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The antitumor activity of tumor-targeted RNA replicase-based plasmid DNA

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The antitumor activity of tumor-targeted RNA replicase-based plasmid DNA

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Dedication

To Gerardo and Rosa Rodriguez for their unconditional love and support

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The antitumor activity of tumor-targeted RNA replicase-based plasmid DNA

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Over the past several decades, there have been numerous attempts to utilize synthetic dsRNA to control tumor growth in animal models and clinical trials. Recently, it has become clear that intracellular dsRNA is more effective than extracellular dsRNA in promoting apoptosis and orchestrating adaptive immune response. To overcome the difficulty in delivering a large dose of synthetic dsRNA into tumors, while avoiding systemic toxicity we propose to deliver a RNA replicase-based plasmid DNA, hypothesizing that the dsRNA generated by the replicase-based plasmid in tumor cells will inhibit tumor growth.

We evaluated the anti-tumor activity of a plasmid (pSIN-β) that encodes the sindbis RNA replicase genes in mice with model tumors (TC-1 lung cancer cells or B16 melanoma cells) and compared it to a traditional pCMV-β plasmid. In cell culture, transfection of tumor cells with pSIN-β generated dsRNA. In mice with model tumors, pSIN-β more effectively inhibited tumor growth than pCMV-β, and in some cases, eradicated the tumors. RNA replicase-based plasmid may be exploited to generate intracellular dsRNA to control tumor growth.

The feasibility of further improving the antitumor activity of the RNA replicase-based plasmid by targeting it into tumors cells was also evaluated. An epidermal growth factor (EGF)-conjugated, PEGylated cationic liposome was developed to deliver the RNA replicase-based plasmid, pSIN-β, into EGFR-over-expressing human breast cancer cells (MDA-MB-468) *in vitro* and *in vivo*. Delivery of the pSIN-β using the EGF receptor-targeted liposome more effectively controlled the growth of MDA-MB-468 tumors in mice than using un-targeted liposome.

Finally the potential of further improving the antitumor activity of the pSIN-β plasmid by incorporating interleukin-2 (IL2) gene into the plasmid was investigated. The resultant pSIN-IL2 plasmid was delivered to mouse melanoma cells that over-express the sigma receptor. The pSIN-IL2 plasmid was more effective at controlling the growth of B16 melanoma in mice when complexed with sigma receptor targeted AA-PEG-liposomes than with the untargeted liposomes. Importantly, the pSIN-IL2 plasmid was more effective than pSIN-β plasmid at controlling the growth of B16 melanoma in mice, and B16-bearing mice that were treated with pSIN-IL2 had an elevated number of activated $CD4^+$, $CD8^+$, and natural killer cells, compared to those treated with pSIN-β.

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Chapter One: Introduction

1.1 Background

Cancer is a group of diseases characterized by uncontrolled growth and metastatic spread of abnormal cells (Society, 2012). Cancer is a disease that is difficult to eradicate and the second most common cause of death in the U.S. (Society, 2012). According to the international agency for research on cancer (IARC) worldwide about 12.7 million cancer cases and 7.6 million cancer deaths occurred in 2008 (Jemal et al., 2011). Treatment varies depending on the type of cancer but surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapies are often used, either in combination or alone. Chemotherapeutic drugs kill tumor cells, but frequently display unwanted toxicities as they lack tumor cell selectivity. Drug resistance is often developed, and ultimately limits the efficacy of chemotherapy in cancer patients due to reduced accumulation of drugs in tumor cells (Arias, 2011; De Palma & Lewis, 2011). The genetic and epigenetic heterogeneity of tumors in combination with the selection of anticancer drugs leads to the overgrowth of drug-resistant variants (Gottesman, 2002). Thus the use of therapies with multiple anti-tumor mechanisms would help circumvent acquired drug resistance. Additionally cancer gene therapy that focuses on using recombinant DNA constructs to augment existing immunotherapeutic and chemotherapeutic approaches seems to be promising (Roth & Cristiano, 1997).

1.2 Cancer gene therapy

Gene therapy has the potential to treat acquired and inherited diseases. A major hurdle to overcome is the development of effective methods for the delivery of a corrective gene into cells. Over two-thirds of approved clinical gene therapy trials are for cancer (Brannon-Peppas, Ghosn, Roy, & Cornetta, 2007; Edelstein, Abedi, Wixon, & Edelstein, 2004; Roth & Cristiano, 1997). Gene therapy is very much in its infancy; further the limited success in clinical trials reflects the need to improve the methodology. Interventions would be classified as gene therapeutics, differing from gene-replacement therapy (Roth & Cristiano, 1997). The most common strategy against syngenic tumors has been to use immunotherapy using recombinant DNA constructs expressing cytokines and lymphokines (Roth & Cristiano, 1997). The major advantage to this approach is the potential to generate a systemic immune response against the tumor. Cytokines stimulate both adaptive and innate immunity (Daud et al., 2008). There are several approaches including the use of tumor infiltrating lymphocytes, tumor cells, or fibroblast that express cytokine genes, or simply to make tumor cells more immunogenic with expression of co-stimulatory molecules such as B7-1 or B7-2 (Sanda et al., 1995).

Cytokine based tumor immunotherapy or vaccination is a promising strategy for cancer gene therapy (F. Sakurai et al., 2003). The aim is to control or even eradicate tumors by intensifying the weak humoral and cellular immune response to tumor antigens (Edelstein, Abedi, & Wixon, 2007). Interferon (IFN) genes have been used both in clinical and preclinical trials. They are administered locally either intratumoral or intraperitoneal with the use of viral or non-viral vectors. Immunotherapy using recombinant DNA vectors that express cytokines such as interleukin-2 (IL2) was

successful with melanoma and renal cell cancer (Roth & Cristiano, 1997). *In vivo* electroporation has been used to intratumorally deliver interleukin-12 (IL-12) plasmid, resulting in a compete tumor regression rate of 80% (Lucas, Heller, Coppola, & Heller, 2002). Clinical phase I and phase II trials have shown that systemic IL-12 caused a significant toxicity, whereas locally delivered IL-12 is less toxic and retains its biological activity (Gollob et al., 2000; Younes et al., 2004) .

Modification of the dominant oncogene or tumor suppressor may influence characteristics that contribute to the malignant cancer phenotype. There have been several studies that utilize tumor suppressor gene replacement or oncogene inactivation (Cai, Mukhopadhyay, Liu, Fujiwara, & Roth, 1993; Mukhopadhyay, Tainsky, Cavender, & Roth, 1991). The ras family of oncogenes is the most common in many human cancers. Transfection with K-ras siRNA or retroviral mutant K-ras was shown to reduce the growth rate of human lung cancers *in vitro* and *in vivo* in nude mice (Georges, Mukhopadhyay, Zhang, Yen, & Roth, 1993; Mukhopadhyay, et al., 1991; Zhang, Mukhopadhyay, Donehower, Georges, & Roth, 1993). Reintroduction of wild-type p53 gene using a retroviral expression vector was found to suppress the growth of H358a human lung cancer cell line (Cai, et al., 1993). Expression of wildtype p53 tumor suppressor gene has been shown to cause regression of human tumor (Edelstein, et al., 2007). More invasive cancers utilize multiple oncogenic pathways. A phase III trial using adenovirus p53 gene therapy against ovarian cancers was terminated early due to no therapeutic benefit, multiple genetic changes in cancer and epigenetic dysregulations probably led to aberrant silencing of genes (Zeimet & Marth, 2003). It may be impossible

to correct for all genetic abnormalities since many are not characterized. Additionally there has been much work evaluation mixtures of siRNA against several oncogenes like MDM2, c-myc, and vascular endothelial growth factor (VEGF) (S. D. Li, Chono, & Huang, 2008). Evaluation of gene therapy constructs aimed for oncogenes or tumor suppressor genes in combination with chemotherapeutic drugs is an important area for future research (S. H. Chen et al., 1995; Fujiwara et al., 1994).

Another modality is gene directed enzyme pro-drug therapy (GDEPT) which relies on vectors that express an enzyme that converts nontoxic prodrug into its toxic metabolite (Pandha et al., 1999). Cells that are transfected with cytosine deaminase (CD) are able to convert the nontoxic pro-drug fluorocytosine (5-FC) to the toxic metabolite fluorouracil (5-FU) (Pandha, et al., 1999). The herpes simplex virus expressing thymidine kinase is used to convert the non-toxic pro-drug ganciclovir into the cytotoxic triphosphate ganciclovir (Edelstein, et al., 2007). This allows non-toxic pro-drugs to be administered at high doses with no side effects as conversion to toxic metabolite would occur only in tumor or tumor microenvironment (Edelstein, et al., 2007).

1.2.1 Viral and non-viral vectors

Vehicles for gene delivery are divided into two major groups; viral and non-viral vectors (Roth & Cristiano, 1997). Viral vectors rely on viruses as efficient gene transfer vehicles. They include retrovirus, adenovirus, adeno-associated virus, herpes simplex virus among others (Hunt, Vorburger, & Swisher, 2007; Roth & Cristiano, 1997). Viral

vectors have high tranduction efficiency and are frequently used as vectors because of ease of large-scale clinical grade production (Hunt, et al., 2007).

Viral vectors dominate clinical gene therapy trials (Xu & Anchordoquy, 2011). Viral vectors integrate into host chromatin leading to long-term gene expression (S. D. Li & Huang, 2007). Since 2007, there have been 1339 gene therapy trials, of which only 25% utilize non-viral vectors (Edelstein, et al., 2007). Limitations of viral vectors include their small capacity for therapeutic DNA and safety issues have stimulated the development of synthetic vectors (Edelstein, et al., 2007). It is generally agreed upon that non-viral vectors are preferable due to less safety concerns, and have become more common (Xu & Anchordoquy, 2011). There are significant safety issues associated with viral vectors including insertional mutagenesis and carcinogenesis (Niidome & Huang, 2002). It has been found that repeated administration of a viral vector leads to an immune response that eliminates the transgene (Niidome & Huang, 2002). Considering these serious limitations non-viral vectors alone or complexed to a cationic carrier are an attractive alternative.

DNA vectors or non-viral vectors are delivered by a physical method or with a chemical carrier such as cationic polymers or lipids to achieve high gene expression levels (Niidome & Huang, 2002). Physical methods such as direct injection, electroporation, bio-ballistic-gene gun, ultrasound, and hydrodynamic techniques have been used to improve transfection efficiency (Niidome & Huang, 2002). Chemical carriers condense the non-viral vectors to protect them from nuclease degradation and improve their delivery to the cytosol and nucleus (Niidome & Huang, 2002). Gene

delivery based on liposomes is one of the most common techniques used. DNA vectors bind to cationic liposomes via electrostatic interaction between the anionic phosphodiester backbones and the positively charged cationic lipids in the liposomes (Xu & Anchordoquy, 2011). The vector bound liposome is referred to as the lipoplex; a particle with a diameter around 100 nm (Guo & Huang, 2012).

A major disadvantage of non-viral vectors complexed to cationic delivery vehicles is that strong interaction with blood components, which tend to lower transfection efficiencies (Xu & Anchordoquy, 2011). Serum proteins bind to charged particles, leading to structural reorganization, aggregation and ultimately dissociation of the delivery vehicle (Yang & Huang, 1998). Nucleases in the serum are known to degrade nucleic acids, which result in a significant decrease in biological activity (Xu & Anchordoquy, 2011). PEGylation offers a solution to overcome serum protein binding. Incorporation of DSPE-PEG into the liposome allows for a delivery vehicle that is sterically shielded from blood components (Torchilin et al., 1994). PEGylation increases circulation time after intravenous administration and is commonly used in animal models (Xu & Anchordoquy, 2011). PEGylated lipid such as DSPE-PEG are conjugated through cationic ethanolamine head groups so the zwitterionic phospholipid is converted to an anionic lipid. Incorporation of DSPE-PEG into a cationic lipid formulation causes a reduction in zeta potential via charge neutralization, this is attributed to the steric stabilization of PEG (Xu & Anchordoquy, 2011).

In conclusion, viral vectors are more efficient and have much higher gene expression *in vivo* compared to non-viral vectors. But safety is an important factor and

clinical trial regulations limit the use of viral vectors because of adverse side effects. It is important to understand the gene delivery barriers to design a more rational delivery carrier for non-viral vectors (S. D. Li & Huang, 2007).

1.2.2 Gene therapy clinical trials in the U.S.

The delivery of non-viral vectors has the potential to develop potent vaccines and novel therapeutics to cure many diseases (Xu & Anchordoquy, 2011). The use of gene therapy in phase I clinical trials have been limited to advanced incurable cancers. Intralesional injections of gene based agents hold potential to prevent local recurrence after surgery. Much remains to be done including safety, efficacy studies, and clinical trials with patients in earlier stages of diseases. Response rates in phase 1 clinical trials utilizing combination therapy appear comparable to single agent chemotherapy (Roth & Cristiano, 1997). Cancer gene therapy has been used to augment existing therapeutic approaches so the limitations in response rate may be a function of the limitations of the existing approaches instead of the gene therapy (Roth & Cristiano, 1997).

Low gene expression and lack of efficacy accompanied with unrealistic expectations regarding gene therapy have caused many biotechnology companies to drop their gene therapy endeavors. However it is important to note that problems still exist in gene therapy including vector design, clinical trial design, delivery mechanisms, and should be kept in perspective. As cancer biology advances in the understanding of mechanisms underlying carcinogenesis so will the advance in gene therapy in the development of novel delivery mechanisms (Roth & Cristiano, 1997).

Currently, there are 26 active and completed clinical trials involving siRNA technology and 42 clinical trials of plasmid DNA based therapeutics in cancer, most of which focused on vaccine therapy (www.clinicaltrials.gov). Non-viral vectors that express cytokines such as IL-12 and IL-2 are the most common vectors found for treatment of cutaneous lymphoma, malignant melanoma, stage II or stage III prostate cancer, and ovarian cancer. Delivery is either intratumoral injection followed by electroporation or intratumoral delivery of complexed vector with DMRIE/DOPE liposomes or PEI polymers.

The most successful lipoplex to reach phase III clinical trials is allovectin marketed by Vical (Daud, et al., 2008; Doukas & Rolland, 2012). Allovectin is a bicistronic plasmid encoding two transgene proteins HLA-B7 and β2M. It is complexed to a cationic liposome composed of DMRIE/ DOPE (Daud, et al., 2008; Doukas & Rolland, 2012). Results from a stage III and stage IVa/IVb melanoma clinical trial showed good safety profile after treatment and a response rate of 11.8% with a median survival of 18.8 months (Bedikian et al., 2010).

The majorities of gene therapy clinical trials to date are phase I or phase I/II. In the last eight years there is in increase in the number of trial advancing to phase III, which potentiate the idea that gene therapy may be moving closer to clinical applications (Edelstein, et al., 2007).

Overall response rate for cancer patients has been less than 20% which is an improvement over the previously evaluated non-viral vectors (Anklesaria, 2000). This is

largely attributed to host immune response to both vectors and transgenes (Harvey et al., 1999).

1.2.3 Route of administration

Systemic administration is useful for the treatment of metastatic tumors (Tada et al., 2001). The extracellular environment is the major obstacle to systemic administration of non-viral vectors complexed to liposomes. Intravenous injection, intra-tracheal instillation, and intra-tissue injection have been investigated (Jenkins et al., 2000; Templeton et al., 1997). It has been found that when lipoplexes are administered intravenously the clearance by the RES and the large amounts of serum nucleases reduce the chance for the plasmid to reach the target (Niidome & Huang, 2002). Generally the lung has the highest transfection efficiency when lipoplexes are administered intravenously (Tandia, Lonez, Vandenbranden, Ruysschaert, & Elouahabi, 2005). Studies have shown that intravenous administration of lipoplexes has high gene expression of up to 10-10,000-fold increase in the lungs compared to other organs (F. Sakurai, et al., 2003).

Several studies have been carried out that assess the use of lipoplexes as systemic delivery vehicles for therapeutic genes (p53, and IL12) for the treatment of malignant tumors. It was found that the antitumor activities were attributed to nonspecific induction of cytokines instead of expression of therapeutic transgenes (F. Sakurai, et al., 2003). Dow *et. al.* has reported that intravenous administration of lipoplexes containing noncoding plasmid DNA can inhibit growth of lung tumors, but it could not inhibit

growth of late metastatic lung tumors (Dow et al., 1999). Nonspecific pro-inflammatory cytokine production probably suppressed early events in tumor metastasis.

The most challenges aspect of gene therapy is the issue of delivery (Zhang, Satterlee, & Huang, 2012). It is important to understand the mechanism of biophysical interaction, which will allow for further optimization of non-viral vectors for systemic gene delivery (Niidome & Huang, 2002). If the lung is not the target, local delivery can avoid the RES uptake, reduce systemic toxicity, and help the delivery system reach the target cells. Intratumoral administration of plasmid DNA allows for transfected cells in the area to express the gene of interest and has been the initial route of delivery in several clinical protocols (Sobol R, 1995).

Systemic delivery is the ultimate goal for metastasizing cancer cells. Based on gene therapy trials it is also the route that induces the highest immune response and potential toxicity. An approach based on gradual increase of systemic exposure has been proposed only after safety with localized delivery is shown (Kirn, Martuza, & Zwiebel, 2001). Once biological activity and safety is demonstrated by the intratumoral route the intra-peritoneal, intra-arterial and finally intravenous administration can be evaluated (Hunt, et al., 2007; Kirn, et al., 2001). Surface visible tumors like such head-and-neck carcinomas and melanomas allow for direct intratumoral administration and is the best route for treatment (Dass, 2002). Ultrasound guided intratumoral administration has been applied to liver, kidney, ovarian, and prostate cancers.

1.2.4 Regulation of transcription with non-viral vectors

Gene expression at the transcriptional level is difficult to regulate. The design of the expression plasmid is critical in determining the level of transgene expressed. Improvement in the design of expression vectors and liposome carriers can ultimately produce more efficient expression for transferred genes and thus a more effective treatment.

A promoter provides that attachment sequence for RNA polymerase to bind and initiate transcription. The selection of a promoter which drives transgene expression in plasmid DNA is critical for effective transfection efficiency in eukaryotic cells. Insertion of different promoters can be screened and validated at the *in vitro* level, however the regulation of the promoter is more difficult to predict *in vivo*. The most commonly used promoter, the cytomegalovirus promoter (CMV) is one of the strongest identified thus far and found to lead to high yields (F. Sakurai, et al., 2003). Various promoters have been constructed and were found to have superior gene expression levels compared to the CMV promoter including human papovavirus (BKV) promoter, the Rous sarcoma virus (RSV) promoter, and the human T-cell leukemia virus (EF-1α/HTLV) promoter (Thierry et al., 1995). These promoters increase steady state transcription and increase translation efficiency through mRNA stabilization. It may be necessary to utilize promoters for specific cancers. Lu *et. al.* has shown that the glyceraldehyde-3-phosphte dehydrogenase (GAPDH) promoter is highly active in breast cancers, and that compared to the CMV promoter showed up to 70 fold increase gene expression *in vivo* (Lu, Zhang, Roberts, Osborne, & Templeton, 2002). Gene delivery systems may require plasmid modifications dependent on the types of tumor.

Tissue specific promoter would allow for expression of heterologous gene *in vivo* and restrict the expression of the gene to the tumor. Melanoma cells synthesizes melanin primarily by malanocytes (Vile & Hart, 1993). The rate-limiting step in the synthesis of melanin is the hydroxylation of tyrosine catalyzed by the enzyme tyrosinase (Parvez et al., 2006). Melanocyte-specific transcription of proteins associated with melanogenesis have been identified as tyrosine related protein (TRP-1) (Jackson, Chambers, Budd, & Johnson, 1991) and TRP-2 (Tsukamoto, Jackson, Urabe, Montague, & Hearing, 1992). Vile *et. al*. demonstrated that the 5' flanking regions of both tyrosinase and TRP-1 genes can direct expression of a heterologous gene in human and murine melanoma cells, while not permitting expression in other cells (Vile & Hart, 1993). The combination of tissue specific promoter such as TRP-1 or tyrosine in melanoma and direct injection of plasmid DNA into tumors can provide new opportunities for targeting gene therapy to specific tumor types (Vile & Hart, 1993). Other tumor specific promoters include carcinoembryonic antigen (CEA) gene in pancreatic carcinoma cells (DiMaio et al., 1994) and human surfactant protein A (HSPA) for non-small cell lung cancers (Smith, Rousculp, Goldsmith, Curiel, & Garver, 1994).

Other components of the vector are also important such as transcription regulatory elements, polyadenylation signal sequence, and enhancers (F. Sakurai, et al., 2003). The different viral promoters and intron sequences within the expression vector was found to affect the efficiency of the liposome-mediated systemic gene expression. Previously it has been reported that cDNA expressed significantly higher levels of transgene when the transgene incorporates a heterologous intron 5' to the coding region (M. T. Huang &

Gorman, 1990). It was also found that analogous transgenes lacking an intron or with a 3' intron are expressed at similar levels (Liu et al., 1995). Additionally it was found that the CMV promoter element is more active than the adenovirus, SV40, and TK promoters in all tissues analyzed (Liu, et al., 1995). In order to make lipoplex-mediated gene therapy more efficient more effort needs to be made in optimizing the transgene expression cassette (F. Sakurai, et al., 2003).

1.2.5 Toxicity

There have not been any treatment related deaths reported with cancer patients in gene therapy clinical trials (Hunt, et al., 2007). Although treatment related severe adverse effects have been reported in clinical studies in other diseases. The fatal cases have prompted investigators to be more cautious in designing and participating in gene therapy trials (Raper et al., 2003). Current preclinical models are inadequate in predicting type, frequency, and severity of toxicity (Hunt, et al., 2007).

Lipoplexes have been shown to be nontoxic in several phase I and phase II clinical trials (Dass, 2002). Tumors are the ultimate target for non-viral vectors; however it is possible that the plasmid DNA can inadvertently circulating throughout the body after direct intra-tumor injection (Lew et al., 1995). Direct DNA injections into tumors for cancer immunotherapy trial in patients with stage IV melanoma appeared to be safe (Lew, et al., 1995). Transgene expression was found to be localized to the site of injection and tumor regression was observed in 1 of 5 patients with stage IV melanoma (Nabel et al., 1993). It was found that plasmid DNA (pVCL-1005) injected intravenously

persisted for at least 6 months post injection with no detectable protein expression in tissues retaining the highest amount of plasmid (Lew, et al., 1995).

The human HLA-B7 gene was introduced into subcutaneous melanoma tumors with the use of DNA-liposome complexes in a human clinical study (Nabel, et al., 1993). The gene was expressed and localized to the site of injection and no apparent toxicity or anti-DNA antibodies were detected (Nabel, et al., 1993). It was found that one patient responded to this treatment with both local and distant tumor regression, more studies need to be done in order to conclude that this treatment is consistently therapeutic (Nabel, et al., 1993).

Toxicity is dependent on the mode of administration. Intra-tracheal administration of lipoplexes has induced acute systemic inflammatory response and causes macrophage and neutrophil infiltration into the lungs of mice (Freimark et al., 1998). The lipoplexes were found to be highly toxic when orally administered resulting in dramatic hypothermia in mice (Filion & Phillips, 1997). Large aggregate formation caused mycocardial damage and tissue ischaemia in the intra-venous route (Dass, 2002). In terms of lung expression by the intravenous route, gene expression was transient and decreased by 1 1og per week (S. Li, Wu, et al., 1999). This may be due to neutralizing antibody production, cytokine-mediated promoter shutdown, or apoptosis of expressing cells (Dass, 2002).

1.3 Double-stranded RNA therapy

Double-stranded RNA (dsRNA) is produced by most viruses during their replication cycle and has a molecular pattern related to viral infection. Ds-RNA may be produced as a replicative intermediate or as an overlapping bi-directional transcript (Karpala, Doran, & Bean, 2005). Ds-RNA of natural origin, isolated from fungal viruses, or synthetically prepared polyinosine-cytosine, poly (I:C), mimic the biological actions of viruses with high interferon induction (Parr, Wheeler, & Alexander, 1973).

1.3.1 Anti-tumor mechanism

DsRNA triggers a number of antiviral responses that ultimately alter the program of the cell. They include intracellular mechanisms like dsRNA-associated protein kinase (PKR) and oligoadenylate synthetase (OAS) which shut down protein translation and lead to apoptosis (Karpala, et al., 2005). Induction by dsRNA also upregulated IFN-stimulated genes and inflammatory elements (Karpala, et al., 2005). Ds-RNA has both intracellular recognition proteins and extracellular antiviral mechanisms (Karpala, et al., 2005). Extracellular exposure to dsRNA is mediated by TLR3, a pattern recognition receptor. Ds-RNA bound to TLR3 induce the expression of cytokines IFN-β, IL-6, IL-12, and TNF-α (Karpala, et al., 2005). The interaction of dsRNA and TLR3 plays an important role in the immunostimulatory activity of dsRNA (Cui, Le, Qiu, & Shaker, 2007).

Ds-RNA has indirect anti-tumor mechanism related to induction of type I IFN, IFN α/β (Chawla-Sarkar et al., 2003). Type I IFNs are known to be induced by viruses directly (Karpala, et al., 2005). IFNs interfere with viral replication by modulating PKR

and OAS pathway which hinder cellular transcription (Karpala, et al., 2005). Many of the dsRNA stimulated genes can by induced by type I IFNs (Karpala, et al., 2005; Sen & Sarkar, 2005). Four families of transcription factors are known to be activated by dsRNA they include NF-κB, IRF-3, c-Jun, and ATF-2 (Sen & Sarkar, 2005). These transcription factors induce transcription of genes like IFN-β and immune response initiators (Sen & Sarkar, 2005).

Huang *et. al*. investigated poly (I:C) treated human peripheral blood mononuclear cells in terms of global pathway activation (C. C. Huang et al., 2006). Poly (I:C) challenge was found to elicit gene expression changes, similar to acute viral infection (C. C. Huang, et al., 2006). Gene regulation patterns reveal distinct immediate early, early-to-late, and late clustering. Early responses were found to be for innate immune responses involving TLR-3, NF-κB dependent pathway, and IFN-stimulated pathway (C. C. Huang, et al., 2006).

1.3.2 Clinical trials

The IFN-inducing activity of dsRNA in the form of poly (I:C) has been exploited in many pre-clinical and clinical tumor therapy trials (Le, Yanasarn, Lohr, Fischer, & Cui, 2008). Poly (I:C) was able to inhibit the growth of transplantable rodent tumors (Levy, Law, & Rabson, 1969). Poly (I:C) was found to decrease tumor size and increase survival time of mice bearing various tumor including reticulum cell sarcoma, lymphatic lymphoma, fibrosarcoma, or a human adenovirus 12-induced tumor model (Levy, et al., 1969). However many reports have shown that there is lack of correlation between

interferon induction by poly (I:C) and antitumor effects (M. Sakurai et al., 1990; Weinstein, Gazdar, Sims, & Levy, 1971).

One of the earliest poly (I:C) clinical trials reported by Robinson *et. al.* showed the poly (I:C) administered in multiple doses was not effective in the treatment of patients with large tumor burdens (Robinson et al., 1976). The lack of efficacy of poly (I:C) was due to rapid degradation in human serum by nucleases (Robinson, et al., 1976). There have been several attempts at stabilizing poly (I:C) including addition of poly-L-lysine, which did not lead to any objective response in phase I efficacy studies (Stevenson et al., 1985). Further addition of carboxymethylcellulose (CMC) to poly (I:C) poly-L-lysine was found to resist hydrolysis by primate serum (Levine, Sivulich, Wiernik, & Levy, 1979). Nonetheless poly (ICLC) induced high titers of interferon in the serum of humans (Levine, et al., 1979). It was found that 66% of patients receiving CMC stabilized poly (ICLC) showed regression or stabilization of tumor size (Salazar et al., 1996). The effects of systemic poly (I:C) treatment are inconsistent and higher doses lead to adverse side effects (Le, et al., 2008). Currently poly (I:C) is being used as an adjuvant in many clinical trials for DNA vaccines as well in combination therapy.

1.3.3 Methods to improve poly (I:C)

It is apparent that poly (I:C) needs to be stabilized to ensure cell exposure *in vivo*. Various methods to protect poly (I:C) have been utilized. Poly (I:C) stabilized with poly-L-lysine and CMC were found to decrease tumor protein synthesis *in vivo*. This stabilized poly (I:C) appeared to resist hydrolysis by serum nuclease and was found to be 8 to 10

times more resistant to hydrolysis by pancreatic RNase than poly (I:C) alone (Levy et al., 1975).

Poly (I:C) complexed to cationic liposomes was found to inhibit the growth of tumor cells (Hirabayashi et al., 1999). The therapeutic effect of intravenous delivery of lipoplexes containing poly (I:C), was evaluated in a murine lung metastasis model. A low level of TNFα and undetectable IFN-γ production were reported (F. Sakurai, et al., 2003). Interestingly the anti-metastatic effect of poly (I:C) lipoplexes were due to the IFN-β induction. A single administration of lipoplexes containing either a plasmid that expresses IFN-β or poly (I:C) showed a significant therapeutic effect on tumor metastasis and prolongation of survival time in tumor-bearing mice (F. Sakurai, et al., 2003). Shir *et. al.* have shown that targeting poly (I:C) to breast cancer cells led to regression of pre-established tumors (Shir, Ogris, Wagner, & Levitzki, 2006). Poly (I:C) has been found to enhance tumor response to radiation therapy synergistically as compared to single treatment modalities (Le, Kaurin, Sloat, Yanasarn, & Cui, 2009).

1.4 Replicase-based plasmid (pSIN-β)

Sindbis virus is an alpha virus that contains a single positive stranded RNA encoding its own RNA replicase (Scheiblhofer, Weiss, Gabler, Leitner, & Thalhamer, 2006; Strauss & Strauss, 1994). An anti-sense RNA is transcribed, and it functions as a template for the synthesis of sense RNA. RNA-dependent RNA polymerase activity was found on the nonstructural protein (nsP4) (M. L. Li & Stollar, 2004; Rubach et al., 2009). Sindbis viral vectors deficient in replication genes have been shown to efficiently target

and kill tumor cells *in vivo* (Lundstrom, 2001; Tseng et al., 2004; Venticinque & Meruelo, 2010). However, concerns regarding uncontrolled vector propagation and toxicity suggest that non-viral based vectors may offer a safer alternative (S. D. Li & Huang, 2007). Previously, the replicase genes (nsp1-4) from sindbis virus have been cloned into a plasmid and placed under the control of cytomegalovirus (CMV) promoter, hence referred to as the pSIN-β plasmid (Scheiblhofer, et al., 2006). When transfected into cells, the replicase genes are expressed, and the resultant replicase complex allows the formation of intracellular dsRNA (Diebold et al., 2009; Scheiblhofer, et al., 2006).

The pSIN-β plasmid was originally designed as a new generation vaccine in which antigen expression was controlled by an alphaviral replicase-enzyme complex with the goal of amplifying RNA production and to obtain high levels of antigen expression (Driver et al., 1998; Leitner, Bergmann-Leitner, Hwang, & Restifo, 2006). The replicase acts as an RNA polymerase amplifying mRNA (Leitner, et al., 2006). It was found that the replicase-based constructs were very immunogenic (Leitner, Ying, Driver, Dubensky, & Restifo, 2000). The generation of dsRNA species from the RNA amplification triggers anti-viral defense pathways in transfected cells mimicking the effects of a viral infection (Leitner, et al., 2006). DsRNA activates the PKR pathway which results in the apoptotic death of the cell (Leitner et al., 2003). Type I interferons are involved in innate immune response against viral infections (Leitner, et al., 2006). IFN α/β are produced in response to dsRNA in cells (Leitner, et al., 2006).

1.5 Liposomes as plasmid delivery vehicles

The first nanoscale drug delivery systems developed were lipid vesicles later described as liposomes (Bangham, Standish, & Watkins, 1965). They form vesicles through self-assembly of amphiphilic lipids and excipients. The bilayer of lipids are based on hydrophobic interactions in parallel packing with the hydrophilic head groups positioned towards the aqueous environment (Alexis, Pridgen, Langer, & Farokhzad, 2010). Liposomes are not rigid. They are fluid-like particles that form complex supramolecular assemblies (Balazs & Godbey, 2011). The physiochemical properties can be controlled based on the lipids that are included in the formulation. Properties such as surface charge and size can be controlled by mixing various lipids.

1.5.1 Formulation

Various lipids are included to impart the cationic characteristics that are necessary for efficient non-viral plasmid delivery. Liposome are generally prepared with FDA approved distearoylphosphatidylethanolamine (DSPE), HSPC, Egg PC, or DSPC (Alexis, et al., 2010). Cationic lipids used include [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), and diooctadecylamidoglycylspermine (DOGS) (Balazs & Godbey, 2011). These cationic lipids impart distinct characteristics to the lipoplex complex, which will affect the association with the cell, uptake, and disassociation from the endosome (Balazs &

Godbey, 2011). Improvements to these cationic lipids include modification with polyethylene glycol (PEG) for *in vivo* protection.

PEGyated lipoplexes are protected from degradation *in vivo* (Immordino, Dosio, & Cattel, 2006). PEGylation was shown to have steric hindrance which reduces the clearance of lipoplexes due to macrophage uptake and improves stability and longer circulation times in the blood (Alexis, et al., 2010). Attachment of PEG to lipoplex can be done by adsorption onto the liposome surface or covalently attaching PEG to a neutral lipid like DOPE, or DSPE (Alexis, et al., 2010).

A cationic liposome used as a gene carrier typically requires a neutral helper lipid to facilitate the release of the nucleic acid from the lipoplex (Guo & Huang, 2012). Disassociation of the lipoplex occurs because of the ion-pairs destabilizing with the formation of the inverted hexagonal phase and ultimate release of plasmids to the cytoplasm (Hafez, Maurer, & Cullis, 2001). The helper lipid promotes fusion with the endosomal membrane and it facilitates mixing with anionic lipids that allow DNA to be released from the delivery vehicle within the cells (Xu & Anchordoquy, 2011). Helper lipids are also required to stabilize the cationic liposome suspension as cationic lipids repel each other. Liposomes formulated without helper lipids have lower rates of transfection (Dass, 2002). Membrane destabilization hexagonal conformation is brought about in acidic pH (Alexis, et al., 2010).

Typically dioleoylphosphatidylethanolamine (DOPE) is used, but it was found that using cholesterol instead increased the amount of gene expression (Liu et al., 1997). It has been shown that incorporation of cholesterol into the liposome can promote

efficient *in vivo* gene delivery (Liu, et al., 1997). When DOPE is used the activity of vectors decreases by 100 to 1000 fold, while including cholesterol significantly enhances the *in vivo* activity (Liu, et al., 1997). Cholesterol is more commonly used as a helper lipid compared to DOPE because DOPE promotes fusion with erythrocytes in blood, which would accelerate the removal of lipoplexes from circulation (Xu & Anchordoquy, 2011). Cholesterol is a more efficient neutral lipid for systemic DNA delivery. High cholesterol content increases the stability of the liposomes by stabilizing bilayers and complexes in the plasma against mechanical breakage due to adsorption of plasma components (Templeton, et al., 1997).

The mechanism of transfection of lipoplexes into cells and the release of cargo is dependent on the liposome formulation (Tros de Ilarduya, Sun, & Duzgunes, 2010). Positive charged lipoplexes interact with the negatively charged cell surface components and enter cells by endocytosis or endocytosis-like mechanism (Tros de Ilarduya, et al., 2010). Once inside cells the pH of the endosome drop from pH 7.0 to 5.5, which causes a conformational change in the lipoplexes, and plasmid bound on the lipoplexes is released from the early endosome into the cytosol. Once near the perinuclear region the plasmid enters the nucleus after nuclear membrane disintegration or by transport through nuclear pores (Tros de Ilarduya, et al., 2010).

1.5.2 Targeting for cancer

The understanding of cancer biology, tumor microenvironment, signaling pathways, and metastatic evolution have improved our understanding and paved the way

for better drug delivery advances. Targeted therapies are designed to recognize cancer specific targets and destroy or slow the growth of cancer cells while avoiding normal cells. In 1909 Paul Ehrlich proposed the "magic bullet concept" in which drugs would go straight to their intended cell-structural targets (Strebhardt & Ullrich, 2008). His paradigm of a rationally targeted strategy has revolutionized modern cancer research. The use of a magic bullet in a genetically complex situation may be difficult. In reality the magic bullet concept fails to explain the drug interaction with tissues, and other non-cancer cells that lead to unacceptable side effects. The translation of cancer targeted pre-clinical approaches to clinical trials has been poor, and this may reflect the multi-dimensional complexity of cancer (Bae & Park, 2011). Drugs have been targeted to proteins such as matrix metalloproteinase inhibitors, epidermal growth factor receptors, transferase inhibitors, and angiogenesis growth factors to name a few (Alexis, et al., 2010). The idea of targeted nanoparticle therapy began with the development of immunoliposomes in 1980 (Alexis, et al., 2010). It is important to note that drug targeting doesn't imply the drug or delivery system actively searching for the target. It describes a ligand-receptor interaction occurring in close proximity ~0.5 nm (Bae & Park, 2011). There is an increased probability of the drug delivery system to be taken up by cancer cells if there is a molecular interaction between the cancer cell and the drug delivery system. There are two general approaches to receptor-mediated targeting. One is to target tumor microenvironment, the other is to target tumor cell surface for intracellular delivery (Alexis, et al., 2010).

In order to increase the feasibility for tumor targeting more understanding of systemic delivery of liposomes is necessary. The dynamic feature of tumor spatial and temporal heterogeneity as well as diffusional barrier in solid tumors need to be determined in order to engineer drug delivery systems that can overcome the current limitation of liposomes (Bae & Park, 2011).

1.5.3 Limitations

Lipoplexes that have high expression efficiency *in vitro* rely on monolayers that don't necessarily depict the *in vivo* situation (Xu & Anchordoquy, 2011). Lipoplexes that are stable in physiological media may have high tranfection rates *in vitro* and low transfection efficiencies *in vivo* or vice a versa. Differences between cell culture and *in vivo* gene delivery lead may lead to inefficient screening. A formulation developed for intra-tumoral administration may not function when used for intravenous administration (S. Li, Tseng, et al., 1999). An optimized liposome formulation may lead to high transfection efficiency in certain cell lines, but lack gene expression in others. Adjustments of the formulation for the liposomes should be evaluated for route of administration, level of experimentation i.e. *in vitro*, *in vivo*, and lipid composition. Improvement of the lipids, neutral lipid, targeting moieties, and shielding will allow for better transfection efficiencies (Alexis, et al., 2010).

1.6 Objectives

Double-stranded RNA has multiple anti-tumor mechanisms that can be exploited to control tumor growth. There is a reviving interest in taking advantage of the anti-tumor activity of poly (I:C) by improving the delivery of into tumor cells (Shir, et al., 2006). Intracellular dsRNA was found to be more effective compared to extracellular dsRNA in promoting tumor cells to undergo apoptosis and orchestrating the initiation of adaptive immune response (Cui, et al., 2007). Several groups have shown that targeting lipoplexes to tumor cells improved transfection efficiency *in vitro* and *in vivo* (Shir, et al., 2006). Based on these results, the following hypothesis was generated:

Intracellularly generated dsRNA may be more immunogenic than extracellular dsRNA. By using a plasmid DNA (pSIN-β) that generates dsRNA intermediates we hope to augment the anti-tumor response. Targeting the pSIN-β plasmid with surface conjugated liposomes will allow for more specific tumor cell delivery.

The aims of this project were

i. **To determine if the replicase-based plasmid (pSIN-β) can be used to generated dsRNA intracellularly and inhibit tumor growth**. This was determined by evaluating the presence of dsRNA in TC-1 transfected cells. The anti-tumor activity in TC-1 tumor bearing mice treated with pSIN-β complexed to cationic liposomes was also determined. (Chapter II)

ii. **To test the feasibility of targeting the pSIN-β plasmid into tumors that overexpress the epidermal growth factor receptor (EGFR).** This was done by evaluating the targeted lipoplexes *in vitro* with breast adenocarcinoma cell

lines with varying expression levels of EGFR/cell. We also determined if the targeted lipoplexes have a superior anti-tumor activity *in vivo* compared to the non targeted lipoplexes (Chapter III).

iii. **To improve the replicase-based plasmid pSIN-β by cloning IL2 into the plasmid may have improved anti-tumor activity.** We evaluated the benefit of IL2 in the pSIN plasmid *in vivo* using B16 melanoma bearing mice. We also compared the changes in $CD4^+$, $CD8^+$, and $CD49^+$ peripheral blood lymphocyte populations with plasmid treated B16 melanoma bearing mice (Chapter IV).

Chapter Two

Replicase-based plasmid DNA shows anti-tumor activity[1]

2.1 Introduction

Double stranded RNA has multiple anti-tumor mechanisms that may be potentially exploited to control tumor growth. It is known to be pro-apoptotic, anti-proliferative, and anti-angiogenic (Absher & Stinebring, 1969; Chawla-Sarkar, et al., 2003; Fujimura, Nakagawa, Ohtani, Ito, & Aiba, 2006). It is also a potent inducer of type I interferons (IFN-α/β) (Chawla-Sarkar, et al., 2003; Friedrich, Shir, Klein, & Levitzki, 2004), which are pro-apoptotic and immuno-stimulatory as well (Absher & Stinebring, 1969; Chawla-Sarkar, et al., 2003; Cui & Qiu, 2006). Intracellular dsRNA can activate various pathways, including anti- proliferative dsRNA dependent protein kinase (PKR), IFN inducible 2'-5'-adenylate synthetase/Rnase L system, and oligo A synthetase (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Friedrich, et al., 2004; Leitner, et al., 2003), which can lead to apoptosis. Intracellular dsRNA is recognized primarily by retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (Mda5) (Kawai & Akira, 2006; Kumar, Koyama, Ishii, Kawai, & Akira, 2008; Weber, Wagner, Rasmussen, Hartmann, & Paludan, 2006). Extracellular dsRNA recognition occurs by Toll-like receptor (TLR3) membrane bound receptor (Kawai & Akira, 2006; Matsumoto & Seya, 2008).

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[1] Significant portions of this chapter have been published in "BMC Cancer. 2011 Mar 28;11:110."

[2] Significant portions of this chapter have been previously published as "Nanomedicine

Over the past several decades, there had been numerous attempts to utilize synthetic dsRNA such as polyriboinosinic-polyribocytidylic acid, poly (I:C), to control tumors in animal models and clinical trials (Fujimura, et al., 2006; Hirabayashi, et al., 1999; Le, et al., 2008; Pimm & Baldwin, 1976). In general, it was found that synthetic dsRNA only slightly delayed tumor growth (Le, et al., 2009; M. Sakurai, et al., 1990; Weinstein, et al., 1971). Increasing the dose of the synthetic dsRNA to improve its anti-tumor activity is not feasible because of the dose-dependent severe adverse effects (Le, et al., 2009; Okada, Akbar, Horiike, & Onji, 2005; Weinstein, et al., 1971). Recently, there is a reviving interest in exploiting the anti-tumor activity of synthetic dsRNA by improving the delivery of dsRNA into tumor cells (Shir, et al., 2006). For example, Shir *et al.* (2006) reported the total regression of implanted human breast cancers or glioblastoma in mouse models when poly (I:C) was intratumorally injected and targeted into the tumor cells using epidermal growth factor as a ligand (Shir, et al., 2006). Using B16-F10 melanoma in a mouse model, Fujimura *et al.* (2006) reported the elicitation of tumor-specific CD8+ T lymphocyte responses by peritumoral injection of poly (I:C) (Fujimura, et al., 2006). Others have exploited the immuno-stimulatory activity of dsRNA by immunizing with tumor cells with intracellular synthetic dsRNA (Cui, et al., 2007). It became clear that intracellular dsRNA was more effective than extracellular dsRNA in promoting tumor cells to undergo apoptosis and orchestrating the initiation of adaptive immune responses (Cui, et al., 2007; McBride, Hoebe, Georgel, & Janssen, 2006; Schulz et al., 2005).

Sindbis virus is an alpha virus that contains a single positive stranded RNA encoding its own RNA replicase (Scheiblhofer, et al., 2006; Strauss & Strauss, 1994). An anti-sense RNA is transcribed, and it functions as a template for the synthesis of sense RNA. RNA-dependent RNA polymerase activity was found on the nonstructural protein (nsP4) (M. L. Li & Stollar, 2004; Rubach, et al., 2009). Sindbis viral vectors deficient in replication genes have been shown to efficiently target and kill tumor cells *in vivo* (Lundstrom, 2001; Tseng, et al., 2004; Venticinque & Meruelo, 2010). However, concerns regarding uncontrolled vector propagation and toxicity suggest that non-viral based plasmids may offer a safer alternative (S. D. Li & Huang, 2007). Previously, the replicase genes (nsp1-4) from sindbis virus have been cloned into a plasmid and placed under the control of cytomegalovirus (CMV) promoter (Scheiblhofer, et al., 2006). When transfected into cells, the replicase genes are expressed, and the resultant replicase complex allowed the formation of intracellular dsRNA (Diebold, et al., 2009; Scheiblhofer, et al., 2006). Therefore, we sought to deliver the replicase-based plasmid into tumor cells, hypothesizing that the RNA replicase based plasmid will generate dsRNA inside tumor cells and inhibit the tumor growth. This strategy is advantageous because it would avoid the delivery of a large dose of synthetic dsRNA *in vivo*, which is rather challenging; while there have been cases of successful delivery of DNA into tumor cells (S. D. Li & Huang, 2007; Pirollo et al., 2007; Tan, Whitmore, Li, Frederik, & Huang, 2002). Another advantage of utilizing plasmid DNA is that the unmethylated CpG motifs on the plasmid are also immuno-stimulatory (Gurunathan, Klinman, & Seder, 2000; Manders & Thomas, 2000). CpG motifs were shown to have anti-tumor activity by

activating natural killer cells and by inducing the secretion of cytokines such as IL-6, TNF-α, and IFN-γ (Gurunathan, et al., 2000).

In the present study, a sindbis replicase-based plasmid pSIN-β was used. In the plasmid, the sindbis nsp1-4 genes were under the control of a CMV promoter (Scheiblhofer, et al., 2006). Using a model mouse lung cancer cell line, TC-1, it was shown that when transfected into cells in culture, the pSIN-β generated dsRNA, and the resultant dsRNA seemed to be pro-apoptotic. In mouse model, the pSIN-β significantly inhibited the growth of the TC-1 tumors. Similar anti-tumor activity was also observed when the pSIN-β was used to treat B16 melanoma in mice.

2.2 Materials and Methods

2.2.1 Plasmids

Plasmid pCMV-β was from the American Type Culture Collection (ATCC, Manassas, VA). The pSIN-β plasmid was constructed following a previously described method (Scheiblhofer, et al., 2006). The pSIN-β-Δnsp was constructed in two steps. First, the pSIN-β was digested with *Pst I* (Invitro- gen, Carlsbad, CA), and the resultant fragment was gel extracted and purified using a PureLink Gel Extraction kit (Invitrogen). The DNA fragment was further digested with *Hind III* (Invitrogen). The correct fragment was gel extracted, and the adhesive ends were ligated using T4 DNA ligase (Invitrogen). All plasmids were amplified in *E. coli DH5a* under selective growth conditions.

Plasmid DNA was methylated at CpG sites with CpG methyl transferase (M.SssI) (New England BioLabs, Beverly, MA). The M.SssI methylates at the carbon position 5 of cytosine residues within double stranded recognition sequence. Methylation reaction containing 2 U of methylase per μg of DNA was incubated at 37°C for at least 3 h. The extent of methylation by the M.SssI was determined using a *BstU* I endonuclease assay (Invitrogen). Plasmid was purified from bacteria using a QIAGEN midiprep kit (Valencia, CA). Large scale plasmid preparation was performed by GenScript (Piscataway, NJ).

2.2.2 Cell lines and culture

Mouse lung tumor cells (TC-1, ATCC, CRL-2785) and mouse melanoma cells (B16-F10, ATCC, CRL-6475) were cultured in RPMI 1640 medium (Invitrogen) and

DMEM medium (Invitrogen), respectively. The media were supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml of penicillin (Invitrogen), and 100 μg/ml of streptomycin (Invitrogen).

The ovalbumin (OVA)-expressing B16-OVA cell line was generously provided by Dr. Edith M. Lord and Dr. John Frelinger (University of Rochester Medical Center, Rochester, NY) (Brown, Fisher, Wei, Frelinger, & Lord, 2001). B16-OVA cells were cultured in RPMI 1640 medium supplemented with 5% FBS and 400 μg/ml of G418 (Sigma).

2.2.3 *In vitro* Transfection

TC-1 cells (n = 3) were seeded in 24 or 48 well plates (20 000 cells/well) and incubated at 37°C, 5% CO_2 for 24 h or until 60% confluency followed by transfection using plasmid DNA (0.15 or 0.40 μg as where mentioned) complexed with Lipofectamine® (Invitrogen) following the manufacturer's instruction. The transfection medium was replaced with fresh medium 3 h later.

2.2.4 Semi quantitative RT-PCR

Total RNA was isolated from TC-1 cells (1×10^7) transfected with plasmid using a QIAGEN RNeasy mini kit. On-column DNase digestion was performed using RNase-free DNase set (QIAGEN) to eliminate DNA contamination. The RNA quality was assessed using the OD260/OD280 ratio.

Reverse transcriptase reaction was performed using Invitrogen SuperScript III™ kits (Cat No. 11752-050 or No. 18080-093) with oligo dT primers or sindbis nsp4 gene specific primers (nsp4-1, p4F (5'-CCGGAATGTTCCT- CACACTT-3') and p4R (5'-GGAATGCTTTTGCTCT GG-3')). Polymerase chain reaction was completed utiliz- ing cDNA from the reverse transcription and primer set p4F/p4R, which amplified a 501 base pair fragment of the nsp4 gene. Reactions were conducted using an Eppendorf Mastercycler (Hauppauge, NY) for 30 cycles: 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a 5 min final extension at 72°C. The nsp4 gene fragment was amplified using platinum taq DNA polymerase (Invitrogen). The PCR products (25 µl) were analyzed using agarose gel electrophoresis.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

The presence of dsRNA in TC-1 cells (n = 3) transfected with the plasmid was confirmed using ELISA as previously described with modification (Sloat, Shaker, Le, & Cui, 2008). Briefly, 96-well plates were coated at 4°C overnight with 1 µg total RNA diluted in PBS. Plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20, Sigma-Aldrich, St. Louis, MO) and blocked with 4% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in PBS/Tween 20 for 1 h at 37°C. Plates were washed again with PBS/Tween 20. Monoclonal anti-dsRNA antibody J2 (English & Scientific Consulting Bt. Szirák, Hungary) was added to each well following the removal of the blocking solution. The plates were incubated for an additional 3 h at 37°C. Horseradish peroxidase (HRP) labeled goat anti-mouse IgG2a (5 000-fold dilution Southern Biotechnology

Associates, Birmingham, AL) was added to the wells, followed by 1 h of incubation at 37°C. The presence of bound secondary antibody was detected after a 30 min incubation with 3,3',5,5'-tetramethylbenzidine substrate (TMB) (Sigma-Aldrich). The reaction was stopped by the addition of sulfuric acid (0.2 M, Sigma).

2.2.6 Determination of cell viability

The number of viable TC-1 cells was determined using a 3-(4,5-dimethylthiazol)-2-,5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich) 24, 48, and 72 h after the initiation of the transfection (n = 3) (Le, et al., 2008). Cells treated with sterile PBS were used as a control. Formula used to calculate the relative cell number (%) was: Relative cell number = 100 x number of live cells transfected with pCMV-β (pSIN-β, or pSIN-β-Δnsp)/ number of live cells transfected with sterile PBS.

2.2.7 Preparation of plasmid DNA-liposome lipoplexes

Cationic liposomes were prepared using cholesterol (Sigma-Aldrich), egg phosphatidylcholine (Avanti Polar Lipids, Inc, Alabaster, AL), and 1,2,-dioleoyl-3-trimethylamonium-propane (DOTAP, Avanti) at a molar ratio of 4.6:10.8:12.9 by thin film hydration method followed by membrane extrusion (1, 0.4, and 0.1 μm, sequentially) (Le & Cui, 2006). The final concentration of DOTAP in the liposome was 10 mg/ml. The plasmid-liposome lipoplexes were prepared by mixing equal volumes of plasmid DNA (25 μg in 25 μl) solution and liposome suspension containing 50 μg of DOTAP liposomes. The mixture was allowed to stay at room temperature for at least 15

min before further use. Particle sizes were measured using a Malvern Zetasizer Nano ZS (Worcestershire, United Kingdom). The size of the liposomes was 110 ± 0.6 nm with a polydispersity index (PI) of 0.121. The pSIN-β-liposome lipoplexes were 255 ± 31 nm (PI, 0.177). The pCMV-β-liposome lipoplexes were 249 ± 33 nm (PI, 0.183). The sizes of the two lipoplexes were not statistically different ($p = 0.83$, t-test, $n = 3$).

2.2.8 Animal studies

All animal studies were carried out following the National Institutes of Health animal use and care guidelines. Animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Female C57BL/6 mice (6-8 weeks) were from Simonsen Laboratories (Gilroy, CA) or Charles River laboratories, Inc. (Wilmington, MA). Female athymic nude mice (6-8 weeks) were from Charles River laboratories. Mice were subcutaneously injected with TC-1, B16/F10, or B16-OVA cells (5×10^5) in the right flank. When tumors reached an average diameter of 3-4 mm, the plasmid DNA-liposome lipoplexes were injected subcutaneously peritumorally (s.c., p.t.) for 5 or 10 consecutive days (Le, et al., 2009; Le, et al., 2008; Shir, et al., 2006). The dose of the plasmid DNA was 25 μg DNA per mouse per injection. Tumor size was measured using a digital caliper and calculated using the following equation (Milas et al., 2004): tumor diameter = (Length + Width)/2. To examine whether the nsp genes were expressed *in vivo*, pCMV-β, pSIN-β, or pSIN-β-Δnsp (25 μg) was injected into the gastrocnemius muscles in the hind legs of mice (n =

2). After 24 h, the injected muscle tissues were collected and homogenized using TRIzol reagent (Invitrogen) to isolate total RNA. RT-PCR was performed to amplify nsp4 gene or β-gal gene using the nsp4-1 primers or the β-gal primers (5'-GACGTCTCGTTGCTGCATAA-3'; 5'-CAGCAGCAGACCATTTTCAA-3').

2.2.9 Histology

TC-1 tumors in mice that were treated for 6 consecutive days with plasmids were collected, fixed in formaldehyde, embedded in paraffin, and sectioned. Immunohistochemistry was performed to detect apoptosis using the anti-ACTIVE caspase-3 antibody (Promega, Madison, WI) according to manufacturer protocol. Fifteen random fields per sample at 40 × magnification were scored for cleaved caspase-3. Apoptotic index was determined based on the % of cleaved caspase-3 positive cells found within total cells counted (Le, et al., 2008).

2.2.10 Quantification of IFN-α in mouse serum samples

Mice were subcutaneously injected with 125 μg of plasmid DNA in lipoplexes (DNA/liposomes, 1:2, w/w). Ten h later, serum was collected, and the concentration of IFN-α was determined using a mouse IFN-α (Mu-IFN-α) ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

2.2.11 Statistical analysis

Statistical analyses were completed using ANOVA followed by Fisher's protected least significant difference procedure. A p-value of $\leq$ 0.05 (2-tail) was considered statistically significant.

2.3 Results and discussion

2.3.1 Generation of dsRNA by transfecting pSIN-β into tumor cells

Shown in Figure 2.1 are maps of the plasmids used. In the pCMV-β, the β-galactosidase gene is driven by the CMV promoter. In the pSIN-β, the nsp1-4 genes are driven by a CMV promoter, while the β-galactosidase gene is driven by a sindbis viral subgenomic promoter. The pSIN- β-Δnsp was constructed by deleting the nsp1-3 and part of the nsp4 genes from the pSIN-β. To confirm that the pSIN-β plasmid can produce dsRNA when delivered into cells, TC-1 cells were transfected with pSIN-β, and the total RNA was extracted from the cells 24 h later. The total RNA was reverse transcribed with either oligo dT primers or nsp4 gene specific primers (p4-F or p4-R). The cDNA was then amplified with nsp4-specific primers. A 501 bp nsp4 gene fragment was observed in all samples transfected with pSIN-β, but not in cells transfected with the pCMV-β (Figure 2.2A), indicating the presence of both sense and anti-sense RNA of the nsp4 gene in cells transfected with the pSIN-β. The production of dsRNA in cells transfected with the pSIN-β was further confirmed using ELISA. As shown in Figure 2.2B, TC-1 cells transfected with pSIN-β had an elevated level of dsRNA compared to untransfected cells, whereas cells transfected with pCMV-β and the untransfected cells had a similar level of dsRNA. The dsRNA generated within cells transfected with the pSIN-β seemed to be functional because the number of live cells in samples transfected with pSIN-β decreased gradually with the increase in incubation time, in contrast to the increase in the number of live cells in samples transfected with the pCMV-β (Figure 2.2C). As expected, the pSIN-

β-Δnsp no longer caused a decrease in the number of live cells when transfected into the TC-1 tumor cells (Figure 2.2C), indicating the significance of the nsp1-4 genes for the pSIN-β plasmid to be functional. This observation is in agreement with the finding by Leitner *et al.* (2004), who showed that the survival of BHK-21 cells transfected with a replicase-based plasmid was significantly lower than cells transfected with a conventional CMV promoter-driven plasmid (Leitner et al., 2004). The cell death after transfection with the pSIN-β was likely caused by the pro-apoptotic dsRNA produced by the sindbis RNA replicase complex (Leitner, et al., 2004).

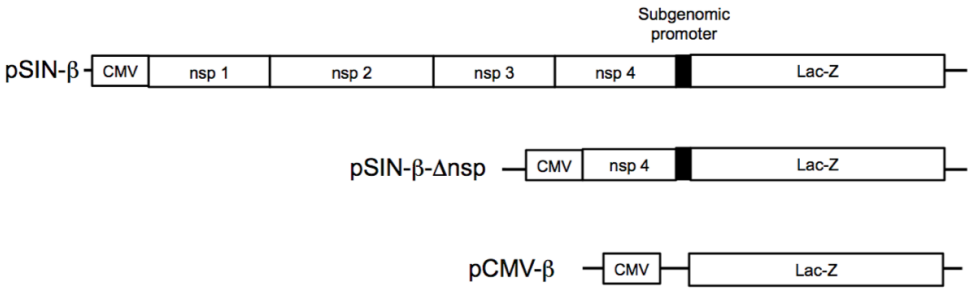

Figure 2.1 A schematic of plasmids used in this study.

CMV, cytomegalovirus promoter; Lac-Z, β-galactosidase; nsp, sindbis virus sequences coding for the nonstructural proteins (nsp1-4). pSIN-β-Δnsp (8,727 bp), pSIN-β (14,869 bp), pCMV-β (7,164 bp).

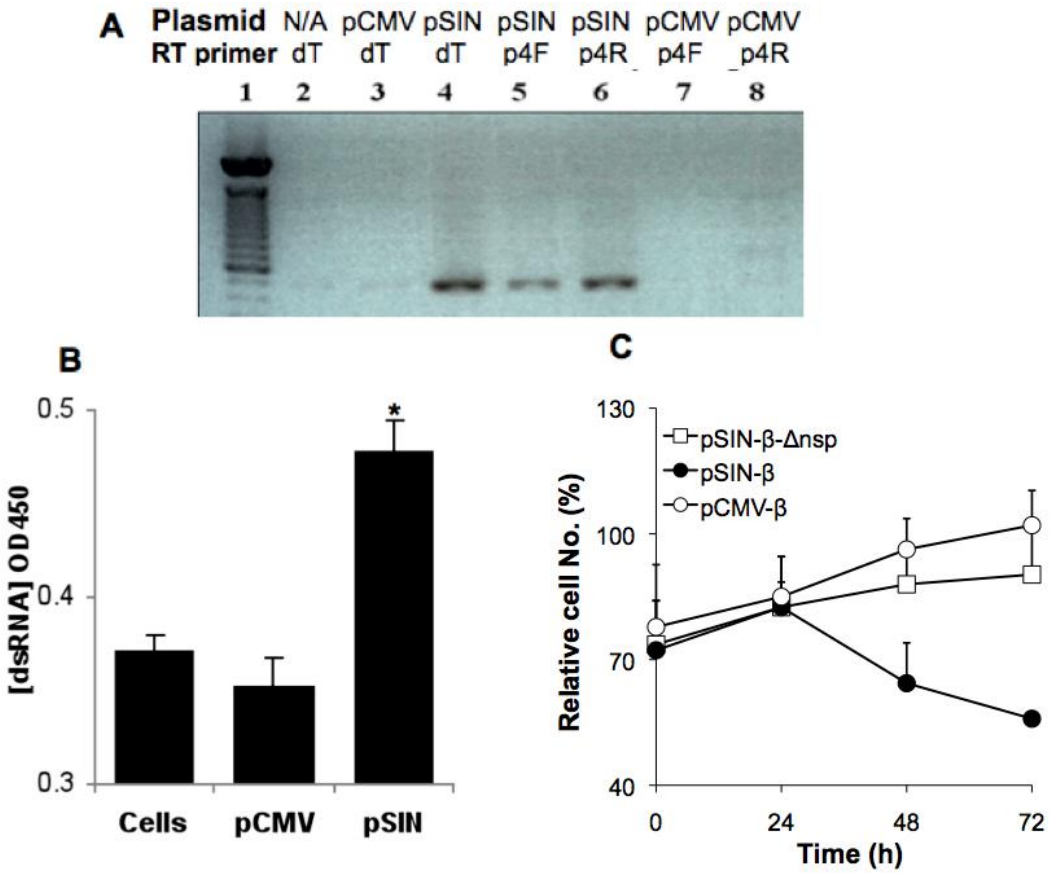

Figure 2.2 Generation of dsRNA in tumor cells transfected with pSIN-β.

(A). TC-1 cells were transfected with pCMV-β (pCMV) or pSin-β (pSIN), or left untreated (N/A). Total RNA was reverse transcribed into DNA with oligo dT primer or primers specific to the nsp4 gene (forward p4F or reverse p4R) before PCR amplification. This experiment was repeated twice with similar results. (B). ELISA confirmed the presence of an elevated level of dsRNA in TC-1 cells transfected with pSIN-β (n = 3). Total dsRNA was isolated from TC-1 cells transfected with pCMV-β or pSIN-β and used to coat ELISA plate. The primary Ab was the J2 anti-dsRNA IgG2a. *, p = 0.004. (C). Transfection of pSIN-β into TC-1 cells inhibited cell growth. TC-1 cells (20 000 cells/well) were transfected with the same amount (0.4 μg) of pCMV-β, pSIN-β, or

pSIN-β-Δnsp (n = 4). Cell numbers were quantified using MTT assay and normalized to cells treated with sterile PBS. Data shown are mean ± S.E.M. **, at 48 and 72 h, the value of the pSIN-β were different from that of the pCMV-β and the pSIN-β-Δnsp ($p < 0.05$).

2.3.2 Treatment of tumor-bearing mice with pSIN-β plasmid caused tumor regression

Prior to carrying out tumor treatment studies, the ability of the pSIN-β to express the nsp1-4 genes *in vivo* was examined. As shown in Figure 2.3 using RT-PCR, nsp4 RNA expression was detected only in mouse muscle tissues injected with the pSIN-β, not in the ones injected with the pCMV-β or the pSIN-β-Δnsp, demonstrating that only the pSIN-β was capable of expressing the nsp genes *in vivo.* The β-gal mRNA was present in all the mouse muscle tissues since the β-gal gene is endogenous (Figure 2.3).

To evaluate the extent to which the pSIN-β can control the growth of tumors pre-established in mice, mouse TC-1 lung cancer cells were seeded in mice. When tumors reached 3-4 mm in diameter, mice were treated with pSIN-β or pCMV-β daily for 10 days. Mice in the negative control group were not treated. As shown in Figure 2.4A, TC-1 tumors grew significantly slower in mice that received the pSIN-β plasmid than in mice that received the pCMV-β plasmid. In fact, 25 days after cell seeding, only 20% or 40% of tumor-bearing mice that were left untreated or received the pCMV-β plasmid, respectively, were alive, but all mice that received the pSIN-β were still alive (Table 2.1). Moreover, on day 25, there was only one mouse in the group that received the pSIN-β plasmid had a tumor of 3.1 mm in diameter, which completely regressed on day 37 (Table 2.1). Clearly, the pSIN-β plasmid was more effective in controlling the growth of the TC-1 tumors than the pCMV-β plasmid. Finally, mice in the negative control group were left untreated because it was shown that repeated peritumoral injection of sterile

PBS or the liposomes did not have any effect on the growth of the TC-1 tumors as compared to mice left untreated (Figure 2.4B), demonstrating that potential inflammations caused by the liposomes alone or by the peritumoral injections per se were not responsible for the anti-tumor activity observed in Figure 2.4A.

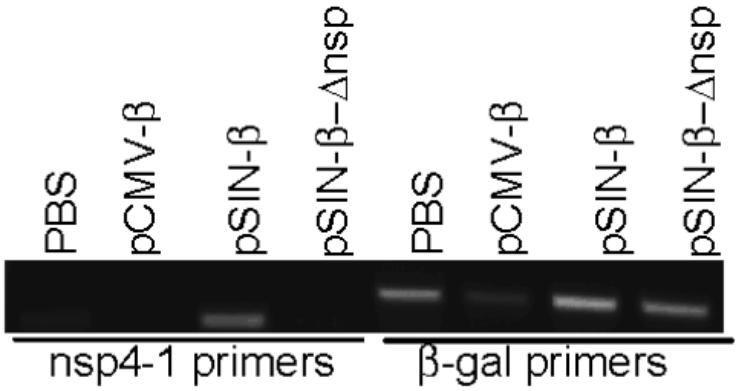

Figure 2.3 *In vivo* expression of nsp4 gene.

Twenty-four h after i.m. injection with PBS, pCMV-β, pSIN-β, or pSIN-β-Δnsp, total RNA was extracted from the muscle tissues and RT-PCR-amplified to detect the expression of nsp4 and β-gal genes.

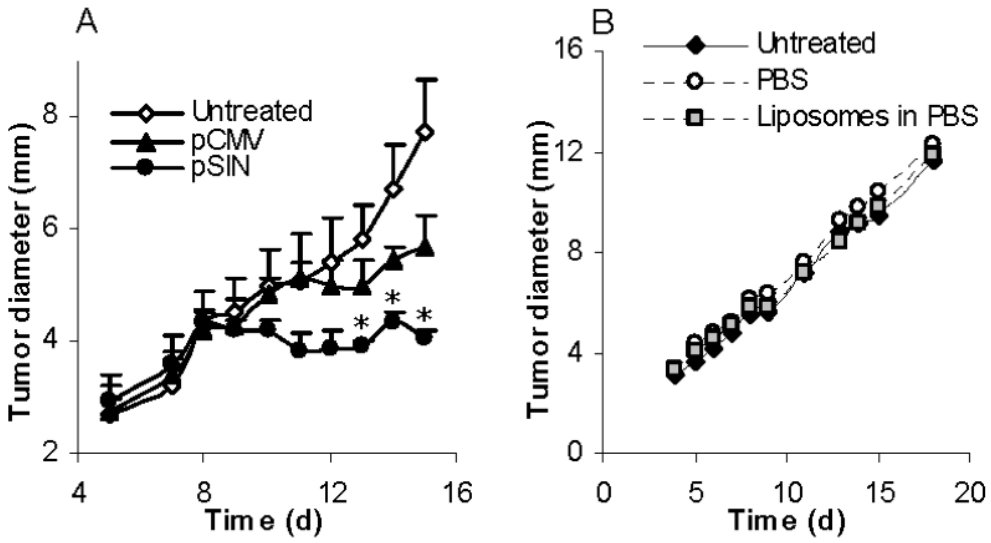

Figure 2.4 Treatment of mice with pSIN-β caused TC-1 tumor regression.

(A). C57BL/6 mice (n = 5) were s.c. implanted with TC-1 tumor cells (5×10^5) on day 0. DNA-liposome lipoplexes were injected (s.c., p.t.) for 10 consecutive days, starting on day 5 (25 μg DNA per day). (*) indicates that on days 13-15 the values of pCMV-β and pSIN-β were different from each other ($p < 0.05$). (B). Peritumoral injection of liposomes alone or sterile PBS did not affect the growth of the TC-1 tumors. Mice (n = 5) with TC-1 tumors were injected (p.t.) with sterile PBS or liposomes in PBS (dose equivalent to that injected in the DNA-liposome lipoplexes) for 10 consecutive days, starting on day 4. Data shown were mean ± S.E.M.

Table 2.1 Treatment with pSIN-β plasmid caused TC-1 tumor regression.

	Untreated	pCMV-β	pSIN-β
No. of mice alive [a]	1/5	2/5	5/5
Tumor size (mm)	9.1	8.9, 6.1	0, 0, 0, 0, 3.1 [b]

Data shown are 25 days after tumor cell seeding into mice.

a. Shown are number of live mice/total number of mice.

b. On day 37, all mice that were treated with the pSIN-β became tumor-free.

2.3.3 The anti-tumor activity from pSIN-β required functional replicase genes nsp 1-4

To understand whether the sindbis replicase genes, nsp1-4, were related to the anti-tumor activity of the pSIN-β plasmid, the anti-tumor activity of the pSIN-β-Δnsp was compared to that of the pSIN-β. When used to treat the TC-1 tumors in mice, the pSIN-β-Δnsp was significantly less effective in controlling the growth of the tumors than the pSIN-β in the beginning (Figure 2.5), demonstrating that the nsp1-4 genes, which were responsible for the dsRNA production, played a significant role in the anti-tumor activity of the pSIN-β plasmid. It needs to be noted that the TC-1 tumor cells are strongly immunogenic, and it was expected the peritumoral injection of pSIN-β-Δnsp to show anti-tumor activity because the plasmid, with CpG motifs, can activate innate anti-tumor immune responses (Whitmore, Li, & Huang, 1999).

As shown in Figure 2.6 more cells in tumors treated with the pSIN-β plasmid underwent apoptosis than in tumors treated with the pCMV-β plasmid. We suspect that the increased apoptosis in tumors that received the pSIN-β plasmid was related to the plasmid's ability to produce dsRNA in transfected cells. However, it is unclear to what extent the apoptosis was caused directly by dsRNA produced by the pSIN-β plasmid. Double stranded RNA is pro-apoptotic (Chawla-Sarkar, et al., 2003), but the type I IFNs induced by dsRNA are pro-apoptotic as well (Chawla-Sarkar, et al., 2003). More over, the unmethylated CpG motifs on the plasmid, the dsRNA per se, and type I IFN are all known to be able to activate innate immunity such as natural killer (NK) cells, which can

cause tumor death (Zamai et al., 2007). In fact, it was shown that subcutaneous injection of the pSIN-β plasmid induced an elevated level of IFN-α in mouse serum samples (Figure 2.7).

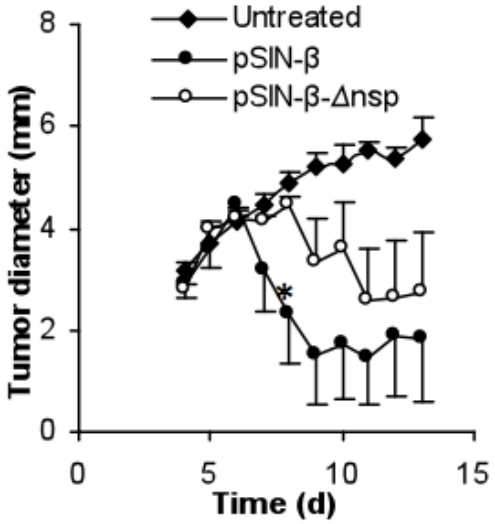

Figure 2.5 Deletion of the replicase genes (nsp1-3 and part of nsp4) from the pSIN-β plasmid significantly decreased the anti-tumor activity of the plasmid.

C57BL/6 mice (n = 4-5) were s.c. implanted with TC-1 tumor cells (5×10^5) on day 0. From days 4 to 13, mice were injected (s.c., p.t.) with lipoplexes prepared with pSIN-β (25 μg) or pSIN-β-Δnsp (25 μg). *, On day 8, p = 0.05, pSIN-β vs. pSIN- β-Δnsp.

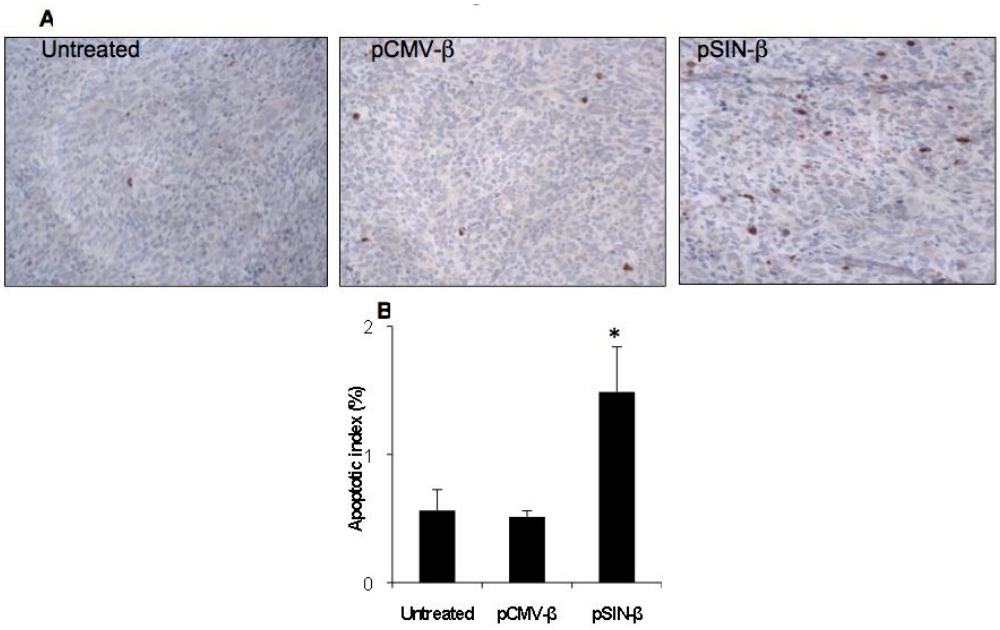

Figure 2.6 Injection with pSIN-β promoted more tumor cells to undergo apoptosis.

(A). Micrographs of tumors stained against anti-caspase- 3 (brown). (B). Apoptotic index. Data shown were mean ± S.E.M. The number of mice in each group was 3-4. (*) Indicates that the value of pSIN-β differed from that of the others (ANOVA, $p = 0.03$).

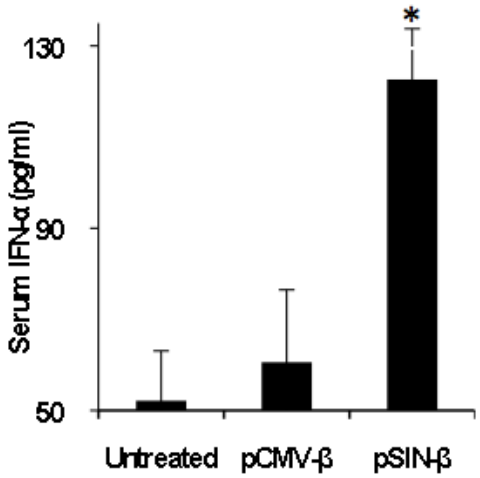

Figure 2.7 The pSIN-β plasmid induced IFN-α production in mouse sera.

IFN-α levels in blood were measured 10 h after injection (n = 4). Data reported are means ± SEM. (*, $p < 0.05$, pCMV-β vs. pSIN-β).

2.3.4. Adaptive immunity contributed to the anti-tumor activity from pSIN-β

TC-1 tumor cells are highly immunogenic in C57BL/6 mice due to the human papillomavirus E6 an E7 genes in the TC-1 cells (Ji et al., 1998). Data from several recent studies have shown that tumor cells with intracellular dsRNA were more immunogenic than tumor cells physically mixed with dsRNA (Cui, et al., 2007; DiCiommo & Bremner, 1998; McBride, et al., 2006; Schulz, et al., 2005; Shir, et al., 2006). Therefore, it was expected that adaptive immune responses have contributed, to a certain extent, to the anti-tumor activity from the pSIN-β plasmid. To test this hypothesis, the same TC-1 tumors established in athymic mice were treated with pSIN-β or pCMV-β. As shown in Figure 2.8 the pSIN-β was no longer more effective than the pCMV-β in controlling the growth of the TC-1 cells, indicating that adaptive immunity contributed to the anti-tumor activity from the pSIN-β plasmid. However, the adaptive immunity was not absolutely required for the pSIN-β to have anti-tumor activity because recent preliminary data in our lab showed that in athymic mice, the pSIN-β caused total regression of pre-established model human tumors when targeted into the tumor cells using a tumor-specific ligand (Rodriguez and Cui, unpublished data).

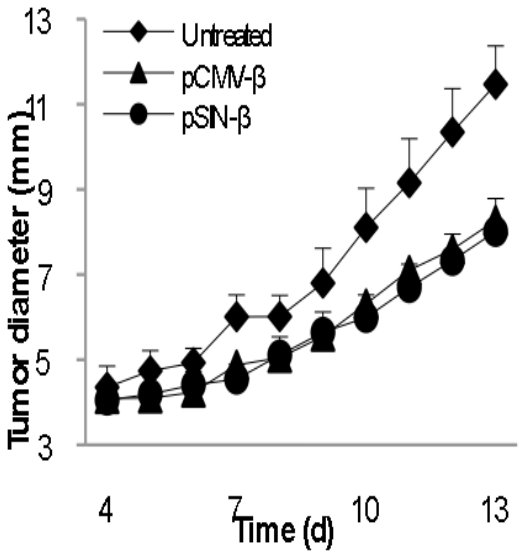

Figure 2.8 The pSIN-β plasmid was no longer more effective than pCMV-β against tumors in athymic mice.

Mice (n = 6-8) were s.c. implanted with TC-1 tumor cells (5×10^5) on day 0. From days 4 to 13, mice were injected (s.c., p.t) with lipoplexes prepared with pSIN-β (25 μg) or pCMV-β (25 μg).

2.3.5 Unmethylated CpG motifs contributed to the anti-tumor activity of the pCMV-β

The anti-tumor effect from the pCMV-β plasmid was likely due to the unmethylated CpG motifs present on the plasmid (Whitmore, et al., 1999). As shown in Figure 2.9 methylation of the pCMV-β depleted the plasmid's ability to inhibit the growth of the TC-1 tumor cells in mice. This is in agreement with a previous report showing that plasmid DNA itself had anti-tumor activity because the unmethylated CpG motifs on the plasmid can activate innate immunity (Whitmore, et al., 1999). Therefore, it is possible that both dsRNA produced by the RNA replicase complex encoded by the nsp1-4 genes and the unmethylated CpG motifs on the pSIN-β plasmid may have contributed to the anti-tumor activity from the pSIN-β.

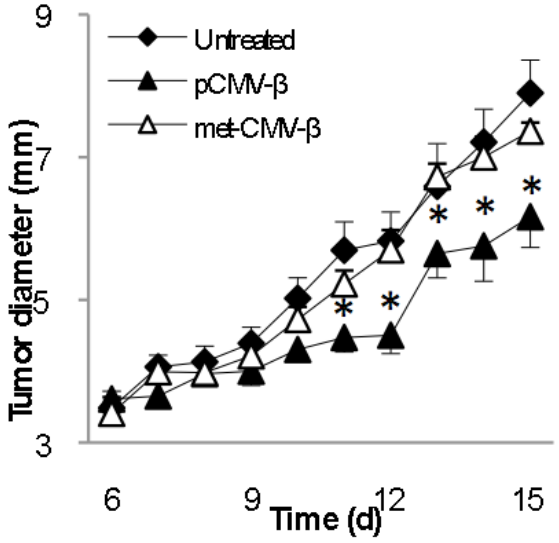

Figure 2.9 Unmethylated CpG motifs contributed to the anti-tumor activity of the pCMV-β.

C57BL/6 mice (n = 5-6) were implanted with TC-1 tumor cells (5×10^5) on day 0. From days 6 to 15, mice were injected (s.c., p.t.) with lipoplexes prepared with unmethylated or methylated pCMV-β (pCMV-β or met-CMV-β, 25 μg). Data shown were mean ± S.E.M. (*) indicates that on days 11 to 15, the values of pCMV-β and met-CMV-β were different from each other ($p < 0.05$).

2.3.6 The pSIN-β plasmid was effective against B16 melanoma in mice as well

To test whether the pSIN-β was effective against tumors other than the TC-1, mice with pre-established B16-F10 or B16-OVA tumors were treated similarly. As shown in Figure 2.10 the pSIN-β plasmid significantly controlled the growth of both poorly immunogenic B16-F10 tumors (Figure 2.10A) and the more immunogenic B16-OVA tumors (Figure 2.10B), indicating that the approach of controlling tumor growth with the replicase-based plasmid was not limited to the TC-1 tumors and likely not limited to highly immunogenic tumors as well. Again, it is not surprising that the pCMV-β also showed anti-tumor activity against the B16 melanoma. In a previous study, McCray *et al.* (2006) showed that intratumoral injection of an empty pcDNA3.1 delayed the growth of B16 tumors, as compared to the injection of saline (McCray et al., 2006). It was also shown that intratumoral injection of the pcDNA3.1 followed by *in vivo* electroporation further improved the anti-tumor activity (McCray, et al., 2006). In the present study, the repeated peritumoral injection of the pCMV-β complexed with cationic liposomes may have improved the non-specific anti-tumor activity from the plasmid.

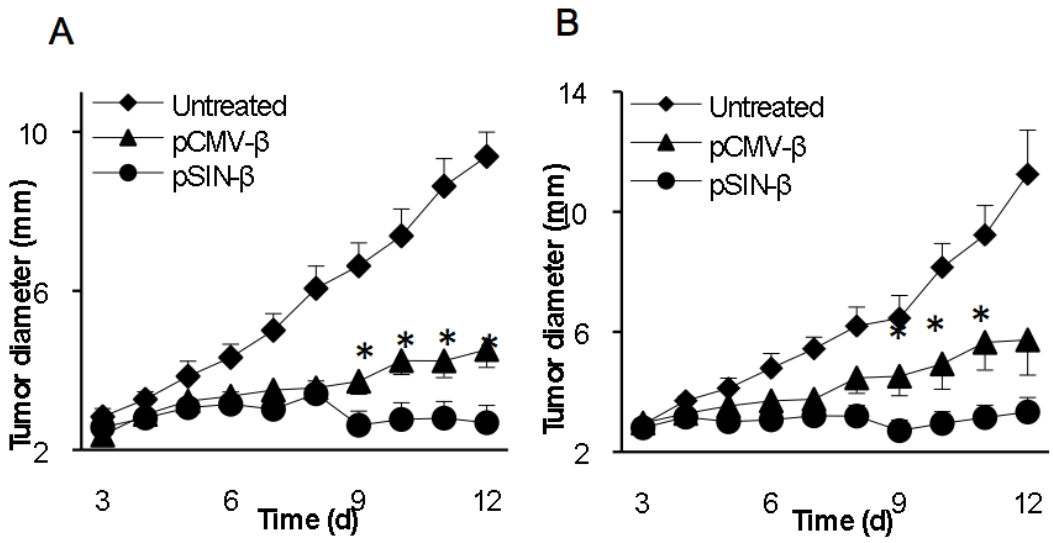

Figure 2.10 pSIN-β was more effective than pCMV-β in controlling the growth of mouse B16-F10 and B16-OVA melanomas as well.

C57BL/6 mice (n = 6-7) were implanted with B16-F10 (A) or B16-OVA (B) cells on day 0. DNA-liposome lipoplexes were injected (s.c., p.t.) for 10 consecutive days starting on day 3 (25 μg DNA per day). (*) indicate that on days 9-12 for B16-F10 (or days 9-11 for B16-OVA), the values of pCMV-β and pSIN-β were different from each other ($p < 0.05$). Data shown are mean ± S.E.M.

2.4 Conclusions

A RNA replicase-based plasmid that did not encode any relevant functional gene was showed to have anti-tumor activity. The anti-tumor activity of the RNA replicase-encoding plasmid was likely due to its ability to allow the transfected tumor cells to produce dsRNA and to activate innate and adaptive immunity. In the present study, for proof-of-concept purpose, the RNA replicase encoding plasmid was dosed to mice by subcutaneous peritumoral injection. Although feasible for tumors such as head and neck cancers, certain non-metastasized melanomas, and brain tumors, peritumoral or intratumoral injection is expected to be difficult to operate for many other solid tumors. We are in the process of developing a liposome-based system to target the RNA replicase encoding plasmid into tumor cells by the intravenous route. Treatment of poorly immunogenic tumors such as B16-F10 melanoma in animal models is a good simulation of conditions observed in cancer patients (Wilcox et al., 2002), and the data in the present study showed that both highly immunogenic and poorly immunogenic solid tumors were receptive to treatment with a RNA replicase based plasmid. Our results suggested a novel approach to cancer molecular therapy.

Chapter Three

Control of solid tumor growth in mice using EGF receptor targeted RNA relicase-based plasmid DNA[2]

3.1 Introduction

Cancer therapy has improved dramatically over the past few years, but chemotherapy remains commonly used in clinics. Traditional chemotherapeutic agents generally have a single tumor-killing mechanism, making it easier to develop resistance and requiring multiple chemotherapeutic agents for combination therapy. An ideal chemotherapy agent would be highly selective for cancer cells and utilize multiple killing mechanisms to ensure tumor cell death.

It is known that double-stranded (dsRNA) molecules have multiple direct and indirect pro-apoptotic, anti-proliferative, and anti-angiogenic activities (Absher & Stinebring, 1969; Chawla-Sarkar, et al., 2003; Fujimura, et al., 2006). Moreover, dsRNA is a known agonist of Toll-like receptor 3 (TLR 3), and the interaction between dsRNA and TLR3 triggers both innate and adaptive immunities (Alexopoulou, et al., 2001; Diebold, et al., 2009; Schulz, et al., 2005). Double-stranded RNA is also a potent inducer of type I interferons (IFN-α/β), which are pro-apoptotic and anti-angiogenic as well (Chawla-Sarkar, et al., 2003; Friedrich, et al., 2004). For decades, synthetic dsRNA, such

[2] Significant portions of this chapter have been previously published as "Nanomedicine (Lond). 2012 Apr;7(4):475-91."

as polyriboinosinic-polyribocytidylic acid (poly (I:C)), had been evaluated in preclinical and clinical trials for cancer therapy (Absher & Stinebring, 1969; Friedrich, et al., 2004; Fujimura, et al., 2006; Hirabayashi, et al., 1999; Le, et al., 2009; Okada, et al., 2005; Pimm & Baldwin, 1976), but the severe adverse effects from high doses of systemic dsRNA limited its clinical application (Absher & Stinebring, 1969; Matsumoto & Seya, 2008; Meier, Myers, & Huebner, 1970; Okada, et al., 2005; Pimm & Baldwin, 1976). Therefore, novel strategies are sought to more effectively take advantage of the multiple anti-tumor mechanisms of dsRNA, while minimizing its adverse effects.

Data from recent studies showed that intracellular dsRNA is more effective than extracellular dsRNA in promoting cells to undergo apoptosis and in orchestrating adaptive immune responses (Cui, et al., 2007; McBride, et al., 2006; Schulz, et al., 2005). Previously, we proposed and demonstrated the feasibility of using an RNA replicase-based plasmid to generate dsRNA intracellularly in the transfected cells to control tumor growth in culture and in mice (B. L. Rodriguez, Z. Yu, W. G. Chung, R. Weiss, & Z. Cui, 2011). The Sindbis viral RNA replicase-based plasmid, pSIN-β, contains cytomegalovirus promoter-driven non-structural protein genes (nsp1-4) that encode the Sindbis viral RNA replicase complex. The β-galactosidase gene in the pSIN-β is driven by a viral subgenomic promoter (B. L. Rodriguez, et al., 2011; Scheiblhofer, et al., 2006). Previous data showed that transfection of cells with a similar alphavirus RNA replicase-based plasmid appeared to mimic cell infection by an alphavirus, because the RNA replicase mediated the production of dsRNA intracellularly (Diebold, et al., 2009; Leitner, et al., 2004). In a previous study, we showed that treatment of tumor-bearing

mice with the pSIN-β plasmid complexed with cationic liposomes peritumorally significantly inhibited the growth of model mouse tumors pre-established in mice and, in many cases, caused complete tumor regression (B. L. Rodriguez, Z. Yu, W.-G. Chung, R. Weiss, & Z. Cui, 2011), suggesting the use of RNA-replicase based plasmid as a novel tumor molecular therapy.

In the present study, in order to further improve the specific killing of tumor cells by the dsRNA produced by the pSIN-β plasmid, we tested the feasibility of targeting the pSIN-β plasmid into tumors cells that over-express EGF receptor (EGFR). EGFR is over-expressed in many cancer cells including glioblastoma, breast cancer, colorectal, head and neck cancer (Ciardiello & Tortora, 2003). For example, Klign *et al*. found that, depending on the method of detection, EGFR was over-expressed in 14-90% of human breast cancer cells with a mean percentage of EGFR positivity of 45% (5,232 patents) (Klijn, Berns, Schmitz, & Foekens, 1992). EGFR consists of an extracellular ligand-binding domain, a single transmembrane spanning region, and an intracellular region containing a kinase domain (Ciardiello & Tortora, 2003). EGFR over-expression is involved in the control of tumor cell proliferation, metastasis, and angiogenesis (Ciardiello & Tortora, 2003). Various EGFR targeting agents have been developed, including those targeting the extracellular domain (e.g., anti-EGFR MAb 225) and tyrosine kinase inhibitors (e.g, ZD1839 (Iressa)) (Baselga, 2000; Ranson et al., 2002). Clinical data of such EGFR targeting agents have been promising, but they are highly dependent on intact EGFR (Ciardiello & Tortora, 2003). We intended to utilize the over-expressed EGFR as the target to more specifically deliver the pSIN-β plasmid into tumor

cells by complexing it with cationic liposomes surface-conjugated with EGF, a known ligand to EGFR. Previously, it was shown that EGF conjugated onto liposomes facilitated the internalization of the liposomes by EGFR-over-expressing tumor cells (Kullberg, Nestor, & Gedda, 2003).

3.2 Materials and Methods

3.2.1 Plasmids and cells

Plasmid pSIN-β was kindly provided by Dr. Richard Weiss (University of Salzburg, Salzburg, Austria). The pEGFP C1 plasmid was obtained from Addgene, Inc. (Cambridge, MA). Plasmids were amplified in *E. coli* DH5α under selective growth conditions and purified using a QIAGEN midiprep kit (Valencia, CA) according to the manufacturer's instruction. Large scale plasmid purification was performed by GenScript (Piscataway, NJ). Human breast adenocarcinoma cells (MDA-MB-468, # HTB-132, MDA-MD-231, # HTB-26, MCF-7, # HTB-22) and human epidermoid carcinoma cells (A431, # CRL-1555) were from the American Type Culture Collection (ATCC) and cultured in DMEM medium (Invitrogen, Carlsbad, CA). EL4/PSA cells, kindly provided by Dr. Pavel Pisa in the Karolinska Hospital Institute (Stockholm), were cultured in DMEM medium as well (Invitrogen). All media was supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (all from Invitrogen). It was shown previously that EGFR expression was not detectable in EL-4 cells (Greta Garrido, 2007). The density of EGFR on MDA-MB-468, MDA-MB-231, and MCF-7 cells was reported to be 1×10^6, $1\text{-}2 \times 10^5$, and 1×10^4 per cell, respectively (M. Hu et al., 2007; Reilly et al., 2000; Walker & Dearing, 1999).

3.2.2 Construction of pSIN-EGFP plasmid

To construct pSIN-EGFP plasmid, the enhanced green florescent protein (EGFP) gene from the pEGFP C1 plasmid was PCR-amplified with primers EGFP F 5'-ACAAGTTCTAGAATGGTGAGCAAGGGCGAG-3' and EGFP R 5'-CCTAGAGCATGCTTACTTGTACAGCTCGTC-3'. The PCR product was digested with *Xba*I and *Sph*I, and the EGFP gene was used to replace the β-galactosidase gene downstream of the subgenomic promoter in the pSIN-β. Positive clones were screened for insert using restriction digestion and further confirmed by DNA sequencing (ABI 3730XL DNA analyzer, Applied Biosystems, Foster City, CA).

3.2.3 Preparation of EGF-conjugated, PEGylated liposomes (EGF-PEG-liposomes)

Cationic liposomes were prepared using cholesterol (Sigma-Aldrich, St. Louis, MO), egg phosphatidylcholine (Avanti Polar Lipids, Inc, Alabaster, AL), and 1,2,-dioleoyl-3-trimethylamonium-propane (DOTAP, Avanti) at a molar ratio of (4.6:10.8:12.9). Lipids were mixed in a 20-ml glass scintillation vial followed by solvent evaporation and the formation of a thin film by placing the vial under a constant stream of nitrogen gas. HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (20 mM, 150 mM NaCl, pH 7.4) was added to the film and incubated at room temperature with intermittent vigorous vortexing for 6-24 h. The liposome suspension was forced through polycarbonate filters (1.0, 0.4, and 0.1 μm, sequentially) using a mini-extruder (Avanti) (Le & Cui, 2006). The final concentration of DOTAP in the liposomes (DOTAP liposomes) was 10 mg/ml.

Recombinant murine EGF was from Peprotech Inc. (Rocky Hill, New Jersey). Prior to the conjugation, EGF was thiolated with 2-iminothiolane (Traut's reagent, Sigma-Aldrich). Protein was diluted in PBS (0.01 M with 2.5 mM EDTA, pH 8.0), followed by the addition of Traut's reagent (20 X molar excess). The mixture was incubated for 1 h at room temperature. The protein was purified/desalted using a PD10 column (GE Biosciences, Piscataway, NJ). Thiolated EGF (0.5 mg) was mixed with 1,2-distearoyl-sn-glycero-3-phophoethanonamine-N-[maleimide(polyethylene glycol) 2000] (DSPE-PEG(2000)-maleimide micelles) (Avanti) at a molar ratio of 8.3:568 in PBS (0.1 M, pH 7.4). The mixture was stirred under nitrogen gas for 24 h at room temperature, mixed with an equal volume of DOTAP liposomes, and incubated at 60 °C for 1 h. Un-conjugated proteins were removed by gel permeation chromatography (GPC, Sepharose® 4B, 5 x 135 mm). The EGF protein concentration in the final EGF-conjugated, PEGylated liposomal preparation (EGF-PEG-liposomes) was determined using a CBQCA Protein Quantitation Kit (Invitrogen). DOTAP concentration in the liposomes was determined following a previously reported method (Wang & Langley, 1977). Briefly, in the presence of chloroform, methyl orange (Fisher Scientific, Pittsburgh, PA) reacts with DOTAP, a cationic lipid, to form chloroform-soluble, yellow colored complexes. The intensity of the yellow color in chloroform is proportional to the concentration of methyl orange/cationic lipid complexes measured spectrophotometrically at 415 nm (Wang & Langley, 1977). EGF-free PEGylated liposomes (PEG-liposomes) were prepared similarly by mixing the equivalent amount of DSPE-PEG(2000)-maleimide micelles with preformed DOTAP liposomes.

The particle size and zeta potential of the cationic liposomes, PEG-liposomes, and EGF-PEG-liposomes were measured using a Malvern Zetasizer® Nano ZS (Westborough, MA).

3.2.4 Preparation of plasmid-liposome complexes (lipoplexes) and their sensitivity to DNase I

The plasmid-liposome complexes (lipoplexes) were prepared by mixing equal volumes of plasmid DNA (25 μg) in solution and various amounts of liposomes in suspension (B. L. Rodriguez, et al., 2011). The mixture was incubated at room temperature for at least 15 min before further use. To evaluate the extent to which the plasmid DNA was protected from DNase digestion by complexing with the liposomes, the lipoplexes were incubated with 2 U/μg of DNase I (Fermentas, Glenn Burnie, MD) in a total volume of 400 μl in Tris-HCl buffer (pH 7.5, 10 mM) containing $MgCl_2$ (2.5 mM). Samples were placed at 37 °C for 1 h, and the reaction was stopped by 10 min of incubation at 60 °C in the presence of EDTA (5 mM). The DNA and liposomes were disassociated from each other by incubation (2 h) at room temperature in the presence of NaCl (2 M). DNA was extracted using phenol/chloroform and analyzed by electrophoresis using 1% agarose gel stained with ethidium bromide (Cui & Mumper, 2002). Band intensities were quantified using the GeneSnap software from Syngene G-box (Syngene, Frederick, MD).

3.2.5 Plasmid DNA uptake assay

Cells (1 x 10^5) were seeded in 24-well plates (n = 6-12) and incubated at 37 °C, 5% CO_2 for 24 h or until 60% confluency. The pSIN-β plasmid was labeled using a *Label* IT® fluorescein nucleic acid labeling kit (Mirus, Madison, WI) according to the manufacturer's instruction. Freshly labeled pSIN-β (0.75 μg) was complexed with the EGF-PEG-liposomes or the PEG-liposomes (DOTAP, 12.9 μg) and incubated for at least 15 min at room temperature. The resultant lipoplexes were added to each well and incubated for 1 h at 37 °C, 5% CO_2. Cells were washed with PBS and lysed using Triton X-100 (0.5% in 20 mM Tris, 100 mM NaCl, and 1 mM EDTA) following by incubation at -80 °C for 1 h. The fluorescence intensity was measured at 492/518 nm in a black bottom plate using a BioTek Synergy® Multi-Mode Microplate Reader (Winooski, CT). To understand whether the uptake of the lipoplexes was mediated by the EGF-EGFR interaction, cells were pre-incubated with free EGF (0.1 mg/ml) at 37 °C, 5% CO_2 for 1 h before the addition of the lipoplexes.

3.2.6 Plasmid DNA uptake detected by fluorescence microscopy

MDA-MB-468 or MCF-7 cells (2 x 10^6) were seeded on poly-D-lysine-coated glass coverslips and incubated in 6-well plates at 37 °C, 5% CO_2 for 24 h. Cells were further incubated in the presence of fluorescein-labeled pSIN-β/EGF-PEG-liposome lipoplexes or PEG-liposome lipoplexes (DNA:DOTAP, 3.75 : 64.7 μg) in reduced growth medium for 1 h at 37 °C. After the incubation, cells were washed twice with PBS

and fixed in 3% paraformaldehyde for 20 min at room temperature. Cells were washed with PBS three times, and coverslips were mounted on slides using a mounting medium (vectashield H-1200 with 4',6-diamidino-2-phenylindole (DAPI)) from Vector laboratories (Burlingame, CA). Cells were viewed using an Olympus BX60 Microscope (Olympus America, Inc., Center Valley, PA).

3.2.7 *In vitro* cell transfection and apoptosis assay

MDA-MB-468 cells (10 x 10^6) were seeded and incubated at 37 °C, 5% CO_2 for 24 h or until 60% confluency followed by transfection using pEGFP C1 or pSIN-EGFP (40 μg) complexed with Lipofectamine® (Invitrogen). After 24 h incubation at 37 ^oC, 5% CO_2, cells were detached using 0.05% trypsin/EDTA and re-suspended in PBS with 2% FBS. GFP positive cells were sorted using a FACSAria II Cell Sorter (BD Biosciences, San Jose, CA), re-suspended in fresh medium, and seeded into a 96-well plate (5,000 cells per well). As a control, untransfected cells were also passed through the cell sorter. Cells were stained 0 and 72 h later using a Guava Nexin kit, which contained annexin V and 7-amino actinomycin D (7-AAD), according to the manufacturer's instruction and analyzed using a Guava Easycyte 8HT Flow Cytometry System (Millipore, Hayward, CA). GFP positive cells were gated and analyzed for annexin V and 7-AAD staining. Analysis was performed using the FlowJo Flow Cytometry Analysis Software (Ashland, OR).

3.2.8 Animal studies

All animal studies were carried out following National Institutes of Health guidelines for animal care and use. Animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Female athymic nu/nu mice (6–8 weeks) were from Charles River laboratories, Inc. (Wilmington, MA). Mice were subcutaneously injected in the right flank with MDA-MB-468 or A431 cells (1×10^7) admixed with BD Matrigel™. When tumors reached an average diameter of 5 mm for the MDA-MB-468 cells and 6.5-7 mm for the A431 cells, the pSIN-β/EGF-PEG-liposome lipoplexes or the pSIN-β/PEG-liposome lipoplexes (DNA:DOTAP, 25:431 μg) were injected subcutaneously peritumorally (s.c., p.t.) for 14 consecutive days (B. L. Rodriguez, et al., 2011). Tumor size was measured using a digital caliper, and tumor diameter was calculated using the following equation: tumor diameter = (length + width)/2.

3.2.9 Hematoxylin and eosin (H&E) staining

Tumors were fixed in formalin and embedded in paraffin prior to sectioning. Sections of 7 μm were cut and stained with H&E.

3.2.10 Immunohistochemical staining for EGFR, CD31, Ki67 and TUNEL assay

MDA-MB-468 tumors were harvested from mice that had been treated for 10 d with the pSIN-β/EGF-PEG-liposome or pSIN-β/PEG-liposome lipoplexes. Tumors were fixed in formalin, embedded in paraffin, and sectioned. After deparaffinization, section was subjected to antigen retrieval by microwaving in sodium citrate (10 mM, pH 6.0) for

20 min, washed in PBS with 0.2% Triton-X 100, and incubated with PBS with 0.1% Tween 20, 5% horse serum albumin (HSA) for 1 h. Slides were incubated for 1 h at 37 °C with anti-EGFR-Alexa fluor 488 (Millipore, diluted 1/1,000) in 5% HSA in PBS. After incubation, slides were washed with PBS. Terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) analysis was performed with an *in situ* cell death detection kit from Roche Diagnostics (Indianapolis, IN). Briefly, the TUNEL reaction mix (50 μl) was placed on the sections and incubated for 60 min at 37 °C. Sections were washed with PBS three times, mounted using the vectashield H-1200 mounting medium, and analyzed using an Olympus BX60 Microscope. Moreover, tumor sections were also stained with antibodies against Ki67 and CD31 as markers of cell proliferation and angiogenesis, respectively, in the Histology Core at the University of Texas M.D. Anderson Center Science Park Research Division (Smithville, TX). Slides were examined under a bright-field microscope. The number of blood vessels per 14.6 mm^2 (n = 13-37 per treatment) and the percent of Ki67 positive cells in an area of 0.04 mm^2 (n = 9 per treatment) were determined.

3.2.11 Statistical analysis

Statistical analyses were completed using ANOVA followed by the Fisher's protected least significant difference procedure. Mouse survival curves were compared using the Kaplan–Meier survival analysis (GraphPad Prism®, La Jolla, CA). A p-value of ≤ 0.05 (two-tail) was considered statistically significant.

3.3 Results

3.3.1 Preparation and characterization of pSIN-β/EGF-PEG-liposome lipoplexes

EGF-conjugated, PEGylated cationic liposomes (EGF-PEG-liposomes) were prepared by mixing cationic DOTAP liposomes with EGF-conjugated DSPE-PEG(2000) micelles followed by 1 h of incubation at 60 ^{o}C. After the removal of the un-conjugated EGF, the concentration of the EGF and DOTAP in the EGF-PEG-liposomes were determined to be 1.94 ± 0.08 μg/ml and 6.9 ± 2.0 mg/ml, respectively. The EGF-free, PEGylated cationic liposomes (PEG-liposomes) were prepared similarly except that an equivalent amount of DSPE-PEG(2000) micelles were not conjugated with EGF before they were mixed with the preformed cationic DOTAP liposomes. The diameter and zeta potential of the EGF-PEG-liposomes were 143 ± 1 nm and 2.2 ± 0.1 mV. The PEG-liposomes were 134 ± 2 nm, with a zeta potential of 13.3 ± 0.7 mV. The liposomes were then complexed with the pSIN-β plasmid to form lipoplexes.

The following experiments were completed to identify the optimal ratio of pSIN-β to liposomes in the lipoplexes. Various amounts of EGF-PEG-liposomes were complexed with a fixed amount of pSIN-β to form different pSIN-β/EGF-PEG-liposome lipoplexes. The particle diameters and the zeta potentials of the resultant lipoplexes are shown in Fig. 3.1A. At the ratio of 8.63:1 (DOTAP vs. pSIN-β, w/w), the lipoplexes appeared unstable and aggregated (Fig. 3.1A), whereas lipoplexes prepared at other ratios

had a smaller size of around 200 nm (Fig. 3.1A). The zeta potential of the lipoplexes peaked at the DOTAP lipid to DNA ratio of 8.63:1 as well (Fig. 3.1A).

The fraction of undigested (intact) pSIN-β plasmid when complexed with increasing amount of EGF-PEG-liposomes, and then digested with DNase I is shown in figure 3.1B. As expected, increasing the ratio of the liposomes to plasmid DNA protected more DNA from DNase I digestion. At the ratio of 17.25:1 (DOTAP vs. pSIN-β), about 70% of the pSIN-β was protected from the DNase I digestion (Fig. 3.1B). At the ratios of 34.5:1 and 86.25:1, almost 100% of the pSIN-β was protected from DNase I digestion.

In order to identify the liposomes (or DOTAP lipid) to DNA ratio that was optimal for transfecting the pSIN-β into tumor cells, the uptake of the pSIN-β in various lipoplexes by the MDA-MB-468 cells was evaluated, and the ratio of 17.25:1 was found optimal based on the highest level of cellular uptake of the fluorescein-labeled pSIN-β at that ratio (Fig. 3.1C). The 17.25:1 ratio was the optimal ratio for the PEG-liposomes as well (Fig.3.1C). Therefore, the lipoplexes prepared at the DOTAP to pSIN-β ratio of 17.25:1 (w/w) were used for further studies. Shown in Fig. 3.1D are the diameters and zeta potentials of the PEG-liposomes and the EGF-PEG-liposomes, before and after complexing with the pSIN-β plasmid at the DOTAP to DNA ratio of 17.25:1 (w/w). For comparison, the diameter and zeta potential of the un-PEGylated DOTAP liposomes are also included (Fig. 3.1D).

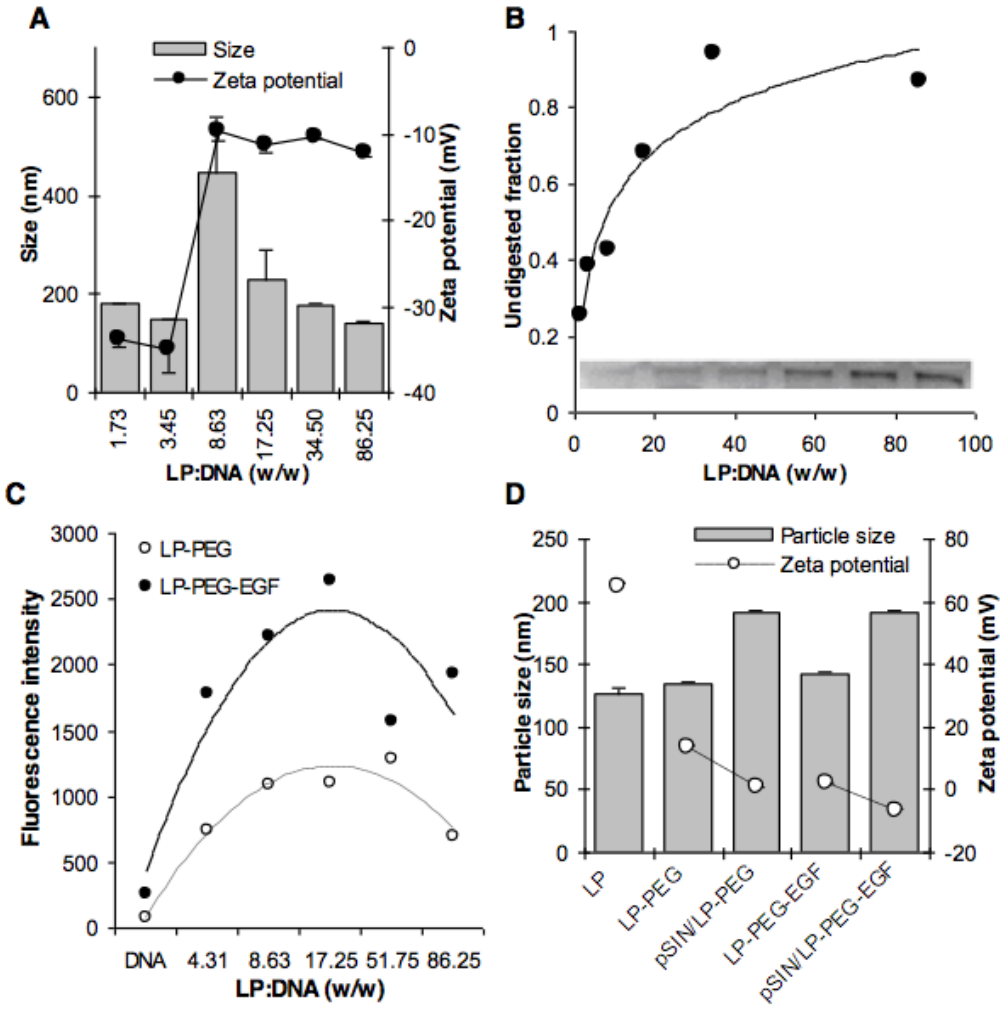

Figure 3.1 Physicochemical parameters of the pSIN-β/EGF-PEG-liposome lipoplexes.

(A). Mean diameter and zeta potential of pSIN-β/EGF-PEG-liposome lipoplexes at various plasmid to liposome ratios. Equal volumes of DNA (25 μg) and EGF-PEG-liposomes were mixed and allowed to incubate at room temperature for at least 15 min. (B). Complexation of the pSIN-β with liposomes protected it from DNase I. pSIN-

β/EGF-PEG-liposome lipoplexes prepared at various plasmid to liposome ratios were incubated with DNase I. DNA was extracted and analyzed using 1 % agarose gel stained with ethidium bromide. Weight ratios of DOTAP lipid to pSIN-β for the DNA bands in the inset are 1.73 (lane 1), 3.45 (lane 2), 8.63 (lane 3), 17.25 (lane 3), 34.5 (lane 5), and 86.25 (lane 6). The experiment was repeated twice with similar results. (C). The uptake of pSIN-β by MDA-MB-468 cells in culture. Cells (1 x 10^5/well) were incubated with fluorescein-labeled pSIN-β complexed with EGF-PEG-liposomes or PEG-liposomes at various ratios for 1 h at 37 °C, washed, and lysed before measuring the fluorescence intensity (492/518 nm). (D). Mean diameter and zeta potential of PEG-liposomes or EGF-PEG-liposomes before and after complexation with pSIN-β at the ratio of 17.25:1 (w/w, pSIN-β, 25 μg). Except in B, data shown are from at least 3 independent determinations. S.E.M. was not included in (C) for clarity.

(LP, liposomes; DNA, pSIN-β; LP-PEG, PEG-liposomes; LP-PEG-EGF, EGF-PEG-liposomes).

3.3.2 *In vitro* uptake of the pSIN-β in lipoplexes by cells expressing different levels of EGFR

The uptake of fluorescein-labeled pSIN-β by cells expressing different levels of EGFR was evaluated after the cells were incubated for 1 h with lipoplexes prepared with the EGF-PEG-liposomes or the PEG-liposomes. The extent to which the pSIN-β was taken up by the EL-4 cells was not dependent on whether the pSIN-β was in the lipoplexes prepared with EGF-PEG-liposomes or with the EGF-free PEG-liposomes, as indicated by the ratio of 1 in Fig. 3.2A. It was previously reported that EGFR expression was not detected in EL-4 cells (Greta Garrido, 2007). However, in the cells that express various levels of EGFR, the ratio of the uptake of the pSIN-β in the EGF-PEG-liposome lipoplexes over that in the PEG-liposome lipoplexes was correlated to the EGFR density on the cells. The ratio was the largest in the MDA-MB-468 cells, followed by the MDA-MB-231 cells, and then by the MCF-7 cells (Fig. 3.2A). It is known, and our RT-PCR data also confirmed, that the relative expression of the EGFR in the cell lines was MDA-MB-468 > MDA-MB 231 > MCF-7 (data not shown).

To further investigate whether the uptake of the pSIN-β/EGF-PEG-liposome lipoplexes was mediated by the binding of the EGF on the lipoplexes to the EGFR on cell surface, cells were incubated with free EGF for 1 h prior to the addition of the lipoplexes. The uptake of the pSIN-β in the EGF-PEG-liposome lipoplexes was significantly inhibited in the MDA-MB-468 cells (Fig. 3.2B), but not in the MCF-7 cells (Fig. 3.2C). Moreover, the uptake of the pSIN-β in the PEG-liposome lipoplexes by both MDA-MB-

468 cells and MCF-7 cells was not significantly affected by pre-incubation of the cells with free EGF (Fig. 3.3C).

Finally, the fluorescent microscopic images in Figure 3.3 showed that after 1 h of incubation, the uptake of the fluorescein-labeled pSIN-β in the EGF-PEG-liposome lipoplexes by the MDA-MB-468 cells was more extensive than by the MCF-7 cells. However, the uptake of pSIN-β was significantly less in both cell lines when it was complexed with the EGF-free PEG-liposomes (Fig. 3.3).

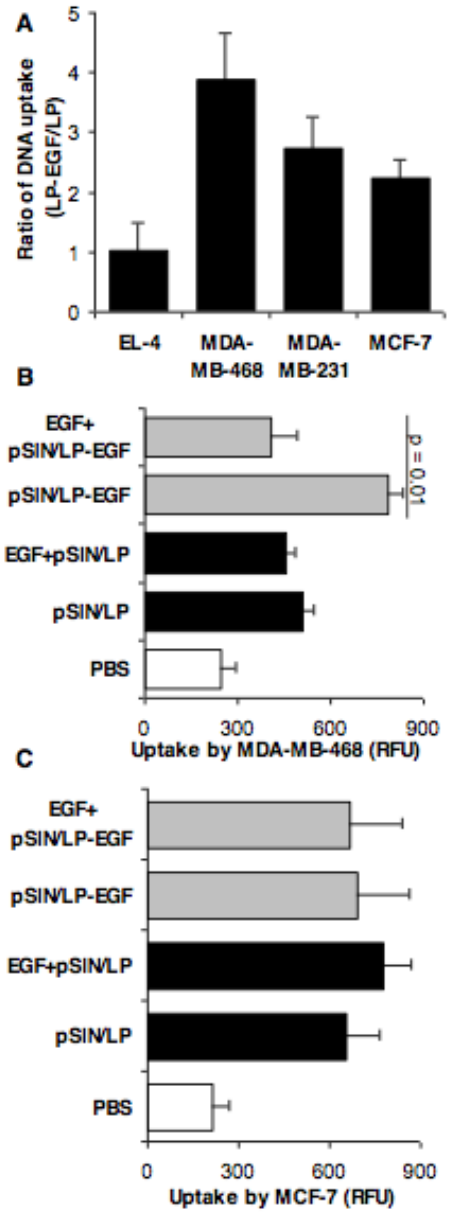

Figure 3.2 *In vitro* uptake of the pSIN-β in EGF-PEG-liposome lipoplexes or PEG-liposome lipoplexes by tumor cells expressing different levels of EGFR.

(A). Cells (1 x 10^5/well) were incubated with fluorescein-labeled pSIN-β (0.75 μg) complexed with EGF-PEG-liposomes (LP-EGF) or PEG-liposomes (LP) (12.9 μg) for 1 h at 37 °C, washed with PBS, and lysed with Triton X-100 to measure fluorescence intensity. Data shown are the fluorescence intensity in cells incubated with pSIN-β/LP-EGF divided by that in cells incubated with pSIN/LP. **(B, C).** Pre-incubation of cells (1 x 10^5/well) with free EGF inhibited the uptake of the pSIN-β/EGF-PEG-liposome lipoplexes by the MDA-MB-468 cells (B), but not by the MCF-7 cells (C). Cells were pre-incubated with EGF (0.1 mg/ml) at 37 °C for 1 h before the addition of the lipoplexes. Data shown are mean ± S.E.M. (n = 6-12).

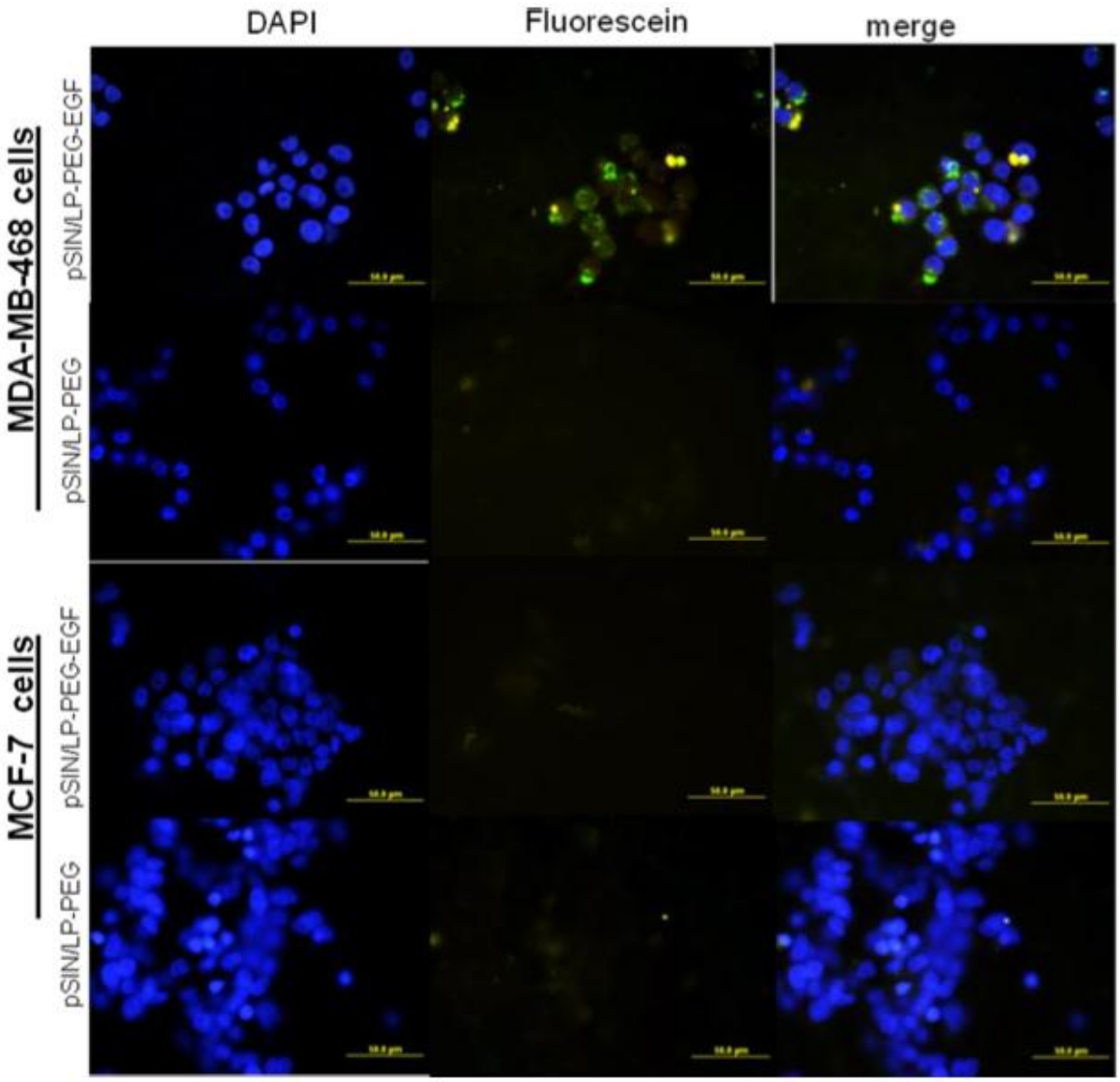

Figure 3.3 Fluorescent images of cells incubated with fluorescein-labeled pSIN-β-liposome lipoplexes.

MDA-MB-468 or MCF-7 cells (2 x 10^6) were incubated with fluorescein-labeled pSIN-β complexed with EGF-PEG-liposomes (LP-PEG-EGF) or PEG-liposomes (LP-PEG) for 1 h at 37 °C. After incubation, cells were washed twice with PBS, fixed in 3 % paraformaldehyde, washed again, and mounted using vectashield medium. Cell nucleus was stained with DAPI (blue) (bar = 50 mm).

3.3.3 The pSIN-β plasmid was more effective at controlling the growth of MDA-MB-468 tumors in mice when complexed with the EGF-PEG-liposomes than with the EGF-free, PEG-liposomes

The anti-tumor activities of the pSIN-β complexed with the EGF-PEG-liposomes or the EGF-free, PEG-liposomes against MDA-MB-468 tumors were evaluated *in vivo*. As shown in Fig. 3.4, the pSIN-β/EGF-PEG-liposome lipoplexes were more effective than the pSIN-β/PEG-liposome lipoplexes at controlling the tumor growth. Starting on day 15, tumors in mice that were treated with the pSIN-β/EGF-PEG-liposome lipoplexes became significantly smaller than in mice that were treated with the pSIN-β/PEG-liposome lipoplexes (Fig. 3.4A). On day 76, tumors in all mice that were treated with the pSIN-β/EGF-PEG-liposome lipoplexes regressed completely. No tumor mass was visible or palpable thereafter. Tumors in mice that were treated with the pSIN-β/PEG-liposome lipoplexes partially regressed. The median survival time for mice treated with sterile PBS was 57 d, 161 d for mice that were treated with the pSIN-β/PEG-liposome lipoplexes (Fig. 3.4B).

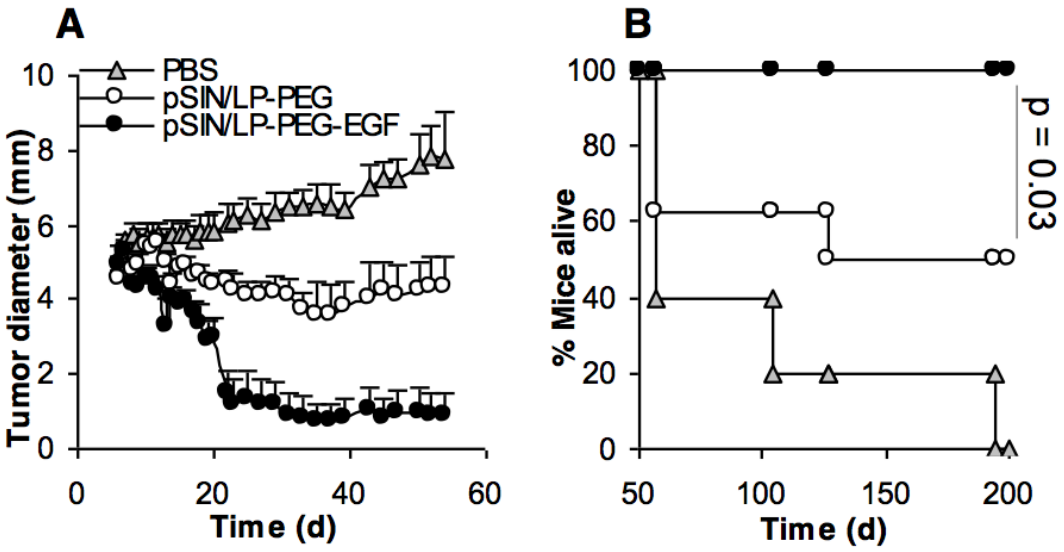

Figure 3.4 pSIN-β was more effective at controlling the growth of MDA-MB-468 tumors in mice when complexed with the EGF-PEG-liposomes.

MDA-MB-468 (1 x 10^7) cells were established in athymic mice (n = 5-8) and treated when tumors reached an average diameter of 5 mm with the lipoplexes for 14 consecutive days. (A). Tumor growth curves. Starting from day 15, the values of the pSIN/LP-PEG-EGF and the pSIN/LP-PEG were different from each other ($p < 0.05$). (B). Mouse survival curves ($p = 0.03$, pSIN/LP-PEG-EGF vs. pSIN/LP-PEG).

3.3.4 Greater pro-apoptotic, anti-proliferative, and anti-angiogenic activities were detected in tumors in mice that were treated with pSIN-β complexed with the EGF-PEG-liposomes than with the PEG-liposomes

MDA-MB-468 tumors in mice were treated for 10 d with pSIN-β/EGF-PEG-liposome lipoplexes or PEG-liposome lipoplexes; tumors were harvested 24 h after the last treatment for histological assays. Extensive apoptosis (i.e., TUNEL positive staining) was detected in tumors in mice that were treated with the pSIN-β/EGF-PEG-liposome lipoplexes, and the apoptotic cells were co-localized with EGFR positive cells (Fig. 3.5). By contrast, significantly fewer apoptotic cells were detected in tumors in mice that were treated with the pSIN-β/PEG-liposome lipoplexes or with sterile PBS (Fig. 3.5). Data in Fig. 3.6 showed that the pSIN-β also induced apoptosis when transfected into MDA-MB-468 cells in culture. The pSIN-EGFP, instead of pSIN-β, was used because the EGFP gene in the pSIN-EGFP allowed the sorting and gating of the transfected cells based on the expression of the EGFP gene. Cells were transfected with pEGFP C1 or pSIN-EGFP plasmids. Twenty-four hours later, GFP positive cells were sorted and cultured for 72 hours. Cells were stained with annexin V and 7-AAD to determine the extent of apoptosis at 0 and 72 h. At 0 h, the percent of viable cells, cells in the early apoptotic stage, or in the late apoptotic stage were not different regardless of the plasmid used for transfection. However after 72 h, all GFP positive cells transfected with the pSIN-EGFP plasmid were in the late apoptotic stage, whereas the percent of viable GFP positive cells that were transfected with the pEGFP C1 plasmid increased by almost three-fold (Fig. 3.6B).

Typical H&E images of MDA-MB-468 tumors after treatment with PBS, pSIN-β complexed with PEG-liposomes, or pSIN-β complexed with EGF-PEG-liposomes were shown in Fig. 3.7A (H&E). A moderate regression of tumors was observed in mice that were treated with pSIN-β complexed with the PEG-liposomes, while a significant regression of tumors was observed in mice that were treated with pSIN-β complexed with the EGF-PEG-liposomes (Fig. 3.7A, H&E). Tumors in mice that were treated with PBS exhibited a high level of Ki67 positive staining, a marker of cell proliferation (Fig. 3.7A); and a lower percent of Ki67 positive cells was detected in tumors in mice that were treated with pSIN-β complexed with the PEG-liposomes. However, the percent of Ki67 positive cells in tumors in mice that were treated with pSIN-β complexed with the EGF-PEG-liposomes was significantly lower (> 50%) than that in mice that were treated with pSIN-β complexed with the PEG-liposomes ($p < 0.001$) (Figs. 3.7A, B). CD31 is a marker of endothelial cells. Anti-CD31 staining revealed extensive vascularization in tumors in mice that were treated with PBS (Fig. 3.7A). The extent of CD31 positive staining was decreased in tumors in mice that were treated with the pSIN-β/PEG-liposome lipoplexes, relative to that in mice that received PBS ($p = 0.003$) (Fig. 3.7C). Finally, a significantly lower extent of CD31 positive staining was detected in tumors in mice that were treated with pSIN-β complexed with the EGF-PEG-liposomes than with the PEG-liposomes ($p < 0.001$) (Figs. 3.7A, C).

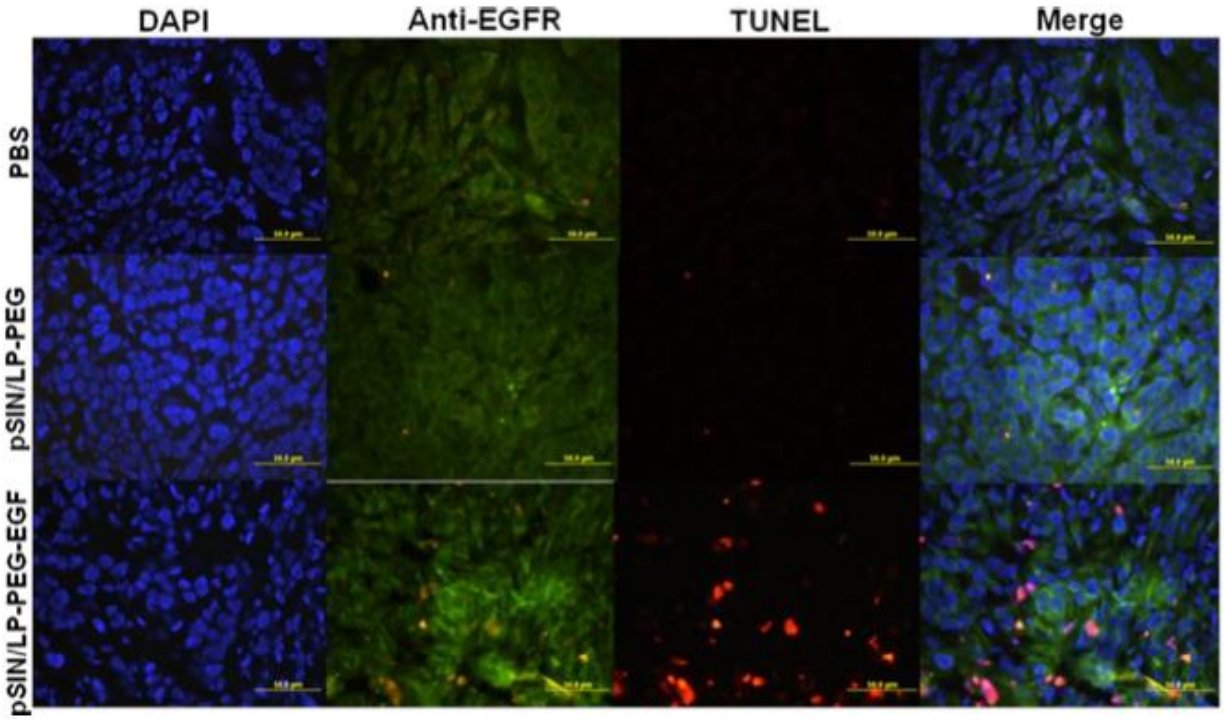

Figure 3.5 pSIN-β induced more apoptosis in MDA-MB-468 tumors in mice when complexed with the EGF-PEG-liposomes than with the PEG-liposomes.

MDA-MB-468 tumors in nude mice (n = 4) were treated with pSIN-β complexed with EGF-PEG-liposomes (LP-PEG-EGF) or PEG-liposomes (LP-PEG) for 10 consecutive days. Tumors were fixed in formalin, embedded in paraffin, sectioned, and stained against EGFR with anti-EGFR alexa fluor 488 (green) and apoptosis using an *in situ* cell death detection kit based on TUNEL analysis (red). Cell nucleus was stained with DAPI (blue), (bar = 50 mm).

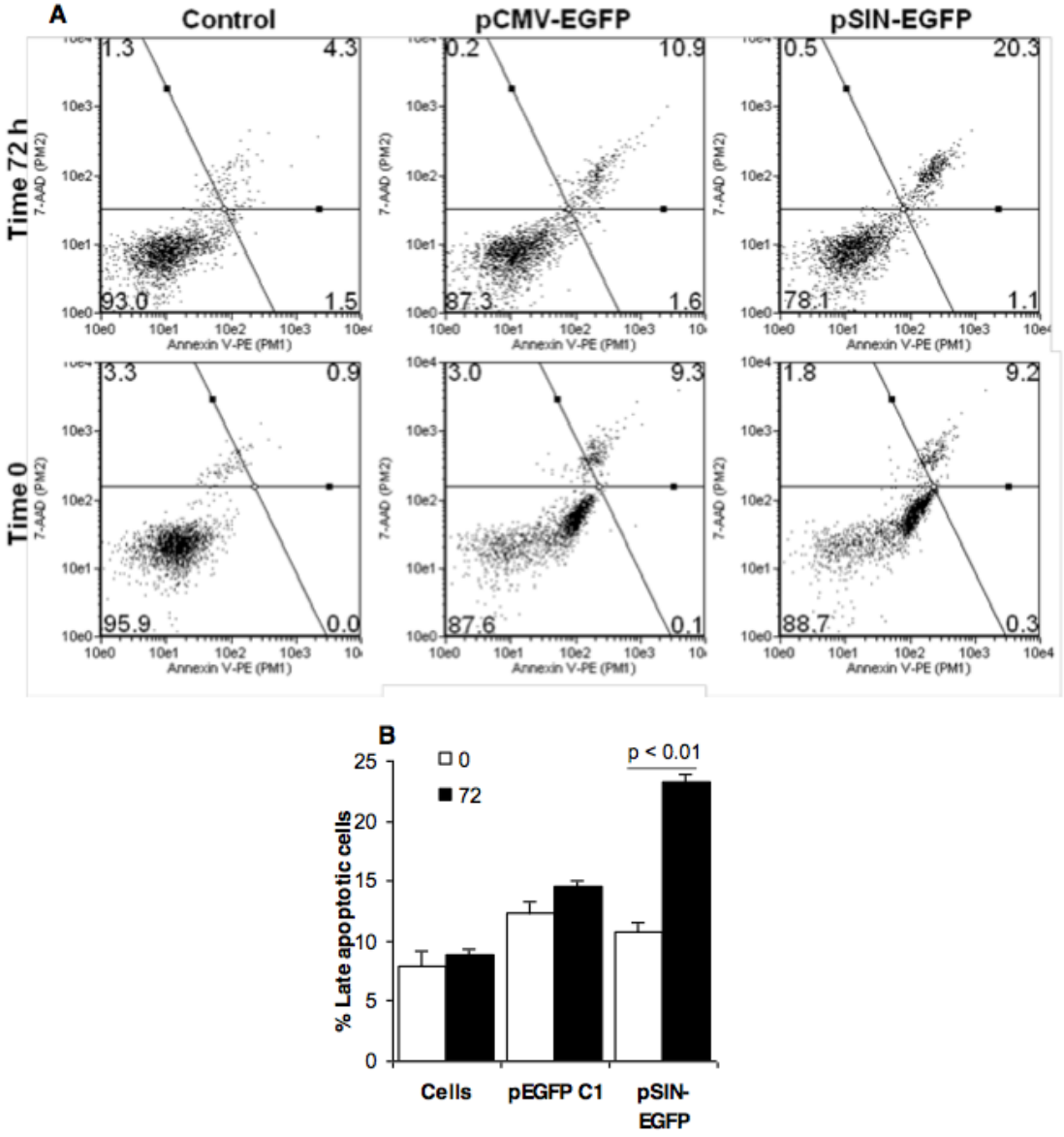

Figure 3.6 pSIN-EGFP induced MDA-MB-468 cells to undergo apoptosis in culture. Cells (1 x 10^7) were transfected with pSIN-EGFP (40 μg) or pEGFP C1 (40 μg) and sorted for GFP positive cells 24 h later. The sorted cells were staining with Annexin V-PE and 7-AAD 0 and 72 h after sorting. (A). Flow cytometry graphs of cells after annexin V and 7-AAD staining. Analysis was performed on GFP positive cells only. Upper right

quadrant represents cells in late apoptotic stage; lower right, cells in early apoptotic stage; lower left, viable cells. Cells in the PBS group were passed through the cell sorter, but not GFP-gated. (B). A comparison of the % of GFP positive cells that were in the late apoptotic stage, early apoptotic stage, or were viable 0 or 72 h after cell sorting. Data shown are mean ± S.D. from three replicates.

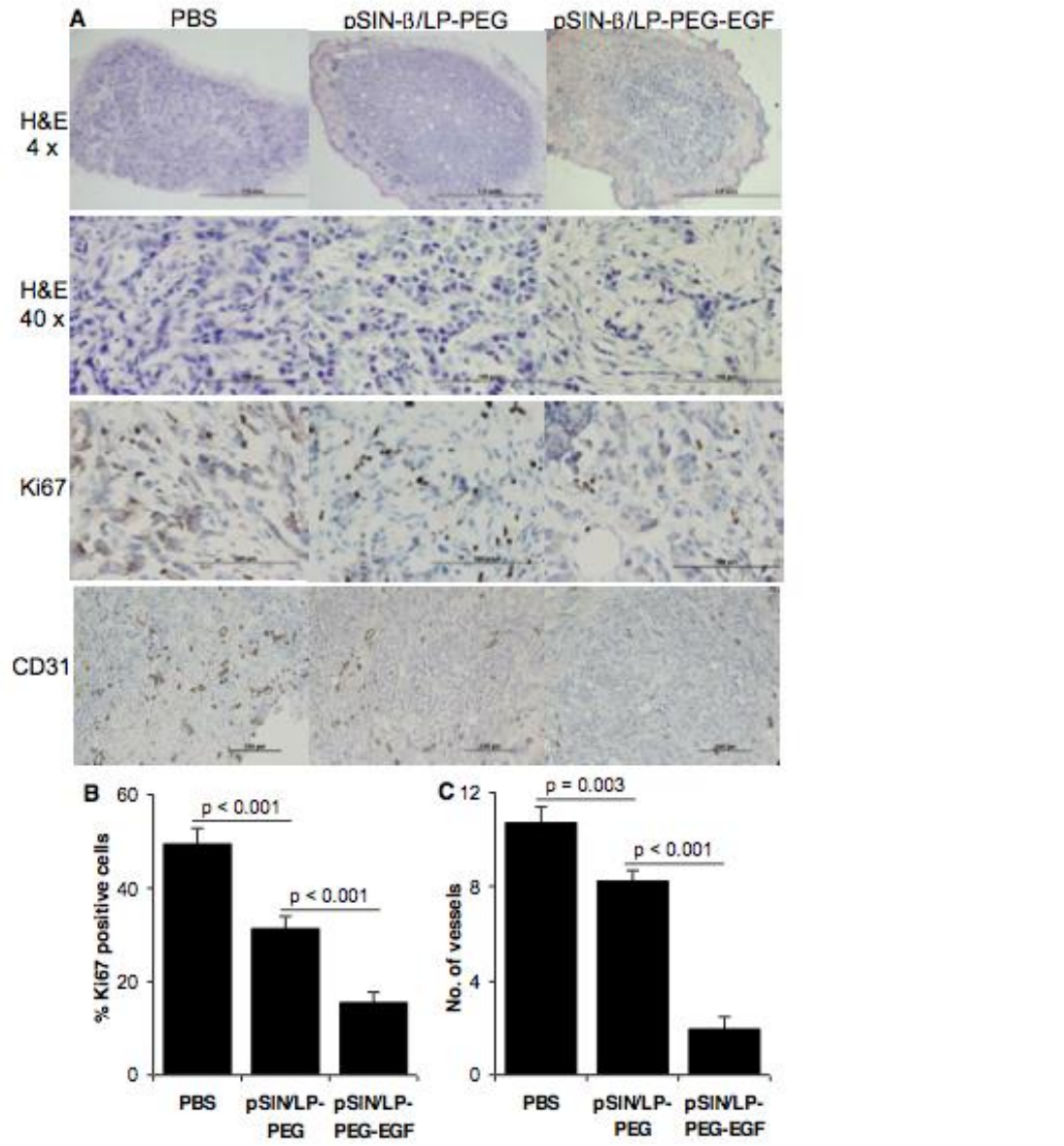

Figure 3.7 Greater anti-proliferative and anti-angiogenic activities were detected in tumors in mice that were treated with pSIN-β complexed with the EGF-PEG-liposomes than with the PEG-liposomes.

(A). Images of MDA-MB-468 tumor tissues after H&E staining or staining with anti-Ki67 or anti-CD31. MDA-MB-468 tumors in nude mice were treated with pSIN-β

complexed with EGF-PEG-liposomes (LP-PEG-EGF) or PEG-liposomes (LP-PEG) for 10 consecutive days before they were harvested, fixed in formalin, embedded in paraffin, sectioned, and stained. (B). The % of Ki67 positive cells in tumors in mice that received different treatments. % of Ki67 positive cells was calculated based on the total number of brown cells divided by the total number of cells. An area of 0.04 mm^2 was analyzed (n = 9). (C). The average number of blood vessels per 14.6 mm^2 (n = 13-37 per treatment).

3.3.5 The pSIN-β plasmid was more effective against the A431 tumors in mice when complexed with the EGF-PEG-liposomes than with the EGF-free PEG-liposomes

It was reported that A431 cells over-express EGFR as well (1-3 x 10^6 per cell) (Carpenter & Cohen, 1990). When left untreated, A431 tumors grew aggressively in nude mice, with a median mouse survival time of 17 days (Fig. 3.8). A431 tumor-bearing mice that were treated with the pSIN-β/PEG-liposome lipoplexes had a median survival time of 15 days (Fig. 3.8). However, the median survival time for A431 tumor-bearing mice that were treated with the pSIN-β/EGF-PEG-liposome lipoplexes was 28 days, significantly longer than the median survival time of mice that were treated with the pSIN-β/PEG-liposome lipoplexes ($p = 0.017$) (Fig. 3.8).

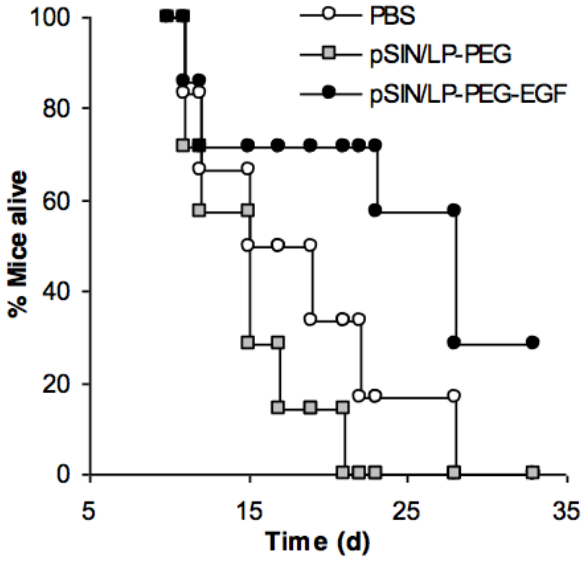

Figure 3.8 A431 tumor-bearing mice survived longer when treated with the pSIN-β/EGF-PEG-liposome lipoplexes than with the pSIN-β/PEG-liposome lipoplexes. Nude mice (n = 6-7) were implanted with A431 cells (1 x 10^7). When tumors reached an average diameter of 6.5-7 mm, treatment with pSIN-β/EGF-PEG-liposome lipoplexes or pSIN-β/PEG-liposome lipoplexes was initiated and continued for 14 consecutive days (pSIN/LP-PEG-EGF vs. pSIN/LP-PEG, p = 0.017).

3.4 Discussion

Previously, it was shown that pSIN-β, a plasmid DNA that encodes the Sindbis viral RNA replicase complex (non-structure proteins 1-4) had anti-tumor activity, probably due to the pro-apoptotic dsRNA generated by the RNA replicase complex expressed in tumor cells transfected with the plasmid (B. L. Rodriguez, et al., 2011). The ability of the dsRNA and the CpG motifs on the plasmid to activate innate immunity likely also contributed to the observed anti-tumor activity (McCray, et al., 2006; B. L. Rodriguez, et al., 2011; Whitmore, et al., 1999). In the present study, the hypothesis that actively targeting the RNA replicase-based plasmid into tumor cells will further improve the anti-tumor activity of the plasmid was tested. It was shown that targeting the pSIN-β plasmid into tumors that over-express the EGFR significantly improved its anti-tumor activity, probably because the targeted pSIN-β induced more tumor cell to undergo apoptosis and exhibited enhanced anti-proliferative and anti-angiogenic activities.

It is well documented that dsRNA has anti-tumor activities. However, data in clinical trials showed that the anti-tumor activity from systemically dosed synthetic dsRNA was weak, inconsistent, and associated with severe adverse effects (Absher & Stinebring, 1969; Meier, et al., 1970; Okada, et al., 2005; Pimm & Baldwin, 1976). Recently, interest in further exploring the anti-tumor activity of dsRNA is revived, largely due to the findings that when delivered intracellularly, dsRNA induced more extensive tumor cell death, and can more effectively activate innate immunity and orchestrate the induction of adaptive immune responses (Cui, et al., 2007; McBride, et al., 2006; Schulz, et al., 2005; Shir, et al., 2006). Instead of utilizing synthetic dsRNA, we

decided to deliver a plasmid DNA that encodes the Sindbis viral RNA replicase genes, and thus enabling the transfected cells to produce dsRNA. It is expected that dsRNA tumor therapy using the RNA replicase-based plasmid DNA has the following advantages: (i) Plasmid DNA is relatively more stable than dsRNA; (ii) Plasmid DNA purified from bacteria has an inherit anti-tumor activity, due to the immuno-stimulatory activity of the unmethylated CpG motifs on the plasmid (McCray, et al., 2006; Whitmore, et al., 1999); and (iii) only cells that are transfected with the RNA replicase-based plasmid will produce dsRNA, and thus being exposed to the dsRNA. The fact that only cells transfected with the RNA replicase-based plasmid will produce dsRNA is important, because the dsRNA is generated endogenously and is expected to be cytotoxic mainly to the cells with the intracellular dsRNA. In other words, delivering RNA replicase-based plasmid DNA into tumor cells can potentially take advantage of the potent anti-tumor activity of intracellular dsRNA, while minimizing the adverse effects caused by the direct injection of synthetic dsRNA. The present study was devised to actively target the RNA replicase-based plasmid into tumor cells to more specifically allow the tumor cells to take up the plasmid, generate intracellular dsRNA, and then undergo apoptosis by committing "suicide".

EGFR is expressed universally, but over-expressed (10-1000-fold) in many tumor cells (Su et al., 2001; Su et al., 2004). It has been exploited as a target to develop a number of novel tumor therapeutics (e.g., Gefitinib and Cetuximab) (Baselga, 2000; Ranson, et al., 2002). EGFR was also used as a target to facilitate the internalization of liposomes surface-conjugated with an EGFR ligand (such as the EGF) by tumor cells that

over-express EGFR (Kullberg, et al., 2003; Yarden, 2001). In the present study, EGF was chemically conjugated onto the cationic liposomal carrier of the pSIN-β plasmid to target the pSIN-β into EGFR-over-expressing human tumor cells *in vitro* and *in vivo*. Murine EGF was used because the *in vivo* studies in the present study were carried out in mice, and data from previous studies showed that murine EGF has similar biological activities as the human EGF (Nakagawa, Yoshida, Hirao, Kasuga, & Fuwa, 1985). Human breast adenocarcinoma cells MDA-MB-468, MDA-MB-231, and MCF-7 were used because they express different levels of EGFR (1×10^6, 2×10^5, and 1×10^4 per cell, respectively) (Reilly, et al., 2000; Walker & Dearing, 1999). *In vitro* data showed that the EGF-conjugated cationic liposomes more effectively delivered the pSIN-β plasmid into EGFR-expressing tumor cells than the EGF-free liposomes, and the extent to which the tumor cells took up the pSIN-β carried by the EGF-conjugated liposomes was correlated to the density of the EGFR on the cell surface. The facilitated uptake of the pSIN-β plasmid in the EGF-conjugated liposomes by the EGFR-over-expressing tumor cells was mediated by the EGF-EGFR interaction, because pre-incubation of the tumor cells with free EGF significantly inhibited the uptake of the plasmid (Fig. 3.2), whereas pre-incubation with free EGF did not significantly affect the uptake of the pSIN-β plasmid complexed with the EGF-free PEG-liposomes (Figs. 3.2B, C). It was noticed that the uptakes of the pSIN-β/PEG-liposome lipoplexes by different cells were not identical. For example, a comparison of data in Fig. 3.2B and Fig. 3.2C revealed that the uptake of the pSIN-β/PEG-liposome lipoplexes by the MCF-7 cells was higher than the uptake of the same

pSIN-β/PEG-liposome lipoplexes by the MDA-MB-468 cells and the MDA-MB-231 cells (data not shown). The pSIN-β/PEG-liposome lipoplexes are physically nanoparticles; the extent to which a specific cell line can take up them is likely determined mainly by the cell's endocytosis activity.

In athymic mice with pre-established human MDA-MB-468 tumors, treatment with the pSIN-β carried by the EGF-conjugated liposomes more effective controlled the tumor growth than with the same pSIN-β carried by EGF-free liposomes (Fig. 3.4). In fact, the EGFR-targeted pSIN-β completely eliminated the MDA-MB-468 tumors in all mice (Fig. 3.4). When the A431 epidermoid carcinoma was used, mice that were treated with the pSIN-β carried by the EGFR-targeted liposomes survived significantly longer than mice that were treated with pSIN-β carried by the un-targeted liposomes (Fig. 3.8). The pSIN-β was unable to cause the regression of the A431 tumors in the present study, likely because a large number of tumor cells were implanted, and the tumors grew aggressively, more than 1 mm a day. Moreover, the pSIN-β treatment was not started until the tumors reached 6-7 mm in diameter.

In vivo, the pSIN-β carried by the EGFR-targeting liposomes induced significantly more tumor cells to undergo apoptosis than the pSIN-β carried by the non-targeted liposomes (Fig. 3.5), which may explain why the EGFR-targeted pSIN-β generated a more potent anti-tumor activity. The enhanced anti-proliferative and anti-angiogenic activities from the pSIN-β complexed with the EGF-PEG-liposomes as shown in Figure 3.7 may have also contributed to the more potent anti-tumor activity from the

pSIN-β/EGF-PEG-liposome lipoplexes. Because immuno-compromised athymic mice were used for the *in vivo* studies, it is unlikely that adaptive immune response had played a significant role in the anti-tumor activity from the pSIN-β plasmid, although data from a previous study showed that in immuno-competent mice, T cell-mediated adaptive immune response contributed to the anti-tumor activity of the pSIN-β plasmid (B. L. Rodriguez, et al., 2011). However, it is expected that the activation of innate immunity by the pSIN-β plasmid, the dsRNA produced by the plasmid, and the type I interferons induced by the dsRNA may have contributed, to a certain extent, to the anti-tumor activity observed.

In the present study, EGFR was used as the target for the delivery of the RNA replicase-based plasmid. EGFR is over-expressed in a variety of tumor cells, but many other targets such as folate receptor or integrins are also over-expressed in many tumors cells and may be potentially exploited to target the RNA replicase-based plasmid into specific tumor cells of interest. Finally, the RNA replicase-based plasmid was given by peritumoral injection, which is not ideal for the administration to many tumors, but tumors such as glioblastoma, head and neck tumors, non-metastasized melanoma and lung cancer are localized and may be ideal tumors for molecular therapy using the RNA replicase-based plasmid by intratumoral or peritumoral injection. Indeed, clinical studies of intratumoral injection for gene therapy have already been performed (Clayman et al., 1998; Hersh et al., 1994; Roth et al., 1996). Tumors in the livers may also be treated by localized injection (Y. H. Hu et al., 2010; Lencioni et al., 2010). The feasibility of

treating orthotopic and spontaneous tumors in mice with the RNA replicase-based plasmid is currently under evaluation.

3.5 Conclusions

Treatment with an RNA replicase-based plasmid that can generate dsRNA in tumor cells transfected with the plasmid represents a promising cancer molecular therapy. Targeting the RNA replicase-based plasmid more specifically into tumor cells further improved the anti-tumor activity of the plasmid, likely by enhancing its pro-apoptotic, anti-proliferative, and anti-angiogenic activities.

Chapter Four

Antitumor activity of tumor-targeted RNA replicase-based plasmid that express interleukin-2 in murine melanoma[3]

4.1 Introduction

Advanced melanoma remains a significant problem although cancer therapy has improved dramatically over the past decades. Melanoma is a malignancy with the worst prognosis, and death rates have been rising faster than those of other cancers (Blank, Hooijkaas, Haanen, & Schumacher, 2011). The median survival time for patients with metastatic melanoma is between 6 and 10 months (Puzanov & Flaherty, 2010). Metastatic melanoma is one of the most resistant cancers to single agents, combination chemotherapy, and immunotherapy.

IL2 therapy is clinically efficacious in patients with advanced melanoma and renal cell carcinoma (Foa, Guarini, & Gansbacher, 1992). IL2 is the only FDA-approved immunotherapeutic agents for patients with metastatic melanoma with an overall response rate of 13-17% (Riker, Radfar, Liu, Wang, & Khong, 2007). IL2 has no direct impact on cancer cells; its antitumor effects are due to its ability to modulate immune responses (Rosenberg, Yang, White, & Steinberg, 1998). IL2 affects cytotoxic $CD8^+$ T-cells, $CD4^+$ T-cells, natural killer (NK) cells, B-cells, and macrophage cells (Ali et al., 2009; Jackaman et al., 2003). Both $CD8^+$ and $CD4^+$ lymphocytes are required for tumor regression in multiple tumor models (Ali, et al., 2009). The short half-life of IL2 *in vivo*

[3] Significant portions of this chapter are under review for publication: Rodriguez B. Leticia *et. al.* (2012).

is a major limitation of IL2 treatment, and toxic side effects are typically observed at high doses. Alternatives to overcome these limitations include continuous infusions (Foa, et al., 1992; West et al., 1987), prolonged low dose daily injection, and local regional injections of IL2 into the tumor area (Cortesina et al., 1988; Foa, et al., 1992).

Introducing IL2 gene into tumor cells may also help overcome the limitation associated with *in vivo* administration of IL2, including side effects caused by high doses of IL2 necessary for antitumor activity (Foa, et al., 1992). Expression of IL2 in tumor cells has been shown to reduce tumor cell tumorgenicity *in vivo* and induce tumor specific antitumor immunity (Fearon et al., 1990; Galanis et al., 1999; Zier & Gansbacher, 1996). Treatment with IL2 expressing plasmid has also been shown to improve the efficacy of IL2 immunotherapy, while avoiding the toxicity associated with high doses of IL2 (Galanis, et al., 1999). Repeated intratumoral injection of IL2 plasmid complexed with liposomes was shown to be safe and well tolerated (Galanis, et al., 1999). The predicted outcome is that tumor cells transfected with IL2 gene will produce IL2 at a level that is low enough to prevent significant side effects to the patients, but sufficient enough to create an antitumor response through the immune system (Foa, et al., 1992).

It is known that double-stranded (dsRNA) molecules have multiple direct and indirect pro-apoptotic, anti-proliferative, anti-angiogenic, and immunostimulatory activities (Absher & Stinebring, 1969; Chawla-Sarkar, et al., 2003; Fujimura, et al., 2006). Double-stranded RNA is also a potent inducer of type I interferons (IFN-α/β), which have pro-apoptotic and anti-angiogenic effects (Chawla-Sarkar, et al., 2003;

Friedrich, et al., 2004). Data from recent studies showed that intracellular dsRNA is more effective than extracellular dsRNA in promoting cells to undergo apoptosis and in orchestrating innate and adaptive immune responses (Cui, et al., 2007; McBride, et al., 2006; Schulz, et al., 2005). Previously, we proposed and demonstrated the feasibility of using an RNA replicase-based plasmid (pSIN-β) to generate dsRNA intracellularly in the transfected tumor cells to control tumor growth in culture and in mice (B. L. Rodriguez, et al., 2011). We have shown that treatment of tumor-bearing mice with the pSIN-β plasmid complexed with cationic liposomes by peritumoral injections significantly inhibited the growth of tumors pre-established in a mouse model and, in many cases, caused complete tumor regression, suggesting the use of RNA-replicase based plasmid as a novel tumor molecular therapy.

The present study is designed to further improve the antitumor activity of the pSIN-β plasmid by incorporating IL2 gene into the plasmid backbone. The resultant pSIN-IL2 plasmid was targeted to melanoma cells that over-express sigma receptors using cationic liposomes. We reason that the IL2 produced by the tumor cells transfected with the pSIN-IL2 plasmid will help improve the antitumor activity of the dsRNA produced by the pSIN.

Sigma receptors are ubiquitously expressed membrane bound proteins that are highly conserved in mammalian systems. The presence of sigma 1 and sigma 2 receptor subtypes was reported in human amelanotic melanoma (Vilner, John, & Bowen, 1995). Anisamide, a benzamide derivative, acts as a ligand to the sigma receptor and is not subtype specific (Hou, Tu, Mach, Kung, & Kung, 2006). Anisamide was previously used

as a functional ligand to the sigma 1 receptor to successfully target liposomes to sigma receptor-overexpressed in melanoma cells (Banerjee, Tyagi, Li, & Huang, 2004; Y. Chen, Bathula, Yang, & Huang, 2010; Le & Cui, 2006).

4.2 Materials and Methods

4.2.1 Plasmids and cells

Plasmid pSIN-β was kindly provided by Dr. Richard Weiss (University of Salzburg, Salzburg, Austria). Plasmid pCMV-β was from the American Type Culture Collection (ATCC, Manassas, VA). The pORF-mIL2 plasmid was from InvivoGen. (San Diego, CA). Plasmids were amplified in *E. coli* DH5α under selective growth conditions and purified using a QIAGEN midiprep kit (Valencia, CA) according to the manufacturer's instruction. Large-scale plasmid purification was performed by GenScript (Piscataway, NJ). The B16-OVA cells were kindly provided by Dr. Edith M. Lord and Dr. John Frelinger (University of Rochester Medical Center, Rochester, NY) and cultured in RPMI 1640 medium supplemented with 5% FBS and 400 μg/ml of G418 (Sigma).

4.2.2 Construction of pSIN-IL2 plasmid

To construct pSIN-IL2 plasmid, the murine interleukin-2 (IL2) gene from the pORF-mIL2 plasmid was PCR-amplified with primers IL2 F (5'-ACA AGT TCT AGA CAC CGG CGA AGG AGG GCC-3') and IL2 R (5'-CCT AGA GCA TGC ATT GAG GGC TTG TTG AGA-3'). The PCR product was digested with *Xba*I and *Sph*I, and the IL2 gene was used to replace the β-galactosidase gene downstream of the subgenomic promoter in the pSIN-β. Positive clones were screened for insert using restriction

digestion and further confirmed by DNA sequencing (ABI 3730XL DNA analyzer, Applied Biosystems, Foster City, CA).

4.2.3 Synthesis of DSPE-PEG-anisamide (DSPE-PEG-AA)

DSPE-PEG-AA was synthesized according to Bangerjee *et. al.* with slight modifications (Banerjee, et al., 2004). Briefly, ρ-anisoyl chloride (3.412 g, 0.02 mol) in 9 mL of benzene at 50°C was added to an aqueous solution of 2-bromo ethylamine hydrobromide (3.73 g, 18.2 mmol) in 27 mL of water. Sodium hydroxide 5% (33.5 mL) was gradually added to the emulsion while shaking and cooling in running water. After 2 h stirring, solid amide was filtered with suction and washed with 0.1 M sodium carbonate. After lyopholization, ^{1}H NMR was taken for dried *N*-(2-bromoethyl)-4-methoxybenzamide, which (50 mg, 0.4 mmol) was then reacted with DSPE-PEG-2000-NH_2 (50 mg, 23.2 mmol) in acetonitrile (2.5 mL) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) (15 μL, 0.1 mmol) at 65-70°C for 8 h. Methanol (2.5 mL) was added to the reaction mixture followed by excess ether (25 mL), and the mixture was kept at -80°C for 24 h. The precipitate was collected after centrifugation, and recrystallization was performed with the addition of methanol (2.5 mL) followed by ether (17.5 mL) at 4°C for 12 h. The overall yield on average was 46%. The product DSPE-PEG-AA was confirmed by ^{1}H NMR and ESI mass spectrometry.

4.2.4 Preparation of anisamide-conjugated PEGylated liposomes (AA-PEG-LP)

Cationic liposomes were prepared using cholesterol (Sigma-Aldrich, St. Louis, MO), egg phosphatidylcholine (Avanti Polar Lipids, Inc, Alabaster, AL), 1,2,-dioleoyl-3-trimethylamonium-propane (DOTAP, Avanti) and DSPE-PEG-(2000)-methoxy (Avanti), or DSPE-PEG-(2000)-AA at a molar ratio of (4.6:10.8:19.6:1.5). To fluorescently label the liposomes, 1,2,-dioleoyl glycerol-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-DOPE) (Avanti 1% w/w) was included in the lipids. Lipids were mixed in a 20-ml glass scintillation vial followed by solvent evaporation and the formation of a thin film by placing the vial under a constant stream of nitrogen gas. The film was hydrated with phosphate buffered saline (PBS) (10 mM, pH 7.4) and sonicated for 1 h, followed with a 24 h hydration at room temperature with intermittent vortexing. The liposome suspension was forced through polycarbonate filters (1.0, 0.4, and 0.1 μm, sequentially) using a mini-extruder (Avanti) (Le & Cui, 2006). The final concentration of DOTAP in the liposomes was 10 mg/ml.

The particle size and zeta potential of the cationic liposomes, PEG-liposomes, and anisamide conjugated PEGylated liposomes (AA-PEG-LP) were measured using a Malvern Zetasizer® Nano ZS (Westborough, MA). Liposomes were mixed with plasmid DNA at various ratios to prepare lipoplexes. The stability of the lipoplexes was determined in simulated biological medium. Briefly, the lipoplexes were diluted in normal saline with 10% FBS, and their sizes were measured immediately (0 min) and after 30 min of incubation at 37°C.

4.2.5 Plasmid DNA uptake assay

B16-OVA cells (1 x 10^5) were seeded in 24-well plates (n = 6) and incubated at 37°C, 5% CO_2 for 24 h or until 60% confluency. The pSIN-IL2 plasmid was labeled using a *Label* IT® fluorescein nucleic acid labeling kit (Mirus, Madison, WI) according to the manufacturer's instruction. Freshly labeled pSIN-IL2 (0.75 μg) was complexed with the AA-PEG-liposomes or the PEG-liposomes (DOTAP, 3.75 μg) and incubated for at least 15 min at room temperature. The resultant lipoplexes were added to each well and incubated for 1 h at 37°C, 5% CO_2. Cells were washed with PBS and lysed using Triton X-100 (in 0.5% in 20 mM Tris, 100 mM NaCl, and 1 mM EDTA) following by incubation at -80°C for 1 h. The fluorescence intensity was measured at 492/518 nm in a black bottom plate using a BioTek Synergy® Multi-Mode Microplate Reader (Winooski, CT).

4.2.6 *In vitro* transfection

B16-OVA cells (2.5 x 10^5 cells/well) were seeded into 24-well plates (n = 3). After overnight incubation, the cells were incubated with pSIN-IL2 complexed with AA-PEG-LP or PEG-LP (DNA : DOTAP, 1:10, w/w) for 10 or 24 h. Briefly 1 μg of plasmid was diluted in 50 μl of serum free medium, and 1 μl of corresponding liposome was diluted in 50 μl of serum free medium. The diluted plasmid DNA samples were mixed with the diluted liposome solution and incubated at room temperature for 15 min. The complexes were added to B16-OVA cell containing wells. The time when the complexes were applied to the cell culture medium was defined as 0 h. After incubation, the

supernatant was collected and analyzed for IL-2 using a mouse IL-2 ELISA kit (BD Biosciences).

4.2.7 Plasmid DNA uptake detected by fluorescence microscopy

B16-OVA cells (2 x 10^6) were seeded on poly-D-lysine-coated glass coverslips and incubated in 6-well plates at 37°C, 5% CO_2 for 24 h. Cells were further incubated in the presence of pSIN-IL2/AA-PEG-liposome lipoplexes or pSIN-IL2/PEG-liposome lipoplexes (DNA : DOTAP, 3.75 μg : 18.75 μg) in reduced growth medium for 1 h at 37°C. After the incubation, cells were washed twice with PBS and fixed in 3% paraformaldehyde for 20 min at room temperature. Cells were washed with PBS three times, and coverslips were mounted on slides using a mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counter stain (vectashield H-1200) from Vector laboratories (Burlingame, CA). Cells were viewed using an Olympus BX53 Microscope with a DP72 digital camera (Olympus America, Inc., Center Valley, PA). Images were acquired using the CellSens dimension imaging software (Olympus America, Inc., Center Valley, PA).

4.2.8 Intracellular trafficking of rhodamine-labeled lipoplexes determined using confocal microscopy

B16-OVA cells (1 × 10^6 cells/well) were seeded in a 35 mm glass bottom dish (Mattek Corporation, Ashland, MA) and incubated overnight. To study the intracellular localization of pSIN-IL2/Rho-AA-PEG-liposome lipoplexes or pSIN-IL2/Rho-PEG-

liposome lipoplexes (DNA : DOTAP, 3.75 μg : 18.75 μg), the cells were incubated at 37°C with the lipoplexes, followed by the addition of 0.2 mM Hoechst 33342 (Ex/Em 345/478 nm) (AnaSpec Inc. Fremont, CA). The cells were viewed live for time indicated, and single focus images were acquired using a Leica TCS-SP5 X Supercontinum confocal microscope with an oil immersion objective (63 × 1.4 NA) (Leica Microsystems GmbH, Mannheim, Germany). Images were processed using the NIH Image J software.

4.2.9 Animal studies

All animal studies were carried out following National Institutes of Health guidelines for animal care and use. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Female C57BL/6 mice (6–8 weeks) were from Charles River laboratories, Inc. (Wilmington, MA). Mice were subcutaneously (S.C.) injected in the right flank with B16-OVA cells (5 x 10^5). When tumors reached an average diameter of 2-4 mm, the lipoplexes (DNA:DOTAP, 25:125 μg) were injected subcutaneously peritumorally (s.c., p.t.) for 5 consecutive days (B. L. Rodriguez, et al., 2011). Tumor size was measured using a digital caliper, and tumor volume was calculated using the following equation: tumor volume = (length x width^2)/2.

4.2.10 Immune cell profiles

The peripheral blood from B16-OVA tumor-bearing mice after treatment with plasmid complexed with AA-PEG-liposomes was collected and immediately mixed with

125 mM EDTA. Peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation following manufacturer's instruction (Lympholyte cell separation media, Cedar Lane, Hornby, Canada). Lymphocytes (1 x 10^6) were blocked with anti-mouse CD16/CD32 for 10 min, washed and further stained with a cocktail of antibodies containing PE-labeled anti-mouse CD4 (Clone RM4-4, cat# 12-0043, eBioscience, San Diego, CA), Pe-Cy5-labeled anti-mouse CD8a (Clone 53-6.7, cat# 15-0081, eBioscience), FITC-labled anti-mouse CD69 (Clone H1.2F3, cat# 11-0691, eBioscience), and APC-labled anti-mouse CD49b (Clone DX5, cat# 17-5971, eBioscience). Stained cells were analyzed using flow cytometry (Guava Easycyte 8HT Flow cytometry System,Millipore, Hayward, CA). Data was analyzed using FlowJo flow cytometery analysis software (Ashland, OR).

Splenocytes were isolated from B16-OVA tumor-bearing mice following treatment with lipoplexes as previously described (Cui & Qiu, 2006). Spleens were removed from each mouse and placed in 10 mM PBS containing 2% FBS. Spleens were homogenized in fresh PBS by passing through a cell strainer using a syringe plunger. Red blood cells were lysed by incubating the cell suspension with a red blood cell lysis buffer (Sigma) for 5 min. at 4°C. Splenocytes were stained with the cocktail of antibodies and analyzed as mentioned above.

4.2.11 *In vivo* expression of IL2 in B16-OVA tumors

B16-OVA tumors were harvested from mice that had been treated for 5 days with plasmids compexed AA-PEG-liposomes. Twenty-four hours after the last treatment,

tumors were removed and flash frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was isolated from tumors by homogenization using TRIzol reagent (Invitrogen) and RNeasy kit (Qiagen) according to manufacturer's instructions. High capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was utilized for the RT-PCR. Real-time PCR of IL-2 gene was carried out using the Power SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) with the following primers: 5'-CCT GAG CAG GAT GGA GAA TTA CA-3' (forward) and 5'-TCC AGA ACA TGC CGC AGA G-3' (reverse). All samples were performed in triplicates, and normalized to β-actin: 5'-TGT GAT GGT GGG AAT GGG TCA GAA-3' (forward) and 5'-TGC CAC AGG ATT CCA TAC CCA AGA-3' (reverse). Data were analyzed using the Applied Biosystems ViiA^{TM} 7 Software (Applied Biosystems).

4.2.12 Hematoxylin and eosin staining

B16 tumors in mice that were treated for 5 consecutive days with plasmids were collected, fixed in formalin, embedded in paraffin, and sectioned. Sections of 7 μm were stained with hematoxylin and eosin (H&E). Slides were scanned and images were acquired using the ScanScope XT (Aperio Technologies, Vista, CA).

4.2.13 Statistical analysis

Statistical analyses were performed using analysis of variance followed by Fisher's protected least significant difference procedure. Mouse survival curves were

compared using the Kaplan–Meier survival analysis (GraphPad Prism®, La Jolla, CA). A p-value of ≤ 0.05 (two-tail) was considered statistically significant.

4.3 Results

4.3.1 Preparation and characterization of pSIN-IL2/anisamide-conjugated liposome lipoplexes

Anisamide conjugated, PEGylated cationic liposomes (AA-PEG-liposomes) were prepared by mixing cationic DOTAP liposomes with anisamide-conjugated DSPE-PEG (2000). The final concentration of DOTAP in the liposomes was 10 mg/ml. The anisamide free, PEGylated cationic liposomes (PEG-liposomes) were prepared similarly except that an equivalent amount of DSPE-PEG (2000) was used instead. The diameter and zeta potential of the AA-PEG-liposomes were 90.3 ± 0.1 nm and 31.1 ± 1.0 mV respectively. The AA-free, PEG-liposomes were 97.8 ± 0.2 nm, with a zeta potential of 24.1 ± 0.4 mV (Fig. 4.1A).

The following experiments were completed to identify the optimal ratio of pSIN-IL2 to liposomes in the lipoplexes. Various amounts of AA-PEG-liposomes were complexed with a fixed amount of pSIN-IL2 to form different pSIN-IL2/liposome lipoplexes. The particle diameters and the zeta potentials of the resultant lipoplexes are reported in Fig. 4.1B. At the ratio of 1:1 and 2:1 (DOTAP vs. pSIN-IL2, w/w), the lipoplexes appeared unstable and aggregated, whereas lipoplexes prepared at other ratios had smaller sizes around 150 nm (Fig. 4.1B).

In order to identify the liposomes (or DOTAP lipid) to DNA ratio that is optimal in transfecting the pSIN-IL2 plasmid into tumor cells, the uptake of pSIN-IL2 in various lipoplexes by the B16-OVA cells was evaluated. The weight ratio of 5:1 to 20:1 (DOTAP: DNA) were found optimum based on the high levels of cellular uptake (Fig. 4.1C). The stability of the lipoplexes at various ratios was evaluated in a simulated physiological medium. The lipoplexes prepared at 5:1 to 30:1 ratio were physically stable within the period tested (Fig. 4.1D). Therefore, the lipoplexes prepared with a DOTAP (in liposomes) to pSIN-IL2 ratio of 5:1 or 10:1 (w/w) were used for further studies.

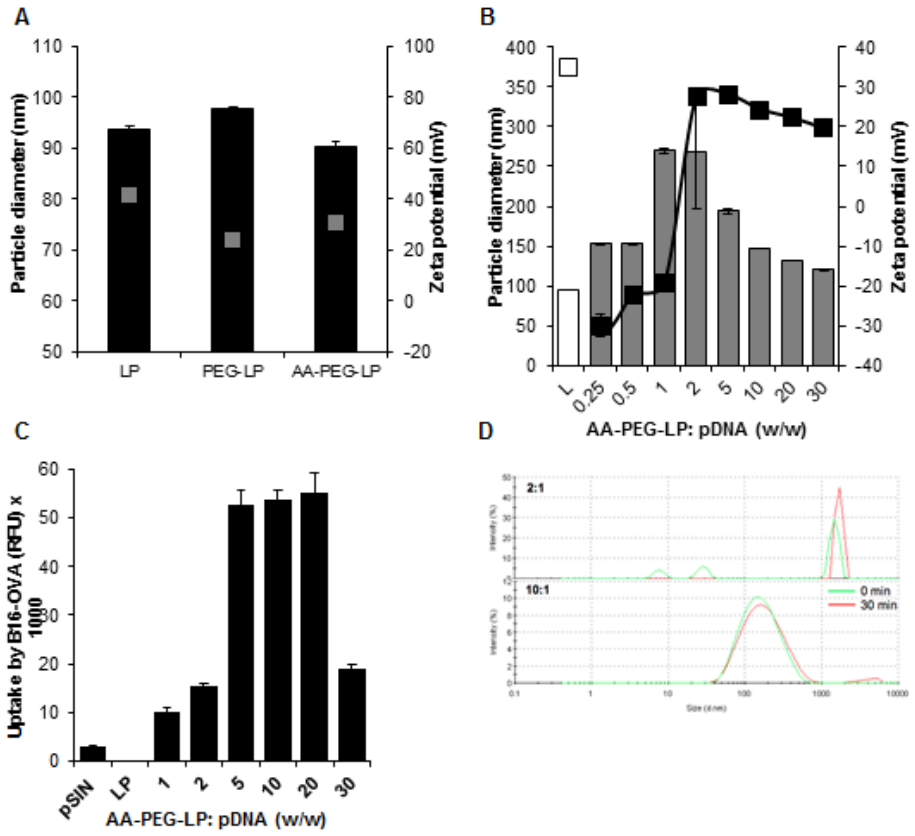

Fig. 4.1 Physicochemical characteristics of lipoplexes prepared by complexing pSIN-IL2 with anisamide-conjugated PEG-liposomes.

(A). Mean diameter (black bars) and zeta potential (grey square) of liposomes, PEG-liposomes, and AA-PEG-liposomes.

(B). Mean diameter and zeta potential of pSIN-IL2-anisamide-PEG-liposome lipoplexes prepared at various ratios (DOTAP : DNA). Equal volumes of DNA (25 μg) solution and AA-PEG-liposome suspension were mixed and allowed to incubate at room

temperature for at least 15 min. Data shown are mean ± standard error of the mean (n = 4).

(C). Uptake of pSIN-IL2 in lipoplexes by B16-OVA cells in culture. Cells (1 x 10^5/well) were incubated with fluorescein-labeled pSIN-IL2 complexed with AA-PEG-liposomes at various ratios for 1 h at 37°C (n = 4).

(D). Overlay of the dynamic light scattering spectra of lipoplexes prepared by complexing AA-PEG-LP with pSIN-IL2 at 2:1 or 10:1 ratios (w/w), immediately after preparation (green) and 30 min (red) after incubation at 37°C in a simulated biological medium.

4.3.2 Expression of IL2 in B16-OVA cells transfected with pSIN-IL2

The expression of IL2 in B16-OVA cells was determined using quantitative ELISA. A high level of IL2 was detected in cells transfected with pSIN-IL2, compared with pSIN-β (Fig. 4.2B). The amount of IL2 produced by the B16-OVA cells transfected with pSIN-IL2 was approximately a third of that produced by the parent plasmid pIL2 (Fig. 4.2B). pSIN-IL2 transfected cells produced IL2 in a dose dependent manner (Fig. 4.2A). Further the level of IL2 expressed in pSIN-β transfected cells was negligible even at high plasmid amounts (Fig. 4.2A).

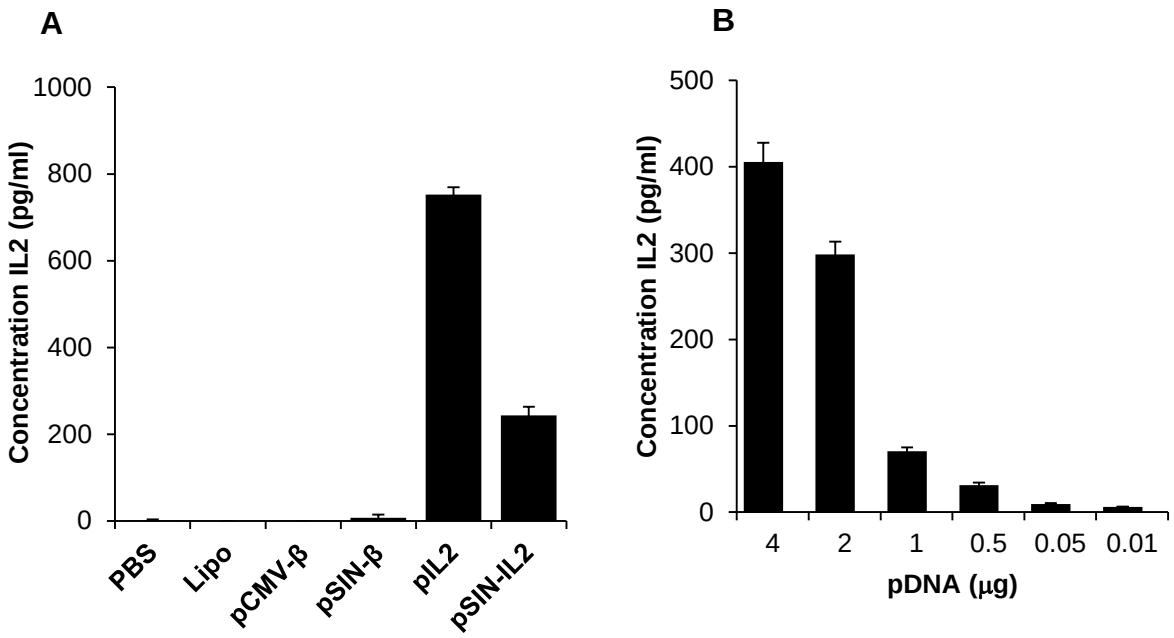

Fig. 4.2 *In vitro* transfection of the pSIN-IL2 in B16-OVA cells

B16-OVA cells (1 x 10^6 cells/well) were seeded into six-well plates (n = 3). After incubation at 37°C, 5% CO_2 for 24 h or until 60% confluency, cells were incubated with 4 μg of plasmid complexed with Lipofectamine® 2000 reagent (Invitrogen) following the manufacturer's instruction. Briefly 4 μg of plasmid was diluted in 250 μl of serum free medium, and 10 μl of Lipofectamine 2000 was diluted in 250 μl of serum free medium. The diluted plasmid DNA samples were mixed with the diluted Lipofectamine 2000 and incubated at room temperature for 15 min. As controls, cells were also treated with sterile PBS, Lipofectamine 2000 reagent alone, or pCMV-β complexed with Lipofectamine

2000. After 24 h of incubation, the supernatant was collected and analyzed for IL-2 using a mouse IL-2 ELISA kit (BD Biosciences). (A). IL2 levels in cells treated with different plasmids. (B). IL2 levels in cells treated with various doses of pSIN-IL2 plasmid.

4.3.3 *In vitro* uptake of pSIN-IL2 in targeted lipoplexes by B16-OVA melanoma cells

The uptake of fluorescein-labeled pSIN-IL2 by B16-OVA cells expressing high levels of sigma receptor was evaluated after the cells were incubated for 1 h with lipoplexes prepared with the AA-PEG-LP or PEG-LP. The uptake of the pSIN-IL2 in the lipoplexes prepared with AA-PEG-LP was significantly higher compared to the PEG-LP (Fig. 4.3A), a 50 % increase. To further investigate whether the uptake of the pSIN-IL2 in the lipoplexes produced IL2, we incubated the lipoplexes with B16-OVA cells and measured IL2 expression. The expression of IL2 was significantly higher in B16-OVA cells transfected with AA-PEG-LP than with the PEG-LP (Fig. 4.3B). The PEG-liposome lipoplex had elevated amount of IL2 as compared to cells transfected with pSIN-IL2 alone. Additionally we did not detect any difference in the viability of the cells after treatment with AA-PEG-LP or PEG-LP for 24 h (data not shown).

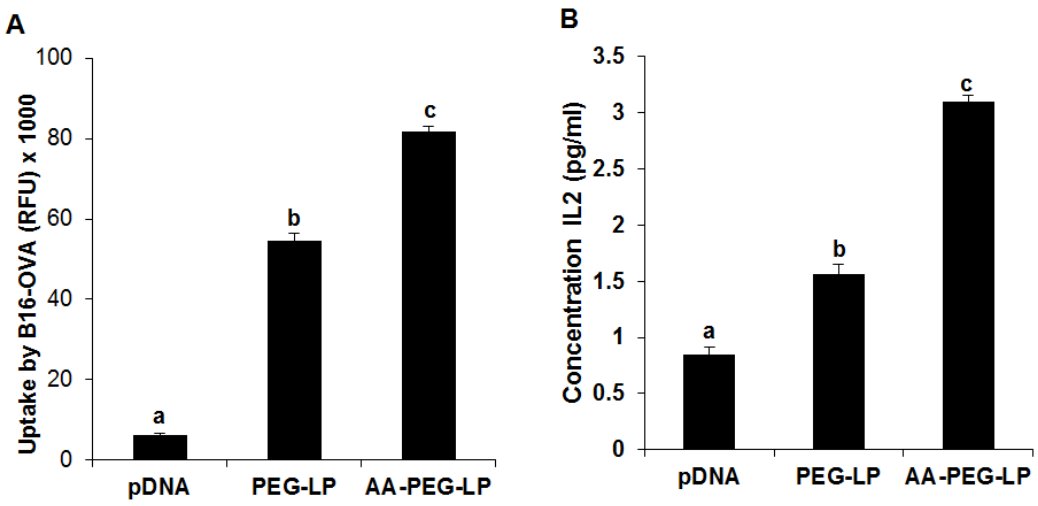

Fig. 4.3 *In vitro* uptake of pSIN-IL2 in AA-PEG-LP or PEG-LP lipoplexes by B16-OVA tumor cells.

(A). Cells (1 x 10^6/well) were incubated with fluorescein-labeled pSIN-IL2 (0.75 μg) complexed with AA-PEG-LP or PEG-LP (DOTAP, 3.75 μg) for 1 h at 37°C. Fluorescence intensity was measured at 492/518 nm (n = 6). Different letters indicate significant differences ($p < 0.05$).

(B). Cells (2.5 x 10^5/well) were incubated with pSIN-IL2 (1 μg) complexed with AA-PEG-LP or PEG-LP (DOTAP, 10 μg) for 24 h at 37°C. The supernatant was collected to analyze IL2 using a mouse IL2 ELISA kit (n = 3). Different letters indicate significant differences ($p < 0.05$).

4.3.4 Intracellular trafficking of lipoplexes in B16 melanoma cells

The cellular distribution of the lipoplexes was examined with fluroscein-labeled pSIN-IL2 complexed with either rhodamine labeled AA-PEG-liposomes, or rhodamine labeled PEG-liposomes. We examined the distribution of pSIN-IL2, liposomes, and the lipoplexes by fluorescence microscopy (Fig. 4.4). The fluorescence emission from the fluorescein-labeled pSIN-IL2 is shown in green, rhodamine labeled liposomes shown in red, and the emission from the lipoplexes in yellow. Overall lipoplexes prepared with AA-free liposomes had less internalization compared to the lipoplexes prepared with AA-PEG-LP (Fig. 4.4). Disassociation of pSIN-IL2 from the Rho-PEG-LP occurred at 180 min whereas disassociation of pSIN-IL2 from the Rho-AA-PEG-LP occurred at 60 min.

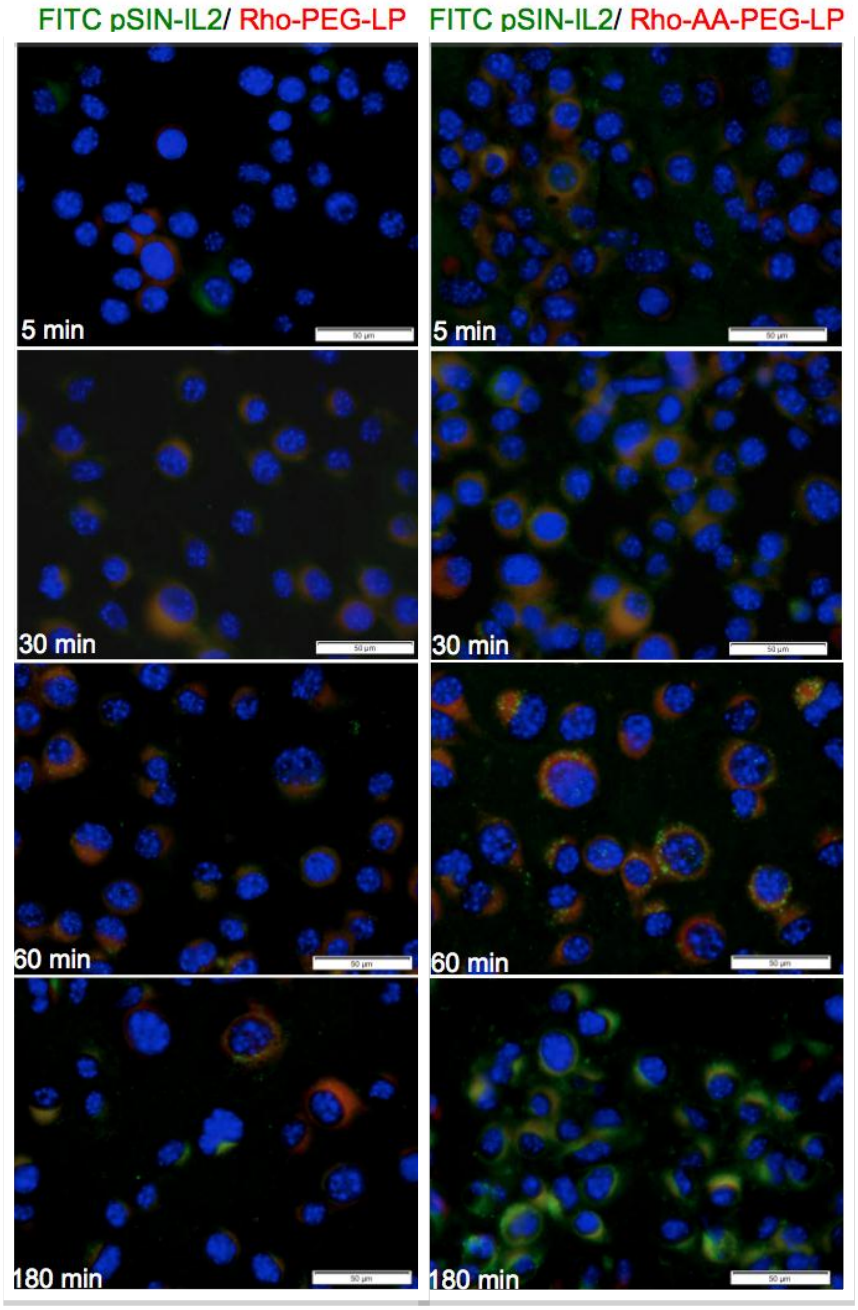

Fig. 4.4 Fluorescence images of cells incubated with fluorescein-labeled pSIN-IL2-rhodamine-labeled liposome lipoplexes. B16-OVA cells (2 x 10^6) were incubated with

fluorescein-labeled pSIN-IL2 complexed with rhodamine-labeled AA-PEG-LP or rhodamine-labeled PEG-LP (DNA: DOTAP, 3.75 μg: 18.75 μg) for up to 180 min at 37°C. Cell nucleus was stained with DAPI (blue) (bar = 50 μm).

4.3.5 Subcellular distribution of lipoplexes

We further evaluated the intracellular localization of the lipoplexes using confocal microscopy. The lipoplexes were prepared using rhodamine liposomes. The lipoplexes prepared with the PEG-LP were primarily located on the plasma membrane at 30 min (Fig. 4.5). By 180 min a large portion of the lipoplexes remained near the plasma membrane with some penetration into the cytoplasm. The lipoplexes prepared with AA-PEG-LP had a significantly higher level of internalization at 30 min, compared with PEG-LP, with some particles remaining on the plasma membrane. However, by 180 min, the lipoplexes were located inside the cells, adjacent to the nucleus.

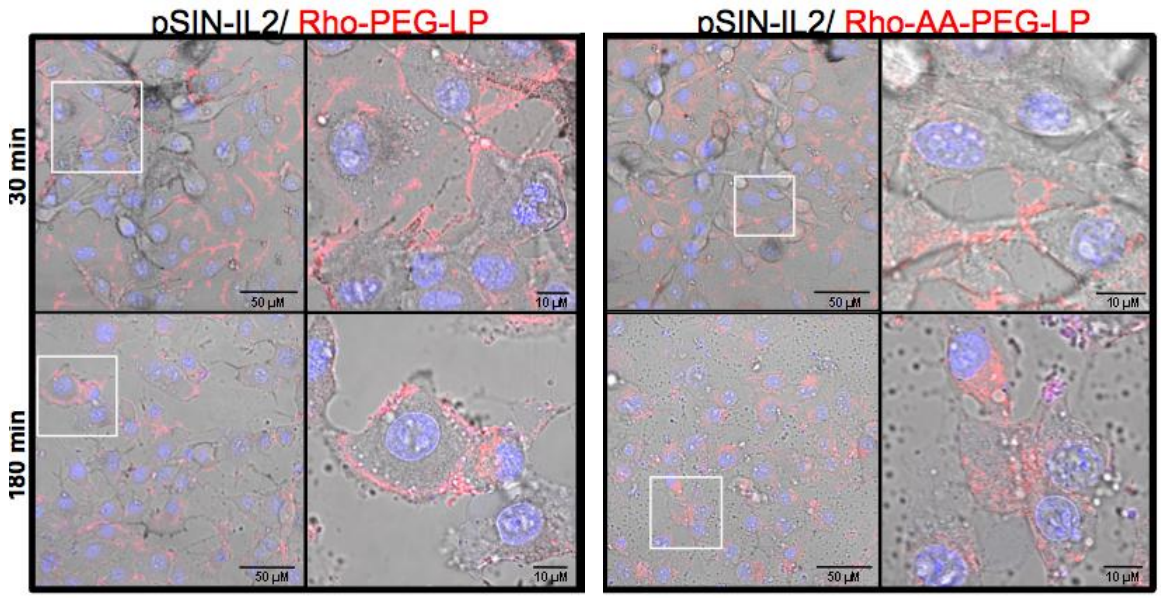

Fig. 4.5 Subcellular distribution of rhodamine-labeled lipoplexes. B16-OVA cells (1 x 10^6) were incubated with pSIN-IL2 complexed with rhodamine-labeled AA-PEG-LP or rhodamine-labeled PEG-LP (DNA : DOTAP, 3.75 μg : 18.75 μg) for up to 180 min at 37°C. The image in the white box is shown at a higher magnification in adjacent image on the right. Cell nucleus was stained with Hoechst (blue).

4.3.6 The pSIN-IL2 plasmid was more effective at controlling the growth of B16-OVA tumor cells in mice when complexed with the AA-PEG-LP than with the PEG-LP

The anti-tumor activity of the pSIN-IL2 complexed with the AA-PEG-LP or PEG-LP were evaluated against B16-OVA tumors *in vivo*. The pSIN-IL2/AA-PEG-liposome lipoplexes were more effective than the pSIN-IL2/PEG-liposome lipoplexes at controlling the growth of the tumors (Fig. 4.6A). Starting on day 13, tumors in mice that were treated with the pSIN-IL2/AA-PEG-liposome lipoplexes became significantly smaller than those treated with the pSIN-IL2/PEG-liposome lipoplexes (Fig. 4.6A). The median survival time for mice treated with sterile PBS was 18 d, 22 d for mice treated with the pSIN-IL2/PEG-liposomes lipoplexes, and 27 d for mice treated with the pSIN-IL2/AA-PEG-liposome lipoplexes (Fig. 4.6B). Toxicity was not observed in terms of body weight loss after treatment with the lipoplexes (data not shown).

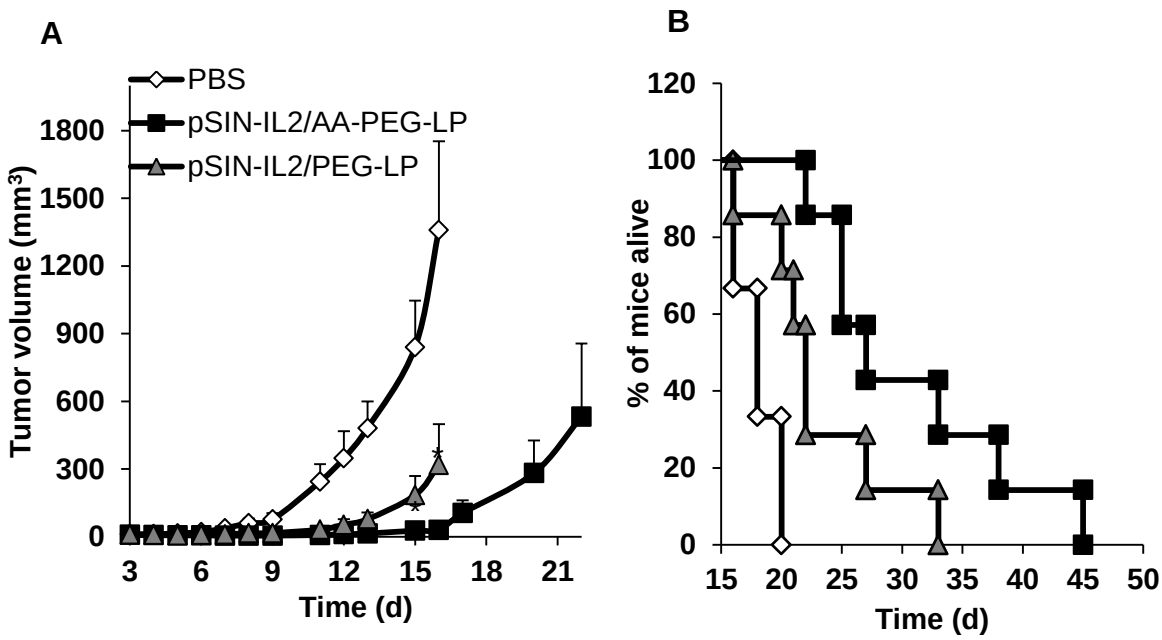

Fig. 4.6. pSIN-IL2 was more effective at controlling the growth of B16-OVA tumors in mice when complexed with the AA-PEG-liposomes.

(A). B16-OVA (5 x 10^5) cells were established in female C57BL/6 mice on day 0 (n = 5-7). Starting on day 3, mice were peritumorally injected with PBS, pSIN-IL2/AA-PEG-LP or pSIN-IL2/PEG-LP for 5 consecutive days. (*) Starting from day 13, the values of the pSIN-IL2/AA-PEG-LP and pSIN-IL2/PEG-LP are different from each other ($p < 0.05$).

(B). Mouse survival curve. ($p = 0.04$, pSIN-IL2/AA-PEG-LP vs. pSIN-IL2/PEG-LP, Gehan-Breslow-Wilcoxon test).

4.3.7 The pSIN-IL2 plasmid was more effective at controlling the growth of B16-OVA tumor cells in mice than the pSIN-β plasmid

To evaluate the extent at which incorporating IL2 gene into the pSIN-β plasmid will improve the anti-tumor activity of the pSIN-β plasmid, B16-OVA tumor cells were seeded in mice. When tumors reached an average of 3 mm, mice were treated with pSIN-IL2 or pSIN-β plasmid complexed with the AA-PEG-LP daily for five days. Tumors grew significantly slower in mice treated with the pSIN-IL2 than in mice treated with the pSIN-β plasmid or the pIL2 plasmid (Fig. 4.7). Mice treated with the pSIN-IL2 plasmid also survived significantly longer than those treated with pSIN-β or pIL2 (Fig. 4.7B). The antitumor activites of the pSIN-β and pIL2 were not different from each other, but were significantly different from the sterile PBS as a vehicle control (Fig. 4.7A).

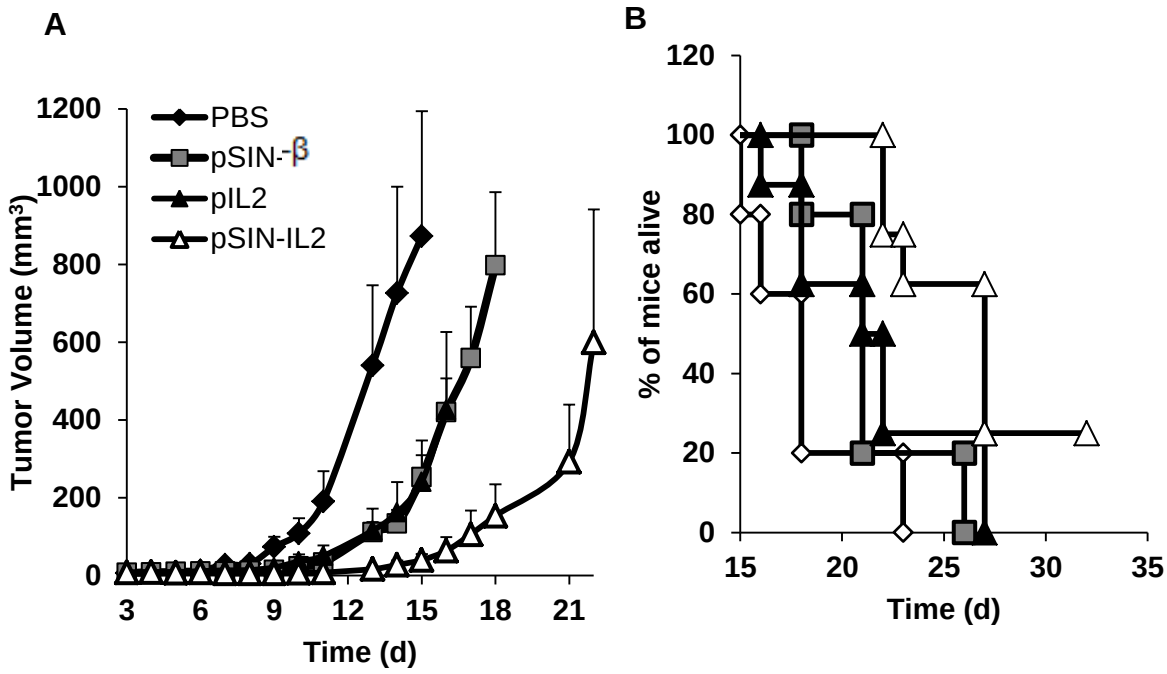

Fig. 4.7 The pSIN-IL2 plasmid was more effective than the pSIN-β plasmid at controlling the growth of B16-OVA tumor cells in mice.

(A). B16-OVA (5×10^5) cells were established in female C57BL/6 mice (n = 5-8) on day 0. Starting on day 3, mice were peritumorally injected with pSIN-IL2, pSIN-□, or pIL2, all complexed with AA-PEG-LP for 5 consecutive days. Starting on day 15, all live mice were treated for an additional 4 consecutive days. Starting from day 7, the values of the pSIN-IL2/AA-PEG-LP and pSIN-β/AA-PEG-LP or pIL2/AA-PEG-LP are different from each other ($p < 0.05$). The values of pSIN-β/AA-PEG-LP and pIL2/AA-PEG-LP are not different from each other on any day ($p > 0.50$)

(B). Mouse survival curve ($p = 0.005$, pSIN-IL2 vs. pSIN-β, $p = 0.03$, pSIN-IL2 vs. pIL2, Log-rank Mantel-Cox test).

4.3.8 Histology

B16-OVA tumors in mice that were treated with sterile PBS had a homogenous epithelial-like cellular appearance (Fig. 4.8). Well-defined blood vessels were present, necrosis was rare, hemorrhage was observed. Further infiltration by inflammatory cells was minimal. Tumors from mice treated with pSIN-β have necrotic areas mainly on the periphery of the tumors, and inflammatory cells were found surrounding the mass. Tumors from mice injected with pIL2 plasmid showed extensive haemorrhagic regions and severe necrosis, and inflammatory cells including neutrophils and T-cells were found on the periphery of the tumors with minor infiltration into the mass. Tumors in mice that were treated with pSIN-IL2 showed extensive central necrotic regions and were highly haemorrhagic. Severe inflammatory cell infiltration was found in both the center and the periphery of the tumors (Fig. 4.8).

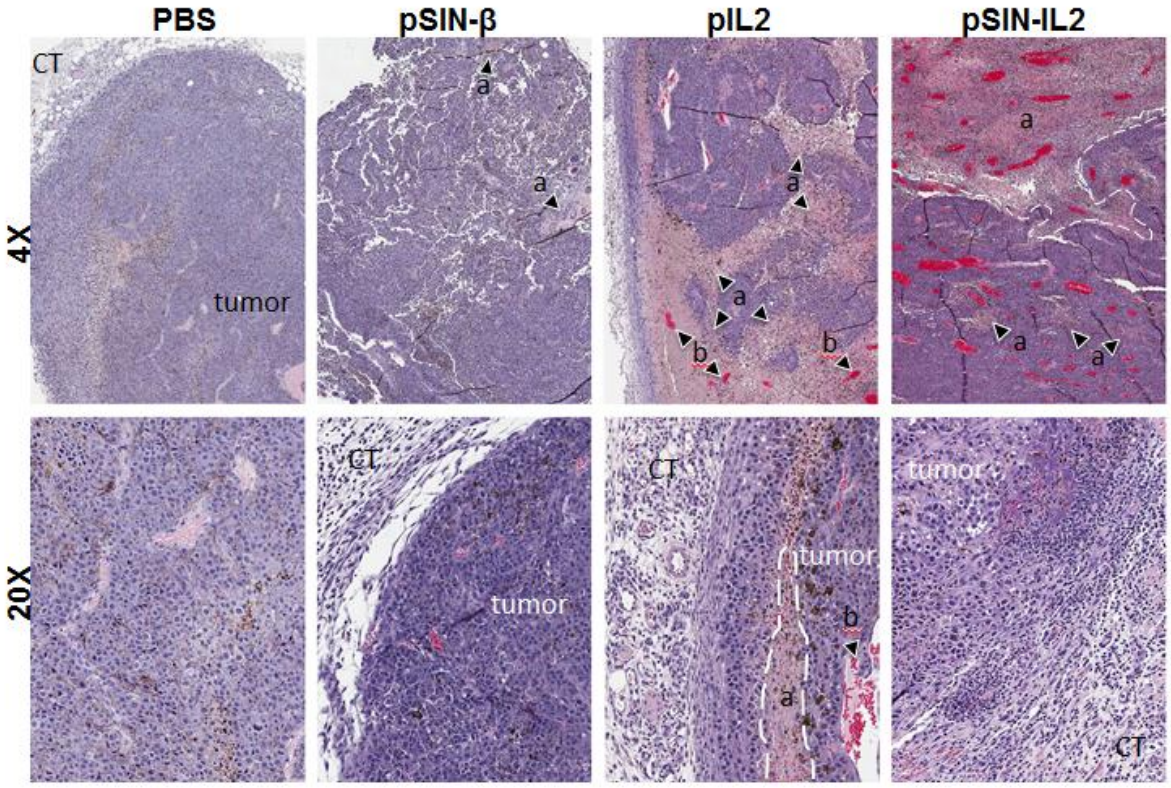

Fig. 4.8 H & E micrographs

H & E micrographs. In pSIN-β, (a) indicates necrotic areas. In pIL2, **(**a) indicates necrotic areas; (b) indicates hemorrhagic regions; and area within the dotted line indicates severe necrosis. In pSIN-IL2, (a) in dotted line indicates extensive central necrotic regions. Tumors were highly hemorrhagic (all red lacunas; note: despite the appearance in the low magnification picture, red blood cells were not contained by vessels), and there is a poorly defined border between connective tissue (CT) and the tumor per se.

Images were taken at 4X and 40X magnification.

4.3.9 Treatment with pSIN-IL2 plasmid increases activated $CD4^+$ T cells, $CD8^+$ T cells, and NK cells in the peripheral blood and spleen of B16-OVA-tumor bearing mice

Peripheral blood and spleens were collected from B16-OVA tumor-bearing mice after 5 treatments with plasmids. Isolated lymphocytes and splenocytes were stained with a cocktail of antibodies containing anti-mouse CD4, anti-mouse CD8a, anti-mouse CD69, and anti-mouse CD49b. CD49 is a marker for cell activation (Ziegler, Ramsdell, & Alderson, 1994). The percentage of $CD49^+/CD69^+$ cells in the PBL of mice treated with the pSIN-IL2 was 8.6 times higher than in mice treated with PBS, and 2.1 times higher than in the mice treated with pSIN-β (Fig. 4.9A). The percentage of $CD4^+/CD69^+$ and $CD8^+/CD69^+$ cells in PBL of mice treated with pSIN-IL2 was 1.6-fold, and 3-fold higher than in the pSIN-β treated mice, respectively (Fig. 4.9A). The percentage of $CD4^+/CD69^+$, $CD8^+/CD69^+$, and $CD49^+/CD69^+$ cells in PBL in mice treated with pSIN-IL2 was not significantly different than from that in mice treated with the pIL2 plasmid (Fig.4.9A). A similar trend was observed for the $CD4^+/CD69^+$, $CD8^+/CD69^+$, and $CD49^+/CD69^+$ cells in the splenocytes of the treated mice (Fig.4.9C, D). Finally, the total $CD4^+$, $CD8^+$, and $CD49^+$ cells were not different among each treatment groups in the PBL and splenocyte samples (data not shown).

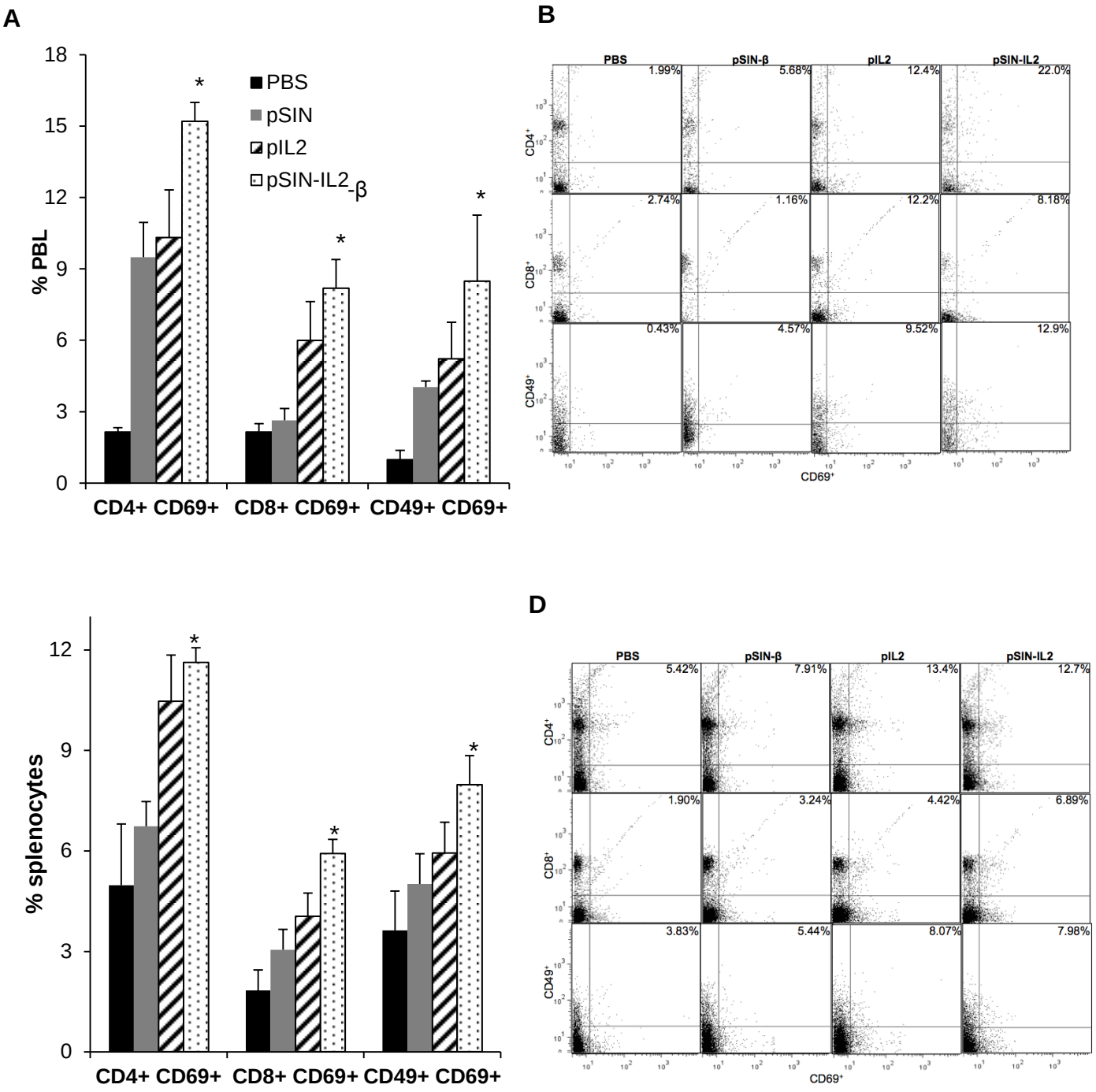
A
B
C
D
PBS
pSIN
pIL2
pSIN-IL2-β
% PBL
% splenocytes
CD4+ CD69+
CD8+ CD69+
CD49+ CD69+
pSIN-β
pSIN-IL2
CD4+
CD8+
CD49+
CD69+
1.99%
5.68%
12.4%
22.0%
2.74%
1.16%
12.2%
8.18%
0.43%
4.57%
9.52%
12.9%
5.42%
7.91%
13.4%
12.7%
1.90%
3.24%
4.42%
6.89%
3.83%
5.44%
8.07%
7.98%

Fig. 4.9 Activation of $CD8^+$ T cells, $CD4^+$ T cells, and NK cells in the peripheral blood and spleens of tumor-bearing mice after treatment with pSIN-IL2 complexed with AA-PEG-LP.

(A). Percentage of $CD4^+$ $CD69^+$, $CD8^+$ $CD69^+$, and $CD49^+$ $CD69^+$ lymphocytes in the PBL of mice treated with PBS, pSIN-β, pIL2, or pSIN-IL2. All plasmids were complexed with AA-PEG-LP. (*) the values of the pSIN-IL2 and pSIN-β are different from each other ($p < 0.05$).

(B). Relative representative dot plots indicating the percentages of activated lymphocytes in PBL of mice treated with PBS, pSIN-β, pIL2, or pSIN-IL2. Square frames represent the gated region based on negative controls.

(C). Percentage of $CD4^+$ $CD69^+$, $CD8^+$ $CD69^+$, and $CD49^+$ $CD69^+$ splenocytes of mice treated with PBS, pSIN-β, pIL2, or pSIN-IL2. (*) the values of the pSIN-IL2 and pSIN-β are different from each other ($p < 0.05$).

(D). Relative representative dot plots indicating percentage of activated splenocytes of mice treated with PBS, pSIN-β, pIL2, or pSIN-IL2. Square frames represent the gated region based on negative controls. In A and C, data shown are mean ± standard error of the mean ($n = 4$-5).

4.3.10 *In vivo* expression of IL2 in B16-OVA tumors

As shown in Fig. 4.10, a significant amount of IL2 mRNA was detected in the B16-OVA tumors treated with pSIN-IL2, but not in tumors treated with pSIN-β. A similar trend for IL2 protein was observed in blood, although not statistically different, likely due to the smaller sample size (data not shown).

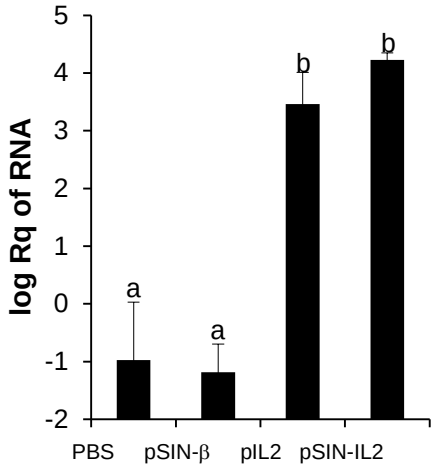

Fig. 4.10 *In vivo* expression of IL2 in B16-OVA tumors

In vivo expression of IL2 in B16-OVA tumors. Twenty hours after the last treatment with PBS, pSIN-β, pIL2, or pSIN-IL2, tumors were collected. Total RNA was extracted from tumor tissues to quantify IL2 expression using qRT-PCR. Data shown are mean ± standard error of the mean (n = 3-4). Different letters indicate significant differences ($p < 0.05$).

4.4 Discussion

We have previously reported that the replicase-based plasmid, pSIN-β, has a strong antitumor activity by enabling tumor cells transfected with it to produce dsRNA, which is pro-apoptotic and activates innate immunity (Absher & Stinebring, 1969; B. L. Rodriguez, et al., 2011). The pSIN-IL2 multigene plasmid construct used in the present study represents an improvement over the pSIN-β plasmid for melanoma treatment. In order to improve the antitumor activity of the pSIN-β, we cloned mouse IL2 into it. A high level of IL2 was detected in cells transfected with pSIN-IL2 (Fig. 4.2), and the level of IL2 produced by the pSIN-IL2 transfected cells was dependent on the dose of the pSIN-IL2 used (Fig. 4.2B). The inclusion of the IL2 gene in the pSIN-β plasmid enabled the plasmid to effectively activate T cells and NK cells in mice (Fig. 4.9), which may explain the significantly stronger antitumor activity of the pSIN-IL2 plasmid in B16-OVA tumor-bearing mice, compared to the pSIN-β (Fig. 4.7)

The antitumor activity of dsRNA have been well documented (Absher & Stinebring, 1969; Friedrich, et al., 2004; Hirabayashi, et al., 1999; Le, et al., 2009; Le, et al., 2008). Polyriboinosinic-polyribocytidylic acid (poly I:C), a synthetic dsRNA, has been used as the standard to evaluate the effect of dsRNA in many animal models as well as in clinical trials (Absher & Stinebring, 1969; Fujimura, et al., 2006; Le, et al., 2009; Okada, et al., 2005). Poly (I:C) was found to slightly delay tumor growth, and it is not practical to increase its antitumor activity by increasing its dose because of the dose-limiting adverse effects (Meier, et al., 1970; Okada, et al., 2005; Pimm & Baldwin,

1976). We have previously reported that the dsRNA-producing pSIN-β plasmid significantly inhibits tumor cell growth both *in vitro* and *in vivo(B. L. Rodriguez, et al., 2011),* likely due to the production of the pro-apoptotic and immunostimulatory dsRNA by tumor cells transfected with the pSIN-β plasmid (B. L. Rodriguez, et al., 2011).

IL2 is approved for use in the treatment of advanced melanoma due to its ability to modulate immune response and stimulate the activation and proliferation of T-cells and NK cells (Riker, et al., 2007). In the present study, we tested whether including IL2 gene into the pSIN-β plasmid will improve the resultant antitumor activity. We found that treatment of tumor-bearing mice with the pSIN-IL2 plasmid significantly increased the percentages of activated $CD4^+$ T cells, $CD8^+$ T cells, and $CD49^+$ NK cells in peripheral blood and splenocytes of the mice, as compared to treatment with the pSIN-β plasmid (Fig. 4.9A, C), indicating that the IL2 gene in the pSIN-IL2 plasmid had helped to incite the activation of circulating T cells and NK cells (Fig. 4.9). H&E micrographs also showed that the pSIN-IL2 plasmid induced an elevated number of infiltrating immune cells both in the center and in the periphery of the tumors, as compared to pIL2 or pSIN-β (Fig. 4.8). The activation of the T cells and NK cells by the IL2 may explain the enhanced antitumor activity from the pSIN-IL2 (Fig. 4.7). It is known that IL2 regulates the Treg lymphocyte balance, which suppresses antitumor immune responses (Shimizu, Yamazaki, & Sakaguchi, 1999; Tanaka, Tanaka, Kjaergaard, & Shu, 2002). Yao and colleagues showed that treatment of mice with an IL2-expressing plasmid led to a reduction of $Foxp3^+$ Tregs populations in peripheral blood (Yao et al., 2011). We found

high levels of IL6, a proinflammatory cytokine, in the serum samples of mice that were treated with pSIN-IL2 (data not shown). It was shown that IL6 decrease Foxp3 expression and down-regulates Tregs *in vitro* and *in vivo* (Korn et al., 2008; Lal et al., 2009), which may also explain the pSIN-IL2 plasmid's ability to activate T cells and NK cells.

The present study is also designed to actively target the RNA replicase-based IL2-expressing plasmid into tumor cells more specifically, therefore allowing the tumor cells to preferentially take up the plasmid, generate intracellular dsRNA and IL2, and then undergo apoptosis by committing "suicide". We chose to target the sigma receptor because it is overexpressed in many tumor cells (Bem et al., 1991; Vilner, et al., 1995). Radiolabeled sigma-receptor ligands have been extensively used as imaging agents in melanoma (John et al., 1993), breast cancer (Caveliers, Everaert, John, Lahoutte, & Bossuyt, 2002), and prostate cancer (John, Vilner, Geyer, Moody, & Bowen, 1999; Kashiwagi et al., 2007). In the present study, anisamide was chemically conjugated onto the cationic liposomal carrier, which was then complexed with the pSIN-IL2 plasmid to target it into sigma receptor-overexpressing murine tumor cells *in vitro* and *in vivo*. Data from previous *in vitro* and *in vivo* studies showed that anisamide has the similar binding affinity to sigma receptors as 4-Iodobenzamide (I123-IDAB), a radiolabeled benzamide, used to visualize sigma receptors *in vivo* (Everaert, Flamen, Franken, Verhaeghe, & Bossuyt, 1997; Megalizzi, Le Mercier, & Decaestecker, 2012). B16-OVA cells were derived from the highly aggressive B16-F10 mouse melanoma cells (Brown, et al., 2001). They express high level of sigma receptors (S. D. Li, et al., 2008). Our *in vitro* data

showed that the anisamide-conjugated cationic liposomes more effectively delivered the pSIN-IL2 plasmid into the B16-OVA tumor cells than the anisamide-free liposomes (Fig. 4.3). Importantly, pSIN-IL2 complexed with the anisamide-conjugated liposomes also more effectively controlled B16-OVA tumor growth in mice than pSIN-IL2 complexed with the anisamide-free liposomes (Fig. 4.6), confirming the feasibility of using anisamide or similar sigma receptor ligands for future targeted gene therapy of melanoma.

Understanding the uptake mechanism and intracellular distribution of plasmid liposome complexes is important in order to design optimal gene delivery systems. The uptake of pSIN-IL2/Rho-AA-PEG-LP complexes were much higher than pSIN-IL2/Rho-PEG-LP after incubation at 30 min (Fig. 4.5). The pSIN-IL2/Rho-PEG-LP complexes appear on the cell surface; with minor internalization at 180 min. The anisamide conjugated on the surface of the liposomes increased the interaction of the lipoplexes with cell membrane as well as the total amount of the lipoplexes entering the cytosol. There is strong correlation between the cellular uptake of complexes and the transfection efficiency of the pSIN-IL2/AA-PEG-LP complexes (Fig. 4.5). Interestingly, we observed that the dissociation of pSIN-IL2 from the pSIN-IL2/Rho-AA-PEG-LP lipoplexes occurred as early as 60 min after the addition of the lipoplexes into the cell culture, whereas the dissociation of pSIN-IL2 from the pSIN-IL2/Rho-PEG-LP lipoplexes did not occur until around 180 min later (Fig. 4.4). Dissociation of the plasmid from the lipoplexes is critical for nuclear translocation of the plasmid. The anisamide on the

lipoplexes facilitated the uptake of the lipoplexes by the B16-OVA cells and thereby increased the expression of the IL2 gene (Fig. 4.3).

4.5 Conclusions

In conclusion, we reported a novel strategy to further improve the antitumor activity the RNA replicase-based pSIN-β plasmid by including an IL2 gene in the plasmid to boost antitumor immunity by activating T cells and NK cells. We also confirmed the sigma receptor as a potential target for future targeted gene therapy of melanoma.

Chapter Five

General Conclusions

The present work represents a departure from the conventional paradigm in tumor dsRNA therapy. Instead of dosing with synthetic dsRNA directly, an RNA replicase-based plasmid was actively targeted into tumor cells, enabling the cells to produce their own dsRNA and then commit 'suicide'. The replicase based plasmid stimulates both innate and adaptive immune response and induces both local regional and systemic anti-tumor effects. The response due to the pSIN-IL2 plasmid can be grouped as transgene related (IL2 and nsp4) and pDNA/LP related. The mechanism of action of this plasmid is expected to be by three ways: the generation of dsRNA that is known to have multiple direct and indirect pro-apoptotic, anti-proliferative, anti-angiogenic, and immunostimulatory activities; IL2 expression that promotes the proliferation of cytotoxic $CD8^+$ T-cells, $CD4^+$ T-cells, and natural killer (NK) cells, and unmethylated CpG motifs that activate innate immunity.

Intracellular dsRNA is more effective than extracellular dsRNA in promoting tumor cells to undergo apoptosis and orchestrate the initiation of adaptive immune responses. We proposed and validated an alternative, indirect dsRNA delivery approach, which involved the delivery of plasmid DNA (pSIN-β) into tumors. The pSIN-β plasmid encodes a replicase complex that generated dsRNA inside tumor cells. We established that dsRNA was produced inside the tumor cells, and further found it to have antitumor activity when administered peritumorally in TC-1 tumor bearing mice. The anti-tumor

activity from the pSIN-β plasmid required functional replicase genes nsp1-4. We also found that unmethylated CpG motifs contributed to the anti-tumor activity of the pCMV-β plasmid.

We further improved the pSIN-β plasmid by targeting it to epidermal growth factor receptor (EGFR) overexpressing cancer cells. We utilized the overexpressed EGFR as the target to more specifically deliver the pSIN- β plasmid into tumor cells by complexing it with cationic liposomes surface-conjugated with EGF, a known EGFR ligand. We evaluated EGF-PEG liposomes and pSIN- β plasmid at various weight per weight ratios in terms of particle size/zeta potential, DNase protection, and MDA-MB-468 uptake. *In vitro* uptake of the pSIN- β lipoplexes by human breast adenocarcinoma cells with varying expression levels of EGFR indicated a direct correlation to reported EGFR density of the cells. We further investigated the effect of pre-incubation with free ligand and found the uptake was inhibited on the cells with the highest amount of EGFR expressed. The pSIN-β plasmid complexed with EGF-PEG liposomes was more effective at controlling the growth of MDA-MB-468 tumors in mice compared to the EGF-free PEG liposomes. We found that that mice treated with the EGF-PEG liposome complexed with pSIN-β had greater pro-apoptotic, anti-proliferative, and anti-angiogenic activities compared to tumor in mice treated with the EGF free lipoplex.

Lastly we improved the pSIN-β plasmid by incorporating interleukin-2 (IL2) into the plasmid. The pSIN-IL2 plasmid was then targeted to melanoma cells that over-express sigma receptors using anisamide conjugated cationic liposomes. *In vitro* uptake was significantly higher with the anisamide conjugated lipoplexes compared to the PEG-

LP alone. The pSIN-IL2 plasmid was more effective in controlling the growth of B16-OVA tumor cells in mice when complexed to the AA-PEG-LP than with the PEG-LP. The cellular distribution of the lipoplexes revealed earlier disassociation of the pSIN-IL2 complexed with the AA-PEG-LP than with the PEG-LP. The intracellular localization of the lipoplex revealed that lipoplexes prepared with AA-PEG-LP had higher levels of internalization compared to the PEG-LP lipoplexes. The pSIN-IL2 plasmid was more effective in controlling the growth of B16 melanoma in mice compared to pSIN-β or pIL2 plasmid. Treatment with pSIN-IL2 plasmid increased activated $CD4^+$ T cells, $CD8^+$ T cells, and NK cells in the peripheral blood and spleen of B16 melanoma-bearing mice.

In conclusion, we reported a novel strategy to generate intracellular dsRNA to control tumor growth; additionally by targeting the plasmid more specifically to tumor cells we further improved the antitumor activity of the plasmid. Incorporation of IL2 gene in the plasmid was found to boost antitumor activity likely by activating T cells and NK cells. The sigma receptor was also confirmed to be a potential target for future targeted gene therapy for melanoma.

Various aspects of the pSIN-IL2 plasmid may be improved upon including the insertion of a tumor specific promoter, cloning of an IRES site in between the replicase complex and IL2 which would allow for both genes to be transcribed by the same promoter making it bicistronic.

There are a considerable amount of parameters that should be taken into account when using a lipoplex system including serum stability, targeting, biodistribution, dose, number of administrations, and mixing parameters. A significant amount of work needs

to be done for scale-up and commercial development. The present work should be viewed in terms of efficacy against specific tumor models. Future work should focus on understanding the mechanism for the delivery system of plasmid DNA, engineering a delivery vehicle that is able to protect plasmid DNA from degradation and prevent interactions with blood components for systemic delivery of plasmid DNA. A targeted lipoplex with sufficient stability until it reaches site of action remains a daunting task. More work is needed for *in vivo* systems and parameters that affect release of plasmid in tumor microenvironment. In the next 5-10 years, we expect more studies further validating and improving the efficacy of this plasmid DNA-based tumor dsRNA therapy, which will provide the underpinnings for future translational studies of this approach.

References

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