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Intracranial Drug Delivery Of Sirna Nanoparticles To Tumor Cells

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Intracranial Drug Delivery of siRNA Nanoparticles to Tumor Cells

A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

Chikezie Eseonu

2011

Abstract

Glioblastoma multiforme, an aggressive malignant tumor, continues to be amongst the most fatal disorders in medicine despite many therapeutic techniques and drug discoveries. Malignant brain tumor cells' ability to invade surrounding brain parenchyma is the main reason for treatment failure and recurrence. Traditional chemotherapy methods have found difficulty accessing the brain due to the blood-brain barrier, while irradiation techniques cause damaging effects on normal regions of the brain. New forms of gene therapy have been found to eliminate tumor cells, while sparing healthy brain tissue; however, with no efficient delivery mechanism gene therapy has been unable to access a large amount of tumor cells to eliminate the tumor mass and prevent the likelihood of recurrence. Local delivery of gene therapy has introduced a new method that looks to solve this problem, by delivering therapy directly to the brain tissue. One method of local delivery is convection-enhanced delivery (CED) of poly(lactic-co-glycolic acid (PLGA) nanoparticles, a biocompatible polymer material. Using a catheter and pump, this intracranial drug delivery method allows for drug-loaded nanoparticles to be released directly into brain tissue in a bulk flow of fluid. This method bypasses the blood-brain barrier and has a large distribution volume which has direct access to the tumor mass.

The efficacy of CED of siRNA nanoparticles on suppressing gene expression in tumor cells was studied in both *in vitro* experiments and *in vivo* rat models. Fisher 344 rats were used as the animal model and 9L gliosarcoma cells, a highly aggressive malignant tumor, labeled with enhanced green fluorescent protein (EGFP) were the tumor source. Small interfering RNA targeting the EGFP gene (siEGFP) were encapsulated into PLGA nanoparticles and used as a gene therapy. Our study shows that siEGFP nanoparticles are capable of causing in vitro and in

vivo gene expression reductions of up to 50 and 60%, respectively. When 9L cells were exposed to small interfering epidermal growth factor receptor (siEGFR), which targets the EGFR gene, *in vitro* growth suppression was observed that reduced 9L cell growth by 89% compared to the untreated control. These gene knockdown results shown in this thesis suggest that siRNA-loaded PLGA nanoparticles provide a great potential means for treating tumors.

Acknowledgements

The author would like to thank Dr. Joseph Piepmeier and Dr. Mark Saltzman for their assistance and guidance with this thesis.

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Introduction

Each year in the United States nearly 16,000 people are diagnosed with primary brain tumors. Sixty percent of the brain tumors diagnosed in the United States are gliomas. Gliomas are a heterogenous mix of neoplastic astrocytes. Amongst these gliomas, glioblastoma multiforme (GBM) is the most common malignant glial tumor accounting for 12-15% of all intracranial neoplasms. This neoplasm is often found in the subcortical white matter of the cerebral hemispheres. In the United States and Europe, the incidence of GBM is 2-3 cases per 100,000 people (1).

There are two forms of Glioblastoma multiforme, primary and secondary. Primary GBM is more prevalent (60% of cases) and consists of a de novo neoplasm that has no histopathologic evidence of a precursor lesion. This type of lesion tends to present in patients over 50 years old. Secondary glioblastoma multiforme often occurs in patients under 45 years old and forms from a slow progression of either a low grade astrocytoma or anaplastic astrocytoma. This progression can take anywhere from one to ten years (2).

The etiology of GBM tumors varies widely. Some of the genetic abnormalities seen in GBM tumors consist of 1) Loss of heterozygosity on the chromosomal arm 10q, 2) mutation in the tumor suppressor gene p53, 3) mutations in the epidermal growth factor receptor gene, which controls cell proliferation, 4) overexpression of the MDM2 gene, 5) overexpression of the platelet-derived growth factor-alpha gene (PDGF-α), which acts as a major growth factor for glial cells, and 6) mutation of the PTEN gene which encodes a tyrosine phosphatase that turns off signaling pathways. Other mutations that are present in the most malignant forms of GBM

tumors are the MMAC1-E1 gene mutation, MAGE-E1 gene mutation, and NRP/B-A nuclear-restricted protein mutation (2).

Mean survival for GBM patients is inversely correlated with age. Glioblastoma multiforme is slightly more common in whites and has a male-to-female ratio of 3:2. Without treatment patients suffering from GBM will die in 3 months. Those undergoing the standard treatment of surgical resection, radiation therapy, and chemotherapy have a median survival of 9 months, with about 10% percent of these patients surviving for up to two years (1).

Treatment options

Glioblastoma multiforme remains a difficult tumor to cure. Despite what is known about the gene mutations of the tumor, multiple challenges remain. Current treatment aims at improving the quality of life of a patient with the standard care consisting of tumor resection followed by a combination of chemotherapy with temozolamide and radiation therapy. However these treatments have their limitations. Poor tumor cell drug uptake, drug metabolism within the cell, and the degree of tumor cell sensitivity to a drug all limit the effectiveness of chemotherapies (1). Surgical resection is often limited depending on the tumor's location and the eloquence of the brain region. In addition, the infiltrative nature of GBM tumors causes the majority of resections to be incomplete which allows infiltrative tumor cells to reform tumor within the near proximity of the resected site (3).

Available chemotherapies have provided modest prolongation of survival and have been the focus of many laboratories. Temozolomide, the current standard, has shown some promise. A phase III randomized study showed that low-dose temozolomide used along with radiation therapy followed by six additional months of temozolomide showed a statistically

significant extended survival length of 14.6 months compared to 12.1 months with radiation therapy alone. Another phase III randomized study looked at the implantation of FDA approved Gliadel wafers (polymer wafers with carmustine) into the tumor resected area of the brain and found prolongation of survival of 13.9 months compared to 11.6 months in the control group that received only radiation therapy (2).

The Blood Brain Barrier and Brain tumor treatment

In addition to tumor insensitivity and invasiveness, many chemotherapies are unsuccessful at treating central nervous system (CNS) disorders because of their inability to cross the blood brain barrier (BBB). This is a specialized system of capillary endothelial cells that creates a semi-permeable barrier that protects the brain from noxious substances while still transporting essential nutrients to the brain. The BBB is composed of an inner layer of endothelial cells with tight junctions, a basement membrane, pericytes, and astrocytes. This serves as both a physical barrier and a biochemical barrier that is able to express peptidases that aid in effluxing drugs from the endothelial capillary cells back into the blood stream. Small molecules that are less than 400 Da and have a high lipid solubility are two characteristics that have been shown to allow for transport across the BBB. Some regions of the CNS, known as circumventricular organs, provide areas where the BBB is absent and the capillary system consists of fenestrated endothelial cells. Areas such as the choroid plexus, pineal gland, neurohypophysis, area postrema are a few examples of this (4).

In order to make chemotherapies effective once introduced into circulation, the BBB must be accounted for and several methods of drug delivery have had to deal with this issue.

Intravenous drug delivery allows for the administration of large amounts of drug into the blood circulation and avoids the first-pass metabolism. However, intravenous administration of therapeutic drugs would result in little accumulation of the drug in the brain as a result of ineffective penetration across the BBB, as well as rapid metabolism of the drug in the plasma, and non-specific binding to plasma proteins (4).

Intraarterial administration of drugs has also been used as a method to access the brain. This method allows for a drug to enter brain vasculature prior to entering peripheral tissue which bypasses first pass metabolism. It is believed that drugs travel arterially into the choroid plexus epithelium, then are transferred into the cerebral spinal fluid (CSF). Often BBB disrupting agents such as bradykinin and mannitol can be used intraarterially to enhance the delivery of drugs to the brain, however, the amount of drug that reaches the brain is difficult to determine with this method (4).

The intranasal route allows a drug to reach the brain by traveling through the nasal mucosa. In this instance, the drug travels from the submucosa of the nose into the CSF. This method is non-invasive and bypasses first-pass metabolism and the BBB completely. The permeability of the nasal epithelium allows for the uptake of drugs tranasally into the brain and allows for the delivery of micro and macromolecules. Intranasal delivery allows for self-administration of small doses without the need of any modifications of the drug, such as coupling to a carrier. Limitations of this method include damage and irritation to the nasal mucosa, rapid clearance of the drug via mucocilia in the nasal cavity, and interruption of drug transport secondary to nasal congestion (4).

Another method used to completely bypass the BBB is through direct delivery of the therapeutics into the CSF of the CNS. This method allows for higher concentrations of drug into the brain, avoids systemic exposure toxicities, and maintains the drug for a longer period of time within the brain. This method can produce variable results depending on where the drug site of puncture is located, rate of drug clearance, CSF production rate, and the drug diffusion rate (4).

Intraparenchymal delivery allows for drugs to be injected as a bolus or an infusion through catheters or microparticles. Bolus injection of a drug into the brain causes slow movement within the parenchyma due to limited diffusion in the brain. Large amounts of drug must be used in order to overcome diffusion limitations to allow for adequate concentrations of the drug to be available to the surrounding parenchyma. In an attempt to overcome diffusion issues, infusion of a drug using convection enhanced delivery (CED) distributes the drug to a wide area of brain tissue (4).

Convection Enhanced Delivery

CED is a method that was developed to overcome the diffusion limitation posed by the brain. CED applies an external force to induce fluid convection within the brain. Small catheters are placed several centimeters into the brain and are used to deliver a therapeutic via a pump. High pressures are used to produce convective flow. CED provides larger distribution volumes when compared to bolus injections. It also provides long infusion times and is a viable method of delivering gene therapy, chemotherapy, and immune therapy. Problems with this procedure are related to its invasiveness and drug distribution limitations that result from the anatomical environment. These limits include backflow and occlusions of the catheter as well

as high intracranial pressures from the catheter infusion. Small air bubbles can form from the infusion and cause damage to brain tissue. The distribution of drug delivery is also unpredictable. The white matter tracks of the brain provide highly conductive routes for the infused drug. With this high conductivity and the backflow in the catheter, much of the drug delivered by CED will not be able to diffuse throughout the brain parenchyma. In addition, perivascular spaces within the brain as well as the wound track of the injection or the space underneath the scalp can collect the infused drug during delivery shifting it away from its target. These factors make it difficult to determine if the infused drug is a consistent concentration throughout its distributive space and may also lead to edema (3).

A recent phase III clinical study compared Gliadel to CED delivery of IL-13PE38QQR in patients who had failed conventional treatment for GBM tumors (surgery, radiotherapy, and chemotherapy). The study showed that there was no significant difference in patient outcome with the median survival following tumor recurrence with the Gliadel and CED being 36.4 weeks and 35.3 weeks, respectively (5). A follow up paper suggested that imprecise catheter placement may have resulted in an absence of benefit in the CED exposed patient population (6).

Compared to bolus injections, CED maintains drug distribution and concentration over a longer duration. Alam et al. looked at the delivery of cytosine arabinoside, an antimetabolic agent used as a chemotherapy, and showed that CED provides for intraparenchymal concentrations that are 100 times greater than intranasal delivery and 1000 to 10,000 times greater than intravenous delivery (4).

Implantable devices that provide controlled release of a drug within the brain are also used to provide direct drug delivery to the brain. These implants are made of either biodegradable or non-biodegradable material and often encapsulate the drug. They are placed into the brain where they release the drug for a predetermined duration. One type of implantable device is the osmotic pump with refillable reservoirs that distributes drugs via a catheter.

For the purposes of this thesis, most of the focus will be on biodegradable, and more specifically polymer implants that are often used to deliver drugs to tumors. These devices are constructed in the form of either wafers or nanoparticles and offer continued release of drugs from a degrading polymer. (4).

Wafer polymers, such as Gliadel, are polyanhydride wafers that contain the chemotherapeutic agent carmustine (BCNU). This device has been FDA approved in the U.S. for implantation for new and recurrent Glioblastoma tumors. Following the resection of a tumor these wafers can be inserted into the resected tumor cavity where they release the chemotherapy at a controlled rate that is proportional to the degradation rate of the specific polymer. The tumor is exposed to 113 times the concentration of BCNU with Gliadel than would be achieved with systemic administration of the drug (7). The localized chemotherapy provided by the wafers prevents a patient from being exposed to the high levels of radiation therapy that can weaken the immune system (3).

Although wafer polymers provide a controlled release system that is capable of sustained release for long durations of time, local penetration of the drug is often restricted by diffusion. The drug's low diffusion coefficient as well as the extracellular matrix (ECM) of the

brain, which presents small pore sizes, and the local environment of the tumor further limit the diffusive abilities of a drug. In addition, many drugs have high rates of elimination. The stated limitations cause a given drug to travel a minimal distance from its starting locus before degrading. For the wafer polymer, the drug distribution is confined proximally to the wafer while the invasive tumor cells extend beyond this distance. One way to bypass this issue is by using a polymer delivery system that is capable of navigating through the pores of the ECM in order to provide better diffusion throughout the brain (3). Nanoparticles provide a smaller delivery vector that may be capable of greater distributive properties.

Nanoparticles

Nanoparticles have become a popular mode of drug delivery to tumors in recent years. With their small size, controlled release capabilities, and the ability to modify the carriers to target selected molecules and receptors, nanoparticles present a new means for tumor therapy. Nanoparticles can be used via direct infusion into the brain or by endcytotic mechanisms that allow them to cross the BBB (4). They can be either nondegradable or biodegradable. The nondegradable nanoparticles function by releasing drug from the nanoparticle matrix which then diffuses into the brain. Biodegradable drug release is determined by the degradation of the polymer matrix in addition to the diffusion of the drug. Biodegradable polymers can be combined to form copolymers that modify the degradation and release pattern of the nanoparticle. Properly designed nanoparticles have the capability of sustained drug delivery for several days to years. Alterations can also be made to the polymer composition to affect release rate and longevity of a drug. Unlike biodegradable wafers, nanoparticles have the ability to completely erode and be cleared from the body. The

degradation of the nanoparticles occurs via hydrolysis, utilizing the large amounts of water in the human body. Nanoparticles are also advantageous to their polymer wafer counterparts because the particles are able to be delivered less invasively to the brain via a burr hole and catheter (3).

Polymer constructs can be altered in order to maximize the release of a drug. For example, the popularly used poly[bis(p-carboxyphenoxy)] propane-sebacic acid (p(CPP-SA)) is hydrolytically unstable when combined with the drug 4-hydroperoxycyclophosphamide (4HC), however, fatty acid dimer copolymers with 4HC provide the advantage of stability over the p(CPP-SA).

Another type of biodegradable polymer that has often been used in local drug delivery is the polylactic-coglycolic acid (PLGA) copolymer, which is a polymer matrix composed of lactide and glycolide polymers. PLGA is a hydrophobic polymers whose monomer components are biocompatible. This biomaterial has been used to make both nanoparticles and wafers and provides the ability to modify the characteristics of these particles that alter size and drug release. Studies dealing with the characterization of nanoparticles have shown that smaller particles, 20nm in diameter or smaller, show more diffusion within the brain than larger particles, greater than 40nm. Surface characteristics can also play a role in volume distribution. Mamot et al showed that particles with neutral or negative charge or that are coated with bovine serum albumin (BSA) or poly(ethylene glycol) (PEG) are able to maximize distribution volume, whereas positive charge has restricted diffusion. (3)

Combining CED with nanoparticles theoretically improves the delivery of drug to the brain. The encapsulation of the drug within the nanoparticle prevents drug reflux which

improves delivery and helps prevent wound dehiscence. Encapsulated drugs can also allow for shorter infusion times using CED. This would cause smaller volumes of infusates and reduce the risk of edema and inflammation. However, combining CED and nanoparticles may also present problems. The polymer nanoparticles have to be large enough to deliver a relevant dose, however small enough to diffuse sufficiently throughout the parenchyma (3).

Small Interfering RNA nanoparticles

In addition to CED, other modifications can be made to polymer nanoparticles to help improve therapy. One of these alterations includes using small interfering RNA (siRNA) as the drug therapeutic. RNA interference is a sequence specific gene silencing application which utilizes a 21-25 base pair double stranded RNA nucleotide sequence, known as siRNA. The siRNA are integrated into RNA induced silencing complex (RISC) in the cytosol of the cell. The RISC complex uses the antisense strand of the siRNA to bind to the complementary messenger RNA (mRNA) strand in the cell. This initiates the initial cleavage and degradation of the specific mRNA sequences (8).

When preparing siRNA as a therapeutic for tumors, multiple steps must be taken. First the siRNA must target mRNA whose downregulation would suppress growth or is integral to the disease process. The siRNA must then be placed in a delivery complex that is capable of stability within an in vivo environment. Finally the siRNA complex must be efficiently delivered to the brain or tumor site in order to effectively knockdown gene expression (8).

Some limitations that are involved with naked siRNA include early extracellular degradation in the serum, often having a half-life of a few minutes to one hour. Some solutions that have tried to improve the stability of siRNA include chemical modifications that alter the

sugar backbone of the siRNA molecule. In addition, polymer or lipid encapsulation have been shown to protect the siRNA from early degradation. Naked siRNA also has a relatively large molecular weight of around 13 kDA and an anionic charge, both which makes it impossible to diffuse freely across the cell membrane. Certain solutions to this problem include conjugating the siRNA to cell penetrating peptides (CPPs) or cholesterol. Encapsulation of the siRNA with a polymer will also allow for endocytosis of the siRNA/polymer complex into the cell. (8)

Although siRNA are beneficial for their specificity in targeting genes, other issues with this type of therapy include nonspecific mRNA binding that can occur through partial mismatch or binding of the sense strand to the mRNA. This nonspecific binding may cause interferon directed inflammation, toxicity to the cell, and unintended effects on the gene target. Chemical modifications to the siRNA including annealing of the guide strand to an additional strand can prevent non-specific effects. (8)

The limited success with the delivery of naked siRNA makes it necessary to develop a delivery vehicle that is capable of transporting and releasing siRNA adequately. Nanoparticles provide a means of achieving this. Peptides, lipids, and polymers are among some of the materials that have been used to deliver siRNA and are described below (8).

The smallest type of nanoparticle (about 10nm) are those where the sense strand of the siRNA is attached to a peptide or small molecule. This modification of the sense strand does not affect the siRNA's ability knockdown a gene, since the antisense portion of the siRNA is what binds to the mRNA. Studies have shown that conjugating siRNA to CPP or PEG have improved in vivo siRNA transfer (8).

Larger nanoparticles allow for easier modifications that can enable cell/tissue specific targeting, longer circulation times, and better prevention of siRNA degradation. Cationic polymers, ranging from 100-300nm have been shown to form electrostatic interactions between the positive charges of the polymer and the negatively charged phosphate groups of the siRNA backbone (8).

Cationic lipid vectors, also ranging from 100-300nm in size, have also shown great promise with knocking down genes using siRNA. Lipid nanoparticles are believed to have a weaker electrostatic bond with the siRNA which allows for easier release of the siRNA into the cytosol. Several lipid vectors have been commercially produced to deliver siRNA to cells. These include Lipofectamine RNAiMAX and DharmaFECT, as well as others (8).

Both cationic lipids and polymers have the complication of toxicity. This can be avoided by using a neutral liposome vector, often smaller than 200 nm in size. These nanoparticles often have hydrophilic cores that contain the siRNA with hydrophobic surfaces that protect the siRNA from degradation from the surrounding environment. The hydrophobic surface also aids with internalization of the siRNA through endocytosis or membrane fusion (8).

Other nanoparticles include Hyaluronic acid nanoparticles, also known as nanogels, which have successfully targeted CD44 receptors in vitro. Poly(d,l lactic-co-glycolic) acid (PLGA) nanoparticles have also been used with siRNA and have shown significant knockdown of genes in vivo with less inflammation in comparison to the lipid equivalents. In addition, calcium carbonate nanoparticles have also shown reduced toxicity with efficacious delivery of siRNA both in vitro and in vivo. Gold nanoparticles have also been shown to extend siRNA half life sixfold compared to naked siRNA (4).

Limitations also exist for siRNA encapsulated nanoparticles. Nanoparticle aggregation can occur due to the positive surface charge of the nanoparticles. This can be addressed by introducing poly(ethylene glycol) (PEG) or certain sugar molecules (cyclodextrin and hyaluronic acid) to the reduce surface charge. By neurtralizing the surface charge the nanoparticles will be capable of circulating for longer periods of time and non-specific interactions between the positively charged nanoparticles and the negative charge of the cell membrane will be minimized. Targeting the nanoparticles to a specific tissue or cell has also been a problem with this delivery vector. Modifications to the nanoparticle complex with ligands or antibodies that recognize specific receptors has helped with this problem. Nanoparticles are also capable of entering the cell via endocytosis (1). However, for the siRNA to be effective it must be released from the nanoparticle and endosome to the cytosol in order for the RISC complex to form. Modifications to the nanopartcle with ligands that specifically target receptors that will mediate endocytosis can enhance nanoparticle internalization. Once within the endosome the nanoparticles must evacuate the endosome to get into the cytosol, which can be achieved by using polymers that contain protonable amines that are capable of disrupting the endosomal membrane or by using polymers that transition from hydrophilic to hydrophobic and can lyse the endosomal membrane. Although methods have been used to address endosomal escape, new studies have shown that while 90-95% of internalized siRNA nanoparticles occurs via endocytosis, it is the remaining 5% that enters the cell through alternative pathways that provides the most functional siRNA within the cytosol (8).

GBM targets for siRNA

Nanoparticle delivery of siRNA has introduced a new means of gene therapy, however, finding the appropriate gene target to suppress tumor growth or induce apoptosis has been a difficult feat. Identifying an appropriate marker for brain tumors and understanding its variations and evolutions are important.

Epidermal growth factor receptor gene is a gene commonly found over-expressed in GBM and is found in about 40% of GBMs patients. Within this population of GBM tumors that over-express the EGFR, 63-75% of them have rearrangements of the EGFR gene which can cause tumors to contain wild type EGFR as well as a mutated form. Amongst these mutations the EGFR variant III, or EGFRvIII, is the most common of the mutated forms and is rarely seen in normal tissue. This variant is often seen in 20-30% of GBM patients without an overexpression of EGFR and 50-60% of patients with an overexpression. This mutation often results from a loss of exons 2 to 7 in the EGFR gene (9).

Type 1 interferon gamma has also shown antitumor activity, however with a short half-life and toxicity occurring with large doses, interferon must be continuously delivered which increases the likelihood of toxicity.

Research with nanoparticles and GBM

Within the last decade an increasing number of biodegradable polymers have been used against Glioblastoma Multiforme. Chemotherapy with cisplatin was added into 6-carboxylcellulose polymer then infused into post-irradiated patients and was found to be extend survival to 427.5 days compared to the control group which survived 211.0 days (10).

A recent Phase I/II clinical trial used CED of a liposomal vector containing the HSV-1-tk gene with systemic ganciclovir to treat glioblatoma. This treatment was shown to have minimal side effects and showed a 50% tumor volume reduction in 25% of the treated patients (11).

The copolymer polifeprosan 20 (1,3 bis(p-carboxyphenoxy) propane and sebacic acid in a 20 to 80 molar ratio with carmustine wafers (Gliadel®) have been shown to have sustained release for up to five days when placed in a tumor resected cavity (4). The Gliadel wafer spans 14mm in diameter, 1mm in thickness and is loaded with 7.7mg of carmustine. Studies have shown improved survival of patients with malignant gliomas who received Gliadel either as a treatment for an initial tumor presentation, recurrence, or as an additional therapy with radiotherapy. There have however been mixed reports about the affects of Gliadel in regards to toxicity, inflammation, infection rates in patients (3). Similar to other wafers, the chemotherapy released by the Gliadel has relatively poor diffusion throughout the brain. To resolve this issue smaller drug carrying polymers that are able to perfuse the brain parenchyma and deliver the chemotherapy can be used. Among the biomaterials that have been used for this purpose, the copolymer poly(lactide-co-glycolide) has shown the most promise for delivering drugs and peptides and will be further explored in the experiments of this thesis.

Statement of purpose specific hypothesis and specific aims of the thesis

Hypothesis:

Convection-Enhanced Delivery of siRNA-loaded Polymer Nanoparticles Produces Effective Gene Knockdown in gliosarcoma tumor cell both in vitro and in vivo.

Specific Aims:

The goal of these experiments was to develop a new effective delivery method for treating tumor cells in rats. Convection-enhanced delivery of siRNA nanoparticles can introduce a new means of eliminating invasive tumor cells and provide an innovative strategy for brain cancer treatment. The enhanced green fluorescent protein (EGFP) transfected into the tumor cells allowed for good visualization of tumor growth and volume quantification after harvesting the brain tissue.

This project was divided into multiple parts with specific objectives:

The first set of experiments consisted of an in vitro analysis of the growth pattern of EGFP 9L gliosarcoma cells that were transfected with EGFP via lentiviral vectors, in order to prove consistent growth patterns between transfected and non-transfected 9L gliosarcoma cells. The next set of experiments aimed to analyze the efficacy of siRNA knockdown of EGFP from the 9L gliosarcoma cells in vitro, using lipid vectors and PLGA vectors.

Experiments then looked at the growth rates of 9L gliosarcoma tumor cells with enhanced green fluorescent protein (EGFP) in Fisher 344 rats in order to establish the in vivo growth rate of the tumor cells. Once the untreated tumor cell growth curve had been established, small interfering enhanced green fluorescent protein (siEGFP) loaded polymer nanoparticles were infused into growing EGFP tumor cells using the convection-enhanced delivery method with the aim of determining the effectiveness of CED of nanoparticles in knocking down gene expression.

The next experiments aimed at identifying a gene within the gliosarcoma cells that would suppress tumor growth. In vitro studies determined the efficacy of siRNA gene

knockdown and growth suppression. Nanoparticles loaded with small interfering RNA targeting epidermal growth factor receptor (EGFR) were used.

Methods

Cell Lines

The 9L rat gliosarcoma cell lines where maintained in a Dulbecco's Modified Eagle Medium (DMEM) solution that was supplemented with 10% FBS and 1% Penicillin-Streptomycin-Fungazone antibiotic.

Cells were split by removing old media and rinsing cells once with 10 mL sterile PBS. Trypsin EDTA 1X (.25%) was used to detach cells. Additional media was added to the cells and they were centrifuged with the supernatant disgarded. The cell pellet was then resuspended in fresh media and added back into a 275 mL flask. Feeding of the cells was done daily. The old media was removed and 12mL of fresh media was added to each flask. The flasks were then incubated at 37°C.

Transfection of 9L gliosarcoma cells

The 9L gliosarcoma cell line was transfected with enhanced green fluorescent protein (EGFP) using the pSicoR plasmid (addgene©) and lentiviral vectors by Dr. Jiangbing Zhou, a member of Dr. Mark Saltzman's lab.

In vitro growth rate analysis

Both the normal 9L cells and EGFP transfected 9L cells were plated in 6-well plates (5x10^4 cells/well) with 3mL culture media per well. Cells were extracted using trypsin and counted using a hemocytometer on day 2 and 3.

Production of siRNA PLGA nanoparticles:

Polylactic-coglycolic acid (PLGA) were loaded with siRNA/polyamines using the double-emulsion solvent evaporation technique. The siRNA was reconstituted in deionized water. The polyamine complexes with siRNA where formed at room temperature for 15 minutes on a rotary shaker. The siRNA (30-300nmoles) was combined with the polyamine at a molar ratio of the polyamine nitrogen to the polynucleotide phosphate (N/P ratio of 8:1). A molecular weight per nitrogen of 85g/mole of spermidine was used. The aqueous solution was then added dropwise to a PLGA polymer solution and dissolved into dicholormethane (2mL) to create the first emulsion. The emulsion mixture is then added dropwise into 4 mL of 5% polyvinyl alcohol (PVA) and sonicated to form the double emulsion. This final emulsion was then poured into aqueous 0.3% (v/v) PVA and stirred for 3 hours, which allows the dicholormethane to dissolve and the nanoparticles to harden. Sonication time and amplitude were optimized to formulate particles with 100nm diameter. Particles were then collected by centrifugation, washed, rapidly frozen, and lyophilized. The nanoparticles contained 300pmol siRNA/mg of nanoparticle.

The siRNA sequences used were siEGFP, sense 5'-GGCUACGUCCAGGAGCGCACCdTdT-3' and antisense 5'-UGCGCUCCUGGACGUAGCCUUdTdT-3'(MW = 14,669.4 g /mol), siEGFR sense 5'-CCGAAUUUAUACACACCAAdTdT-3', and antisense 5'-UUGGUGUGUAUAAAUUCGGdTdT-3' (MW = 13,273 g/mol).

Characterization of Nanoparticle size and siRNA loading

In order to determine the loading efficiency of the PLGA nanoparticles, 5mg of nanoparticles of siRNA nanoparticles were dissolved in 0.5mL of dichloromethane at room temperature for 30 minutes. The nanoparticles were extracted from the organic phase using

0.5mL TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.4). The TE buffer was added to the organic phase and vortexed for 1 minutes, and then centrifuged at 12,000 rpm for 5 min at 4°C. The combined 1 mL aqueous fraction was analyzed for double-stranded RNA content using the QuantIT™PicoGreen™ assay (Invitrogen). A standard curve relating fluorenscence to siRNA concentration was used to determine the amount of siRNA in the nanoparticles.

Particle size was determined using the dynamic light scattering (DLS) feature of the ZetaPals (Brookhaven Instruments) particle size analyzer. Particles were analyzed in a 10µg/mL in 2mL PBS solution. The particle size was also analyzed using an image analysis of micrographs from a scanning electron microscopy (SEM). The samples were coated with 25 nm-thick gold using a quick carbon coater. Using image analysis software the particle diameter and size distribution was determined.

Nanoparticle Delivery to Cultured Cells

Cells were plated at 3x10^4 to 5x10^4 cells per well in a 6-well tissue culture treated plate and left to proliferate. After 48 hours in culture, cells were at 30-50% confluence. Nanoparticle treatment groups and controls were diluted in the DMEM solution with supplements at a concentration of .1mg/mL for the particles. Cells were exposed to 10nM-25µM of siRNA. The treatment groups included siRNA-loaded PLGA nanoparticles, siRNA-loaded lipid nanoparticles, a control with no siRNA. Treatment groups were placed on the cells in 100µL volumes and left for 48, 72, and 120 hours at 37C and 5% CO2. At the end of incubation, the cells were washed with PBS. Cells were then extracted using trypsin and evaluated using FACS or RT-PCR.

FACS analysis

The LSR II Green (BD Biosciences) multilaser multiparameter analysis was utilized. The digital data collection was done using the FACS DIVA software. The 488nm (blue) detector was used to identify the GFP fluorescence. Round bottom 12 x 75 mm polystyrene falcon tubes were used. Cells were extracted and resuspended in 1mL of PBS supplemented with 10% fetal calf serum (FCS), 1% sodium azide.

RT-PCR

RNA was isolated from the 9L gliosarcoma cells using the RNeasy®Mini Kit (Qiagen) based on the manufacturer's protocol. The purified mRNA was reverse transcribed using the iScript DNA synthesis kit (Biorad) to produce cDNA. Real time PCR was conducted on 2µL of cDNA combined with the iQ SyBr Green (biorad) reagents for fluorenscent detection of the PCR product. Primers used for the RT-PCR are: EGFP_forward: 5'- GAAGCGCGATCACATGGT – 3', EGFP_reverse: 5'-CCATGCCGAGAGTGATCC-3'. PCR parameters consisted of 5 minute activation of the DNA polymerase at 95°C, followed by 40 cycles of expression of 95°C x 20 s, 60°C x 30 s, and 72°C x 20s.

Implanting tumors in Rat Brain Tissue

The glioma tumor cell line that was used in these experiments were 9L gliosarcoma cells. These cells induced intracerebral tumors via a craniotomy and using stereotaxis to determine the injection locations in the brain.

During the surgery the rodents received pre-procedural anesthetic intraperitoneal injections of ketamine/xyalzine mixture (80/10 mg/kg), followed by an intraperitoneal analgesic, meloxicam at 0.3 -1.0 mg/mg. Once anesthetized, the scalp of the rodent was shaved using clippers, and the scalp was sterilized using alcohol and betadine wipes. The

animal was restrained on a stereotaxic frame, which held the animal in place using two ear bars and a nose bar. Heart rate and respiratory rate of the animal were monitored manually during the surgery. These intracranial surgeries took approximately 30 minutes per rat. A midline incision was made along the scalp using a scalpel. The pericranium was pushed aside and excess bleeding was wiped off using q-tips until the coronal and sagittal sutures were exposed. Once the injection coordinates are identified, a burr hole was drilled into the skull going -3.5mm posteriorly and 2.3mm laterally to the right from bregma using a high speed drill. A sterile needle punctured the dura and a Hamilton syringe was filled with 1.5µl ($1.5x10^5$ cells) of EGFP-labeled 9L gliosarcoma cells suspended in injection buffer (sterile PBS, $MgCl_2$ (1µg/mL), $CaCl_2$ (1ug/mL), 0.1% glucose). The needle was inserted 5.7mm deep into the cerebral cortex and allowed to equilibrate for one minute in the tissue. The needle was withdrawn about 0.2mm and allowed to equilibrate in order to give a space for the cells to fill. The cells were slowly injected (1.5µl in 1.5mins) into the tissue and given two minutes to settle before the syringe was removed. The burr hole was plugged with bone wax, and the incision was sutured or stapled.

Following surgery the animal was then taken to a recovery cage with food and ibuprofen in drinking water for 48 hours after the surgery. The animal was monitored daily for signs of pain, discomfort, or abnormal movement or behavior. The incision was checked and cleaned if necessary.

Animal Maintenance

Fischer 344 rats were used as a model system for this project because of they were large enough to allow intracranial implantation of polymers of sizes that fall in the range of the

nanoparticles that were used for these experiments. The rats weighed 180-220 grams. Animals were housed in Yale Animal Resources Center (YARC) facilities (Malone Engineering Center) and received the standard care.

Harvesting of Tissue and Growth Analysis

Following tumor implantation, animals were maintained for various amounts of time: 1-15 days. Animals were euthanized by carbon dioxide overdose according to YARC procedure, and the brain was removed and frozen directly on dry ice. The brain was then stored in a -80°C freezer before sectioning. The brains were sectioned into thin slices of known thickness by a microtome machine and then fluorescent images were taken of the coronal brain sections. The tumor showed up as a green image because of the EGFP, and the tumor volume was calculated from the area of green signal found on each slice and the given width of the slice.

Intracranial implantation of siRNA nanoparticles

The infusion procedure of the nanoparticles utilized convection enhanced delivery and was the same as the 'Implanting tumors in Rat Brain Tissue' procedure with a few alterations. A nose cone was added to the stereotaxis frame and 1-4% of isoflurane was added to the oxygen to maintain anesthesia for the longer procedure. The CED technique took longer because the infusion time and volume of nanoparticles infused (about 20ul) was large. A catheter probe was used to administer the nanoparticles and fluid into the brain tissue, substituting for the Hamilton syringe. The nanoparticles were re-suspended in 20µL sterile PBS buffer solution (supplemented with MgCl2 (1ug/mL), CaCl2 (1ug/mL), 0.1% glucose). A sterile needle was used to puncture the dura and a syringe with the catheter probe was filled with the suspended nanoparticles. The probe was then inserted into the right hemisphere of the cerebral cortex,

just anterior to the putamen for one minute to equilibrate with the tissue. The nanoparticles were slowly injected (1.5µl in 1.5mins) into the tissue and given two minutes to settle before the probe was removed. The burr hole was plugged with bone wax, and the incision was stapled. Typical surgeries took about one hour per animal.

Production of the Lipid nanoparticles

The siRNA was incubated with Lipofectamine™, a cationic lipid, in order to form a lipid complex that was infused into the brain tumor. Established protocols for this procedure are available at www.invitrogen.com/RNAiMAX.

Statistical Analysis

Experimental conditions were compared using a two tailed t-test to evaluate whether a significant difference existed between conditions. P-values less than 0.05 were considered to be significant.

The PLGA and Lipid nanoparticle conditions were corrected with their respective controls in the in vivo charts.

All experiments were conducted by the author unless otherwise stated.

Tumor fluorescent volume is a term used in this thesis that refers to the overall fluorescence generated by the 9L gliosarcoma cells. When exposed to small interfering EGFP (siEGFP), the actual tumor cell volume remains unchanged, but the siEGFP exposed tumor cells have a lower tumor fluorescent volume (Figure 1).

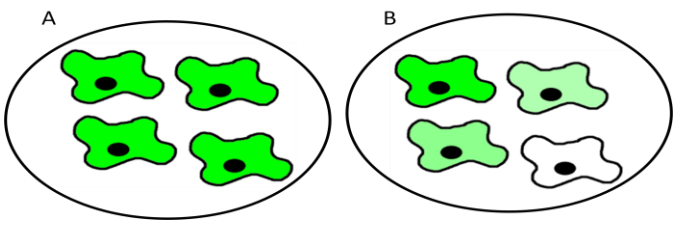

Figure 1: A, represents a group of EGFP transfected 9L gliosarcoma cells that have not been exposed to siEGFP, while B, represents a group of EGFP transfected 9L gliosarcoma cells after exposure to siEGFP. Specimen A and B have equal overall tumor volume, but specimen A has a higher tumor fluorescent volume than specimen B.

Results

EGFP transfected cell proliferation assay

A proliferation assay compared regular 9L gliosarcoma passage 5 cells to 9L passage 5 cells that were transfected with EGFP with a lentiviral vector in order to determine whether the transfected cells had a similar growth rate as the regular cells. On day two the transfected 9L cells showed a similar growth rate to the normal 9L cells with the standardized mean cell count being 9 ±2.2 and 10.1 ±2.1 respectively (Figure 2). The two tailed t-test showed a p-value of 0.68. The day three time points of the transfected cells showed a mean standardized cell growth of 18.6 ±2.9 and the normal 9L cells showed a mean of 22.6 ±3.1. The two tailed t-test showed a p-value of 0.13 (n=3).

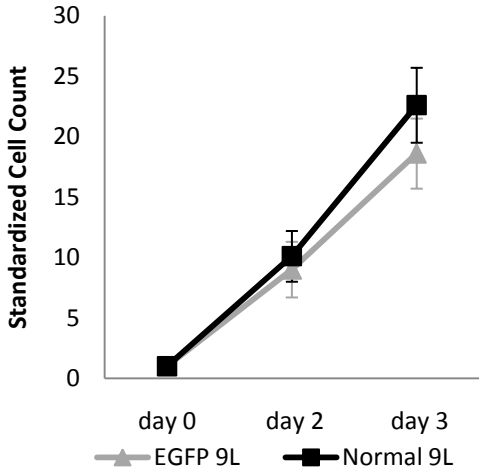

Figure 2: The EGFP transfected 9L gliosarcoma cells show a similar growth patterns as a non-transfected 9L gliosarcoma cells over a three day period. The p-value for both timepoints was p>0.05

Characterization of the siRNA

The PLGA nanoparticles loaded with siEGFP or siEGFR were analyzed by scanning electron microscopy and dynamic light scattering to characterize the size. The data generated from the DLS was used to create a histogram to calculate the average diameter of the nanoparticles. For the siEGFP-loaded PLGA nanoparticles the mean diameter was 110 ±24nm (n=100). The minimum and maximum diameters were 58.7 and 182.7 nm. This was supported by the SEM analysis. The histogram of the siEGFR-loaded nanoparticles showed nanoparticles

with a mean diameter of 109 ±20nm (n=103). The minimum and maximum diameters were 73.6nm and 176.5nm, respectively (Figure 3). The SEM confirms the size of the nanoparticles.

In vitro siRNA nanoparticle knockdown

An in vitro study was conducted that looked at the efficacy of siRNA knockdown of the EGFP using the PLGA nanoparticles. The EGFP transfected 9L gliosarcoma cells were plated on 6-well plates and exposed to three conditions: siEGFP-loaded PLGA nanoparticles, scrambled siRNA PLGA nanoparticles, and no nanoparticles. The cells were exposed to 10nM of siRNA within the nanoparticles. Over a four day period the siEGFP-loaded nanoparticles showed significant knockdown under visual microscopy. The PLGA nanoparticles with scrambled siRNA

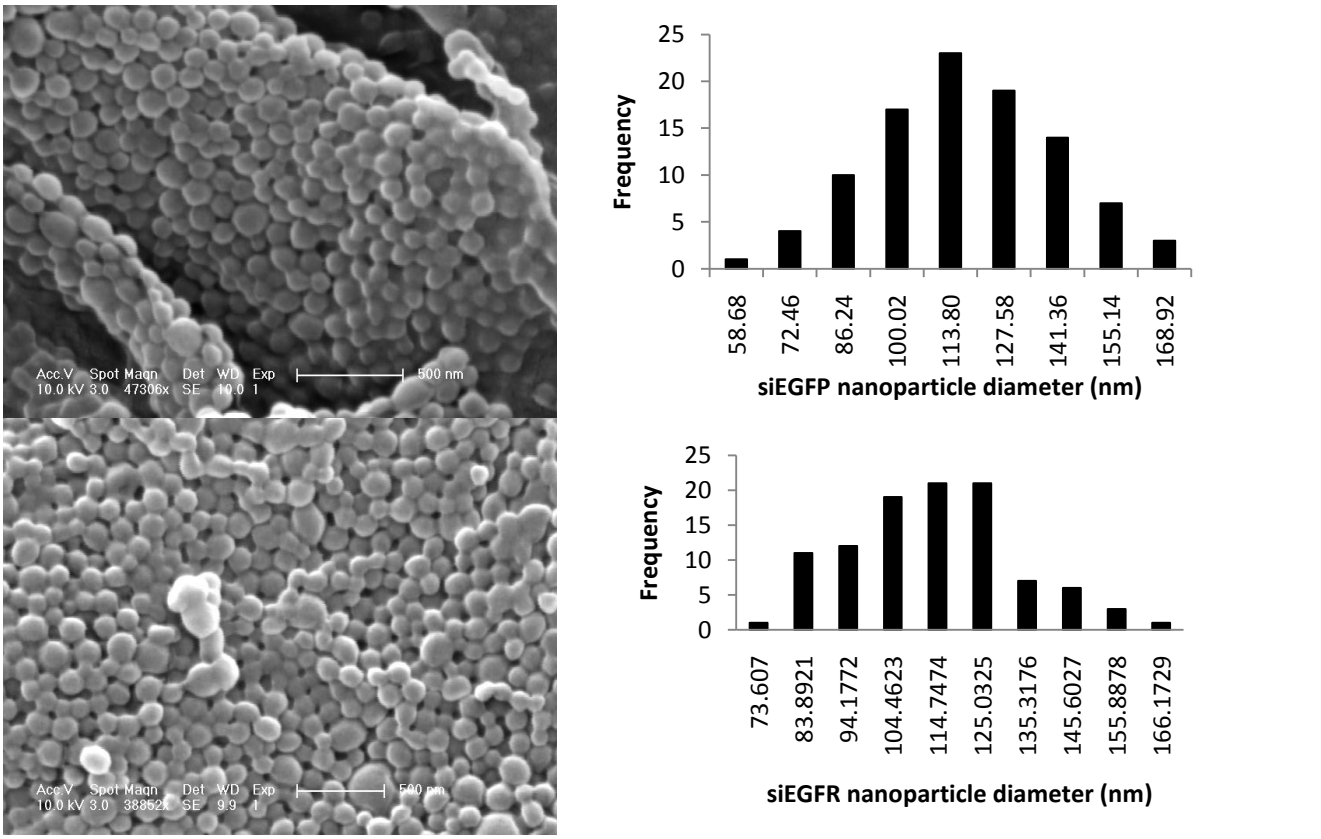

Figure 3: Top left, scanning electron micrographs (SEM) of the siEGFP nanoparticles prepared using spermidine as a complexing agent. Top right, histogram of siEGFP-loaded PLGA nanoparticle diameters with a mean value of 110 ±24nm. Bottom left, SEM image of the siEGFR nanoparticles. Bottom right, histogram depicting the diameters of siEGFR-loaded PLGA with a mean value of 109 ±20nm. Bar = 500nm.

showed minimal knockdown (Figure 4). FACS and RT-PCR were used to quantify the level of fluorescence generated from the cells at the three time points (Figure 5). The FACS analysis showed the siEGFP-loaded nanoparticles were capable of diminishing the majority of the 9L population fluorescence (♦) and possessed a small population of complete knockdown (❍). The RT-PCR analysis looking at the level of EGFP expression showed a 45%

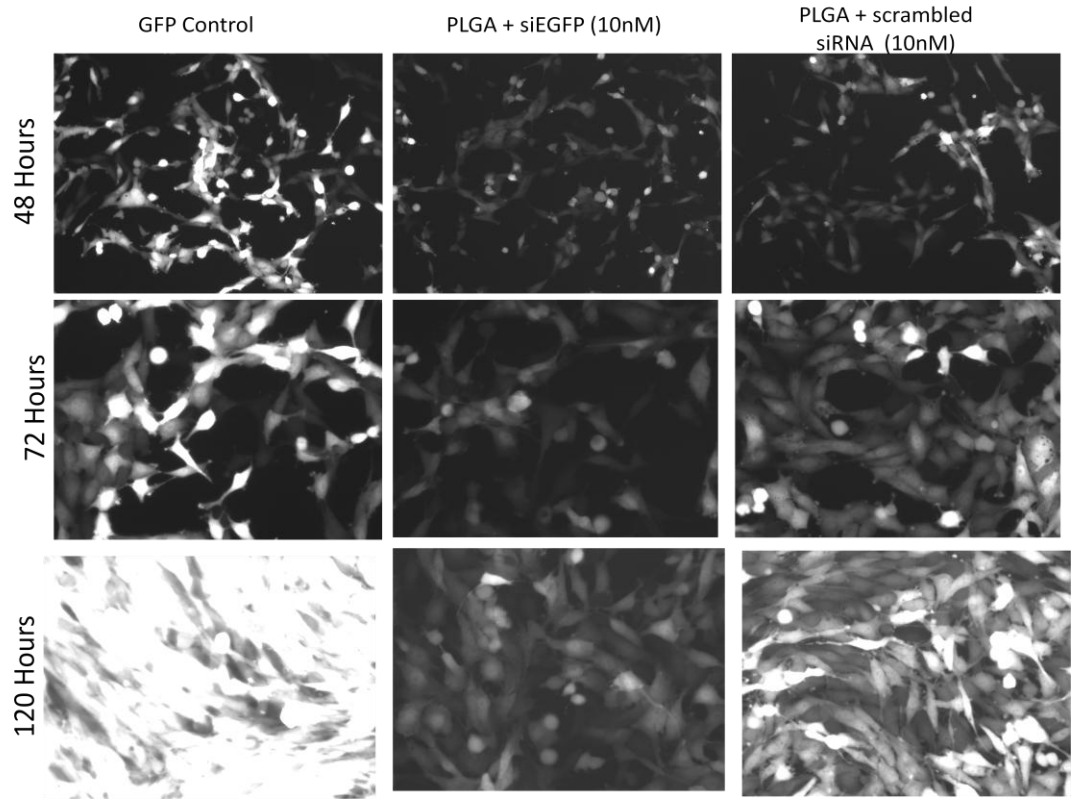

Figure 4: In vitro images of the enhanced green fluorescent protein 9L gliosarcoma cells that were grown to 30% confluency and were then exposed to the siRNA nanoparticles for three different timepoints.

green fluorescent reduction by the siEGFP compared to the control on day two. The scrambled siRNA nanoparticles had no significant difference in the level of fluorescence compared to the control ($p=0.79$, $n=6$). On day 3, the siEGFP reduced fluorescence by 53% compared to the control. The scrambled siRNA nanoparticles had no significant difference in the level of fluorescence ($p=0.06$). Day 5 results were similar with the siEGFP-loaded nanoparticle condition showing a 50% EGFP expression reduction and the scrambled siRNA had no significant EGFP reduction compared to the control ($p=0.07$).

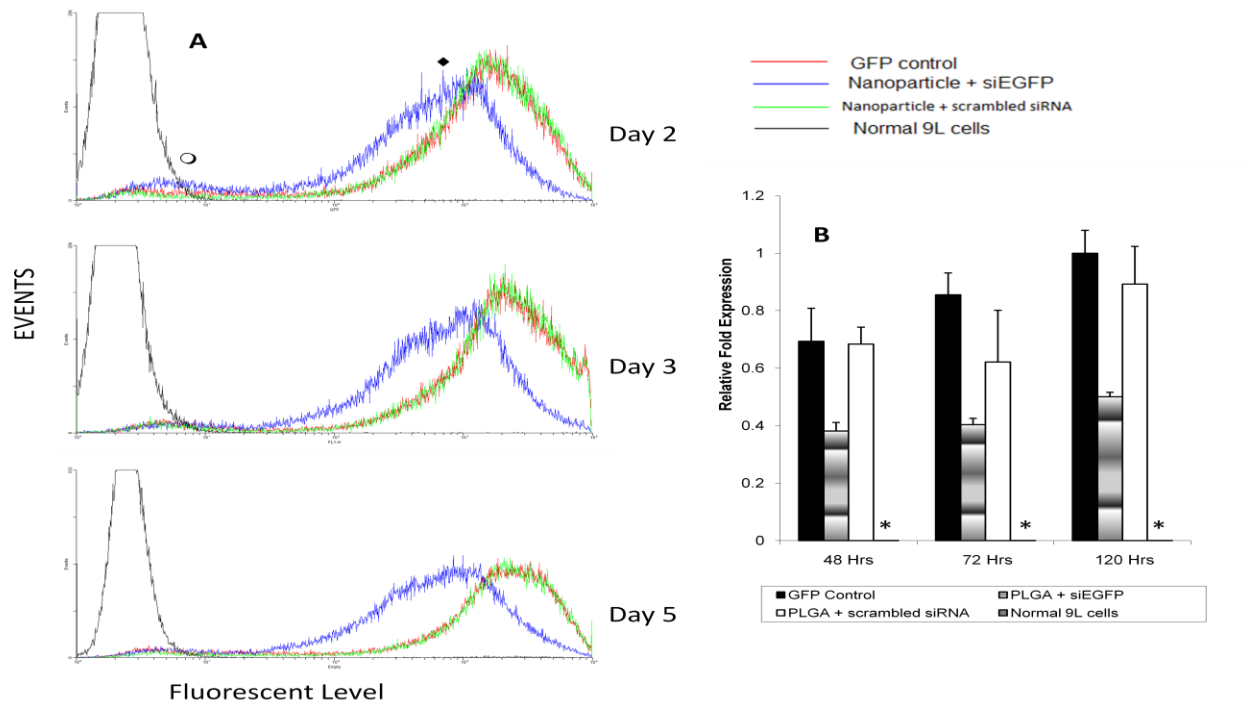

*Figure 5: Nanoparticles delivering siEGFP are internalized and cause sustained gene silencing in cultured cells, in vitro. A, FACS analysis shows EGFP expression was reduced in cultured cells exposed to the siEGFP nanoparticles (◆) and a small population showed complete knockdown (❍). B, RT-PCR results of the siEGFP nanoparticles knockdown on cultured cells. The * mark is above the bar for the normal 9L cells EGFP expression (n=3).*

In vivo 9L EGFP gliosarcoma cell knockdown

The Fischer 344 rats were intracranially injected with 9L gliosarcoma cells which were allowed to grow for five days. On day 5, either lipid or PLGA nanoparticles were injected intratumorally and tumor fluorescent growth was analyzed on day 8, 11, and 15 (Figure 6). On day 8, the siEGFP-loaded PLGA nanoparticles showed significant knockdown of the EGFP fluorescence, in vivo, with a 60% reduction of the fluorescence (p-value= 0.009, n= 3). The mean tumor fluorescent volume of the 9L cells exposed to siEGFP-loaded nanoparticles were $3.8\pm1.6mm^3$ compared to the control which was $9.6\pm2.9mm^3$. The siEGFP-loaded lipofectamine also showed some EGFP knockdown with a tumor fluorescent volume of $6.7\pm4.4mm^3$ (p-value = 0.03, n= 3). Compared to the lipid nanoparticles, the siEFGP-loaded PLGA nanoparticles showed 30% more reduction of the 9L cells green fluorescence than the lipid vectors (p-value =

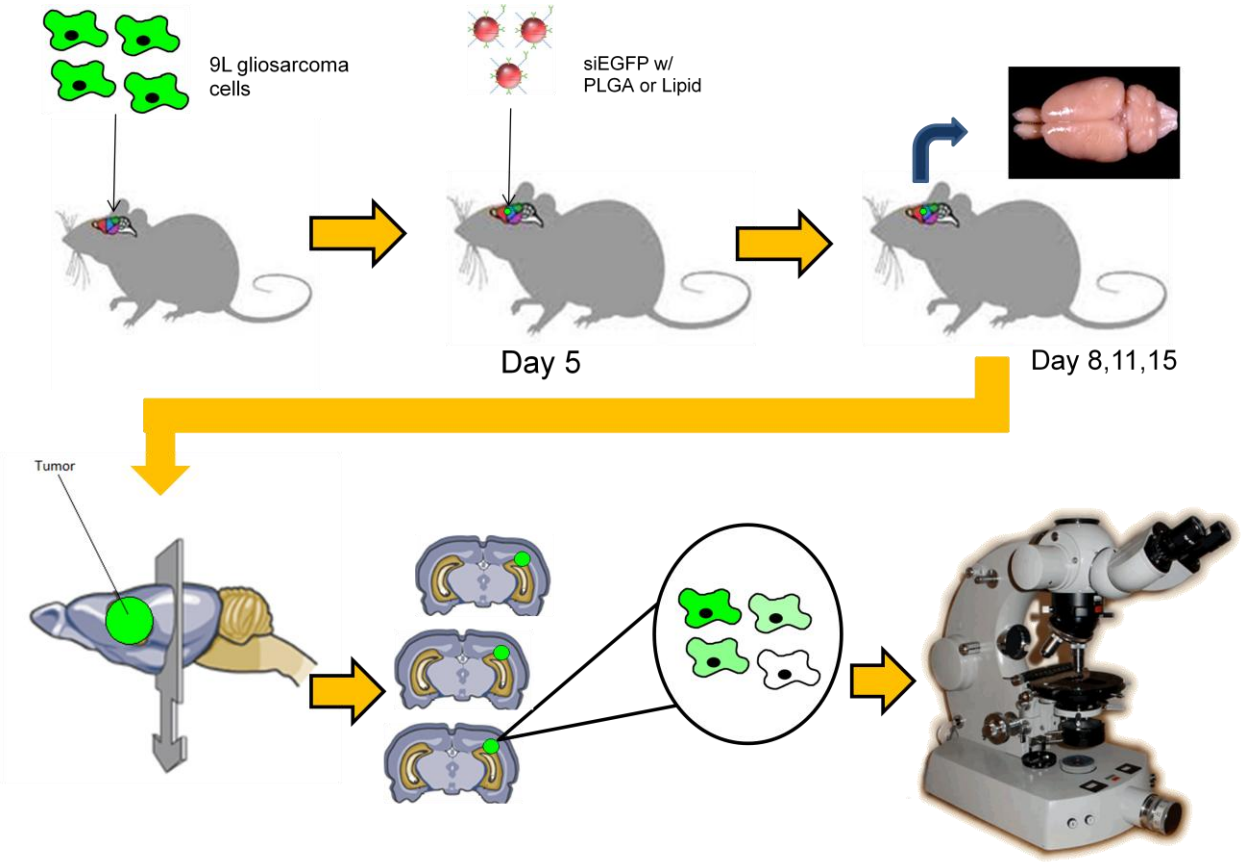

Figure 5: The Fischer 344 rats were injected with 9L gliosarcoma cells which were allowed to grow in vivo for 5 days. Nanoparticles (either lipid or PLGA) were injected into the rat brain, intratumorally, on day five and remained in the brain for an additional three, six, or ten days. The rats were sacrificed and the brain was harvested and cut into coronal sections. The cells exposed to the siEGFP had various levels of green fluorescent knockdown. A microscope was used to analyzed the total tumor fluorescent volume.

0.04). On day 11 however the tumor fluorescent volumes showed no significant difference with the siEGFP-loaded PLGA nanoparticles (p-value = 0.26) and the siEGFP-loaded lipid nanoparticles (p-value = 0.13) compared to the control. Day 15 showed similar results with the no tumor fluorescent volume difference with the siEGFP-loaded PLGA nanoparticles (p-value = 0.75) and siEGFP-loaded lipid nanoparticles (p-value = 0.11) (Figure 7).

In vitro knockdown of 9L gliosarcoma cell growth

A gene target capable of suppressing growth of 9L gliosarcoma cells was identified to test out the knockdown capabilities of siRNA nanoparticles. Epidermal growth factor receptor gene was the target for siRNA knockdown. Small interfering epidermal growth factor receptor

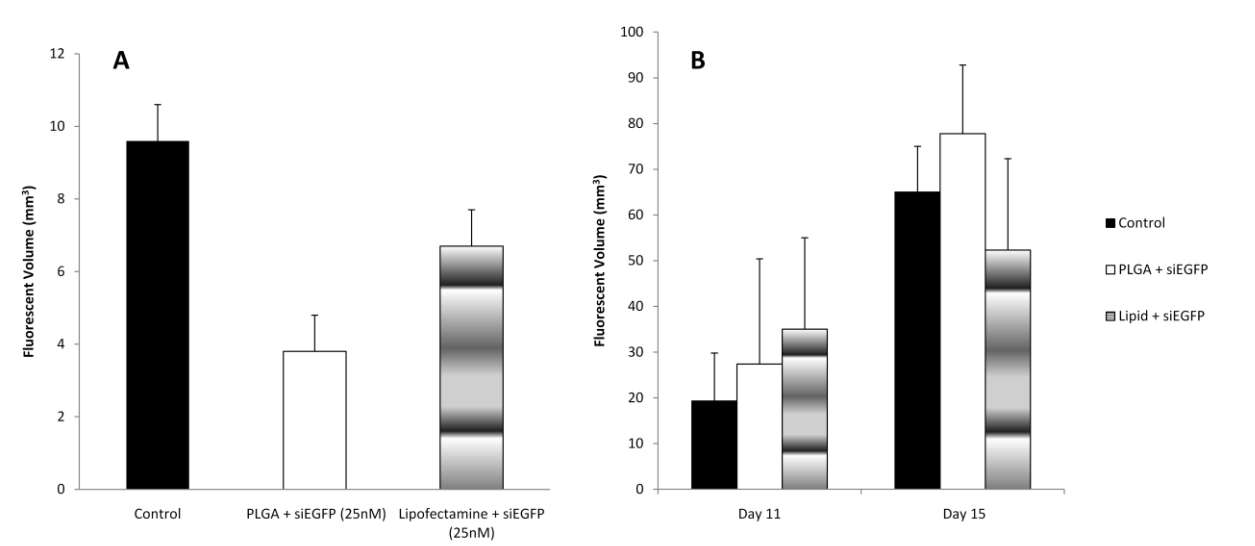

Figure 7: A, Day 8 of the in vivo Tumor fluorescent volume. The siEGFP-loaded PLGA nanoparticles (25nM) showed significant green fluorescent knockdown (p=0.009). The lipid nanoparticles also show significant tumor fluorescent reduction (p=0.03). B, Day 11 and 15, showed no significant difference in fluorescence compared to the control.

(siEGFR) was encapsulated by PLGA. In addition, nonspecific siRNA (scrambled siRNA) was also encapsulated in PLGA. The 9L cells were plated on 6 well plates and grown for 48 hours. At this time the cells were exposed to the siEGFR or scrambled siRNA nanoparticles. Two days after exposure to the siEGFR significant suppression of growth was seen in the the 9L gliosarcoma cells with a 66 percent reduction of growth (p value =0.048, n = 3). The scrambled siRNA did not show any significant suppression of the 9L growth (p value = 0.21, n = 3). Three days after siRNA exposure, the siEGFR showed an 89 percent reduction of growth compared to the control (p value= 0.022). The scrambled siRNA did not show any growth suppression (p-value = 0.32) (Figure 8,9). Time constraints prevented an in vivo study of the effects of siEGFR-loaded nanoparticles on 9L cells from being conducted.

Discussion

The reported experiments explored the efficacy of siRNA nanoparticles both in vitro and in vivo. The EGFP transfected 9L gliosarcoma cells were shown to have consistent growth with

the normal 9L cells and proved to serve as an adequate tumor cell model for the analysis of the

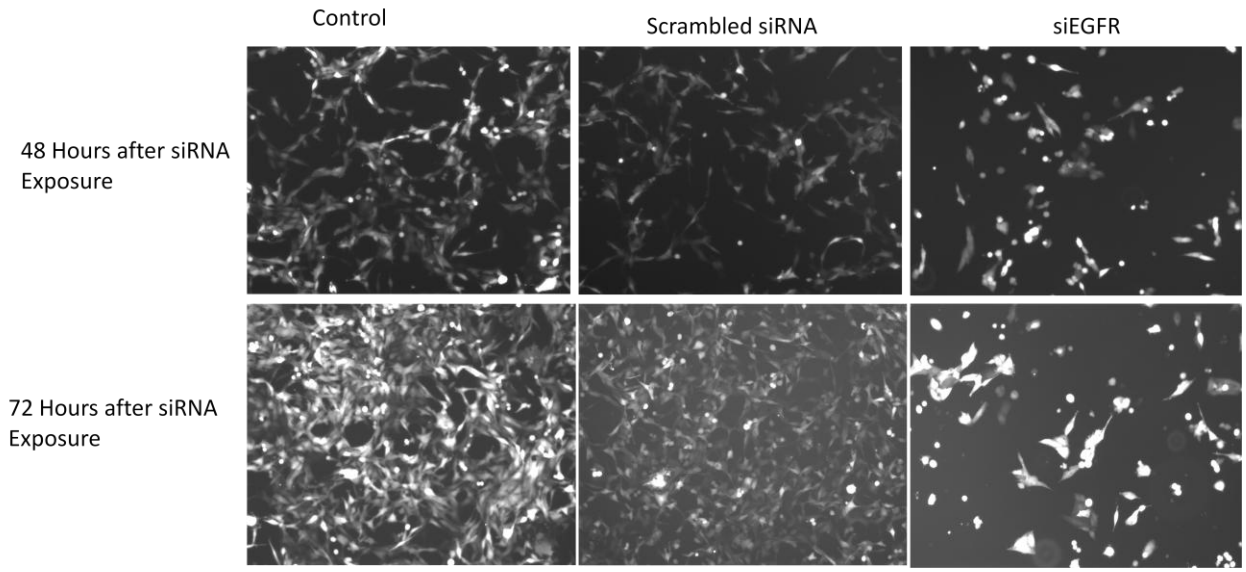

Figure 8: Microscopic images of 9L gliosarcoma cells after exposure to siEGFR and scrambled siRNA. The siEGFR reduced the growth of the 9L cells by 66% two days after exposure and 89% three days after. The scrambled siRNA showed no significant growth reduction.

efficacy of siRNA knockdown. The proliferation analysis showed no significant difference between the growth rate of the 9L transfected cells compared to the non-transfected cells, which allowed the green fluorescent transfected cells to be used for growth assessment studies.

Both the small interfering enhanced green fluorescent protein (siEGFP) and the small interfering epidermal growth factor receptor (siEGFR) were able to encapsulate into a PLGA copolymer with a diameter of around 110nm. Although the extracellular space fluid filled pores in a Fischer 344 rat are around 38-64nm diameter and optimal nanoparticle diffusion within the rat brain would require a diameter within this range, with the pressure from the convection enhanced delivery and the particles' distribution origin starting from within the tumor, the nanoparticles were still capable of diffusing (12).

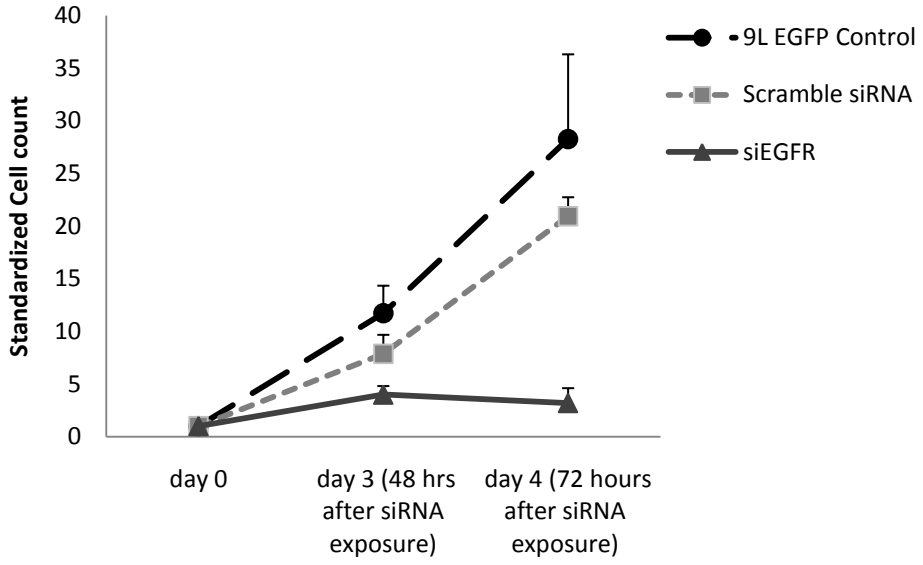

Figure 9: 9L gliosarcoma cells were plate on 6-well plates and allowed to grow for 24 hours and then exposed to siEGFR and scrambled siRNA nanoparticles. Two days after exposure to the siRNA the siEGFR showed significant suppression of 9L growth (p=0.048) while the scrambled siRNA did not (p=0.21). Day 4 showed a 89% growth reduction of the siEGFR condition compared to the control (p=0.022). (The scrambled siRNA contained small interfering enhanced green fluorescent protein, siEGFP).

In vitro studies of the siEGFP nanoparticle knockdown compared to the control and scrambled siRNA showed significant knockdown of the green fluorescent signal over a five day period. The FACS and RT-PCR studies determined that the majority of EGFP transfected 9L gliosarcoma cells had underwent a significant reduction in green fluorescence, however only a small population of cells had underwent complete knockdown. The small number of complete knockdown cells could be indicated by the low concentration (10nm) of siEGFP that was used in the in vitro studies.

In vivo studies produced similar results as the in vitro study, showing significant knockdown of the green fluorescence with siEGFP-loaded PLGA nanoparticles. The PLGA nanoparticles were compared to lipid nanoparticles which have been shown to have efficacious siRNA delivery capability, however are considered to be neurotoxic (13). Three days following in vivo exposure of the 9L tumor to siEGFP-loaded PLGA nanoparticles, there was a significant knockdown compared to both the control and the lipid nanoparticles. The sixty percent green fluorescent reduction by the siEGFP-loaded PLGA nanoparticles showed that the siRNA as a drug delivery mechanism has potential of suppressing gene expression with specificity.

However, at six and ten days following in vivo introduction of the siRNA, the PLGA polymers did not show any significant knockdown of the EGFP in the 9L cells. Similar to the in vitro studies, the nanoparticles were likely to not contain a large enough dose of the siEGFP. Loading PLGA nanoparticles with siRNA has been shown to be difficult and often results in nanoparticles with low siRNA concentration (14). Using the common preparation technique of the double emulsion solvent evaporation method, it is difficult to have a high siRNA loading efficiency. This is a result of the low molecular weight of the siRNA which allows for the nuclei acid to leak out of the inner water phase of the nanoparticle into the environmental outer water phase during the preparation. The hydrophilic nature of the siRNA along with the electrostatic repulsion forces of the phosphate groups of the nucleic acid and the anionic acid groups of the polymer causes the siRNA to leak out. This makes it difficult to have high encapsulation efficiency and causes quick release of the siRNA once it is exposed to the hydrophilic environment, which may be the cause of the early fluorescent knock down, but no subsequent knock down in the following timepoints.

Strategies to address this issue might be to change the spermidine complexing agent to another cationic material such as dioleyltrimethylammoniumpropane (DOTAP) or polyethyleneimine which have stronger bonds to siRNA. However, these materials may delay the release of siRNA.

With evidence of efficacious gene suppression of the green fluorescence in the 9L cells, the next experiments looked at a gene target that would suppress cellular proliferation. Small interfering epidermal growth factor receptor (siEGFR) was selected for in vitro studies and showed significant suppression of cellular growth.

EGFR has been shown to be present in 40% of Glioblastoma patients and has been shown to be a viable target for growth suppression. Although only a portion of GBM patients over express the epidermal growth factor receptor on their cell surface, intracellular EGFR affects signal transduction cascades, such as the MAPK, Akt, and JNK pathways all which are involved in DNA synthesis, cellular proliferation, and cell adhesion. The intracellular activity of the siEGFR may offer additional suppressive effects that extend beyond solely inhibiting the receptor function on the outer cell surface, however no studies have determined whether siEGFR may inhibit intracellular transduction cascades integral to cellular proliferation in tumor cells that do not over express epidermal growth factor receptors.

Although this study showed that suppression of the EGFR gene inhibits growth, new studies have shown that the deletion of the nuclear factor of K-light polypeptide gene enhancer in B–cells inhibitor-α (NFKB/A), a gene whose product functions to inhibit EGFR, can cause tumorigenesis in patients who lack the excessive EGFR activation that is commonly seen in a portion of GBM patients (15). It is believed that this mutation in addition to the over expression of EGFR may represent a majority of GBM patients and provides a new potential gene to investigate.

The experimental results showed that PLGA delivery of siRNA is capable of gene knockdown, however the short term duration of suppression requires that additional work needs to be done to reduce the diameter of the PLGA nanoparticles to a size under 70nm where the nanoparticles may diffuse through the extracellular space of the brain with less obstruction. In addition to reducing the size, utilizing different complexing agents that may be capable of preventing quick leakage of siRNA out of the nanoparticle may also be warranted.

EGFR has been shown to be a viable gene target for growth suppression, however future studies using a mixture of many siRNA gene targets within the nanoparticles may provide a more effective suppression of growth. Additional work will also need to investigate whether siEGFR has suppressive effects on 9L cells in vivo.

The last decade has seen numerous advancements in the use of siRNA and nanoparticles as therapeutics for gene silencing. Delivery of siRNA using the PLGA matrix allows for efficient tumor and brain penetration as well as good cellular uptake, protection of the siRNA from RNase activity, and an alterable and controllable degradation character that can allow for specific targeting and sustained release. PLGA is a delivery vector that has shown itself to be efficacious with in vitro and in vivo delivery of siRNA and may provide the means of effectively treating brain tumors.

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