Combined gene therapy of siRNA and apoptosis inducing factor generated from a single transcriptional event

Hsin-I (Cindy) Hung

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Approved by School of Pharmacy
Advisor: Leaf Huang, Ph.D. Professor,
Xiao Xiao, Ph.D., Professor,
Moo J. Cho, Ph.D., Associate Professor,

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I would also like to thank Taiwan government for awarding me 3 years scholarship and giving me the opportunity to come to the US to study. Special thanks to Dr. Anthony

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ABSTRACT

Hsin-I (Cindy) Hung

Combined gene therapy of siRNA and apoptosis inducing factor generated from a single transcriptional event

(Under the direction of Leaf Huang, Ph.D.)

The goal of this project is to test the hypothesis that a single transcriptional event driven by RNA polymerase II promoter driven siRNA expression plasmid that utilized RNA splicing mechanism. During this event, a specific siRNA to a target oncogene, forms from the spliced intron; meanwhile a therapeutic protein is established after rejoining of the exon fragments. In this case, the well-known, strong RNA polymerase II (CMV) promoter was used to produce an siRNA against several well known oncogenes and a therapeutic protein, Apoptosis Inducing Factor (AIF), as a dual therapeutic to induce tumor cells apoptosis simultaneously from a single plasmid construct. By using this splicing approach with 2 different targeting mechanisms (i.e., oncogene inhibition by siRNA and therapeutic protein expression), we expect to see a synergistic effect from our novel RNA polymerase II promoter-driven plasmid. It was demonstrated that the plasmid construct achieved up to 40 percent siRNA inhibition, as well as increased apoptosis of cells transfected after cloning of the therapeutic exon component. In addition, by using a DNA-vector driven siRNA expression system, we can reduce the cost of producing siRNA and increase the duration of siRNA efficacy to achieve long term effects of gene silencing. Thus, by utilizing the

combination of these 2 different targeting mechanisms from the RNA splicing process, we expected that the strategy will bring us a new affordable and promising way for efficient cancer cell targeting and treatment. Combined with the consistent and satisfactory lipid-based delivery system previously described by our lab (Li & Huang, 1997; Li et al, 2008; Li & Huang, 2006), we anticipate a greatly improved cancer targeting therapy.

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ABBREVIATIONS

AIF:	Apoptosis inducing factor
B16F10:	Murine melanoma cells with GL3 transduced
EGFP:	Enhanced green fluorescent protein
EGFR:	epidermal growth factor receptor
EM22:	pEGFP-N1 plasmid removed PstI site from MCS and added PstI site within EGFP by silent mutagenesis, (pEGFP-PstI+PstI)
EMC:	pEGFP-PstI+PstI+artificial Intron+control scramble siRNA
EM-E6:	pEGFP-PstI+PstI+artificial Intron+HPV E6 siRNA
EM-EGFR:	pEGFP-PstI+PstI+artificial Intron+EGFR siRNA
EM-intron:	pEGFP-PstI+PstI+artificial Intron
EML6:	6th clone of pEGFP-PstI+PstI+artificial Intron+Luciferase siRNA
EML8:	8th clone of pEGFP-PstI+PstI+artificial Intron+Luciferase siRNA
EM-VEGF:	pEGFP-PstI+PstI+artificial Intron+VEGF siRNA
HHI-80:	AIF inserted ahead of EGFP gene in the pEGFP-N1 (MCS 15 clone)
HPV:	Human papillomavirus
SPL:	EGFP replaced by AIF in the pEGFP-N1 (Rep 23 clone)
STS:	Staurosporine
VEGF:	Vascular endothelial growth factor

Introduction

RNA interference (RNAi) is a powerful cellular mechanism to destroy target specific mRNAs. In 1995, Guo and Kemphues (Guo & Kemphues, 1995) first found that either strand of sense or antisense RNA alone is efficient to suppress gene expression in worms. Subsequently, Fire et al. (Fire et al, 1998) further found that double-stranded RNAs (dsRNAs) were more effective at producing interference than either strand individually. Fire and coworkers (Fire et al, 1998) then coined the term "RNA interference (RNAi)" to describe the inhibition of gene expression by dsRNAs. RNAi was first discovered in plants and lower eukaryotic organisms but was later observed as an important genetic regulatory mechanism in mammalian cells (van Rij & Andino, 2006). The mechanism of RNAi involves a multistep process to generate the small interfering RNAs (siRNAs), which act as triggers for targeted RNA degradation (Dykxhoorn et al, 2006). In brief, first, an RNAse III type enzyme, Dicer, will cleave double stranded RNAs into short 21-23 base pair duplexes. Then, the antisense strand of RNA is incorporated into a protein complex, RISC (RNA induced silencing complex), which has helicase, endonuclease activity. The antisense strand RNA will then guide the whole protein complex to its target complementary sequence, followed by endonucleolytic cleavage of the target mRNA and recycling of RISC.(Dykxhoorn et al, 2006; Steinman et al, 2004; Tiscornia et al, 2003; Wang et al, 2008). One particular benefit of RNAi is that siRNAs have not been found to activate interferon pathway genes in mammalian cells, largely owning to their short length, thereby making this a powerful tool for sequence specific knockdown of RNAs.

siRNAs are typically generated either by chemical synthesis or DNA-vector-mediated methods. While chemically synthesized siRNAs are frequently used and have shown some advantages such as high transient inhibition efficiency and easy to conduct, they also have some major drawbacks: cost and short half-life (Fewell & Schmitt, 2006). Chemically synthesized siRNAs generally last 2-5 days in *in vitro* systems and provide only transient knockdown activity, which together with their high cost make this approach unsuitable for the analysis of the long-term effects of gene silencing. While DNA-vector-mediated methods are time-consuming, laborious and with extra nuclear barrier concern (Dykxhoorn et al, 2006; Wang et al, 2008), it is looked to as an alternate, affordable means to achieve long-term effects of gene silencing.

Non-viral DNA-vector-mediated siRNA can be driven either by RNA polymerase II or RNA polymerase III promoters. RNA polymerase III promoter such as U6, H1, and tRNA have been widely used in non-viral DNA-vector-mediated siRNA studies because of their high driving efficiency (Kawasaki & Taira, 2003; Tiscornia et al, 2003; Xia et al, 2004). In addition, the constructs containing RNA polymerase III promoter are small, simple and easily inserted into viral vectors. However, those RNA polymerase III constructs tend to have minimal cell specificity, and their high driving activity increases the possibility of off-target and non-specific silencing effects, which in turn could trigger an interferon response and cellular toxicity (Bridge et al, 2003; Jackson et al, 2003). In contrast, many RNA polymerase II promoters, such as the CMV promoter, are known to be successfully in direct cell or tissue-specific gene silencing and transgenic animal studies (Lin SL, 2003). Furthermore, RNA polymerase II promoter directed synthesis of short hairpin RNA (shRNA) mimic the well-known natural miRNA synthesis and therefore,

could be an efficient strategy to study RNAi silencing (Greber & Fussenegger, 2007; Lin SL, 2003; Wang et al, 2008). In order to overcome the limitations of the currently commonly used RNA polymerase III promoter driven siRNA expression system, we made an effort to study the RNA polymerase II promoter driven siRNA expression system. Here, by taking advantage of the splicing mechanism from an RNA polymerase II promoter driven shRNA-forming plasmid, we expect to achieve a synergistic cancer treatment by using 2 complimentary mechanisms that include an oncogene targeted siRNA from the intron and a therapeutic protein from the exons. Together with the well-established delivery methods from our lab, we hope to achieve a better targeted cancer treatment.

Materials and Methods

Cell lines and drug treatments

B16F10 cells, murine melanoma cells, were obtained from the American Type Cell Collection (Manassas, VA) and were stably transduced with GL3 firefly luciferase gene using a retroviral vector from Dr. Pilar Blancafort's lab at the University of North Carolina at Chapel Hill. TC1 cells were obtained from ATCC. Both cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmids

A commercial available EGFP containing plasmid, pEGFP-N1was purchased from (Clontech, Mountain View, CA) as parental plasmid for all the subsequent modification. Silent mutagenesis was carried out by using site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions with a pair of complementary primers to create Pst I site on the pEGFP-N1. Silent mutagenesis primers were: 5' - CCT GCT GGA GTT CGT GAC CGC TGC AGG GAT CAC TCT CGG CAT GGA C - 3' and 5' - GTC CAT GCC GAG AGT GAT CCC TGC AGC GGT CAC GAA CTC CAG CAG G - 3' Artificial intron fragment was synthesized by company (Integrated DNA Technology, Inc.). Sequence of anti-HPV E6 siRNA, anti-EGFR siRNA, anti-VEGF siRNA, control scramble siRNA, and anti-Luciferase siRNA were adapted from the literature (Shih et al, 2002; Song et al, 2005; Steinman et al, 2004; Zhang et al, 2005) and synthesized

(Integrated DNA Technology, Inc., Coralville, IA) as templates for the PCR reaction. PCR was performed using the following PCR primers (Integrated DNA Technology, Inc.) and an ABI PRISM 7900HT PCR machine (Applied Biosystems, Foster City, CA): for anti-HPV E6 siRNA , 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CGC TTG CAG T - 3' and 5' - TTC CTT ACT AGT AGT ACT TCG CGA GGT ACC TTG GAA AAA AGC TTG CAG TAC ACA CAT TCC - 3' ; anti-EGFR siRNA, 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CAG GAA TTC G - 3' and 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA AAG GAA TTC GCT CC - 3'; anti-VEGF siRNA, 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CGC ACT CCA G - 3' and 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA AGC ACT CCA GGG CTT CAT - 3'; control scramble siRNA, 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CAC GTG ACA C - 3' and 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA AAC GTG ACA CGT TCG G - 3'; anti-Luciferase siRNA, 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CGT CGA AGT ACT C - 3' and 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA ATC GAA GTA CTC AGC GTA AGC - 3'. All the PCR cloned siRNAs fragments were subcloned into the intron on modified pEGFP-N1 plasmid with MluI and NruI restriction sites. All the cloned fragments and constructs were sequence verified by the UNC genome center, sequence facility. A commercial RNA polymerase III promoter driven siRNA expression plasmid was purchased from Genescript as pRNA-siRNA system in the study (Genescript).

RNA Extraction and RT-PCR

RNA was extracted by RNeasy kit (QIAGEN). Immediately after extraction, 1 to 2 μg of RNA was used for one-step RT-PCR synthesis with SuperScript II (Invitrogen) and different primers: AIF primer 5′- AAG GAA CCC GGG TGC GTG GAA GGA AAA GGA AGG AGC GGG AGC TTC CG -3′ and 5′- TTC CTT GCG GCC GCC GCC AGG GAT GGA AAA GTG CTT GTG TAT TCC ACG A - 3′. RT-PCR cloned AIF fragment from B16F10 cell line was subcloned into pEGFP-N1 plasmid with XhoI and AgeI restriction sites.

Luciferase reporter assay

B16F10 cells were seeded in 96-well dishes and transfected using Lipofectamine 2000 (Invitrogen) with 300 ng of different EM-intron plasmids. After 48 h transfection, cells were trypsinized and 2 x 10^5 cells were collected. Those collected cells were incubated with 100 μl lysis buffer (0.05% Triton X-100 and 2 mM EDTA in 0.1 M Tris-HCl) at room temperature for 10 min. Ten microliters of lysate was mixed with 100 μl substrate (Luciferase Assay System, Promega Co., Madison, WI) and the luminescence was measured by a plate reader. The protein concentrations of the samples were determined by using a protein assay kit (Micro BCA^{TM} protein assay kit, Pierce, Rockford, IL). Luciferase activity of a sample was normalized with the protein content and expressed as percent luminescence intensity compared to the positive control, EM-scramble siRNA.

MTT assay

B16F10 cells grown in 96-well plates with 10% FBS DMEM were incubated to reach at 30-

40% confluence. The cells were then washed with PBS and 50ul/well of FBS free DMEM was added. A 0.3ug quantity of various DNA constructs in 50ul of FBS free DMEM containing 1 μl of Lipofectamine 2000 (Invitrogen) was added to the medium in each well. After transfection for 48 h, the cells were observed by fluorescent microscope and then 5ul MTT substrate was added. An MTT stock solution (5 mg of MTT/ml of distilled water) was filter sterilized and kept for no more than 2 weeks at 4°C. To start the coloring reaction, stock solution was added to growing cultures (final concentration, 0.5 mg/ml). The mixture was incubated for 1 h and the tubes were vortexed. Lysed cells and debris were pelleted (15,000 × *g*, 5 min), and 100 μl of the supernatant was transferred into a 96-well plate. The OD was measured with a spectrophotometer (SPECTRAmax PLUS, Molecular Devices) at 570 nm. A blank with propanol alone was measured and subtracted from all values.

Statistical analysis

All statistical analyses were performed by the one-way ANOVA or a two-tailed student t-test. Data were considered statistically significant when P value was less than 0.05.

Results and Discussion

Insertion of an artificial intron containing siRNA cloning sites within EGFP by silent mutagenesis created PstI site

A new strategy for utilizing specially designed plasmid constructs that take advantage of RNA splicing mechanism was proposed by Lin and collegues (Lin SL, 2003). In this approach, after splicing, the RNA polymerase II leads to the production of an oncogene targeted siRNA from the spliced intron. Furthermore, a functional protein is formed by the exons resulting from splicing (Fig.1). We used pEGFP-N1 plasmid (Clontech) containing EGFP as report gene to serve as the parental plasmid to facilitate all subsequently subcloning. In order to apply the aforementioned strategy in our study (Lin SL, 2003), the original pEGFp-N1 plasmid was modified allowing the minimum required splicing elements to be inserted into the EGFP to create an artificial intron (Fig. 1). First, the PstI site was removed from the multiple cloning site (MCS) of the original pEGFP-N1plasmid (pEGFP-N1-PstI) and then a new PstI site was created within EGFP gene by silent mutagenesis of the pEGFP-N1-PstI plasmid (pEGFP-N1-PstI+PstI, or EM22) (Fig. 2A) Both pEGFP-N1-PstI and EM22 plasmids were sequenced to verify the relocation of the site as well as subjected to restriction enzyme digestion by Pst1, to confirm fragment formation (Fig. 2B). After the PstI site was relocated into the EGFP gene to form the EM22 plasmid, the minimum splicing required elements containing a donor splicing site, a acceptor splicing site, a branch point and a poly-pyrimidine tract as an artificial intron were inserted into the EGFP gene ofEM22 (Fig. 3A). The EM22 plasmid containing the artificial intron within the EGFP gene is referred to as EM22-intron. After the EM22-intron was

sequence verified, its splicing ability was further examined by investigating EGFP levels as observed by fluorescent microscopy. Based on our results (Fig. 3B), it is apparent that both EM22 (silent mutagenesis) plasmid and EM22-intron plasmid turned green, indicating the splicing mechanism worked in our constructed EM22-intron plasmid. Moreover, not only forward inserted intron plasmids turned green but also those reversed- inserted introns turned green, which strongly supports that the splicing mechanism was successfully carried out in our EM22-intron plasmids.

Following the verification of EM22-intron plasmids splicing, siRNA sequences for known cancer related genes, such as HPV E6, EGFR, VEGF, and control scramble, were inserted into our EM22-intron plasmid. These were verified to be into the forward facing intron plasmids (data not shown). The schematic of this step is shown in Fig. 4A and resulting PCR product of various siRNAs are shown in Fig. 4B. EM22-intron with HPV E6, EGFR, VEGF, and scramble siRNAs insertions, respectively, were sequence verified and tested their splicing ability by fluorescent microscopy (Fig.5). We wish to overcome the limitations of RNA polymerase III promoter driven siRNA expression system and to compare their siRNAs suppressive activity with the RNA polymerase II promoter driven siRNA expression system. We have constructed our novel RNA polymerase II promoter driven siRNA plasmid which utilizes RNA splicing mechanism as EM-intron –siRNA system. A RNA polymerase III promoter driven siRNA expression plasmid was purchased from (genescript) as pRNA-siRNA system. Both of these 2 siRNA expression plasmid systems (Fig.6) were successfully inserted with HPV E6, EGFR, VEGF and scrambled siRNAs individually with sequence verified and fluorescent microscopy examined of the EGFP activity.(Fig 4 and Fig 5) (data of RNA polymerase III system not shown). Our

constructs containing RNA polymerase II promoter driven EM22-intron system have shown successful intron splicing mechanism as well as produced the functional protein (e.g., EGFP). Therefore, we further examined the gene silencing activity from the siRNA spliced into the intron region. The B16F10 mouse melanoma cell line, transduced with GL3 firefly luciferase gene, was chosen. The luciferase was stably transduced using a retroviral vector by Dr. Pilar Blancafort's lab at the University of North Carolina at Chapel Hill. This plasmid was designed to assess the inhibition activity by the anti-Luciferase siRNA. We inserted anti-Luciferase siRNA into an artificial intron of EM22-intron system (Fig. 7A). After the sequence was verified, EM22-intron–Luciferase constructs were transfected into B16F10 cells and analyzed for their ability to suppress luciferase activity. Based on the data shown in Fig. 7B, we found that two specific clones, EML6 and EML8, showed inhibition activity. EML8 was found to down-regulate the luciferase activity about 40% compared to EM22-intron ($P<0.01$, $N=45$).

Cloning of Apoptosis Inducing Factor as therapeutic protein

Our EM22-intron siRNAs system has successfully shown splicing activity that has enabled us to obtain a functional protein from the re-joined exon, previously described as EGFP (Fig. 3B and Fig. 5) and also active suppression from the siRNA from the intron (Fig. 7). Therefore, in order to obtain a synergistic effect in treating cancer cells, the AIF gene was cloned, in an effort to further induce cells into apoptosis. We designed a pair of AIF primers based on the PubMed (AF100927). RT-PCR was performed on cell extract from both B16F10 and Tc1 cell lines. We cloned a 1.93KB fragment of AIF from B16F10 cell line but not from the Tc1 cell line (Fig. 8A) and inserted it into the EGFP-N1 plasmid to

form SPL, and HHI-80 plasmids, respectively (Fig. 9). The SPL plasmid (Rep23 clone) contained the cloned AIF in place of EGFP in the pEGFP-N1 plasmid (Fig. 9A). The HHI-80 (MCS15 clone) has cloned AIF inserted upstream of the EGFP fragment (Fig. 9B). Both SPL and HHI-80 were sequenced and restriction digestion confirmed predicted size fragments (Fig. 8B). We observed the AIF transfected B16F10 cells by light microscopy (Fig. 10A). The SPL (e.g., AIF) transfected cells displayed obvious cell death, even in the absence of STS, an apoptosis inducer (Fig. 10A). MTT assays were conducted to analyze the cell growth inhibition activity of both SPL and HHI-80 AIF constructs and compared them to EM22-intron-HPV E6 siRNA (Fig. 10B). The preliminary data suggest that both SPL and HHI-80 can inhibit cell growth around 15-20%. With the addition of STS, both SPL and HHI-80 can reach up to 25% to 30% cell growth inhibition (Fig. 10B).

Overall, it has been shown that the novel RNA polymerase II promoter driven constructs (EM22-intron) successfully demonstrated the ability to correctly splice a reporter gene. As shown in Fig. 3B, both for forward-inserted and reverse-inserted introns are able to be correctly excised. It is likely that the forward-inserted intron seems to have higher splicing ability owning to the PPT (Poly-Pyrimidine Tract) element located much far from the promoter, and thus enable the RNA polymerase to go farther. In contrast, the PPT element of the reversed-inserted intron is closer to the promoter and the pyrimidine-rich sequences of the PPT tend to act as a stop signal for the transcription reaction. Therefore, it is not surprising that the forward-inserted intron has higher splicing activity. Replacing this reporter gene with the apoptosis inducing factor (AIF), demonstrated increased cell death as compared to control cells (i.e. up to 30% increase). Furthermore, it was demonstrated that siRNA sequences inserted into the introns were able to down-regulate gene activity up to

40% compared to control sequences. Owning to lack of better ways to only analyze with those transfected cells, our results were based on the whole population of the sample cells. Therefore, we believed that by examining only those plasmid transfected cells, we will be better able to accurately measure the down-regulation ability of our EM-intron constructs. This system may provide interesting therapeutic options for future cancer therapeutic research.

Table1. Primer Sequence

Gene	Primer sequences
Mouse AIF	Forward 5' - AAG GAA CCC GGG TGC GTG GAA GGA AAA GGA AGG AGC GGG AGC TTC CG - 3' Reverse 5' - TTC CTT GCG GCC GCC GCC AGG GAT GGA AAA GTG CTT GTG TAT TCC ACG A - 3'
VEGF siRNA	Forward 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CGC ACT CCA G - 3' Reverse 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA AGC ACT CCA GGG CTT CAT -
HPV E6 siRNA	Forward 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CGC TTG CAG T - 3' Reverse 5' - TTC CTT GCG GCC GCC GCC AGG GAT GGA AAA GTG CTT GTG TAT TCC ACG A - 3'
EGFR siRNA	Forward 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CAG GAA TTC G - 3' Reverse 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA AAG GAA TTC GCT CC - 3'
Control siRNA	Forward 5' - AAT TAA TGA TCA CGA TCG ACG CGT GGA TCC CAC GTG ACA C - 3' – Reverse 5' - TTC CTT ACT AGT AGT ACT TCG CGA AAG CTT TTG GAA AAA AAC GTG ACA CGT TCG G
EGFP-Pst I Mutagenesis	Forward 5' - CCT GCT GGA GTT CGT GAC CGC TGC AGG GAT CAC TCT CGG CAT GGA C - 3' Reverse 5' - GTC CAT GCC GAG AGT GAT CCC TGC AGC GGT CAC GAA CTC CAG CAG G - 3'

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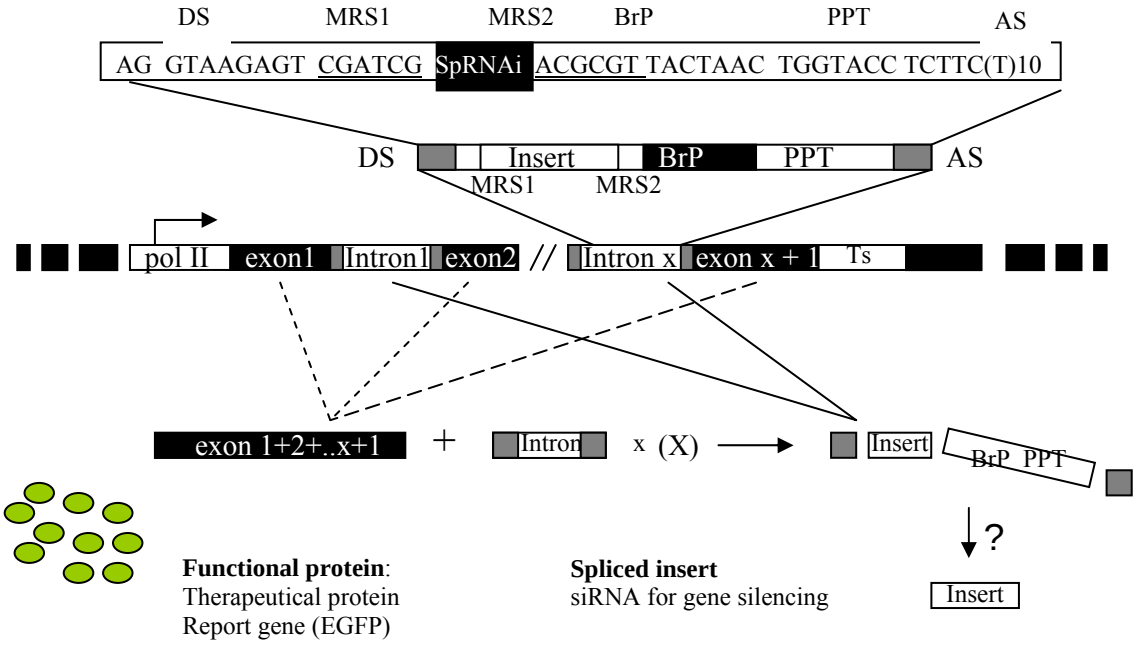
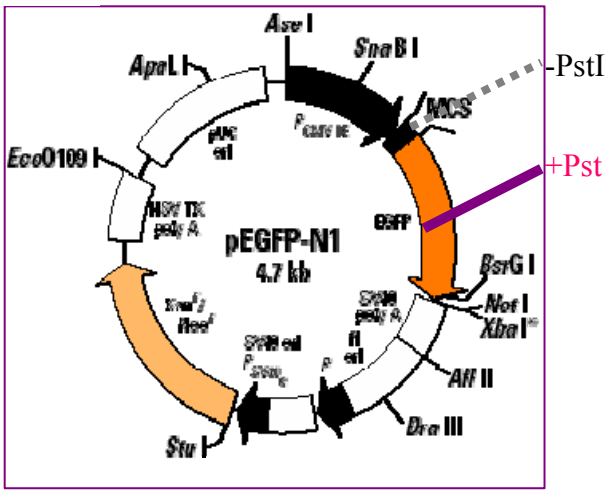


DS= Splicing Donor Site
AS=Splicing Acceptor Site
Ts= Termination Sequence Code

MRS= Multiple Restriction Site
BrP= Branch Point
PPT=Poly-Pyrimidine Tract

Fig.1 Schematic strategy of RNA polymerase II driven siRNA and functional protein. A new strategy for utilizing specially designed plasmid constructs that take advantage of RNA splicing mechanism was proposed by Lin and collegues (Lin SL, 2003). In this approach, after splicing, the RNA polymerase II leads to the production of an oncogene targeted siRNA from the spliced intron and a functional protein from the exon.

A.



B.

pEGFP-N1 pEGFP-N1-PstI pEGFP-N1+PstI+PstI

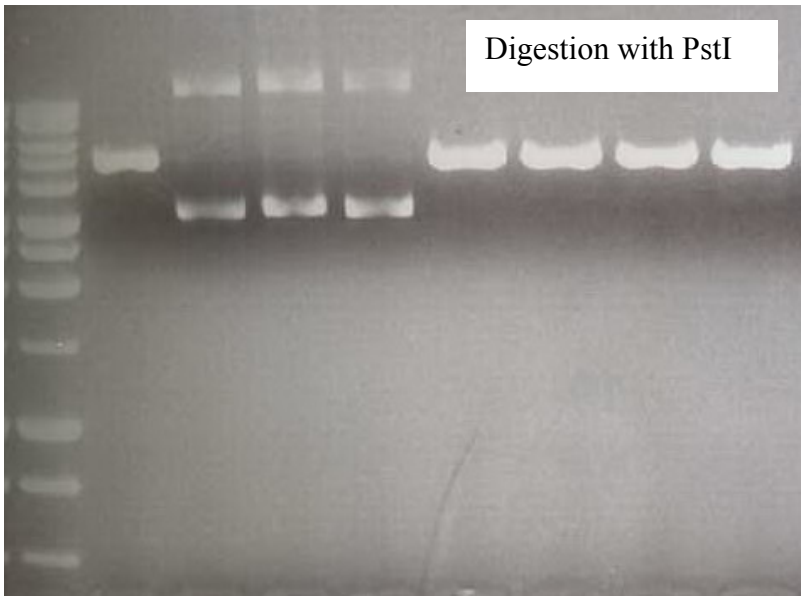
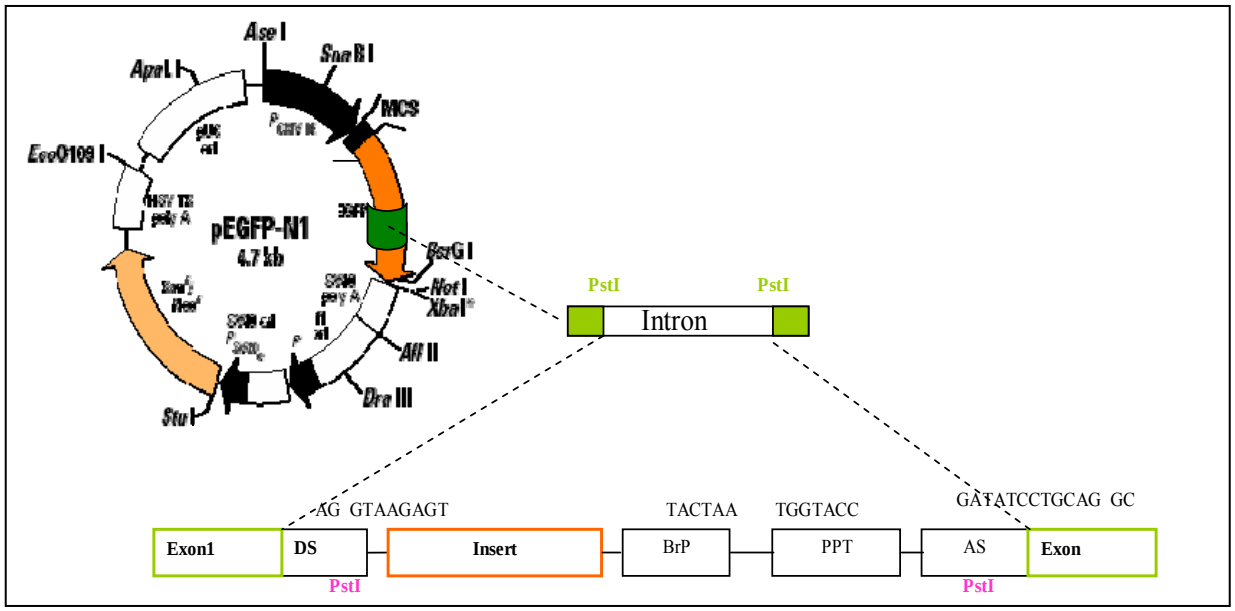
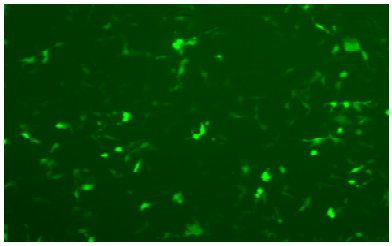


Fig.2 Relocation of PstI site within EGFP
The PstI site from the multiple cloning sites was removed by ScaI and XhoI and then religated to form a XmnI site. (pEGFP-N1-PstI). A new PstI site within the EGFP was created by site-directed mutagenesis of the pEGFP-N1-PstI to form pEGFP-N1-PstI +PstI. Datas shows different clones of each group constructs.

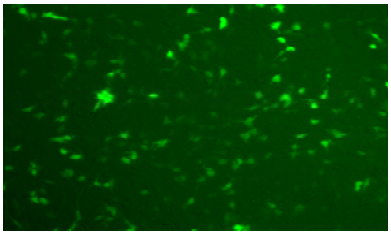
A.



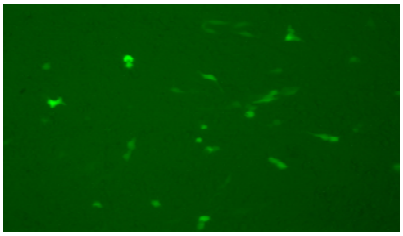
B.



EGFP

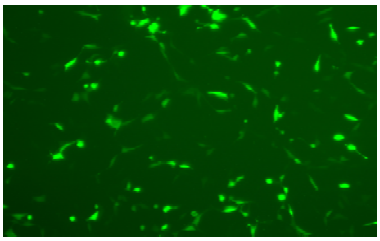


EM22 (-Pst I +Pst I)



EM22- Intron Reverse

Intron reverse

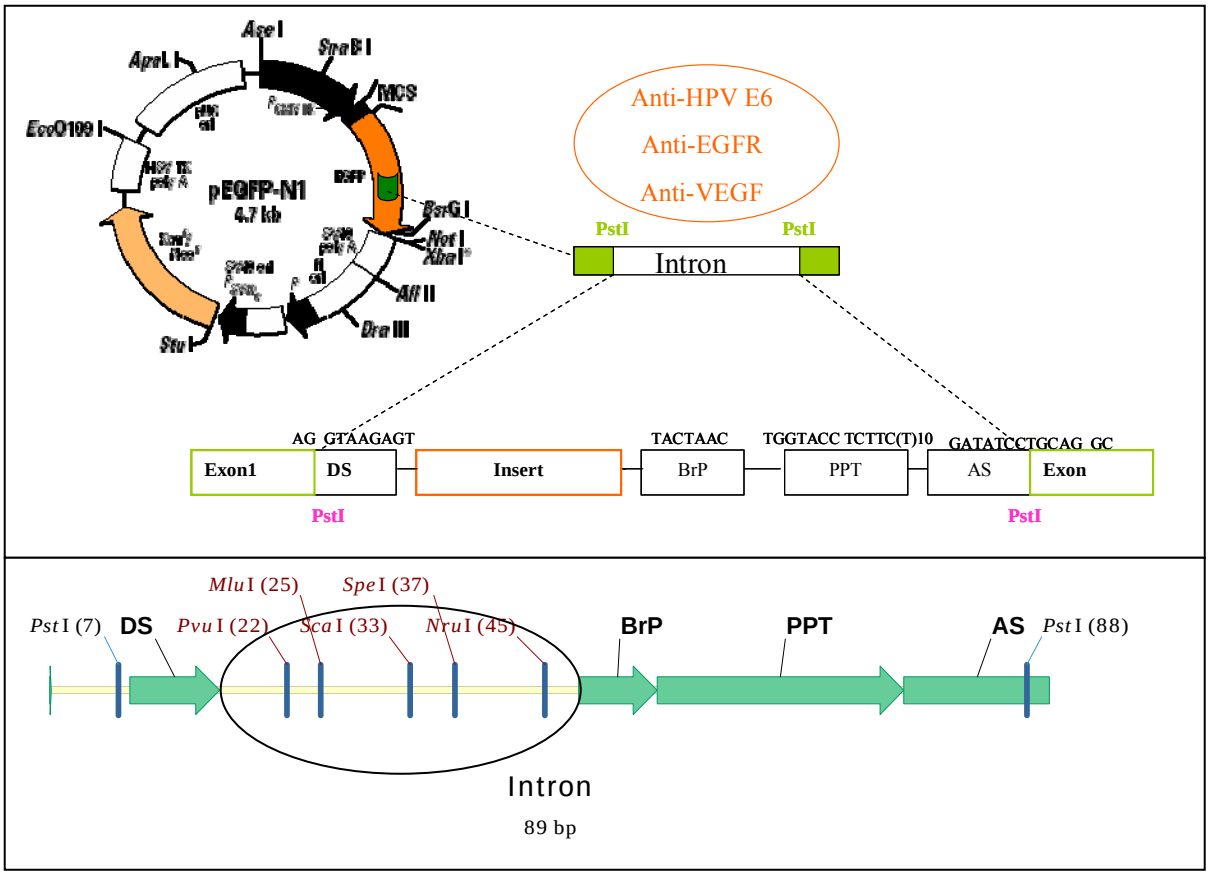


EM22- Intron Forward

Intron forward

Fig.3 Insertion of artificial intron into the newly created PstI site within EGFP (A) Schematic representing of Intron insertion into newly created PstI site. (B) Splicing ability of both EM-PstI +PstI and EM-intron was examined by fluorescent microscope

A.



B.

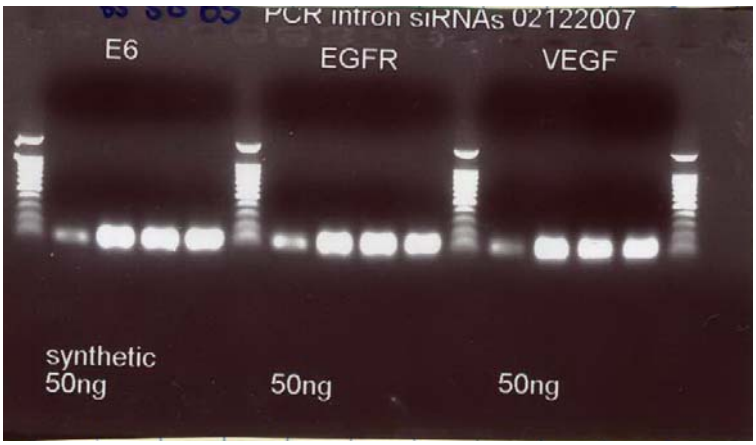


Fig.4 Insertion of various siRNAs into the intron of EM system
(a) Schematic representing of HPV E6, EGFR, VEGF siRNAs insertion into EM-Intron system. (B) PCR product of each siRNA fragment with the originally synthetic one as positive control

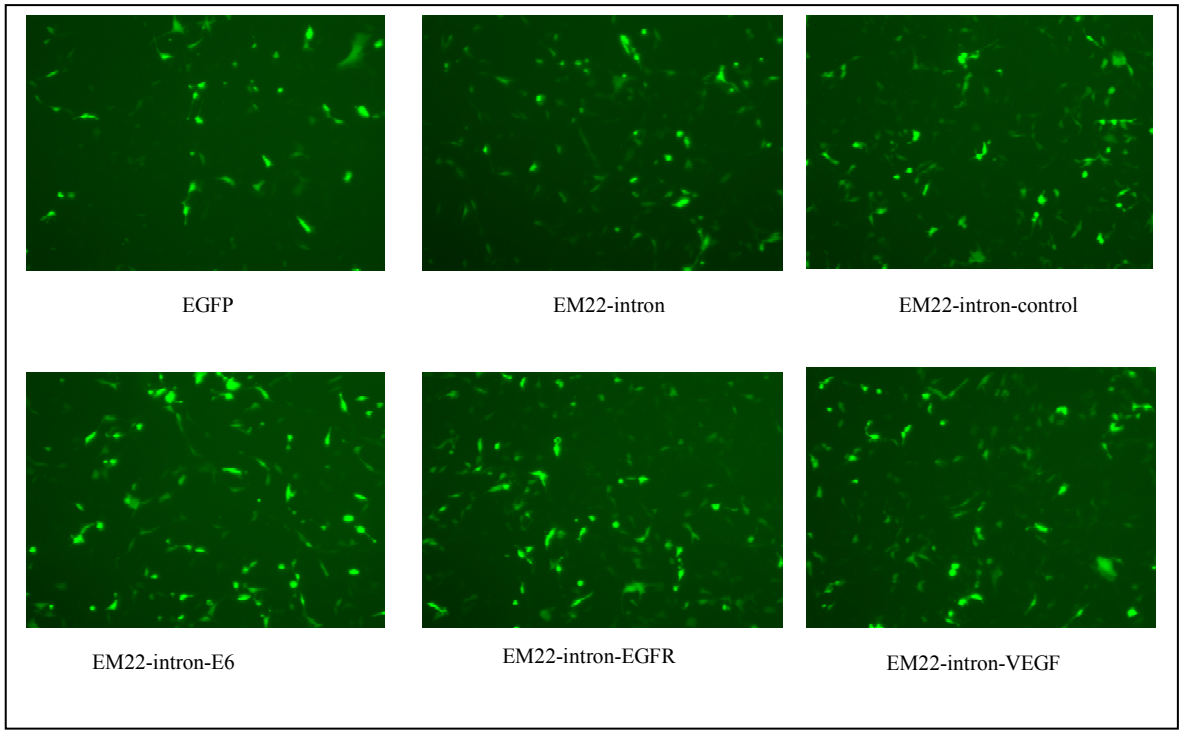


Fig.5 Analysis of EGFP function with several EM-intron constructs
EGFP, EM-intron, EM22-intron-control siRNA, EM22-intron-HPV E6 siRNA, EM22-intron-EGFR siRNA, EM22-intron-VEGF siRNA were tested their splicing ability by examining with fluorescent microscope

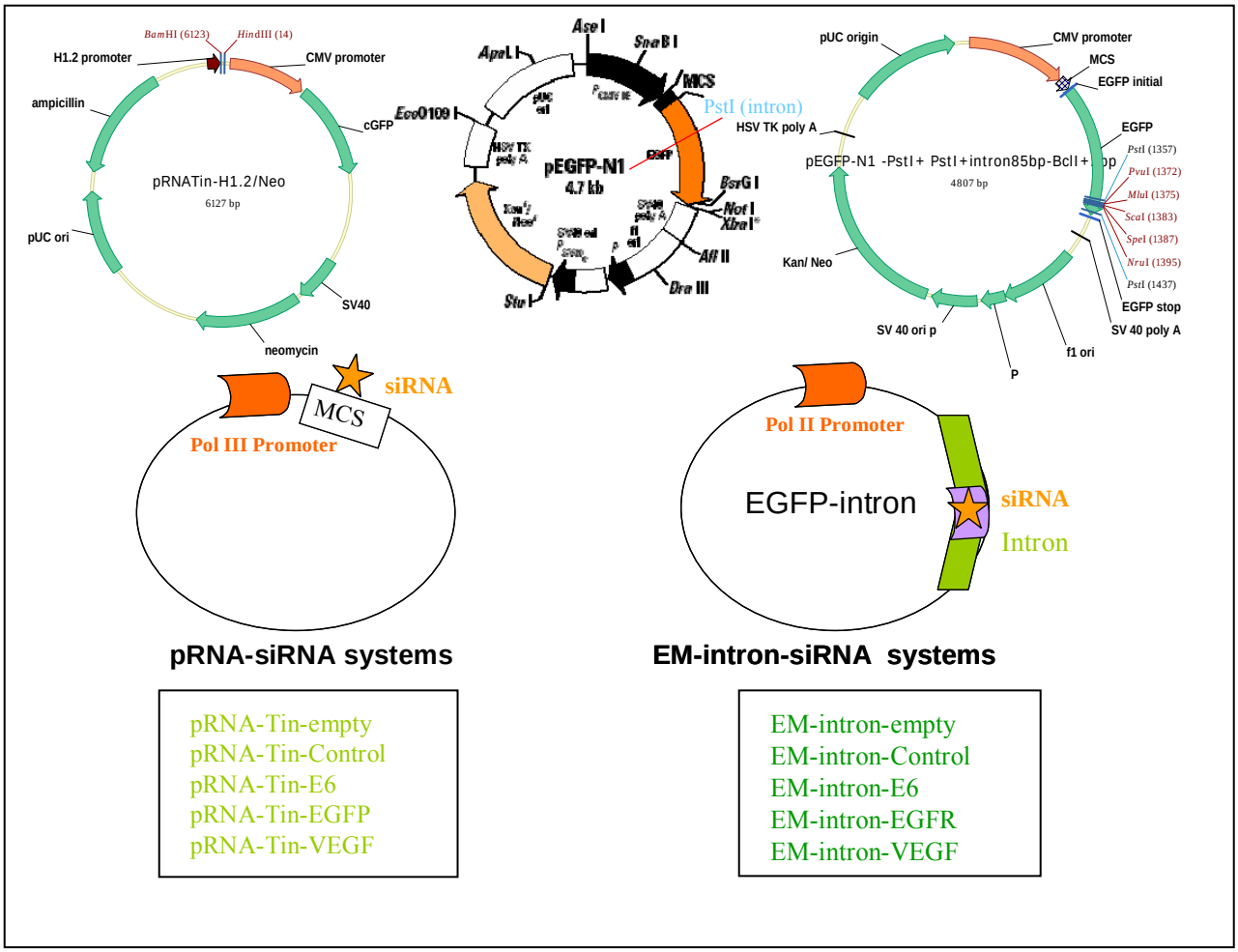
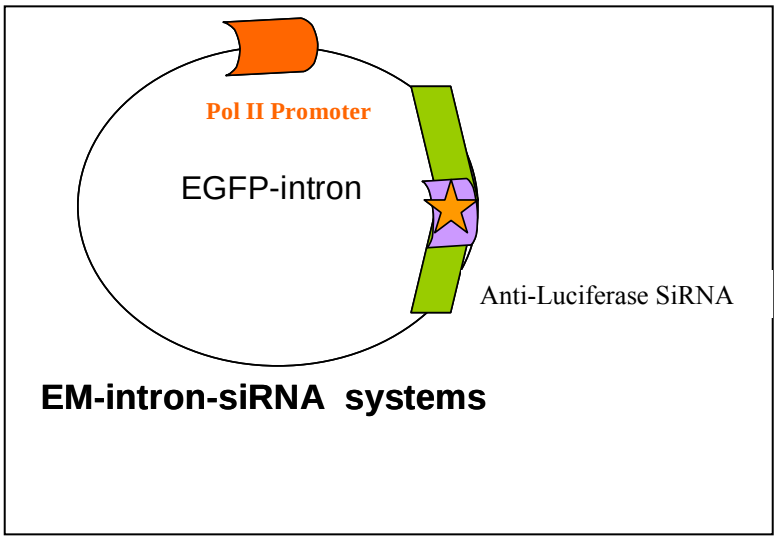


Fig.6 Schematic representing of RNA polymerase III vs RNA polymerase II promoter driven constructs A commonly used RNA polymerase III promoter driven siRNA expression plasmid was purchased from genescript as pRNA-siRNA system to compare the siRNA expression activity with our RNA polymerase II promoter driven siRNA expression plasmid which utilize the RNA splicing mechanism. Both system were successfully inserted with control siRNA, HPV E6 siRNA, EGFR siRNA , VEGF siRNA and were all sequence verified.

A.



B.

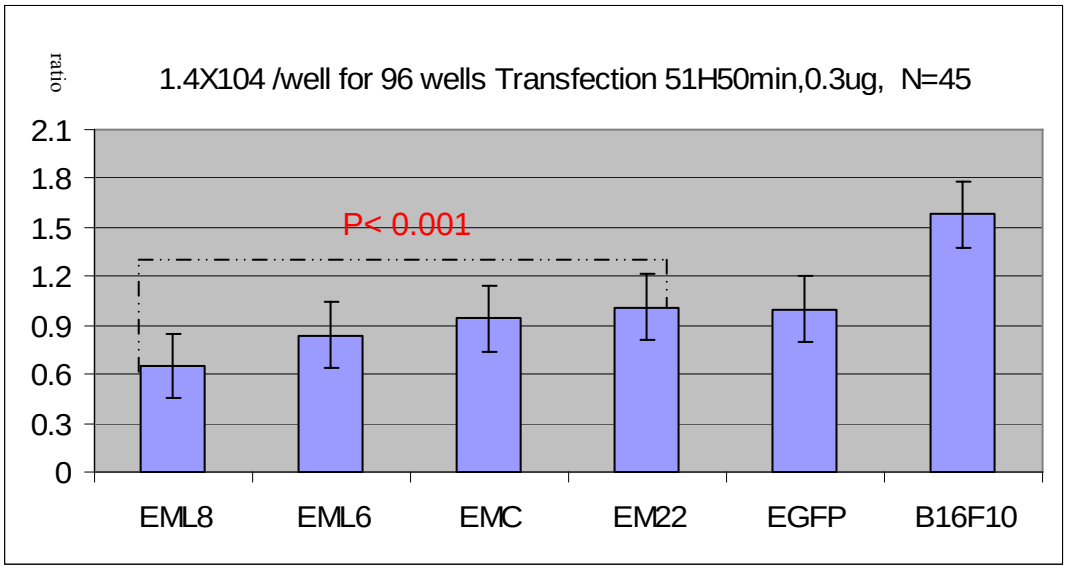
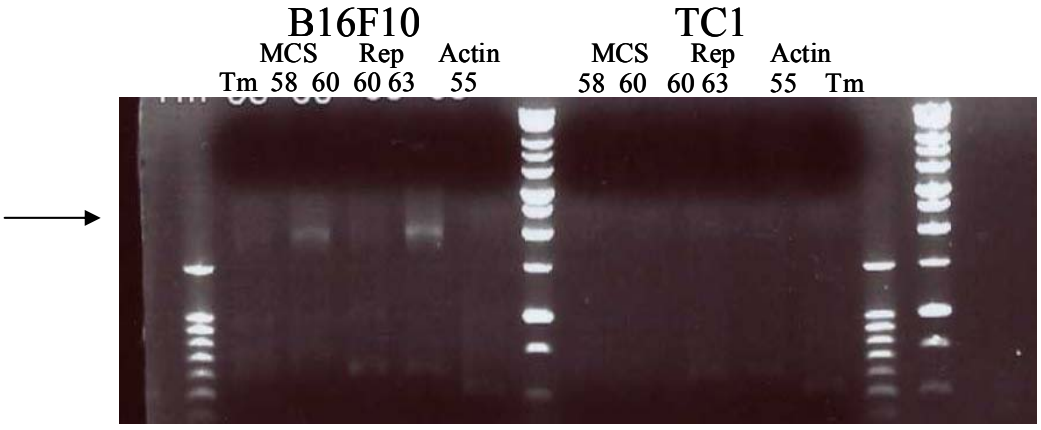


Fig.7 Analysis of Luciferase RNAi efficiency by luciferase assay
(a) Schematic representing of EM-Intron Luciferase siRNA construct. (B) Luciferase activity assay of different EM-Intron constructs. The result was normalized with the whole cell protein and further compared with the EM22 (EM-Intron) as 100 percent.

A



B.

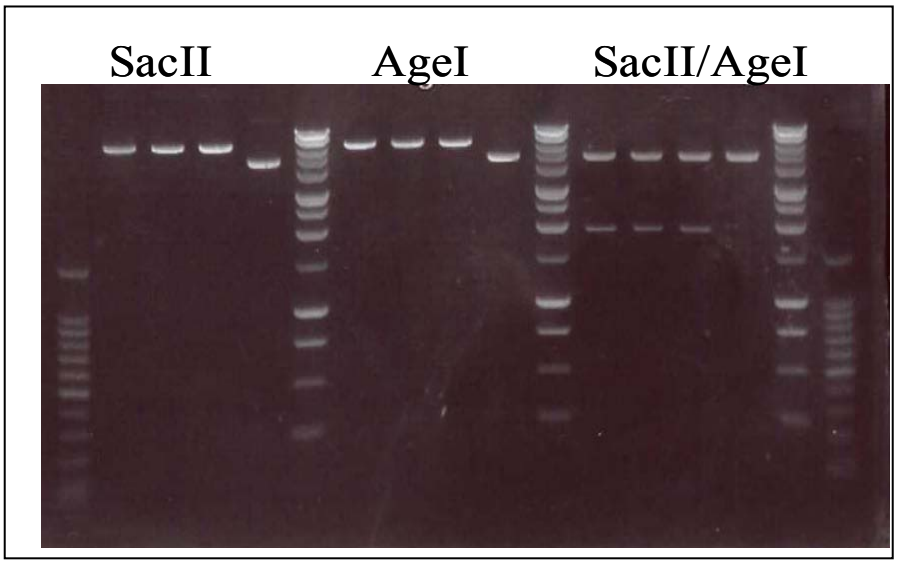
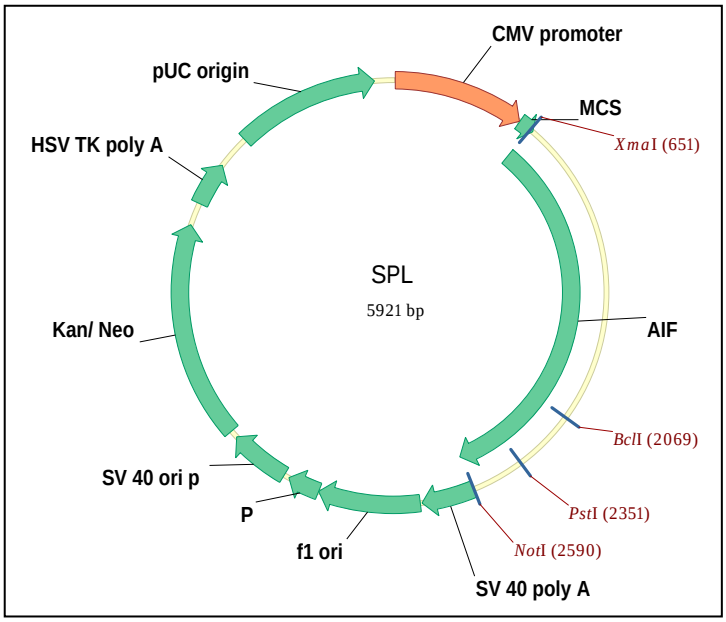


Fig.8 RT-PCR cloning AIF(a) RT-PCR cloning AIF of cell lysate from both B16F10 and TC1 cell lines with SPL, HHI-80, actin primer pairs. (B) Cloned 1.93kb AIF from B16F10 cell line was inserted into pEGFP-N1 plasmid to form SPL and was enzyme digestion confirmed by predicted fragment size. The SPL construct was then sent to sequence verified.

A.



B.

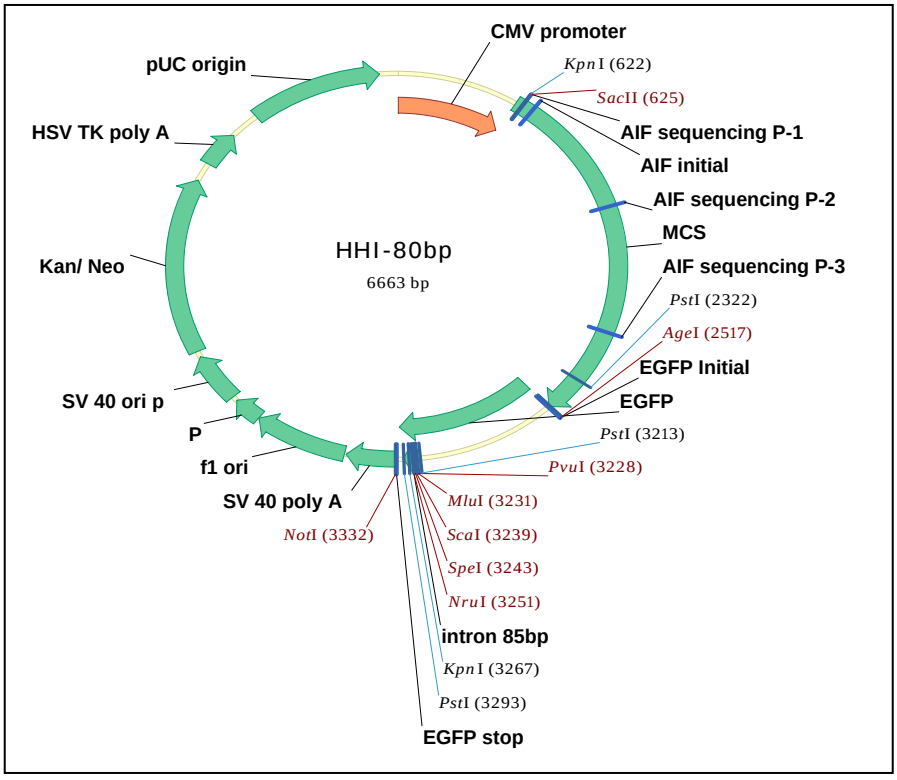
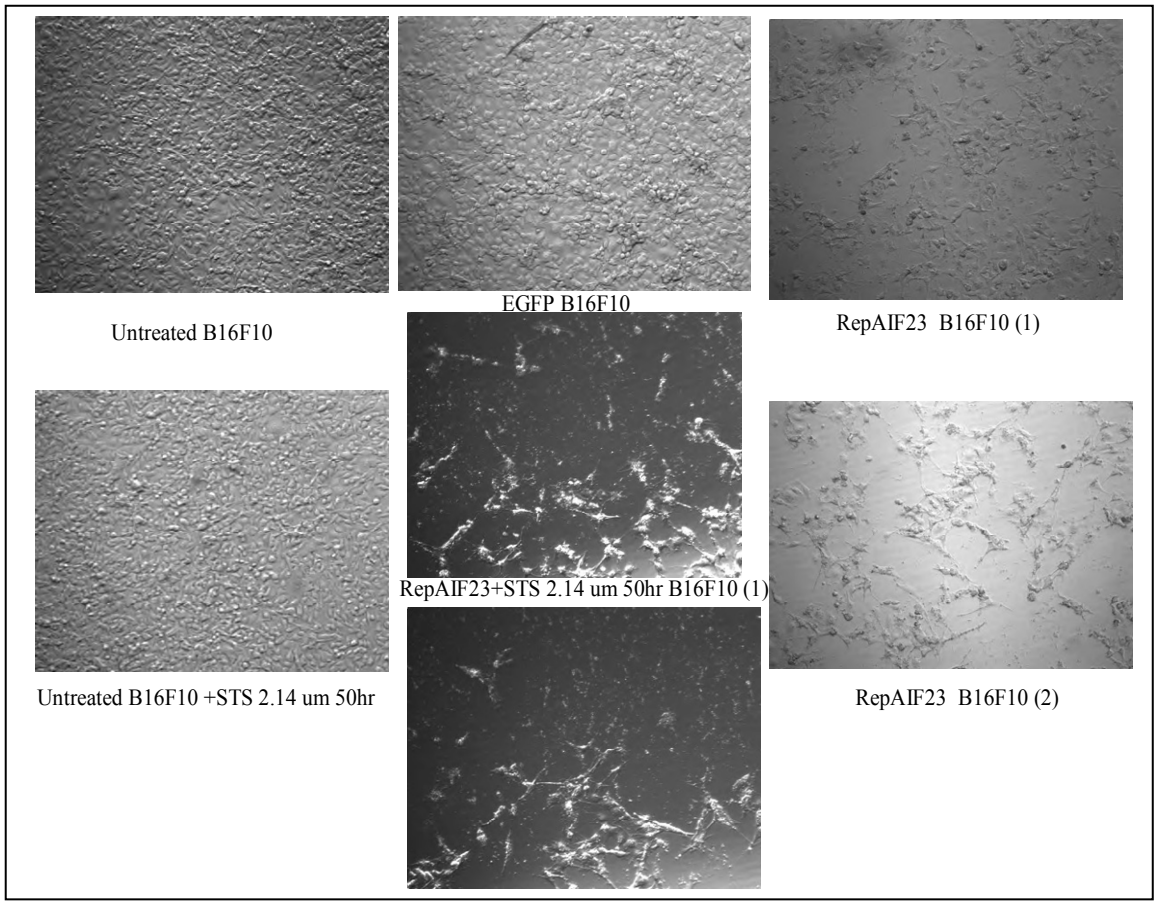


Fig.9 Schematic representing of AIF cloning constructs: SPL and HHI-80 Cloned 1.93kb AIF from B16F10 cell line was inserted into pEGFP-N1 plasmid to form SPL (replaced EGFP) and HHI-80 (inserted ahead of EGFPgene). Both SPL and HHI-80 were sequence confirmed

A



B.

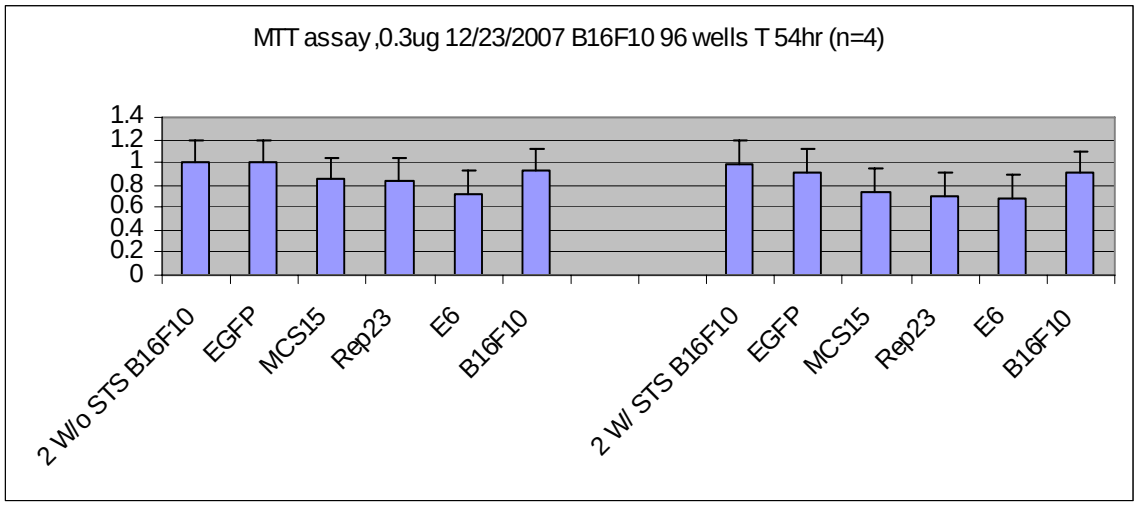


Fig.10 Functional analysis of AIF
(A) SPL transfected B16F10 cell line was observed with light microscope to exam the cell killing ability either with or without STS, apoptosis inducer, (B) MTT assay of cell viability with EGFP, SPL, HHI-80, EM-Intron-HPV E6 plasmid transfected B16F10 cell line

References

Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* **34**(3)**:** 263-264

Dykxhoorn DM, Palliser D, Lieberman J (2006) The silent treatment: siRNAs as small molecule drugs. *Gene Ther* **13**(6)**:** 541-552

Fewell GD, Schmitt K (2006) Vector-based RNAi approaches for stable, inducible and genome-wide screens. *Drug Discov Today* **11**(21-22)**:** 975-982

Greber D, Fussenegger M (2007) Multi-gene engineering: simultaneous expression and knockdown of six genes off a single platform. *Biotechnol Bioeng* **96**(5)**:** 821-834

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* **21**(6)**:** 635-637

Kawasaki H, Taira K (2003) Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res* **31**(2)**:** 700-707

Li S, Huang L (1997) In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Ther* **4**(9)**:** 891-900

Li SD, Chono S, Huang L (2008) Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. *J Control Release* **126**(1)**:** 77-84

Li SD, Huang L (2006) Surface-modified LPD nanoparticles for tumor targeting. *Ann N Y Acad Sci* **1082:** 1-8

Lin SL, Chang D, Wu DY, Ying SY (2003) A novel RNA splicing-mediated gene silencing mechanism potential for genome evolution. *Biochem Biophys Res Commun* **310**(3)**:** 754-760

Scherr M, Eder M (2007) Gene silencing by small regulatory RNAs in mammalian cells. *Cell Cycle* **6**(4)**:** 444-449

Shih SC, Robinson GS, Perruzzi CA, Calvo A, Desai K, Green JE, Ali IU, Smith LE, Senger DR (2002) Molecular profiling of angiogenesis markers. *Am J Pathol* **161**(1)**:** 35-41

Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, Feng Y, Palliser D, Weiner DB, Shankar P, Marasco WA, Lieberman J (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* **23**(6)**:** 709-717

Steinman HA, Sluss HK, Sands AT, Pihan G, Jones SN (2004) Absence of p21 partially rescues Mdm4 loss and uncovers an antiproliferative effect of Mdm4 on cell growth. *Oncogene* **23**(1)**:** 303-306

Tiscornia G, Singer O, Ikawa M, Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci U S A* **100**(4)**:** 1844-1848

Wang MB, Helliwell CA, Wu LM, Waterhouse PM, Peacock WJ, Dennis ES (2008) Hairpin RNAs derived from RNA polymerase II and polymerase III promoter-directed transgenes are processed differently in plants. *RNA* **14**(5)**:** 903-913

Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, Davidson BL (2004) RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat Med* **10**(8)**:** 816-820

Zhang X, Chen ZG, Choe MS, Lin Y, Sun SY, Wieand HS, Shin HJ, Chen A, Khuri FR, Shin DM (2005) Tumor growth inhibition by simultaneously blocking epidermal growth factor receptor and cyclooxygenase-2 in a xenograft model. *Clin Cancer Res* **11**(17)**:** 6261-6269

Zhou H, Xia XG, Xu Z (2005) An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res* **33**(6)**:** e62

Zou GM, Yoder MC (2005) Application of RNA interference to study stem cell function: current status and future perspectives. *Biol Cell* **97**(3)**:** 211-219