pEGFP and pEGFP-SMAR transfected and G418 selected cells into either DH5α or supercompetent cells did not produce any colonies on Kanamycin-containing plates. This result, however, cannot strongly argue for the plasmid integration because of the low efficiency of transformation in general.

**Testing the Novel p2TR-LANA-GFP-SMAR Vector in 293 Cells**

The novel vector which combines 2TR-LANA and SMAR element was tested in 293 cells for GFP expression stability throughout several cell passages. It was previously shown that the p2TR-LANA-GFP plasmid establishes long term GFP expression in a subpopulation of GFP positive cells. The establishment of long term GFP expression was rare and approximately 3% of cells harbored 3-10 copies of episomal 2TR-LANA-GFP plasmid on average. [48] We expected that the combination of LANA and SMAR element would enhance the episomal maintenance of plasmids when compared to p2TR-LANA-GFP. This hypothesis was tested by transfecting cells with those plasmids and comparing GFP expression over time. It was repeatedly found that p2TR-LANA-GFP-SMAR transfected cells completely lost GFP expression as early as passage three and four whereas its non-SMAR control, 2TR-LANA-GFP, established stable GFP expression in a rare subpopulation (Figure 4-8). Even after the first passage, p2TR-LANA-GFP-SMAR transfected cells became rounded and less attached to the plate which is thought to indicate the cell death. It is important to note that control

cells transfected with p2TR-LANA-GFP also displayed the same cell phenotype; however, GFP positive cells were still present at later passages in those controls.

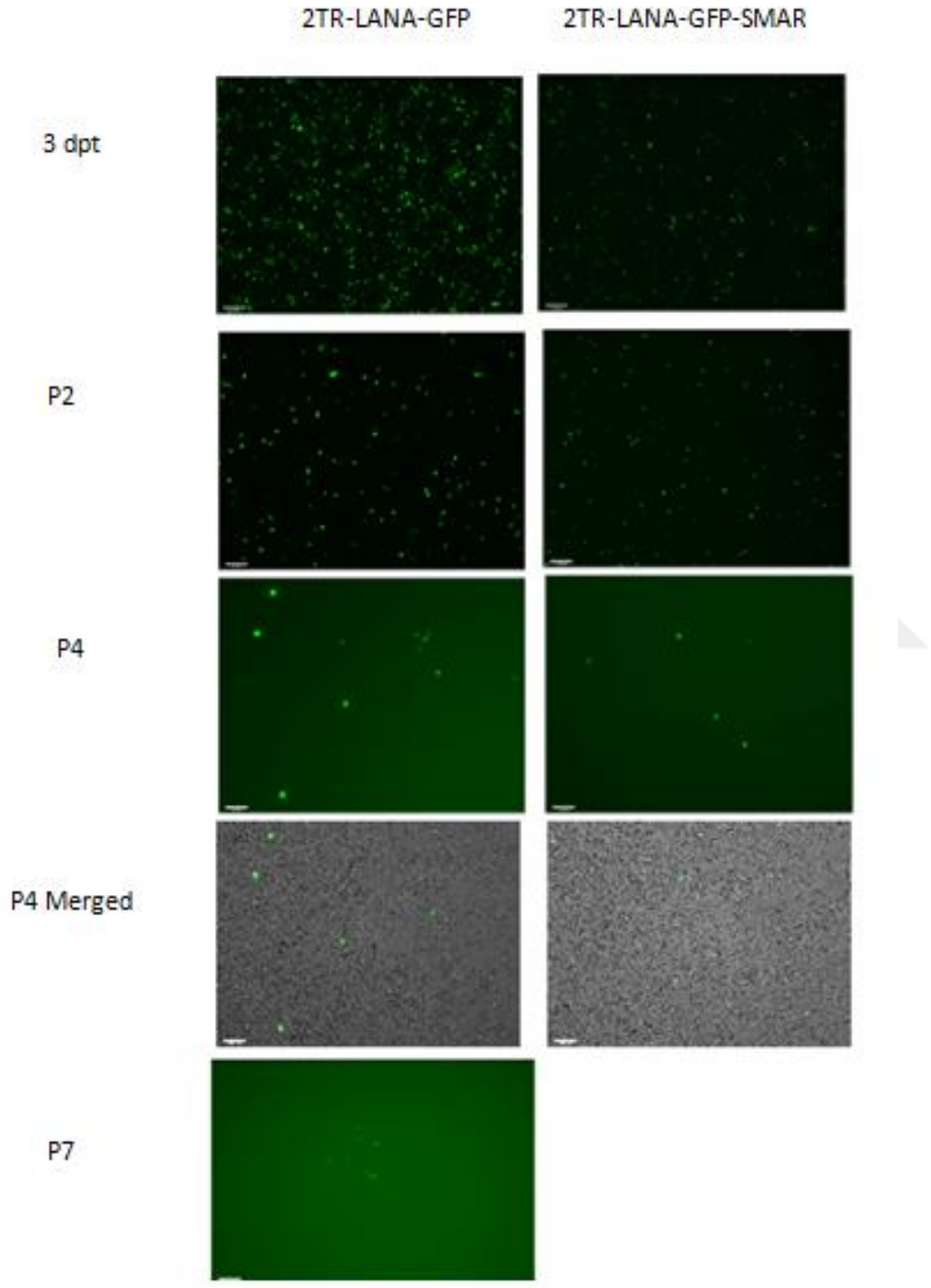


Figure 4-8. GFP expression loss in 293 cells transfected with 2TR-LANA-GFP (left) and 2TR-LANA-GFP-SMAR (right). At passage 7 (P7), no GFP positive cells were detected on 2TR-LANA-GFP-SMAR transfected plates.

Inherently, the transfection efficiencies for two plasmids were different. Therefore, we sorted cells by FACS to begin with a 100% GFP positive cell population. In three different sorting experiments, only one time was p2TR-LANA-GFP-SMAR transfected cells able to attach and grow on plates. In contrast, the non-SMAR controls yielded viable cell populations after each sorting. FACS results revealed that 24% of the cells were GFP positive after transfection with p2TR-LANA-GFP-SMAR while GFP positive cell percentage was 46 % for p2TR-LANA-GFP transfected cells. After sorting cells were seeded onto 24 well plates and passaged twice a week to monitor GFP expression. Figure 4-9 shows the loss of GFP expression over passages after FACS sorting. For p2TR-LANA-GFP-SMAR transfected cells, GFP expression was completely lost at passage six and no signal was detected by microscopy afterwards (Figure 4-9 A). GFP positive cell numbers were also gradually declining in p2TR-LANA-GFP transfected plates initially; however, a sub-population of cells was still GFP positive as seen at passage 16 in Figure 4-9 B.

**Examination of LANA Speckle Distribution for p2TR-LANA-GFP-SMAR**

The long term passaging experiments showed that the LANA and SMAR combination causes a quick decline in GFP positive cells in a relatively short time. After transfection with p2TR-LANA-GFP-SMAR, cells became rounded up and less attached to the plate, indicating cell death. And generally three or four passages later, all GFP positive cells disappeared. We hypothesized that the two episomal maintenance factors might have been competing for binding sites in the nucleus. It is known that SMAR elements are associated with the nuclear scaffold and LANA tethers the TR-containing plasmids to chromosomes. Therefore, we wanted to test our hypothesis by examining the LANA speckle distribution in the nuclei of p2TR-LANA-GFP-SMAR transfected cells.

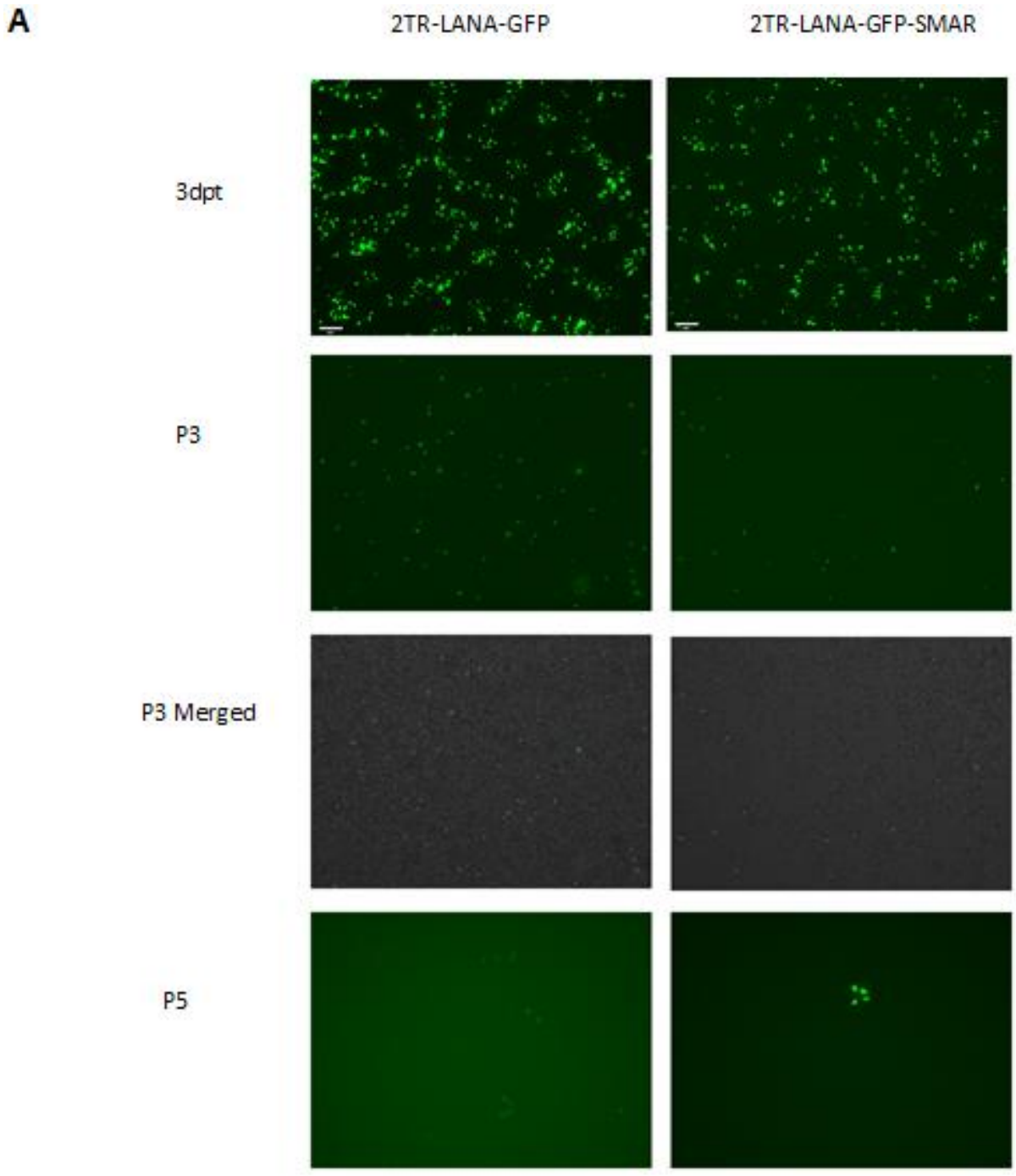


Figure 4-9. (Continued on the next page) Loss of GFP expression for 2TR-LANA-GFP and 2TR-LANA-GFP-SMAR plasmids in cells after FACS. A) Comparison of 2TR-LANA-GFP and 2TR-LANA-GFP-SMAR through five passages. B) Stable GFP expression was established in a subpopulation of cells transfected with p2TR-LANA-GFP.

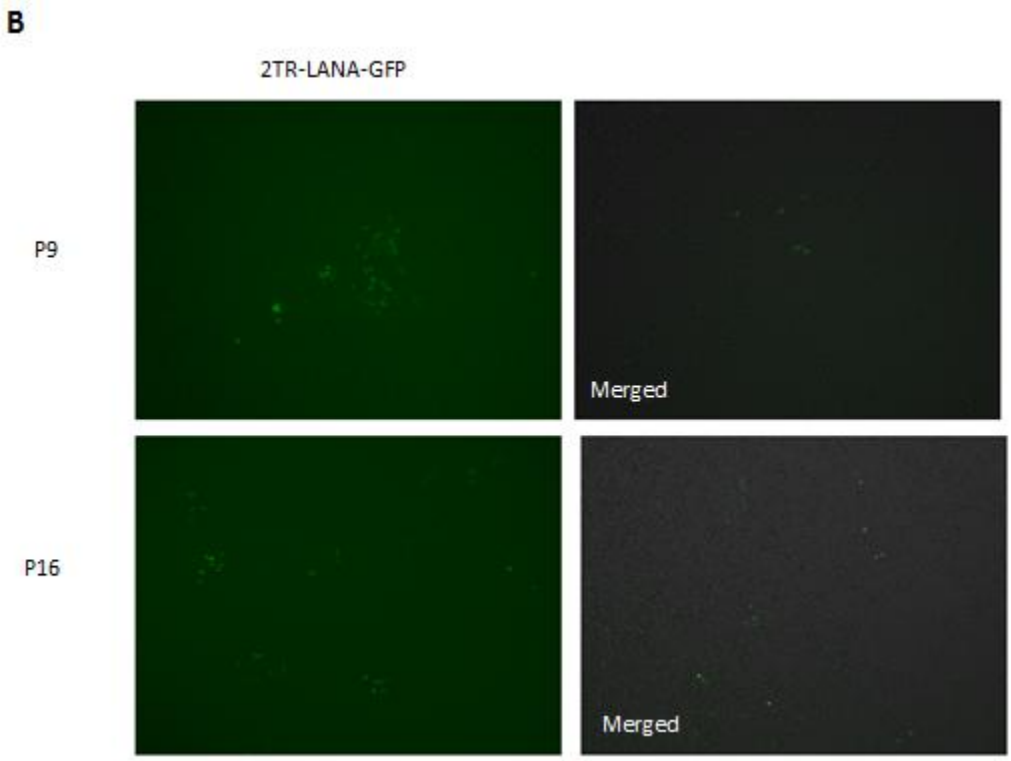


Figure 4-9. (B) Loss of GFP expression for 2TR-LANA-GFP and 2TR-LANA-GFP-SMAR plasmids in cells after FACS.

When KSHV infected cells were stained for LANA, LANA displays a discrete speckle distribution. [53] Figure 4-10 shows the discrete LANA speckle distribution. In this set ofexperiments, a LANA C-terminal-RFP fusion protein encoding plasmid (pDsRed-LANA) was used for determining changes in LANA speckle distribution. LANA binds to TRs through its C terminal region. Therefore, red speckles seen in the nucleus points to the localization of TR elements within the transfected cells. We wanted to examine the effect of SMAR and LANA containing plasmids on three different cell lines: SLK, Hep2 and 293 cells. However, due to the poor adherence of 293 cells and poor fluorescence signal in Hep2 cells, data is unsatisfying on those cell lines and only SLK cell line data is presented. Basically, SLK cells were co-transfected with p2TR-LANA-GFP-SMAR and

pDsRed-LANA. As a control non-SMAR plasmid (2TR-LANA-GFP) was also co-transfected with pDsRed-LANA.

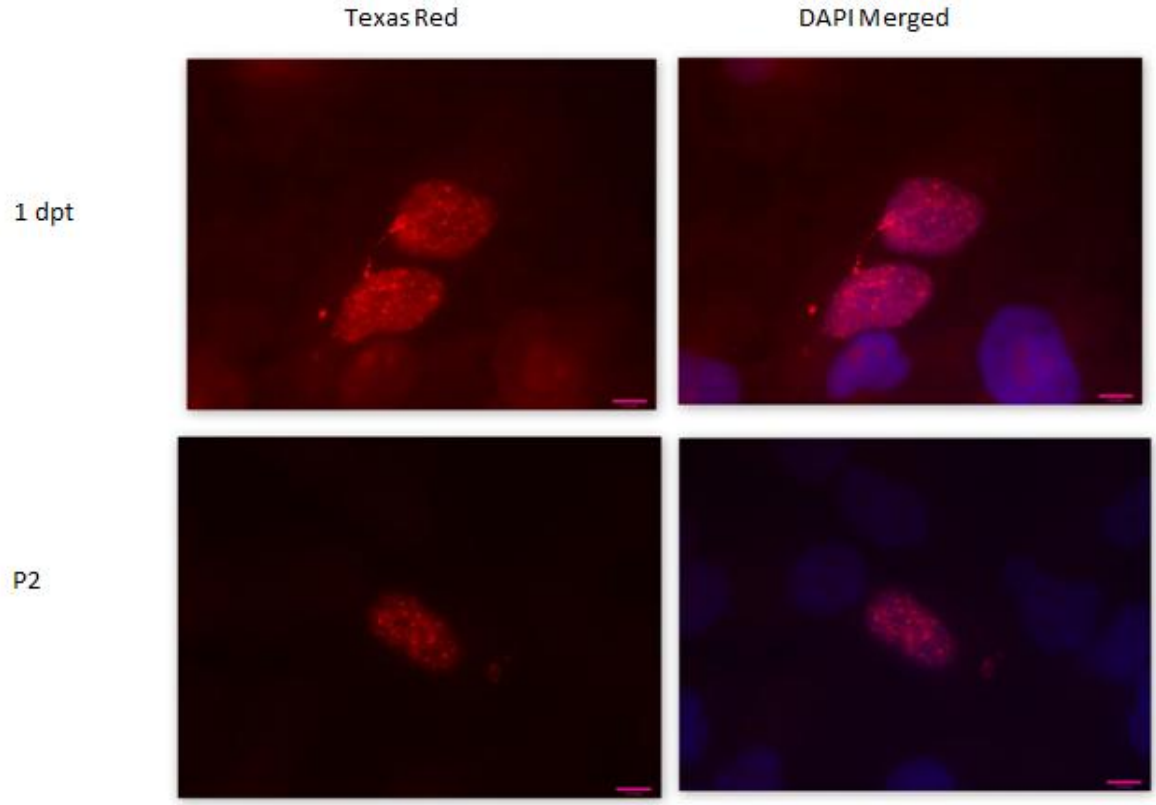


Figure 4-10. LANA speckle distribution at different time points.SLK cells only received pDsRed-LANA plasmid.

Slides were prepared at different time points after transfection. In most of the cells transfected with p2TR-LANA-GFP-SMAR, LANA speckle distribution displayed a normal pattern at day 1 post-transfection (Figure 4-11 A). However, it was also observed that in some of those cells speckle distribution started to differ from normal pattern (Figure 4-11B). Arrows indicates abnormal LANA staining which was not detected in controls. The accumulation sites indicated by arrows did not overlap with DAPI staining. When cells underwent several cell divisions, the speckle distribution change become more obvious as seen in Figure 4-12.

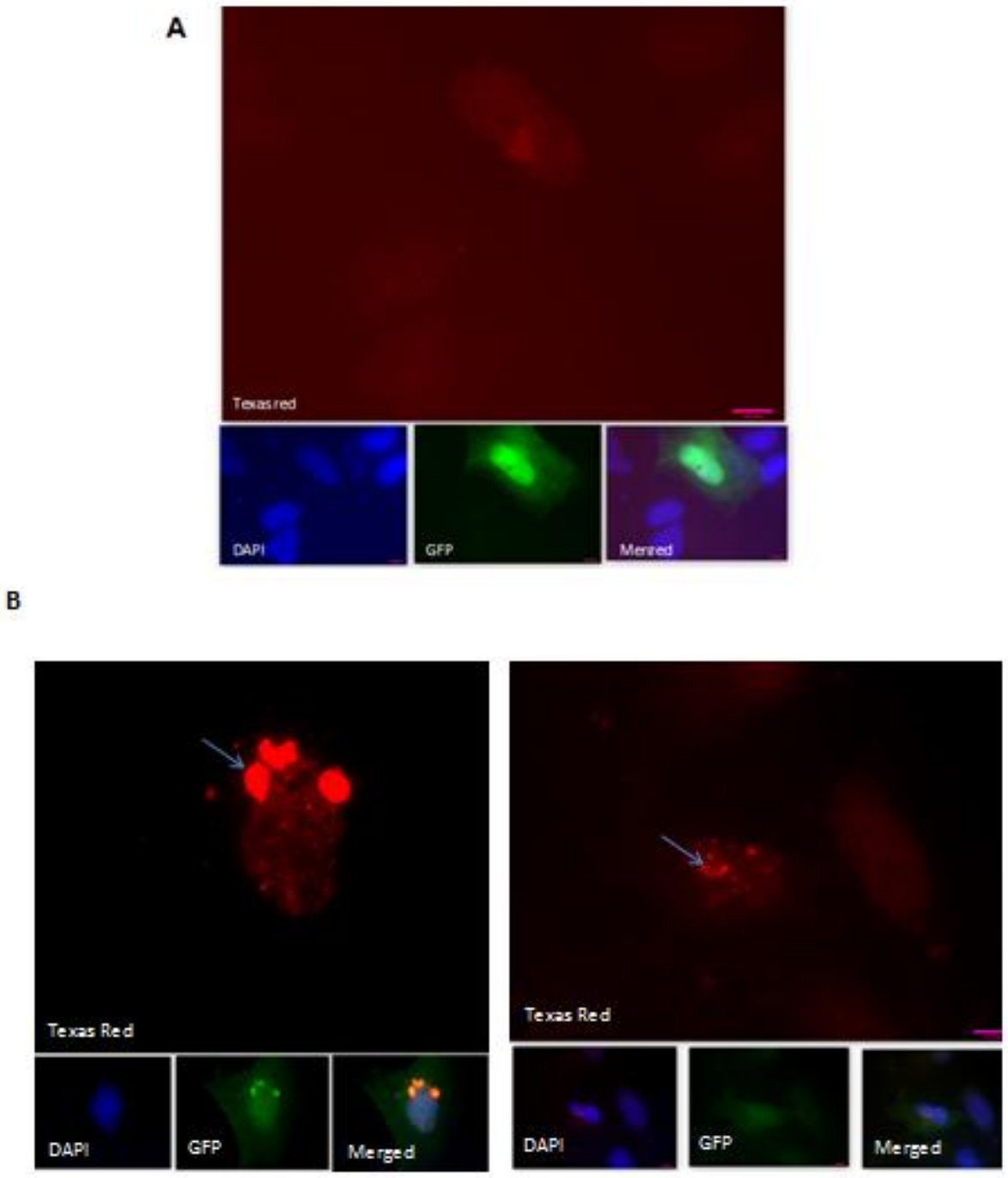


Figure 4-11. LANA speckle distribution in p2TR-LANA-GFP-SMAR transfected cells at day 1 post-transfection. A) Normal speckle distribution was observed. B) Nuclei of cells displayed abnormal LANA binding accumulation indicated by blue arrows.

The speckles showed a disordered pattern at day 3 post-transfection. When cells were passaged one time after transfection, nuclei of the cells started to compartmentalize unevenly. Blue arrows in Figure 4-12 point out the abnormal nucleus structures. When cells reach passage three, 2TR-LANA-GFP-SMAR recipient cells displayed a multi-nucleated phenotype. It is important to note that speckles were mainly located at peripheral regions in the nucleus at passage three (indicated by green arrows). It is indicative that plasmids are positioned by SMAR elements to the nuclear matrix. This phenotype appears after cells divide several times.

These results indicate that the presence of both LANA and SMAR in the same plasmid is detrimental to the cells and causing cell death. As a control we also examined the LANA speckle distribution in p2TR-LANA-GFP transfected cells. The initial experiments showed that non-SMAR control plasmid transfected cells also displayed rounded cell phenotype to a certain degree. Figure 4-13 shows that speckle distribution stays normal over several cell divisions in non-SMAR control plasmid transfected cells. It was observed that even though some cells are dying (indicated by blue arrow in P2), LANA speckles displayed normal pattern. No multi-nucleated cells were observed in p2TR-LANA-GFP transfections. Therefore, it can be concluded that neither LANA nor SMAR causes cell death when only SMAR or only TR-LANA-containing plasmids are transfected alone However, the combination of both on the same plasmid causes cytotoxicity. In summary, the data suggest that 2TR-LANA-SMAR containing vector is apparently not suitable for long term gene expression in gene therapy, as it seems to cause cell death.

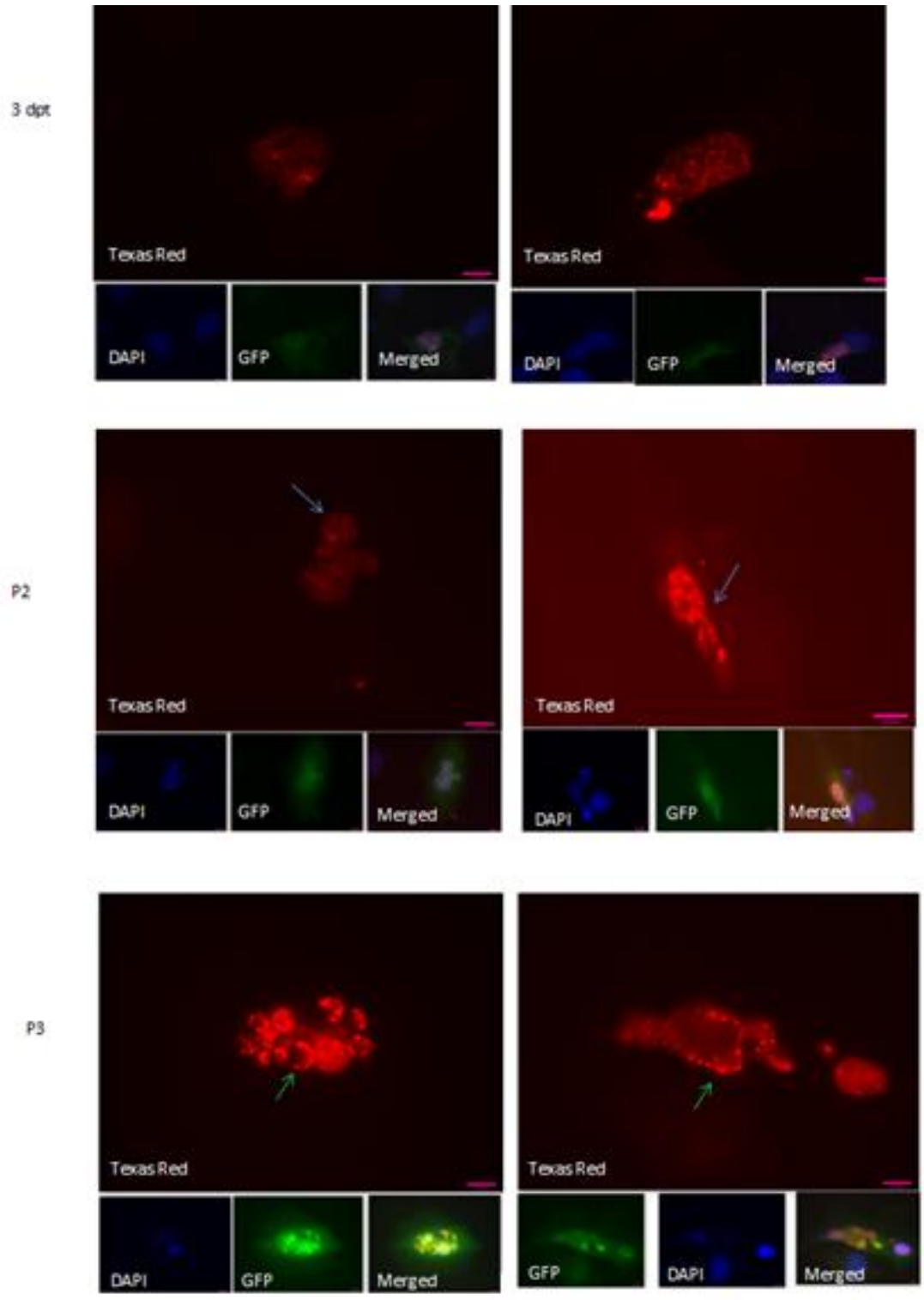


Figure 4-12. Changes in LANA speckle distribution over time. Blue arrows indicate that nucleus compartmentalization started and green arrows indicate that speckles locate to peripheral zones in the nucleus.

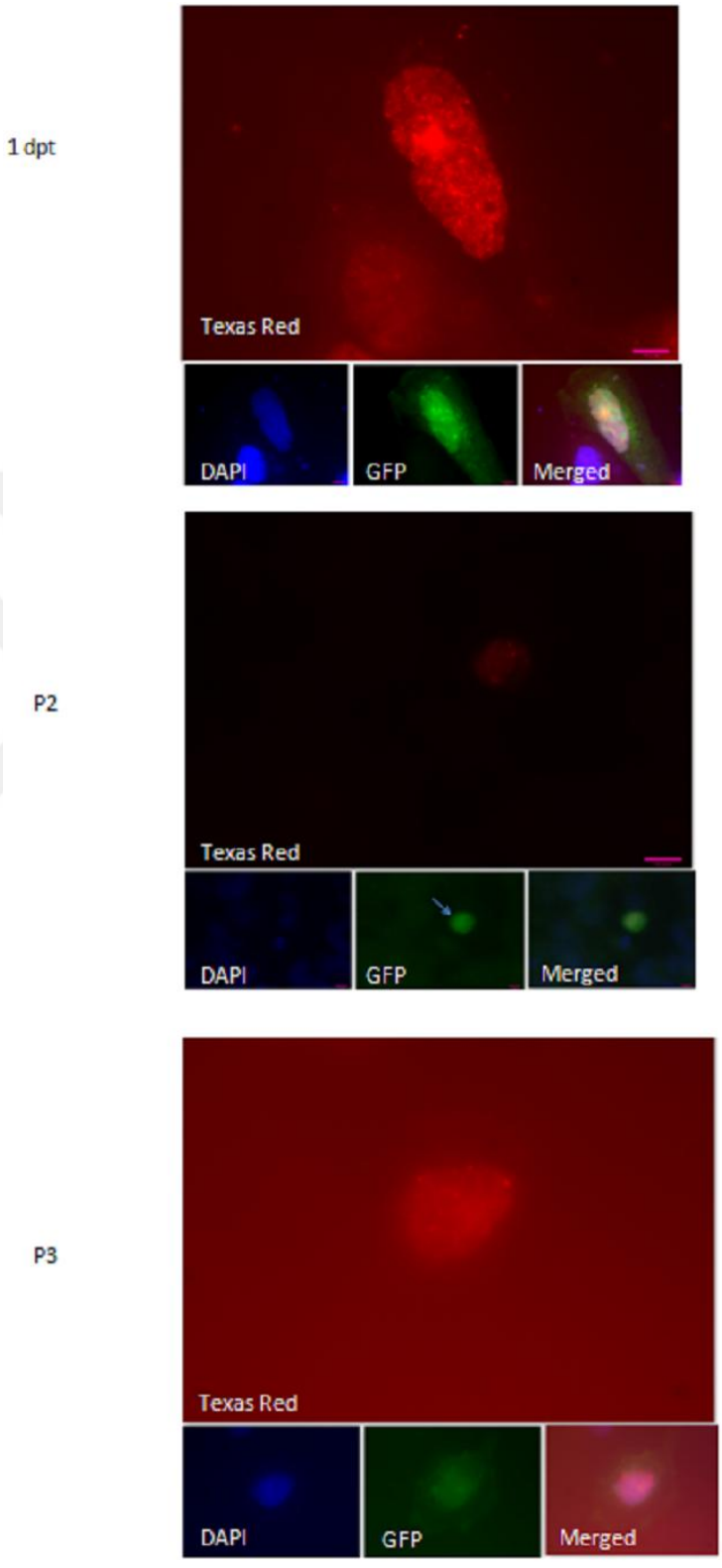


Figure 4-13. LANA speckle distribution in p2TR-LANA-GFP transfected cells at different time points. Blue arrow indicates a rounded cell.

## Testing SMAR Element-Containing Vectors in Vivo

One of the easiest and quickest ways of testing vectors in experimental mice is to perform hydrodynamic injections. Thus, we tested pEGFP and pEGFP-SMAR vectors for the GFP expression in the liver over time by hydrodynamic tail vein injections (HTV). For this study, 20 $Pah^{enu2}$ heterozygous male mice were used. 10 mice injected with SMAR vector (pEGFP-SMAR) and the other 10 mice received non-SMAR (pEGFP) control. Mice were sacrificed at different time points as described in Materials and Methods, and liver sections were analyzed for GFP expression. We expected to see a decline in the number of GFP expressing cells for non-SMAR control vector whereas SMAR-containing vector should maintain stable GFP expression levels. The results were unsatisfying and liver transduction efficiency was quite low when compared to the previous reports (Figure 4-14).[12, 13]

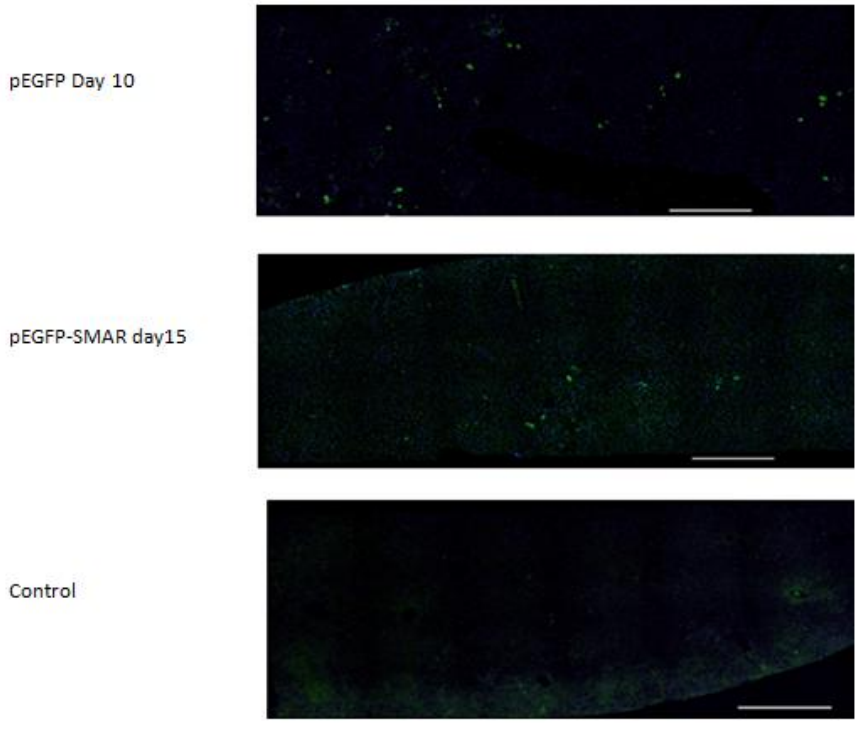


Figure 4-14. GFP expression from liver sections after hydrodynamic injections. Images were taken at 20X and stitched together. Scale bar indicates 2 mm.

There were more GFP positive cells in the livers of mice injected with pEGFP than those injected with pEGFP-SMAR plasmid.

### Testing mPAH Encoding Vectors for PAH Activity

After construction of mPAH vectors with and without SMAR element, we wanted to test those vectors for Phenylalanine hydroxylase activity. Transfections and PAH activity assays were performed as described in Materials and Methods. The results in Figure 4-15 indicates that the constructed vectors, mPAH-C1 and mPAH-SMAR, yielded specific PAH activity which was comparable to parental vector (CB-mPAH). However, other PAH activity assays performed only with CB-mPAH (indicated with * in Figure 4-15) showed that CB-mPAH gives the same specific activity with liver in general. This low specific activity was most likely due to the poor transfection efficiency and it should not be attributed to the low expression from the constructed plasmids.

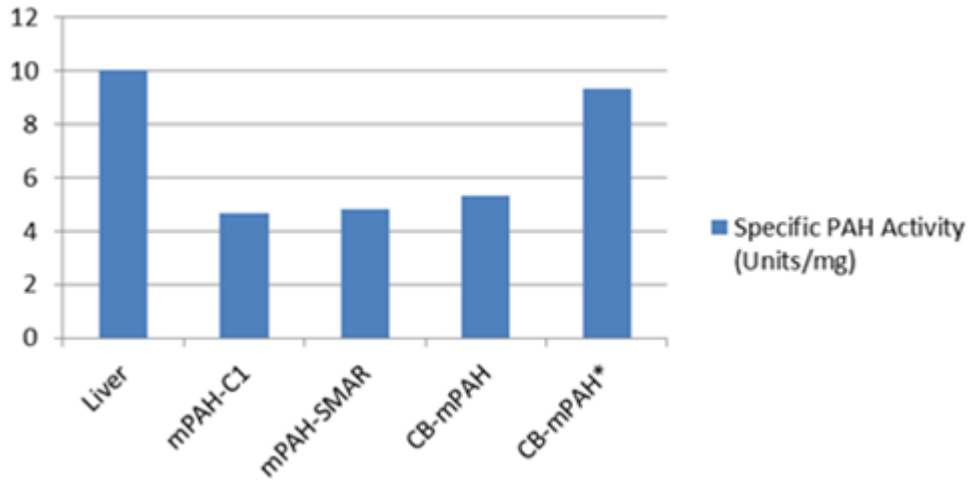


Figure 4-15. Specific mPAH activity. * is from different experimental setups for mPAH activity.

# CHAPTER 5
# DISCUSSION AND FUTURE DIRECTIONS

In the context of gene therapy, long term transgene expression is desirable for many diseases. The safety issues with retrovirus-derived vectors and poor stability of non-viral integrating vectors in growing cells have led to attempts to develop non-integrating, non-viral vectors which can maintain and replicate episomally for long periods. [7, 25] Several candidate vectors have been proposed for episomal maintenance and replication. [44] Among them, SMAR element-based vectors were evaluated for long term gene expression in this thesis. Additionally, we have designed and tested a novel vector which combines the SMAR element with the minimal replicator of KSHV.

## SMAR Is Not Sufficient for Long Term Gene Expression in 293 Cells

SMAR elements were mainly studied in the presence of an initial selection by previous studies. [33, 34, 36] In order to consider SMAR elements in a realistic vector design for gene therapy as stated in Specific Aim 1, we wanted to determine whether the SMAR provided any advantage to long term stability of vectors in the absence of selection pressure in 293 cells. Our results indicated that there was no difference in long term GFP expression for SMAR and non-SMAR vectors. The loss of GFP expression after FACS sorting revealed that inclusion of SMAR did not offer any advantage to the vector for long term stability in 293 cells (Figure 4-4). GFP positive cells seen at passage 12 and later are most likely results of genomic integration. Transformation of HIRT extracts prepared from non-selected cells at passage 12 did not yield any colonies in plasmid rescue assays. Southern blot analysis for non-selected cells was inconclusive and plasmids were not detected on either extrachromosomal or genomic blots. This was probably due to the low percentage of GFP positive cells at later

passages (roughly ~5-10%). Ideally for future experiments, GFP positive cells at passage 12 or later can be sorted and analyzed for the plasmid integration or episomal maintenance in the absence of selection. By preparing probes with high specific activity, this question can be addressed. Additionally, the question whether plasmids are integrated or in episomal state can be elucidated by a PCR-based approach. Previously, it was shown that Dpn I-based PCR assays with HIRT extracts can be performed to understand the replication properties of plasmids.[48] Therefore, it will be useful to adopt a similar approach in order to find out whether SMAR is sufficient for replication.

As previous studies showed that SMAR-based vectors can replicate in several *in vitro* systems, we wanted to test the replication ability of SMAR vectors in 293 cells after applying an initial selection. Stably GFP expressing cells were obtained following a ten-day initial selection pressure. 2 months after removing the selection reagent, cells were still GFP positive (Figure 4-5). In order to understand replication status of plasmids, we performed Southern blot analysis with extrachromosomal and chromosomal DNA extracts. However, plasmids were not detected in either of the DNA extracts. This was possibly due to the low specific activity of probes. The specific activity of the probes, calculated according to the manufacturer's manual, was ranging between ~$1x10^8$ dpm/μg to $2x10^8$ dpm/μg, whereas "good" specific activity for a probe should be around $2x10^9$ dpm/μg. Therefore, Southern blot analysis was unable to detect the plasmids from extracts. It is important to note that in later trials, 0.2 ng control plasmids were detected with the low specific activity probes. By Avogadro's number, 0.2 ng DNA for ~5kb plasmid is equal to approximately $4x10^7$ plasmid copies. Therefore, it should have

been easily detected in Southern blots if GFP positive cells harbored two plasmids per cell. Since probes prepared for hCA2 gene as an internal control could not detect the single copy gene in the genome (Figure 4-7), it is hard to reach a conclusion for the integration status of the SMAR and non-SMAR plasmids. To address this question, it will be necessary to prepare probes with high specific activity. As discussed above, Dpn I-based PCR assays can also serve as an alternative tool to reach a conclusion about replicative features of SMAR-based vectors.

**LANA-SMAR Combination Does Not Provide Long Term Gene Expression**

In order to provide the novel gene therapy vector with the ability of efficient episomal maintenance and faithful partition, we designed a plasmid which combined SMAR element with the minimal KSHV replicator. Ideally, TR-LANA-SMAR combination would provide long term transgene expression, persist as an episome in the cells without integration, and faithfully segregate to daughter cells. As previously introduced in Specific Aim 2, the novel vector was tested in 293 cells by means of monitoring GFP expression over time. It was found that p2TR-LANA-GFP-SMAR transfected cells quickly lost GFP expression whereas its non-SMAR control plasmid established long term GFP expression in a subpopulation of cells (Figure 4-9). We then hypothesized that the competition between LANA and SMAR element in terms of binding to different nuclear locations is the cause of quick loss in GFP expression. This hypothesis was tested in Specific Aim 3. We examined whether LANA binding locations differ in the nuclei of cells transfected with LANA and SMAR-combining vectors. Even though LANA speckle distribution was normal at the beginning, multinucleated cell phenotype was observed after several cell divisions. Speckles were located mainly at the peripheral regions of the nucleus (Figure 4-12). This result suggested that plasmids were

positioned by the SMAR element to the nuclear matrix. It is known that SMAR elements are bound by SAF-A protein, which organizes chromatin in the nucleus by attaching DNA loops to the nuclear matrix. [30] For further elucidation, it will be useful to examine SAF-A binding distribution in the cells transfected with LANA-SMAR vector. A co-localization study for LANA and SAF-A binding sites will further confirm that the presence of SMAR element and LANA-binding TR elements on the same plasmid causes a disordered nuclear organization which eventually gives rise to multi-nucleated cells.

Furthermore, this hypothesis was also supported by the normal distribution of LANA observed in non-SMAR control vector (p2TR-LANA-GFP) transfected cells. Even though some of the cells transfected with non-SMAR control vector displayed dying cell phenotype, LANA speckle distribution was normal in those cells. It was previously reported that LANA itself causes genomic instability through inhibiting P53. [54] However, in this study, Si et. al. observed this effect in constitutively LANA expressing cells which were obtained after application of an 8-week selection pressure. It is arguable that LANA expression can give rise to multinucleated cells after a relatively long period of time. However, the disordered nuclear organization and multinucleated cell phenotype observed in LANA-SMAR transfected cells happened very quickly after transfection. Thus, our results imply that LANA and SMAR combination is detrimental to the cells. For these reasons, the novel vector we have designed is, unfortunately, not feasible for gene therapy applications.

### A Minicircle-based Vector Proposal for Future Experiments

One of the important developments in the field of non-viral and non-integrating gene therapy vector design is the development of minicircles. In several *in vitro* systems

and an *in vivo* system, SMAR-based minicircles were studied for long term transgene expression. [30, 31] Both reports showed that removal of bacterial sequences enhances the stability of the SMAR vectors. Thus, minicircle strategy might be useful to design episomally stable vector systems. SMAR minicircles can be tested for long term maintenance and gene expression in tissue culture by means of GFP reporter expression. Thanks to the available methods for gene delivery into experimental animals, an *in vivo* system such as mice can be utilized to test the long term stability of the vectors. Indeed, we performed hydrodynamic tail vein injections to compare SMAR and non-SMAR vectors. However, due to the aged mice we used for the experiment, the results were inconclusive (Figure 4-14). As discussed in the introduction of this thesis, an approach which combines ORMOSIL nanoparticles and hepatocyte-targeting strategy will provide a good experimental system to evaluate SMAR minicircles in vivo. It seems plausible that encapsulating vectors in nanocarriers and modifying those nanocarriers with targeting ligands will increase the transduction efficiency. Such an approach would make it easier to assess the benefits of SMAR minicircles in an experimental animal model.

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Mehmet Kara was born in Odemis, Turkey. He graduated from Ortaklar Anatolian Teacher Training High School, Aydin Turkey. He enrolled at Istanbul University where he earned a bachelor of science. degree in molecular biology and genetics in 2007. He came to the United States in 2008 to attend a language school at Indiana University to learn English. He enrolled as a master's student at the University of Florida to obtain a degree in medical sciences in 2009. After graduation from master's program, he continued his academic career to obtain a PhD, in Interdisciplinary Program at University of Florida.