2010

Identification of a tumor-targeting-peptide and development of a tumor-targeted-cytokine vector for systemic treatment of primary and metastatic malignancies

Jeffry Cutrera
Louisiana State University and Agricultural and Mechanical College

IDENTIFICATION OF A TUMOR-TARGETING-PEPTIDE AND DEVELOPMENT OF A TUMOR-TARGETED-CYTOKINE VECTOR FOR SYSTEMIC TREATMENT OF PRIMARY AND METASTATIC MALIGNANCIES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Comparative Biomedical Sciences

by
Jeffry Cutrera
B.S., Louisiana State University, 2005
December 2010

Acknowledgements

As Goose says in *Top Gun*, the list of people who I need to thank is "long but distinguished." I must first thank Dr. Shulin Li for being such a wonderfully supportive mentor. Throughout my graduate program, his guidance and advice were always there to motivate and teach me. He has made my tenure in this program a truly excellent experience, and I am excited to have the opportunity to continue my post-doctoral training with him.

I also owe a debt of gratitude to the members of my committee: Dr. Steven Barker, Dr. John Battista, Dr. Shisheng Li, Dr. James Miller, and Dr. George Strain. Your timely advice, constructive criticism, and assistance were greatly appreciated and always helpful. Likewise, Dr. Gary Wise, the head of the department, was an inspirational professor and leader from whom I learned a lot.

I also need to thank countless others from the School of Veterinary Medicine and LSU, especially the following people: Dr. Arthur Penn for showing me the truth about horse slides, Ms. Marilyn Deitrich for helping with flow cytometry, Dr. Azeem Hasan for analyzing our protein samples, Ms. Sherry Ring for processing tissue sections, Ms. Cecilia Koon for providing excellent service in DLAM, and the entire administrative staff in the CBS Department for performing all the tasks I did not even know needed to be done. Also, I am very thankful to my good friends and colleagues Ms. Danielle Tatum, Mr. Jeffrey Cardinale, and Ms. Maria Cochran for helping me through the tough times and making the fun times that much more enjoyable.

Many thanks for helping me with my project and putting up with me also go to the current and former members of our lab. Mr. Mike Flanagan, Mr. Blake Johnson, Ms. Jiemiao Hu, Dr. Scott Reed, Dr. Mehrnoosh Tashakori, Dr. Boyu Zhang, and Ms. Nan Zhang all made my project possible. Dr. Denada Dibra deserves special appreciation. Her insightful input,

intelligence, abilities, and especially her patience when helping me with *in vitro* experiments were invaluable. Another special thanks to Ms. Xuexing Xia for preparing the plasmids vital to my experiments and teaching me countless lab techniques.

My friends outside of school were also instrumental in helping me complete my program. Specifically, I am extremely grateful to my longtime friends Mr. Jon Forester, Mr. and Mrs. Joshua Hebert, Mr. and Mrs. Dane Lejeune, Mr. Matt Loup, and Mr. Tommy Overton. Their support throughout my graduate career and beyond has always and will always be appreciated.

Finally, and most importantly, I want to thank my family for helping me become who I am today. I must thank my brothers and sisters-in-law Mr. and Mrs. Dylan Cutrera and Mr. and Mrs. Vincent Cutrera for always being there for me. I am most thankful to my parents Mr. Lucien Cutrera and Mrs. Mary Lou Cutrera for their unwavering support and unending confidence. I am truly a lucky person to have such a loving and supportive family.

Table of Contents

Abstract

Advances in cancer therapies continue to be improved, yet cancer continues to be one of the deadliest diseases in the world. Harnessing the power of the body's immune system to attack cancer is a promising strategy that can further improve therapies for neoplastic diseases. As part of this strategy, cytokines such as interleukin (IL) 2 and interferon α are currently accepted cancer treatments, and other cytokines such as IL12 and GM-CSF also show potential as new treatments. Clinical trials with these cytokines have shown less than acceptable therapeutic efficacy and toxicities, but tumor-targeting motifs can improve these effects. Both antibodies and peptides specific for tumor antigens have been used in recombinant protein and gene therapy systems to increase the intratumoral cytokine accumulation and decrease systemic toxicities. Still, these treatments have not been capable of overcoming the obstacles for clinical acceptance. The hypothesis tested in this dissertation is that inserting tumor-targeting peptide coding sequences into IL12 plasmid DNA will create a novel systemic gene therapy approach which will increase the antitumor efficacy and decrease toxicity for cancer treatments. To accomplish this goal, a reporter gene mediated screening strategy was developed to identify a peptide which can target multiple tumor models. While preparing this method, it was discovered that these peptides can have a strong effect on the activity of the conjugated reporter gene. Once this strategy was finalized, the peptide VNTANST was found to increase the intratumoral accumulation of the reporter gene in five tumor models including a human xenogeneic model. The VNTANST coding sequence was then inserted into an IL12 plasmid to examine the antitumor efficacy. In breast adenocarcinoma, squamous cell carcinoma, and colon carcinoma models, VNTANST-IL12 plasmid DNA treatments distal from the tumor site increased tumor

inhibition and, in two models, prolonged survival. Also, these treatments reduced the development of metastatic lung tumors in a spontaneous metastatic model. As expected, these tumor-targeted IL12 treatments decreased the level of liver toxicity compared to wild-type treatments. The receptor for VNTANST was identified as vimentin, which is a potentially powerful target for human cancers.

Chapter 1

Passive and Active Tumor Homing Cytokine Therapy*

Introduction

In 2008 approximately 565,500 people will die from and more than 2 million people will be diagnosed with cancer in America alone. While continued progress in conventional therapies such as surgery, radiation, and chemotherapy is being maintained and the 5-year survival of cancer patients is steadily increasing for most types, cancer is still one of the leading causes of death in America, second only to cardiovascular disease [1]. Most cancer deaths are attributed to metastatic disease which can be distributed throughout the body [2] and residual tumors which remain after treatment with conventional therapies [3]. Thus, new cancer treatment strategies must be able to destroy not only the primary tumors but also all of the metastatic or residual tumor cells which remain after conventional treatments. One of the most promising strategies to accomplish this feat is directly targeting immunostimulatory agents to tumor cells or tumor microenvironments.

The ability of the immune system to attack tumor cells was first hypothesized by Paul Erlich in 1909, but the discovery of cytokines, intracellular signaling proteins produced by immune cells, and their ability to increase the immune response towards cancer really boosted the study of immunomodulation for cancer therapy [4]. Among these cytokines were Tumor Necrosis Factor (TNF)α [5], Interleukin (IL)2 [6], and IL12 [7]. Interestingly, the biological properties of these cytokines vary tremendously yet all have since shown potential for treating malignancies. For instance, TNFα has direct cytotoxic effects against tumor cells as well as activating antitumor immune responses [8], IL2 induces proliferation of B, T, and NK cells [9], and IL12 induces Interferon (IFN) γ in T and NK cells [10]. While these and other cytokines are pleiotropic, many have overlapping functions such as the ability of IL2 and IL12 to enhance the cytotoxicity of Natural Killer (NK) and activated T cells [11] and synergistically upregulating

the other's receptors via independent signaling pathways [12]. In fact, very quickly after the IL2 gene was purified in substantial amounts clinical trials in cancer patients began [13, 14].With the continued elucidation of these potential antitumor qualities, several cytokines have been and continue to be clinically evaluated for the treatment of many types of cancer.

Unfortunately, several clinical trials implementing systemic delivery of recombinant cytokines for the treatment of cancer failed to produce positive results and produced severe toxicities. For early IL2 clinical trials the only benefits were seen in patients with renal cell carcinoma (RCC) and malignant melanoma. While only a select few patients benefited from the treatments, toxicities ranging from the severe, such as hypotension, vascular leak, and respiratory insufficiencies, to the problematic, like nausea, emesis, diarrhea, etc., limited the levels of cytokines that could be delivered and, therefore, the antitumor effects [4]. Similar results were seen in clinical studies using systemic delivery of recombinant IL12 and TNFα. So, the next steps to overcome the barriers of systemic cytokine therapies were locoregional delivery of the cytokines and combinational treatment regimens. Unfortunately, the benefits of these treatment strategies have continued to be undermined by the toxicity [4, 8, 10, 12]. Despite the low response rates and high potential for toxicity, IL2 is clinically approved in the United States, Canada, and the European Union for the treatment of RCC [4].

Nonetheless, the potential benefits of cytokine therapy for the treatment of cancer are present but there are still several obstacles that need to be overcome. With systemic cytokine treatments, the level of cytokine is increased systemically which leads to most of the toxicities and even death in some cases. Also, the increase in cytokine level was generally not high enough at the target area (i.e. tumor site) to elicit an immune response capable of creating a therapeutic benefit. Another problem with the treatments was the rapid clearance of the cytokine through the

body's normal excretory and biotransformation systems. Even with locoregional administrations the minimal level of cytokine concentrations for therapeutic benefits was hard to maintain as well as the fact that most tumors are not available for noninvasive administrations [4, 8, 10, 12]. So, there is a need for treatments that can reach sites distal from the site of administration, reach an immunologically active concentration of the cytokine, and maintain that concentration long enough to induce a therapeutic response.

One such administration technique that could improve the efficacy of cytokine treatments is gene therapy. Local delivery of cytokine-encoding genes is capable of producing therapeutic levels of cytokines at the site of delivery with only slight increases in systemic cytokine levels. Also, the local concentration of cytokines will persist so the immune system can be properly activated. Furthermore, for difficult-to-reach sites of administrations, the frequency of treatment is lower so the therapy is less invasive. Toward this purpose, several types of vectors including multiple viral and non-viral vectors have been tested in animal models as well as clinical trials [2, 4, 15, 16]. There is still much debate about which vector is the best for the treatment of cancer in humans because they all have varying advantages and disadvantages. Several cytokine genes in various vectors have already been investigated via systemic and locoregional administration in preclinical and clinical trials including IL2, IL12, TNFα, IFNα, and many more. Another method for local cytokine delivery is adoptive transfer of cells that have been transfected with cytokine producing genes. These cells can be delivered by either intratumoral (i.t.) or peritumoral (p.t.) injection, where they will then produce and secrete the cytokines in the tumor microenvironment [17-20]. Regrettably, most of these cytokine treatment approaches with a few exceptions have yet to successfully overcome enough barriers to meet the requirements necessary to become clinically approved treatments for cancer [4, 15].

A very promising solution to the aforementioned obstacles for cytokine therapy of cancer is targeted delivery of the cytokines to the tumor and tumor microenvironment. For the purpose of this review, targeting entails any method that enhances the delivery, retention, and biological activity of the cytokines at the tumor site. Toward this goal, researchers have developed a wide array of strategies ranging from the passive targeting via the enhanced permeability and retention effect of tumors to the active targeting of tumor and tumor microenvironment antigens. This review will focus on the development, action, progress, and future directions of targeting cytokines to tumor sites for immunomodulatory treatments.

Passive Targeting with Poly(Ethylene Glycol)

First described in 1986, tumor environments are characterized by fenestrated vasculature and poor lymphatic drainage [21]. Now termed enhanced permeability and retention (EPR), this tumor-specific trait can be exploited to accumulate high concentrations of systemically delivered treatments. In brief, intravenously delivered agents collect in the tumor tissue by passively transversing large gaps between endothelial cells in the tumor vasculature. Once present in the tumor tissue, these agents remain due to the poor drainage of the lymphatics in the tissue; therefore, the concentration of these agents continues to increase. Most systems that attempt to exploit the EPR effect are nanoparticle colloidal drug carrier systems which consist of repeating elements conjugated to therapeutic modalities. When these systems are introduced into the blood circulation, they are readily incapacitated by opsonization and then rapidly removed by the mononuclear phagocytic system (MPS) and the reticulo-endothelial system (RES), so the nanoparticels are not present in circulation long enough to utilize the EPR and create an antitumor effect [21, 22].

Several investigators have worked to develop new stealth nanoparticles that can avoid these systems. Several stealth particles were discovered including poly(acrylamide), polysaccharides, and poly(vinyl alcohol). The most widely used and successful technique is the addition of poly(ethylene glycol) (PEG) units onto the surface of particles which is known as PEGylation. PEG has a structure of $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$. This structure encompasses a chemically inert polyether backbone. Another key component of PEG is the terminal hydroxyl groups which can be used for conjugation to an extensive list of agents. *In vivo*, these stealth nanoparticles have longer half-lives in circulation which leads to an increased invasion of tumor vasculature and accumulation in the tumor site. This stealth characteristic of PEG is determined by several different factors including particle size and surface characteristics. Optimization of these factors has led to the increased efficacy of cytokine-nanoparticle conjugates [23].

IFNα has long been known to be critical in antitumor therapies in addition to its direct antitumor effects [24]. Several clinical trials have shown the potential for IFNα to be used as a treatment for tumors, but the effective dose is hard to reach due to toxicities of systemic treatment. Also, the short elimination half-life ($T_{1/2}$) of IFNα necessitates daily administrations to see any therapeutic effect [25]. PEGylation of IFNα elongates its $T_{1/2}$ in humans from 4 to 16 h for unmodified IFNα to 61 to 100 h. Likewise, the absorption $T_{1/2}$ increased from 2.3 h to approximately 50 h. Several other factors are increased with the PEGylation of IFNα making the treatment of cancer with IFNα more plausible [26].

Indeed, one of the first phase I clinical trials using PEGylated IFNα-2B (one form of IFNα) (PEGIFNα-2B) in patients with chronic myeloid leukemia (CML) showed a dramatic increase in the efficacy of treatments. In this study, the MTD of PEGIFNα-2B was defined as 7.5 to 9 μg/kg weekly. This dose level given weekly is equivalent to 3 times the dose level of IFNα-

2B in previous CML studies with no increase in toxicities. Furthermore, 53% of patients who were suffering from active disease at the start of the trial showed a therapeutic response. Of the 27 patients in the study who had failed previous IFNα therapy, 13 had a favorable response to this treatment. Also, this study used subcutaneous (s.c.) administrations and the PEGIFNα-2B was readily absorbed with increasing serum concentrations through week 1, but not by week 4. This early clinical study showed the safety, ease of administration, and efficacy of treatment with PEGIFNα-2B [25].

Preclinical and clinical studies with different types of PEGylation conjugated to various cytokines has steadily continued; therefore, the quality of PEG used to modify cytokines has improved. One of the best modifications to PEGylation of cytokines was the creation of poly(methoxypolyethyleneglycol-cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) nanoparticles (PEG-PHDCA). This new formulation is a more rapidly degradable copolymer with higher stealth capabilities. Similar to the original PEG-IFNα studies, PEG-PHDCA conjugated to TNFα increased the T_h of TNFα in the blood circulation. Also, accumulation at the tumor site was increased at 6 h after intravenous injection [27]. So, the PEG-PHDCA increased the length of activity for TNFα, but the characteristics that created this increase were not well understood.

The study of how these characteristics and the formulation of the nanoparticles helped to increase their tumor-targeting capabilities. First, studies to analyze the effects of molecular weight and particle size of surface modifications of PEG-PHDCA conjugated to TNFα resulted in optimized parameters for increased circulation time and tumor accumulation. These studies revealed that nanoparticles with smaller PHDCA particle sizes (~80 nm) with larger molecular weights (5 kD) decreased serum protein adsorption which results in decreased phagocytosis, increased circulation time, and higher accumulation in the tumor compared to other tissues. The

smaller particle size allows a higher density of MePEG chains which increases the interaction between the particles and water molecules which prevents the adsorption of serum proteins [23, 28].

Another use of PEG for targeting cytokines to tumors is conjugation to the shell of virus vectors loaded with cytokine-coding genes. Since one of the major limits to viral gene-therapy is the antibody response to the vectors, PEGylation of the vectors can help reduce detection by the the humoral immune system. Indeed, intravenous injection of PEGylated adenovirus vectors (PEG-Ad) in mouse models results in reduced CTL production and elongated viral gene expression from 4 to 42 d [29]. Furthermore, PEG-Ad encoding TNFα (PEG-Ad-TNF) has several benefits over unmodified vectors (Ad-TNF). First, the $T_{1/2}$ of PEG-Ad-TNF was 12-fold longer than the unmodified. Second, PEG-Ad-TNF gene expression in the tumor and liver tissue was 35-fold higher and 20-fold lower than Ad-TNF, respectively. The PEG-Ad-TNF also showed increased antitumor activity compare to Ad-TNF. These results show that there is potential for treating tumors with systemic administration of PEG-Ad loaded with cytokine genes [30].

Exploiting the EPR effect for targeting cytokines to tumors can improve the efficacy while reducing the toxicity. This effect is caused by the nature of solid tumors and their effects on the physiologic conditions of the tumor environment. Adding PEG and different variations of PEG can increase the circulation time of cytokines allowing for increased concentrations in the tumor environment and decreased concentrations in toxicity causing tissues such as the liver. While these effects can be seen in solid tumors, better modalities to exploit more specific tumor-associated characteristics for targeting the cytokines in the tumor environment can further

increase the effectiveness and decrease the toxicity of these treatment strategies for tumor therapy.

Active Ligand Targeting with Tumor-homing Peptides

In addition to the EPR effect, tumors have many other unique attributes which can be exploited for targeting cytokines. To exploit these traits, several active targeting strategies have been developed such as peptides that home to tumor environments. With the advent of *in vivo* biopanning with peptide phage display technology, vast amounts of peptides can be expressed on the surface of phage and then analyzed for their ability to bind to ligands in the different tissue types [31]. Using this technique, several peptides have been isolated, and new ones continue to be discovered, that preferentially target tumors [32]. Of these tumor-targeting peptides, the most widely studied and most successful peptides do not only target the tumors themselves but also target motifs that are expressed due to unique qualities of the tumor environment. Specifically, these peptides target proteins that are upregulated in tumor vasculature and lymphatic vessels [33].

Peptides that contain the amino acid sequence Arg-Gly-Asp (RGD) were one of the first targeted peptides discovered via biopanning. These peptides target integrins that are upregulated during angiogenesis but bind most specifically to the integrins αvβ3 and αvβ5. Along with their ability to bind to these integrins, RGD peptides are also capable of internalizing into the cells, causing cell death, and interrupting the development of more vasculature. There are several different RGD peptide variants, including ACDCRGDCFCG (RGD4C), which have different beneficial abilities [33, 34]. By using knockout mice to isolate the effects of the RGD4C peptide, it was shown that RGD4C has antiangiogenic effects. Using multiple angiogenesis assays in these knockout strains, RGD4C repeatedly demonstrated antiangiogenic capabilities [35]. While

these peptides have beneficial properties in addition to their targeting abilities, it is their interactions with integrins which have been used for targeting, imaging, and treating tumors in preclinical models [33-39].

Among these techniques, conjugating RGD sequences to cytokines has shown to increase the therapeutic effect compared to the wild-type (wt) cytokines. RGD-cytokine conjugates can be produced by expression of the fusion protein by recombinant DNA technology or chemical coupling of the purified peptide to purified cytokines. By these methods, several fusions of different RGD peptides with murine and human cytokines have been created, and these fusions have been examined *in vitro* and *in vivo*. Most RGD-cytokine conjugates maintain both the binding specificity of the RGD peptide and the biological activity of the conjugated cytokines [36, 37, 39].

One of the most widely studied RGD-cytokine conjugates are those using TNFα. Several different groups have analyzed the RGD binding ability of these conjugates *in vitro*, and they determined that conjugating RGD peptides does not affect the affinity of the peptide for its receptors [36, 37, 39]. Likewise, the activity of the conjugated TNFα was not lower than the wt cytokine as demonstrated through fibroblast cell viability assays [37, 39]; however, cell viability studies using a cell line that expresses the receptor intregrins specific for RGD showed that the conjugated TNFα does have increased toxicity to these cells. Also, incubating the cells with excess RGD peptide prior to addition of the TNFα and RGD-TNFα decreased the activity of the RGD-TNFα and not wt TNFα. So, the increased activity is due to the binding of the RGD-TNFα to its receptors on the cell surface. Similar results were found by exploiting another attribute of TNFα: ICAM-1 induction. FACS analysis revealed that RGD-TNFα increased the expression of ICAM-1 more than TNFα, and preincubation with free RGD peptide reduced the ICAM-1

expression level to that of the wt TNFα while not reducing the level of ICAM-1 induced by the TNFα [36]. The results from these *in vitro* studies demonstrate the potential for using RGD-TNFα conjugates in preclinical models.

Indeed, different groups have demonstrated that RGD-TNFα does have increased antitumor activities *in vivo*. Since TNFα is used to increase the efficacy of chemotherapeutic treatments, one group studied the effect of using RGD4C-TNFα in conjunction with melphalan, a commonly used chemotherapeutic agent. They found that a single treatment of RGD4C-mTNFα (0.3 ng i.p.) when administered in conjunction with one treatment of melphalan (50 ng i.p.) was able to reduce tumor growth in a syngeneic RMA lymphoma model when administration of either agent alone induced no antitumor effects. Also, while the addition of RGD4C-TNFα to the chemotherapeutic treatment increased antitumor efficacy, there was no corresponding change in animal weight revealing that there is no increase in toxicity with the combinatorial treatments [36]. More recently, another group has shown that treatments of RGD4C conjugated to human TNFα (RGD4C-hTNFα) can improve the antitumor efficacy of hTNFα in a nude mouse model. In these reports, nude mice bearing xenogenic MDA-MB-435 tumors were treated with 5 consecutive daily i.v. injections of saline, hTNFα, or RGD4C-hTNFα (0.25 mg/kg). Compared to the saline treated group, treatment with hTNFα and RGD4C-hTNFα resulted in tumor volume reductions of 24% and 72%, respectively, by 15 d after treatment. The investigators also monitored animal weight, and there was no difference among the groups throughout the experiment. Additionally, *ex vivo* staining for CD31 and TUNEL revealed that the RGD4C-hTNFα treatments resulted in selective cytolysis of αvβ3-positive tumor cells and tumor vessels while the saline and hTNFα treatments did not cause any integrin dependant cytolysis [38]. These *in vivo* results continue to show the potential for using RGD peptides to increase the

antitumor effect of TNFα while reducing the toxicity which is normally seen in TNFα treatments.

RGD peptides have also been shown to improve the antitumor efficacy of other cytokines. Similar to RGD4C-TNFα *in vitro* studies, the RGD4C-IL12 conjugate mrIL12vp binds to the $\alpha_v\beta_3$-positive cell line M21 (human melanoma) and not to the $\alpha_v\beta_3$-negative cell line Saos-2 (human osteosarcoma) while the nontargeted mrIL12 did not bind to either cell line. Furthermore, *in vivo* toxicity studies with i.p. injections of 0.025 to 0.5 μg/d of mrIL12 resulted in readily observable toxicities such as loss of appetite, weight loss, trouble breathing, and even sudden death after seven days, and comparable levels of mrIL12vp did not cause any such toxicity. Also, when NSX2-tumor-bearing mice were treated with PBS, mrIL12, or mrIL12vp continuously for 3 weeks (1 μg/d), there was a nearly two-fold reduction in tumor volume with the targeted therapy compared to both PBS and nontargeted IL12 therapies. So, RGD4C can also increase the efficacy of IL12 for treating tumors while simultaneously decreasing the toxic effects normally seen with IL12 treatments [35]. All of the aforementioned experiments employing conjugates of RGD peptides and cytokines show that the RGD-integrin interactions can be exploited to enhance the antitumor efficacy and reduce the toxicity of cytokine tumor therapies.

Another set of peptides that target tumor environments are peptides containing the amino acid sequence Asn-Gly-Arg (NGR). Similar to RGD peptides, NGR peptides were first identified via *in vivo* biopanning [31] and preferentially bind to a ligand, aminopeptidase N (CD13), which is upregulated on tumor vessels during angiogenesis [40]. CD13 is also found in other tissues such as kidney and myeloid cells, but the NGR peptides do not bind to CD13 found in normal tissues [41]. While there are several different NGR-containing sequences that have been used to

target to tumor environments, disulfide-bridged cyclic NGR peptides (CNGRC) show an increased affinity for CD13 and increased antitumor activity, greater than 10-fold higher, when coupled to TNFα compared to a linear NGR peptide (GNGRG) [42]. So, the cyclic-NGR peptides have a lot of potential to be used as tumor targeting vehicles for cytokines.

After the discovery that NGR peptides do indeed target an angiogenesis marker in tumor vasculature, the natural next step was to investigate its ability to facilitate the delivery of antitumor therapies. To this end, NGR was first coupled the murine TNFα (NGR-TNFα) and characterized via *in vitro* cytotoxicity assays. These experiments using L-M mouse fibroblast cells revealed that coupling the NGR peptides to murine TNFα did not affect the folding, oligomerization, or binding to TNFα receptors. After these proof-of-concept experiments, NGR-TNFα was administered to C57/Bl6 mice bearing syngeneic RMA-T lymphoma tumors. The LD_{50} values of TNFα and NGR-TNFα were 60 μg and 45 μg, respectively, which are not very different; however, the antitumor effects were drastically different. Impressively, 1 μg of NGR-TNFα delayed tumor growth better than 27 μg of TNFα 14 days after a single i.p. administration 10 days after tumor inoculation. Also, some mice treated with less than the LD_{50} of NGR-TNFα were completed cured of tumors while no mice treated with any amount of TNFα were completely cured of tumors. The body weights of mice from these experiments were monitored to identify the difference in toxicity from NGR-TNFα compared to TNFα. The efficacy:toxicity ratio of NGR-TNFα was 14 times higher than those of TNFα. Similar experiments were performed using syngeneic B16F1 tumors to analyze the effects of multiple treatments. With treatments at days 11, 17, and 19 after tumor cell inoculation, NGR-TNFα was 12 to 15 times more effective than the same doses of TNFα while the toxicities were comparable [43]. These

studies reveal the increased antitumor activity and decreased toxicity of NGR-TNFα vs. TNFα which shows the potential for NGR-TNFα in the treatment of tumors.

NGR-TNFα was evaluated also for its ability to enhance the antitumor efficacy of chemotherapeutic agents. Similar to RGD-TNFα, a single administration of subnanogram levels of NGR-TNFα combined with a single dose of melphalan (50 μg) creates a synergistic decrease in tumor volume with no corresponding increase in toxicity as determined by animal weights. Interestingly, only 0.1 ng of NGR-TNFα is needed to see a significant reduction in tumor volume while no difference is seen in tumor volumes below 0.3 ng of RGD4C-TNFα [36]. More recent studies have shown that pretreatment with NGR-TNFα as opposed to simultaneous treatment greatly increases the efficacy of the chemotherapeutic agents. In several different tumor models and with different chemotherapeutic drugs, a pretreatment with 0.1 μg NGR-TNFα increased the efficacy of the drugs without increasing the toxicity seen with any of the drugs or NGR-TNFα alone. Notably, the increase in efficacy of each drug in combination with NGR-TNFα was different depending on the tumor model used [44, 45]. Interestingly, NGR-TNFα does not increase the cytotoxicity of doxorubicin in *in vitro* cytotoxicity assays using the prostate cancer tumor cell line TRAMP-C1, but the *in vivo* antitumor effect of doxorubicin with NGR-TNFα was significantly increased. This observation demonstrates that NGR-TNFα does not directly increase the cytotoxicity of doxorubicin but instead primes the tumor environment to increase the effect of the doxorubicin on tumor growth [46]. In addition to the direct antitumor activity of NGR-TNFα, it can also synergistically increase the efficacy of chemotherapeutic drugs.

Recent studies have shown that NGR also increases the antitumor efficacy of the human cytokine IFNα2a by targeting to the tumor environment. *In vitro* studies of EC migration and tube formation using IFNα2a and the NGR-IFNα2a conjugate revealed that the conjugate had

consistently increased antiangiogenic effects at all tested concentrations as determined by a reduction of tube formation by HUVEC in matrigel. Similarly, treatment with NGR-IFNα2a to HUVEC induced with bFGF reduced the ability of the cells to migrate through matrigel more than IFNα2a. Also, in two different xynogeneic tumor models in nude mice daily i.p. treatments of NGR-IFNα inhibited tumor growth at levels comparable to those seen with 2- to 5-fold higher doses of IFNα2a. At all treatment doses, no toxicities were observed with either formulation [47]. Further toxicity studies were performed on mice, rats, and monkeys using pharmacologically active doses. Following single doses ranging from 50 to 674 times higher than the clinical dose for adults resulted in no toxicities evidenced by no abnormalities in general signs and animal weights for all species as well as food consumption, urinalysis, hematological examinations, and blood biochemical examinations in monkeys. For repeated-dose studies, rats were administered daily i.m. injections with $1.5x10^6$, $4.5x10^6$, or $1.25x10^7$ U/kg for 90 days and monkeys were given $7.5x10^5$, $2.25x10^6$, or $6.75x10^6$ U/kg for 47 days. In rats, there were no changes in body weights, food intake, or general health and activity. There were some minor changes in some hematological and clinical chemistry parameters, but all levels returned to normal levels after the administrations. The results in the monkey studies were similar with no deaths occurring during the treatments and most of the clinical and pathology changes returned to normal after the administrations were discontinued [48]. These preclinical efficacy and toxicity data indicate that targeting IFNα2a to tumors using NGR is a very plausible safe and effective treatment for malignant diseases in humans.

Effort continues to be made to discover more peptides that can target tumor environments and to improve the targeting abilities of the peptides. The secret to improving these peptides may not only be based on discovering new peptides [32] but also on improving the current peptide

modalities. To such an end, modifying peptides to increase the amount of ligand interactions they can utilize in the tumor environment would be extremely beneficial. Recently, a shortened version of RGD4C, CDGRC, when fused to murine IFNα and delivered via i.m., i.t., or i.d. injections of plasmid DNA followed by electroporation maintains its ability to anchor in tumor environments and produce antitumor immune responses. This peptide sequence shares homology with NGR peptides as well as RGD peptides so its array of potential ligands is increased to create better homing abilities. Additionally, it has a shorter amino acid sequence than RGD4C so it is less likely to cause a humoral response to the recombinant fusion protein [49]. Further modifications of these peptides will continue to improve the efficacy of peptide-cytokine treatments for malignancies and possibly propel them into clinical settings.

An alternative method to reduce toxicity and increase efficacy of cytokines is to deliver cytokine genes to tumors. Virus vectors have been studied extensively to deliver genes to various tissue types. Modifying the tropism of the viral vectors to preferentially transduce cells in the tumor or tumor environment would help to sequester the effects of the cytokines and minimize systemic toxicity. To accomplish this goal, viruses armed with cytokine genes are modified to express tumor-targeted peptides on the capsid fibers and home to the tumor vasculature to deliver their payloads. To date, viral vectors have been successfully modified with RGD peptides for the delivery of various genes to the tumor environment with high success [50]. So, it appears to be a potential strategy for the delivery of cytokine genes to the tumor environment.

One of the first viral vectors used to attempt this feat were adenovirus modified to express RGD on the capsid fiber (AdRGD). These vectors were much more efficient at transducing melanoma cells both *in vitro* and *in vivo*. Also, i.t. injections of these vectors loaded with TNFα-coding DNA (AdRGD-TNFα) resulted in more hemorrhagic necrosis and inhibition

of tumor growth compared to injection of the conventional vector loaded with TNFα genes. However, the TNFα produced from the transduced cells would leak into the circulation resulting in typical TNFα-induced toxicity [51, 52]. Similarly, i.t. injections of AdRGD loaded with IL12 (AdRGD-IL12) resulted in increased transduction of melanoma cells *in vitro*, and in a syngeneic melanoma model tumor reduction compared to conventional IL12 loaded vector at a dose level a magnitude of order less than the non-targeted vector. Different from AdRGD-TNFα, there were no toxic side effects at therapeutic doses, but loss of body weight was seen with doses that were high enough to almost completely reduce tumor volume. Furthermore, combinations of AdRGD-TNFα and AdRGD-IL12 resulted in complete tumor regression in all treated mice and there were no detectable cytokine levels in the serum [53]. Although these results are exciting for cytokine-loaded-viral vectors to transduce tumor cells, these data do not show any tumor-targeting of the vectors, but further modifications of the vector capsid fibers as well as the cytokine genes will increase the effectiveness of the vectors and allow them to be used for systemic delivery of tumor-targeted cytokine viral vectors.

Active Targeting with Tumor-targeted Antibodies

By far the most investigated method to target cytokines to tumors is the fusion of antibodies to cytokines to create immunocytokines. Similar to the peptide-cytokine fusions, these immunocytokines actively target antigens on the tumor cells or in the tumor microenvironment and enhance the direct antitumor effects of the cytokines or activate an antitumor immune response against the tumors. On the other hand, antibodies can be developed to specific antigens in the tumor environment whereas most tumor-targeted peptides are derived from *in vivo* biopanning so the ligand is not necessarily known. Also, several factors hamper the ability of antibodies to successfully be used to improve therapies. First, they are larger molecules with a

full IgG antibody having a molecular weight of approximately 150 kDa. Second, the original antibodies used for therapeutic purposes were created by murine hybridoma technology so the Fc fragments were not fully compatible with the human immune system. Lastly, because they originated from murine origins, the antibodies elicit an immune response against the antibodies themselves which in turns reduces the therapeutic effect. For these reasons, the antibodies for therapeutics had to be modified to have any potential to be successful in humans [54, 55].

Several different approaches have been used to overcome these initial problems for using antibodies in therapeutic settings, and these approaches differ depending on what the antibody is used for in the therapy. For most cytokine-antibody conjugate therapies the only region of the antibody that needs to be functioning properly is the variable region. So, removal of the Fc fragment can reduce the size and therefore the immunogenicity of the antibody while maintaining its ability to bind to a specific antigen; however, the resulting fragment is still of murine origin and still immunogenic. Other modifications of antibodies create chimeric antibodies which are created by combining human portions of antibodies to murine variable regions to reduce the immunogenicity [54, 55]. Regardless of which derivative of an antibody is used, the main goal remains to maintain the specificity of the antibody and the immunomodulatory effects of the cytokine to get the highest antitumor response with the lowest possible toxic side effects. To date, tumor specific antibodies have been generated for a vast array of tumor environment specific ligands, and these antibodies have been conjugated to a vast array of cytokines.

As in peptide-cytokine therapies, immunocytokines specific for changes specific in tumor vasculature have proven highly successful. One of the first antigens for these immunocytokines utilized the recombinant human single chain antibody L19 which is specific for the domain B

isoform of fibronectin. In addition to being found only in tissue undergoing angiogenesis such as tumors and the endometrium, an interesting characteristic of this isoform is that the amino acid sequence is identical in mouse, rat, rabbit, dogs, humans, and several other mammals; therefore, the specificity of L19 is consistent in all these species [56]. L19 conjugated to IL2 (L19-IL2) was the first L19 immunocytokine used to for biodistribution and antitumor studies in mice [57].

These first studies found that the L19-IL2 conjugate did indeed target the tumor neovasculature and increased the antitumor effects of IL2. Radiolabeled L19-IL2 and the nonspecific conjugate D1.3-IL2 were i.v. administered to nude mice bearing F9 teratocarcinoma tumors. After 24 hours, the tumor:blood ratio of the L19-IL2 was 33 whereas the D1.3-IL2 was less than one. Also, microautoradiography showed that the L19-IL2 accumulated around the tumor vasculature in a similar manner to L19 alone proving that the immunocytokine has similar binding affinity for its antigen. They also analyzed the antitumor effects in several tumor cell lines in both syngeneic and xyngeneic models. In all models tested, tumor growth was significantly reduced with treatment of L19-IL2 compared to D1.3-IL2 and saline treated groups. In addition to a reduction in tumor volume, increases in tumor-infiltrating lymphocytes and necrotic area as well as reduction in the level of mitoses were seen with the L19-IL2 treatments. Also, treatment with a mixture of non-conjugated IL2 and L19 did not show any significant increases in any of the above mentioned antitumor immune responses [57]. This first study of an L19-cytokine fusion therapy led the way for several other cytokine conjugates.

L19 was then fused to IL12 to improve the efficacy and reduce the toxicity of the cytokine treatment for malignancies. In the recombinant L19-IL12 protein the p35 and p40 subunits were fused with a linker to maintain the proper folding of the heterodimer which was then attached to the N-terminus of L19. As in L19-IL2, this formulation retained the *in vitro*

bioactivity of the cytokine and the *in vivo* biodistribution of the antibody. To determine the antitumor activity, two syngeneic tumor models, C51 colon adenocarcinoma and F9 teratocarcinoma, were implanted into Balb/c and 129Sv mice, respectively, and then treated with 2.5 μg of L19-IL12, HyHEL10-IL12 (a non-specific antibody fusion), or saline every 48 h starting 4 days after tumor implantation. Throughout the treatments tumor volume and animal weights were noted. In both tumor models, tumor growth was drastically reduced with L19-IL12 treatment with at least a 4-fold decrease in tumor volume on the last day. Other signs that reveal the increased antitumor effects of L19-IL12 included increased tumor infiltration of immune cells and increased levels of IFNγ in the tumor and serum which were not seen in either of the control groups. However, in both models there were signs of IL12 induced hepatotoxicity as well as antibody production against the components of both fusion antibodies. When compared to treatment with IL12, the L19-IL12 treatments performed better than wt IL12 even at dose levels as low as 20 times less than the wt cytokine. Also, even when treatments were started as late as 7 days after tumor inoculation there was an 82% reduction in lung metastases compared to saline treated groups [56]. These results are very promising for L19-IL12 to be used in clinical settings.

Several other L19-cytokine conjugates have been created and studied including those with IL15, GM-CSF [58], IFNγ [59], and TNFα [60, 61] further proving the potential for immunocytokines in cancer therapy. In addition, antibodies with different specificities have been created and tested for their ability to target cytokines to tumor environments. Antigens for these cytokines include EGF receptor (ch225) [62, 63], ganglioside GD_2 [62, 64-66], HER2 [67, 68], KSA [9, 69], and many more [54]. However, continual improvement in the design of immunocytokines is needed to develop clinically applicable formulations as evidenced by the

IL12 induced hepatotoxicity described in the L19-IL12 study above. For these reasons, different derivations of immunocytokines have been created and studied.

One subsequent investigation involved the therapeutic potential of two new L19-IL12 derivatives. The first is a disulfuide linked homodimer composed of two L19-IL12 immunocytokines [IL12-SIP(L19)], and the other is a single IL12 with a single-chain L19 component linked to each subunit of IL12 (L19-IL12-L19). The biodistribution pattern of IL12-SIP(L19) was very similar to L19-IL12 with tumor uptake of less than 1% of the initial dose, tumor:organ accumulation ratios of approximately 5:1, and high levels of liver uptake; however, L19-IL12-L19 exhibited a much improved pattern with 19% and 9% tumor uptake at 4 and 24 h, respectively, and tumor:organ ratios of approximately 10:1 to 20:1 at 24 h. Also, L19-IL12-L19 performed comparably to wt IL12 and L19-IL12 in lymphocyte proliferation assays. Single *in vivo* injections up to 80 µg did not cause any weight loss greater than 5%. Antitumor efficacy studies revealed that indeed the L19-IL12-L19 is the superior agent. Three separate experiments with a single dose of 20 µg, 4 doses every 3 days of 5 µg, or 3 doses every three days of 20 µg and 40 µg revealed that IL12-(SIP)L19 retarded tumor growth and extended survival time more than the group treated with saline, and L19-IL12-L19 was even more effective than doses at and half of the IL12 molar equivalent of IL12-(SIP)L19 [70]. These results show that modification of antibodies can increase the performance of immunocytokines for cancer therapies.

Since it has been proved that immunocytokines and modifying the antibody regions of immunocytokines can increase the therapeutic efficacies of cytokines for tumor therapies, further avenues to create stronger therapies are continuing to be examined. One such method is using combinations of immunocytokines to create synergistic therapeutic responses. Several combinatorial cytokine therapies using recombinant wt cytokines have already been proven to be

more efficacious than treatments with the individual cytokines such as combinations of IL12 with several different cytokines [12]. To this end, combinations of immunocytokines as well as versions of immunocytokines wielding two cytokines have been investigated.

One such investigation looked at the benefit of using the L19 antibody conjugated to both IL12 and TNFα (ILT). *In vitro* bioactivity studies showed that ILT was able to maintain the function of both cytokines; however, *in vivo* studies revealed that ILT lacked any significant tumor targeted properties. Experiments to explore the antitumor effects confirmed that ILT showed almost no ability to reduce tumor growth, but coadministrations of L19-IL12 and L19-TNFα were extremely potent for inhibiting tumor growth. In a syngeneic F9 teratocarcinoma model, simultaneous delivery of 2 μg of each immunocytokine completely halted tumor growth while delivery of either agent alone had almost no effect [71]. Another bifunctional immunocytokine was more successful. This human immunocytokine, DCH, consists of two single chain antibodies specific for epithelial cell adhesion molecule (Ep-CAM) with IL2 and GM-CSF. *In vitro* assays revealed that both cytokines retain their activities and the antibodies retain their specificities compared to non-fused versions of each [72]. For *in vivo* experiments, a murine version of DCH (mDCH) was created which was similar in activity and binding affinity to the original DCH. Treatments with mDCH in syngeneic models did result in some inhibition of tumor growth, but it was not dependant on the tumor targeting ability of the antibody because a non-specific immunocytokine conferred the same level of tumor inhibition. Also, treatments with the combination of the single cytokine versions of the anti-Ep-CAM immunocytokines resulted in similar levels of tumor inhibition [73]. These results demonstrate that combinations of immunocytokines to exploit the synergistic effects of cytokines can create therapies with high antitumor efficacy, but the combination of cytokines onto the same antibody has yet to show any

further benefit. Further exploration and development of immunocytokine combinations and dual-cytokine immunocytokines is needed before any significantly beneficial therapies will emerge.

Conclusions

Malignant diseases will always be a concern for humanity, and improving treatments will always be needed. The most promising developments for treating these diseases are modulating the immune system to identify the malignant tissue as a disease and implementing a corresponding immune response. To accomplish this goal, cytokines are a valuable resource that can be developed into therapies; however, the innate pleiotropic effects of cytokines makes them a double-edged sword that can cause damage and toxicity as bad as if not worse than the malignancy itself. So, the knowledge to properly wield this sword is paramount to utilizing cytokines as treatments for malignancies. Fortunately, the current technology as well as developing technological advancements is exponentially increasing to allow us to safely and effectively use these cytokines in the near future to treat malignant diseases.

Beginning with the discovery of the EPR effect of the tumor environment, modifications of different cytokines with PEG derivations have allowed targeting to the tumor to reach effective concentrations in the tumor while reducing toxic concentrations in sensitive organs. Also, PEG as also increased the ability of viruses to specifically deliver cytokine-gene payloads to the tumor environments to achieve reduction in tumor volume while simultaneously reducing toxicity seen with non PEGylated viruses. While this passive targeting strategy helped to increase the functionality of cytokine therapies, more active techniques are needed to increase the cytokine therapies to mainstream clinical applications.

Specifically targeting ligands in the tumor environment with tumor environment-specific peptides is one such active method. These peptides have the ability to not only reach the tumor

environment but also exhibit antitumor effects such as interrupting angiogenesis. But the true value of these peptides lies in their ability to transfer more destructive agents such as cytokines and viruses loaded with cytokine genes to the tumor environment. The discovery of RGD, NGR, and their derivatives has truly helped expand the use of cytokines in cancer therapies. These peptides can target various tumor types because they target ligands in the tumor environment and the tumor cells. But these universal targeted peptides may not be beneficial to all patients. The use of *in vivo* biopanning to discover potential ligands has been shown to identify patient specific ligands in biopsies [74]. This strategy has the potential to make patient-specific peptide-cytokine conjugates which will be extremely effective.

Likewise, the use of antibodies has cytokine carriers has proven to be very beneficial for cytokine therapies. These antibodies are very specific for tumor antigens and can be manufactured for any antigen and to be less immunogenic in patients. Also, immunocytokines maintain their specificity for their antigens as well as the bioactivity of the cytokines. These tumor-specific antibodies can also carry cytokine genes loaded in viral vectors to concentrate the cytokine activities in the tumor environment while reducing toxic side effects elsewhere. There is still much room for improvement in these therapies but promising results and preclinical experiments continue to increase the efficacy of these treatments.

Tumor-targeted cytokine treatments are still years away from being a primary treatment for cancer, but continued research into how to modify current treatment strategies will help bring that goal closer every year. Also, the development of new strategies is promising improved treatment strategies. For instance, using mesenchymal stem cells (MSC) to deliver cytokines to the tumor environment are already appearing on the horizon. Several groups have used local delivery of MSC loaded with cytokine genes to decrease tumor volumes, but the successful

treatment of tumors with systemic MSC delivery has yet to be realized [17, 19]. Current and future development of these tumor-targeted cytokine therapies is crucial for the continuing improvement of immunomodulatory cancer treatments.

Statement of the Problem and Specific Aims

Improving cytokine gene therapy to deliver cytokines to distally located, metastatic, and residual tumors is a promising strategy for improving tumor-targeted cytokine therapies. Results from our lab have shown that intratumoral delivery of IL12 plasmid DNA results in tumor eradication of 40% in a model of squamous cell carcinoma, but systemic delivery via intramuscular administration has no effect [75]. Unfortunately, directly treating the tumors, especially metastatic and residual, is rarely a viable option; therefore, developing a gene therapy strategy that can be delivered systemically to produce a product that accumulates in the tumor environment will increase the antitumor effects while decreasing the toxic side effects.

The targeting agent with the most promise for use in this strategy is a tumor-targeting peptide. While several tumor-targeted peptides have been identified, none have been shown that can target an *in vivo* produced gene product, and phage display libraries are not viable for this strategy. So, a new screening strategy was needed, and any potential idiosyncrasies involved in this strategy needed to be understood. So, we inserted tumor- and tissue-targeted peptides directly before the stop codon in a reporter gene, but there were several inconsistencies when the reporter gene activity was measured. Understanding these conflicts was critical for creating this reporter gene screening strategy to identify the best tumor-targeted-peptide to be used in this gene therapy approach.

Once the reporter gene method is perfected, the best tumor-targeted peptide can be identified, and the therapeutic benefits of systemic treatments with the tumor targeted-IL12 gene

therapy can be assessed. To achieve these goals, several experiments were performed to fulfill the following aims:

Aim 1: Establish a method to identify peptides capable of targeting an *in vivo* produced gene product to the tumor environment;

Aim 2: Identify the best tumor targeted peptide for use in this strategy;

Aim 3: Determine the therapeutic benefits of using the systemic tumor-targeted-IL12 gene therapy; and

Aim 4: Identify the receptor for the peptide.

References

1. Society AC (2008). Cancer Facts & Figures 2008. American Cancer Society, Atlanta.

2. Bazan-Peregrino M, Seymour L, Harris A (2007). Gene therapy targeting to tumor endothelium. *Cancer Gene Therapy* **14**: 117-127.

3. Haaga JR, Exner AA, Wang Y, Stowe NT, Tarcha PJ (2005). Combined tumor therapy by using radiofrequency ablation and 5-FU-laden polymer implants: evaluation in rats and rabbits. *Radiology* **237**: 911-918.

4. Li CY, Huang Q, Kung HF (2005). Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol* **2**: 81-91.

5. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* **72**: 3666-3670.

6. Morgan DA, Ruscetti FW, Gallo R (1976). Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* **193**: 1007-1008.

7. Kobayashi M, *et al.* (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* **170**: 827-845.

8. Jiang YY, Liu C, Hong MH, Zhu SJ, Pei YY (2007). Tumor Cell Targeting of Transferrin-PEG-TNF- Conjugate via a Receptor-Mediated Delivery System: Design, Synthesis, and Biological Evaluation. *Bioconjugate Chem* **18**: 41-49.

9. Xiang R, *et al.* (1997). Elimination of established murine colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy. *Cancer Res* **57**: 4948-4955.

10. Del Vecchio M, *et al.* (2007). Interleukin-12: biological properties and clinical application. *Clin Cancer Res* **13**: 4677-4685.

11. Soiffer RJ, Robertson MJ, Murray C, Cochran K, Ritz J (1993). Interleukin-12 augments cytolytic activity of peripheral blood lymphocytes from patients with hematologic and solid malignancies. *Blood* **82**: 2790-2796.

12. Weiss JM, Subleski JJ, Wigginton JM, Wiltrout RH (2007). Immunotherapy of cancer by IL-12-based cytokine combinations. *Expert Opin Biol Ther* **7**: 1705-1721.

13. Rosenberg SA, *et al.* (1989). Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* **210**: 474-484; discussion 484-475.

14. Atkins MB, *et al.* (1993). Randomized phase II trial of high-dose interleukin-2 either alone or in combination with interferon alfa-2b in advanced renal cell carcinoma. *J Clin Oncol* **11**: 661-670.

15. Loisel-Meyer S, Foley R, Medin JA (2008). Immuno-gene therapy approached for cancer; from in vitro studies to clinical trials. *Frontiers in Bioscience* **13**: 3202-3214.

16. Schatzlein AG (2003). Targeting of Synthetic Gene Delivery Systems. *Journal of Biomedicine and Biotechnology* **2003**: 149-158.

17. Hamada H, *et al.* (2005). Mesenchymal stem cells (MSC) as therapeutic cytoreagents for gene therapy. *Cancer Sci* **96**: 149-156.

18. Nakamura K, *et al.* (2004). Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* **11**: 1155-1164.

19. Reiser J, Zhang XY, Hemenway CS, Mondal D, Pradhan L, La Russa VF (2005). Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases. *Expert Opin Biol Ther* **5**: 1571-1584.

20. Stagg J, Lejeune L, Paquin A, Galipeau J (2004). Marrow stromal cells for interleukin-2 delivery in cancer immunotherapy. *Hum Gene Ther* **15**: 597-608.

21. Matsumura Y, Maeda H (1986). A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs. *Cancer Res* **46**: 6387-6392.

22. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K (2000). Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *Journal of Controlled Release* **65**: 271-284.

23. van Vlerken LE, Vyas TK, Amiji MM (2007). Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. *Pharm Res* **24**: 1405-1414.

24. Brassard DL, Grace MJ, Bordens RW (2002). Interferon-alpha as an immunotherapeutic protein. *J Leukoc Biol* **71**: 565-581.

25. Talpaz M, *et al.* (2001). Phase 1 study of polyethylene glycol formulation of interferon {alpha}-2B (Schering 54031) in Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood* **98**: 1708-1713.

26. Matthews SJ, McCoy C (2004). Peginterferon alfa-2a: a review of approved and investigational uses. *Clin Ther* **26**: 991-1025.

27. Li YP, *et al.* (2001). Stealth polycyanoacrylate nanoparticles as tumor necrosis factor-alpha carriers: pharmacokinetics and anti-tumor effects. *Biol Pharm Bull* **24**: 662-665.

28. Fang C, Shi B, Pei Y-Y, Hong M-H, Wu J, Chen H-Z (2006). In vivo tumor targeting of tumor necrosis factor-alpha-loaded stealth nanoparticles: Effect of MePEG molecular weight and particle size. *European Journal of Pharmaceutical Sciences* **27**.

29. Croyle MA, Chirmule N, Zhang Y, Wilson JM (2001). "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. *J Virol* **75**: 4792-4801.

30. Gao J-Q, *et al.* (2007). Effective tumor targeted gene transfer using PEGylated adenovirus vector via systemic administration. *Journal of Controlled Release* **122**: 102-110.

31. Pasqualini R, Ruoslahti E (1996). Organ targeting in vivo using phage display peptide libraries. *Nature* **380**: 364-366.

32. Craig R, Li S (2006). Function and Molecular Mechanism of Tumor-Targeted Peptides for Delivering herapeutic Genes and Chemical Drugs. *Mini-Reviews in Medicinal Chemistry* **6**: 109-120.

33. Enback J, Laakkonen P (2007). Tumour-homing peptides: tools for targeting, imaging and destruction. *Biochem Soc Trans* **35**: 780-783.

34. Maubant S, *et al.* (2006). Blockade of alpha v beta 3 and alpha v beta 5 integrins by RGD mimetics induces anoikis and not integrin-mediated death in human endothelial cells. *The American Society of Hematology* **108**: 3035-3044.

35. Dickerson EB, *et al.* (2004). Enhancement of the Antiangiogenic Activity of Interleukin-12 by Peptide Targeted Delivery of the Cytokine to {alpha}v{beta}3 Integrin. *Mol Cancer Res* **2**: 663-673.

36. Curnis F, Gasparri A, Sacchi A, Longhi R, Corti A (2004). Coupling Tumor Necrosis Factor-{alpha} with {alpha}V Integrin Ligands Improves Its Antineoplastic Activity. *Cancer Res* **64**: 565-571.

37. Ma D, *et al.* (2007). Purification and characterization of RGD tumor-homing peptide conjugated human tumor necrosis factor alpha over-expressed in Escherichia coli. *J Chromatogr B Analyt Technol Biomed Life Sci* **857**: 231-239.

38. Wang H, *et al.* (2008). Integrin-targeted imaging and therapy with RGD4C-TNF fusion protein. *Mol Cancer Ther* **7**: 1044-1053.

39. Wang H, Yan Z, Shi J, Han W, Zhang Y (2006). Expression, purification, and characterization of a neovasculature targeted rmhTNF-alpha in Escherichia coli. *Protein Expr Purif* **45**: 60-65.

40. Pasqualini R, *et al.* (2000). Aminopeptidase N Is a Receptor for Tumor-homing Peptides and a Target for Inhibiting Angiogenesis. *Cancer Res* **60**: 722-727.

41. Curnis F, *et al.* (2002). Differential Binding of Drugs Containig the NGR Motif to CD13 Isoforms in Tumor Vessels, Epithelia, and Myeloid Cells. *Cancer Research* **62**: 867-874.

42. Colombo G, *et al.* (2002). Structure-activity relationships of linear and cyclic peptides containing the NGR tumor-homing motif. *J Biol Chem* **277**: 47891-47897.

43. Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A (2000). Enhancement of tumor necrosis factor [alpha] antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). *Nat Biotech* **18**: 1185-1190.

44. Curnis F, Sacchi A, Corti A (2002). Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. *J Clin Invest* **110**: 475-482.

45. Sacchi A, Gasparri A, Gallo-Stampino C, Toma S, Curnis F, Corti A (2006). Synergistic Antitumor Activity of Cisplatin, Paclitaxel, and Gemcitabine with Tumor Vasculature-Targeted Tumor Necrosis Factor-{alpha}. *Clin Cancer Res* **12**: 175-182.

46. Bertilaccio MT, *et al.* (2008). Vasculature-targeted tumor necrosis factor-alpha increases the therapeutic index of doxorubicin against prostate cancer. *Prostate* **68**: 1105-1115.

47. Meng J, *et al.* (2007). High-yield expression, purification and characterization of tumor-targeted IFN-alpha2a. *Cytotherapy* **9**: 60-68.

48. Meng J, *et al.* (2008). Preclinical safety evaluation of IFN[alpha]2a-NGR. *Regulatory Toxicology and Pharmacology* **50**: 294-302.

49. Craig R, Cutrera J, Zhu S, Xia X, Lee YH, Li S (2008). Administering plasmid DNA encoding tumor vessel-anchored IFN-alpha for localizing gene product within or into tumors. *Mol Ther* **16**: 901-906.

50. Temming K, Schiffelers RM, Molema G, Kok RJ (2005). RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. *Drug Resistance Updates* **8**: 381-402.

51. Okada Y, Okada N, Mizuguchi H, Hayakawa T, Mayumi T, Mizuno N (2003). An investigation of adverse effects caused by the injection of high-dose TNFalpha-expressing adenovirus vector into established murine melanoma. *Gene Ther* **10**: 700-705.

52. Okada Y, *et al.* (2002). Tumor necrosis factor alpha-gene therapy for an established murine melanoma using RGD (Arg-Gly-Asp) fiber-mutant adenovirus vectors. *Jpn J Cancer Res* **93**: 436-444.

53. Okada Y, *et al.* (2004). Optimization of antitumor efficacy and safety of in vivo cytokine gene therapy using RGD fiber-mutant adenovirus vector for preexisting murine melanoma. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1670**: 172-180.

54. Ortiz-Sanchez E, Helguera G, Daniels TR, Penichet ML (2008). Antibody-cytokine fusion proteins: applications in cancer therapy. *Informa Helthcare* **8**: 609-632.

55. Schrama D, Reisfeld RA, Becker JC (2006). Antibody targeted drugs as cancer therapeutics. *Nature Reviews* **5**: 147-159.

56. Halin C, *et al.* (2002). Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. *Nat Biotechnol* **20**: 264-269.

57. Carnemolla B, *et al.* (2002). Enhancement of the antitumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. *Blood* **99**: 1659-1665.

58. Kaspar M, Trachsel E, Meri D (2007). The Antibody-Mediated Targeted Delivery of Interleukin-15 and GM-CSF to the Tumor Neovasculature Inhibits Tumor Growth and Metastasis. *Cancer Research* **67**: 4940-4948.

59. Ebbinghaus C, *et al.* (2005). Engineered vascular-targeting antibody-interferon-gamma fusion protein for cancer therapy. *International Journal of Cancer* **116**: 304-313.

60. Balza E, *et al.* (2006). Targeted Delivery of Tumor Necrosis Factor-alpha to Tumor Vessels Induces a Therapeutic T Cell-Mediated Immune Response that Protects the Host Against Syngeneic Tumors of Different Histologic Origin. *Clinical Cancer Research* **8**: 2575-2582.

61. Borsi L, *et al.* (2003). Selective targeted delivery of TNFalpha to tumor blood vessels. *Blood* **102**: 4384-4392.

62. Becker JC, Pancook JD, Gillies SD, Mendelsohn J, Reisfeld RA (1996). Eradication of human hepatic and pulmonary melanoma metastases in SCID mice by antibody-interleukin 2 fusion proteins. *Proc Natl Acad Sci U S A* **93**: 2702-2707.

63. Reisfeld RA, Gillies SD, Mendelsohn J, Varki NM, Becker JC (1996). Involvement of B Lymphocytes in the Growth Inhibibion of Human Pulmonary Melanoma Metastases in Athymic nu/nu Mice by an Antibody-Lymphotoxin Russion Protein. *Cancer Research* **56**: 1707-1712.

64. King DM, *et al.* (2004). Phase I Clinical Trial of the Immunocytokine EMD 273063 in Melanoma Patients. *Journal of Clinical Oncology* **22**: 4463-4473.

65. Metelitsa LS, Gillies SD, Super M, Shimada H, Renolds CP, Seeger RC (2002). Antidisialogaglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. *Blood* **99**: 4166-4173.

66. Schrama D, *et al.* (2001). Targeting of Lymphotoxin-alpha to the Tumor Elicits and Efficient Immune Response Associated with Induction of Peripheral Lymphoid-like Tissue. *Immunity* **14**: 111-121.

67. Dela Cruz JS, Trinh KR, Morrison SL, Penichet ML (2000). Recombinant anti-human HER2/neu IgG3-(GM-CSF) fusion protein retains antigen specificity and cytokine function and demonstrates antitumor activity. *J Immunol* **165**: 5112-5121.

68. Peng LS, Penichet ML, Morrison SL (1999). A Single-Chain IL-12 IgG3 Antibody Fusion Protein Retains Antibody Specificity and IL-12 Bioactivity and Demonstrates Antitumor Activity. *The Journal of Immunology* **163**: 250-258.

69. Ko YJ, *et al.* (2004). Safety, pharmacokinetics, and biological pharmacodynamics of the immunocytokine EMD 273066 (huKS-IL2): results of a phase I trial in patients with prostate cancer. *J Immunother* **27**: 232-239.

70. Gafner V, Trachsel E, Neri D (2006). An engineered antibody-interleukin-12 fusion protein with enhanced tumor vascular targeting properties. *International Journal of Cancer* **119**: 2205-2212.

71. Halin C, *et al.* (2003). Synergistic Therapeutic Effects of a Tumor Targeting Antigody Fragment, Fused to Interleukin 12 and to Tumor Necrosis Factor alpha. *Cancer Research* **63**: 3202-3210.

72. Schanzer JM, Baeuerle PA, Dreier T, Kufer P (2006). A human cytokine/single-chain antibody fusion protein for simultaneous delivery of GM-CSF and IL-2 to Ep-CAM overexpressing tumor cells. *Cancer Immun* **6**: 4.

73. Schanzer JM, Fichtner I, Baeuerle PA, Kufer P (2006). Antitumor activity of a dual cytokine/single-chain antibody fusion protein for simultaneous delivery of GM-CSF and IL-2 to Ep-CAM expressing tumor cells. *J Immunother* **29**: 477-488.

74. Krag DN, *et al.* (2006). Selection of tumor-binding ligands in cancer patients with phage display libraries. *Cancer Res* **66**: 7724-7733.

75. Li S, Zhang L, Torrero M, Cannon M, Barret R (2005). Administration route- and immune cell activation-dependent tumor eradication by IL12 electrotransfer. *Mol Ther* **12**: 942-949.

Chapter 2

Enhancement of Reporter Gene Detection Sensitivity by Insertion of Specific Mini-Peptide-Coding Sequences*

*Reprinted with permission from Nature Publishing Group

Introduction

Gene therapy is on the forefront of many areas of biomedical research and therapeutics. The use of gene therapy ranges from the utilization of reporter genes for noninvasive monitoring and gene distribution [75-77] to the application of therapeutic genes to treat several diseases such as cancer [78-81] and blood disorders [82, 83]. Advancements in the development of both viral and non-viral delivery systems for gene therapy result in continuous improvements in the efficacy of gene therapy, but several barriers remain which are blocking gene therapy from becoming widely successful.

Secreted alkaline phosphatase (SEAP) is a widely used reporter gene which is known for its resistance to high temperature and L-homoarginine, unlike endogenous alkaline phosphatases. SEAP is used for a variety of *in vitro* and *in vivo* assays including interferon activity [84], efficacy of antidepressants [85], and endoplasmic reticulum stress [86, 87]. With current strategies, the low level of gene expression of SEAP as well as other reporter genes affects the use of reporter genes and limits their uses to *in vitro* and small animal models [75-77, 86, 88]. Similarly, therapeutic applications of gene therapy are hindered by having trouble acquiring therapeutically significant levels of the gene products [89, 90]. Although improvements in gene delivery and vector design can increase the total level of gene expression, these advancements do not improve the sensitivity per molecule of the gene product.

To increase the level of gene expression, investigators have focused on increasing gene delivery. For instance, several methods to increase the delivery of genes with viral vectors are currently used. The addition of Poly(ethylene glycol) (PEG) motifs to the capsid fibers of viral vectors, a process termed PEGylation, can increase the viral half-life by protecting the viral particles from enzymatic degradation and increasing the circulation time. Also, PEG can protect

the viral vectors from cell- and humoral-mediated immune responses. By improving the *in vivo* viral properties, PEG can increase the level of gene delivery by the viral vectors [89]. Also, modifying the tropism of the viruses by the addition of tissue-targeted ligands can increase the viral load at specific sites which increases the delivery of genes at the desired location [89, 91, 92]. Similarly, plasmid delivery can be enhanced by delivery via gene gun, electroporation, ultrasound, and many other techniques [90].

Besides gene delivery, the level and duration of gene expression may be one of the main limiting factors for successful gene therapy. One of the most important elements that can affect both of these factors is the promoter. The most commonly used promoter was developed from the immediate early promoter of human cytomegalovirus (CMV) because it can increase the level of expression in a wide range of cell types; however, the expression generated from the CMV promoter consistently decreases beginning one to two days after transfection [93]. Other promoters can increase the level of expression based on the tissue that is transfected. For instance, the muscle creatine promoter can increase expression of SEAP in muscle tissues as well as inhibiting both cell- and humoral- mediated immune responses [94]. To create more sustained expression, synthetic promoters have been developed by combining promoters and enhancer elements from different sources. One such promoter is the CAGG promoter which consists of the CMV enhancer, β-actin promoter, and a chicken β-actin/rabbit β-actin globin composite intron [82]. Composite promoters can be developed by using high throughput methods using random assembly of promoter elements to create improved promoters [95, 96]. Other methods that are used to increase the level and duration of gene expression include reducing the amount of unmethylated CpG dinucleotides [80, 97], using codon optimization to remove rarely used codons from plasmid DNA [98, 99], and decreasing the size of the vectors by using DNA

fragments [100]. These approaches are all valuable, but current strategies have not overcome all the hurdles to gene therapy that are caused by the low level of gene expression.

To complement these conventional approaches and increase the detection sensitivity of each reporter molecule, here we report a novel concept—increasing the activity of the gene product. Insertion of mini-peptide-coding sequences prior to the stop codon in SEAP-coding DNA drastically alters the enzyme activity of the resulting SEAP enzymes. This effect occurs in all cell lines tested as well as in two separate murine animal models. The change in detectable levels of the enzymes is not due to a change in amount of the SEAP enzyme present but instead is the result of a change in the activity of the SEAP enzyme.

Materials

Gene Constructs Encoding Wild-type and Peptide Conjugated Proteins. All SEAP gene constructs were generated via direct PCR as previously described [101].

***In vitro* DNA Transfection via Electroporation and Reporter Gene Assays.** Plasmid DNA was transfected into cells using electroporation in the following cell lines: EC40, C2C12, 4T1, Jurkat, and HEK293 (American Type Culture Collection, Manassas, VA). All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DMEM) (Life Technologies, Rockville, MD). Cells were then suspended at a concentration of $1x10^7$ cells per milliliter of Opti-mem medium (Invitrogen, Carlsbad, CA) containing 10 μg/mL of firefly luciferase (Fluc) or *Gaussia* luciferase (Gluc) plasmid DNA (Valentis, Burlingame, CA, and New England Biolabs, Inc., Ipswich, MA, respectively) as transfection efficiency controls. 100 μL of the solution was transferred to individual electroporation cuvettes (n=3) to which 2 μg of experimental plasmid DNA was added. Each cuvette was pulsed with one 75 msec-pulse of 150 V. The cell suspensions and 900 μL DMEM were transferred to individual

wells of 6-well plates, and the medium was collected and replaced on specified days. If needed, cell lysates were collected on the last day by incubating the cells with 200 µL of 1x Cell Lysis Buffer (Promega, Madison, WI), freezing the plates at -80 °C for 20 min, thawing the plates on ice, and collecting the lysate suspensions. All samples were stored at -80 °C until assayed.

Cell lysates and mediums were assayed for SEAP activity using the Phospha-Light SEAP Reporter Gene Assay System (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions. Fluc activity or Gluc acitivity was analyzed by assaying cell lysates with the Luciferase Assay System (Promega, Madison, WI) or cell mediums with the Gaussia Luciferase Assay System (New England Biolabs, Inc., Ipswich, MA) following the manufacturers' instructions. Data is presented as fold comparisons of the concentrations of SEAP per Fluc or Gluc activity in the peptide-SEAP conjugates to the WT-SEAP.

Animal Models, *In Vivo* DNA Delivery via Electroporation, and Serum Collection. Six- to eight-week old Balb/c (in-house animal breeding facility) and nude mice (Charles River Laboratories, Inc., Wilmington, MA) were maintained under NIH guidelines approved by the Institutional Animal Care and Use Committee of Louisiana State University. Plasmid DNA, prepared as discussed above, was diluted in half-strength saline to 5 µg/30 µL, injected into each rear tibialis muscle, and directly followed by electroporation (n=4) as previously described[102]. The electroporation was performed using the following parameters: two 20 msec-pulses of 35 V/mm with a 100-msec interval between pulses. Blood was collected via cheek-bleeding on days 1, 5, 10, and 15 after transfection, placed on ice for 1 h, centrifuged at 3,000 x *g* for 10 min to separate the serum, and the serum was collected and stored at -80 °C until assayed as above.

Western Blot Analysis. 10 µL of serum was subjected to SDS-PAGE in a 12% polyacrylamide gel and then transferred to a Trans-Blot Transfer Medium nitrocellulose membrane (Bio-Rad

Laboratories, Hercules, CA). Immunoblotting of the membrane was performed with a 1:500 dilution of the primary anti-Human Placental Alkaline Phosphatase polyclonal antibody (GeneTex, Inc., San Antonio, TX) and a 1:5000 dilution of the secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (GE Healthcare, Piscataway, NJ). The peroxidase signal was generated with the Western Lightning ECL (PerkinElmer, Waltham, MA) and visualized with a Kodak Image Station 440CF using the 1D Image Analysis Software v3.6 (NEN Life science Products, Boston, MA). All blots are representative of at least two repeat blots with similar results.

Statistical Analyses. For all *in vitro* experiments, bars represent mean values of the SEAP/Fluc or Gluc activity divided by the mean WT-SEAP values, and the labels represent the fold-change compared to the control. For the *in vivo* experiments, the bars represent mean values of SEAP activity per microliter of serum divided by the mean WT-SEAP values. One-way ANOVA followed by Dunnett's test was used for statistical analysis, and an asterisk (*) denotes $p<0.05$ comparing the SEAP-peptide mean value to the control SEAP mean value.

Results

Mini-peptide-coding Sequences Conjugated to SEAP Plasmid DNA Profoundly Affect the Activity of the Resulting Enzyme. Phage display technology has been widely used for *in vivo* biopanning to identify peptides that target tumors and other specific tissues [103, 104]. A group of peptides that were identified as tumor-targeted were analyzed for their ability to target tumors via insertion of mini-peptide-encoding segments directly before the stop codon on the C-terminus of SEAP plasmid DNA (**Fig. 2.1**); however, in experiments performed in our lab the majority of the peptides failed to demonstrate any tumor targeting capabilities when fused with the SEAP reporter gene via a genetic engineering method (see Chapter 3).

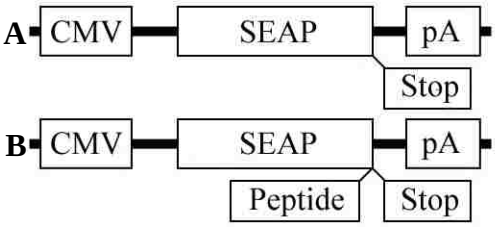


Figure 2.1 Structure of wild-type (WT) and the peptide modified reporter gene plasmid DNA constructs. (**A**) The SEAP control construct. (**B**) Mini-peptide-SEAP fusion gene. Mini-peptide-coding sequences were directly inserted before the stop codon in the same open reading frame. CMV, Cytomegalovirus promoter; SEAP, secreted alkaline phosphatase; pA, polyadenylation signal; Stop, stop codon; Peptide, inserted peptide-encoding sequences.

Surprisingly, the different peptides caused drastic changes in the detected SEAP activity 24 h after transfection in EC40, an immortalized endothelial cell line. These changes ranged from more than a 3-fold increase compared to the wild-type SEAP (WT-SEAP) to almost undetectable activity (**Fig. 2.2A**). There are some changes in the SEAP activities from cell lysates which correlates to the previously known tumor targeted peptides which are based on the RGD motif; however, there are no significantly different SEAP activities in the cell lysates (**Fig. 2.2B**). So, the extreme changes in activity are not due to the peptides binding to the cell or the enzyme not being secreted from the cell. From this broad screening, we were able to conclude that the peptides that best enhanced the SEAP activity were CWDDWLC, TAASG, GSL, and RGD.

To further investigate whether the increased activities by inserting these peptides are cell specific and whether insertion of these peptides results in a longer duration of increased expression, these selected peptide-SEAP fusion gene constructs were then transfected into murine muscle cells (C2C12), and the expression was determined for multiple days instead of just one day after the transfection. A plasmid DNA coding for *Gaussia* luciferase (Gluc), which is a secreted luciferase originating from the marine copepod *Gaussia princeps* [105], was included in the transfection to correct for transfection efficiency. After transfection of these cells, the medium was collected and replaced on days 1, 2, 3, and 4 and analyzed for SEAP and Gluc

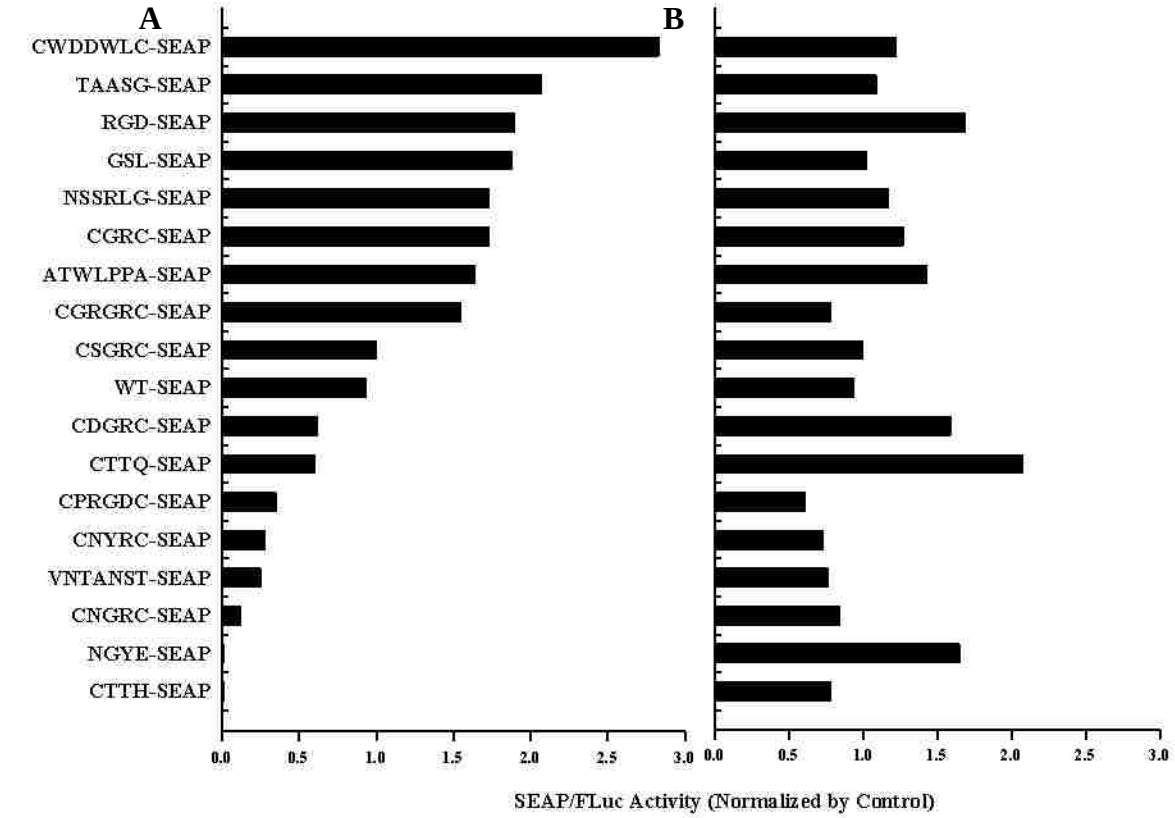


Figure 2.2 Differences in SEAP activity from transfection of endothelial cells with peptide-modified SEAP reporter fusion genes. The magnitude of secreted (**A**) and cell retained (**B**) SEAP activities from EC40 cells that were transfected with SEAP or SEAP fusion genes. Firefly luciferase (FLuc) plasmid DNA was co-transfected for normalization of the transfection efficiency variation. The abbreviations for each construct represent the single-letter amino acid code except for 'WT' which represents the wild-type SEAP activity. The bars represent the activity of SEAP (corrected for transfection efficiency by the activity of luciferase) using the wild-type SEAP/Luc activity to normalize the data. Bars and abbreviations in all subsequent figures represent the same data presentation unless otherwise noted.

activity (**Fig. 2.3A**). The same as with EC40, one day after transfection the C2C12 cells produced significantly higher peptide-SEAP activities compared to the WT-SEAP with CWDDWLC nearing 3-fold higher activity and TAASG, GSL, and RGD at almost 2-fold higher activities. Furthermore, the fold-increases of CWDDWLC-SEAP compared to WT-SEAP on days 2, 3, and 4 were 2.51, 2.87, and 4.69, respectively. The other peptide-SEAP activities on

days 2, 3, and 4 also remained higher than the WT-SEAP activities with RGD-SEAP elevating to 4-fold higher on day 4. Also, since the medium is replaced each day the increase in the activities is not from residual SEAP remaining from the previous day but new SEAP produced in a 24 h period. These data show that the elevated peptide-SEAP activities are not dependent on a specific cell type and result from newly produced enzyme units.

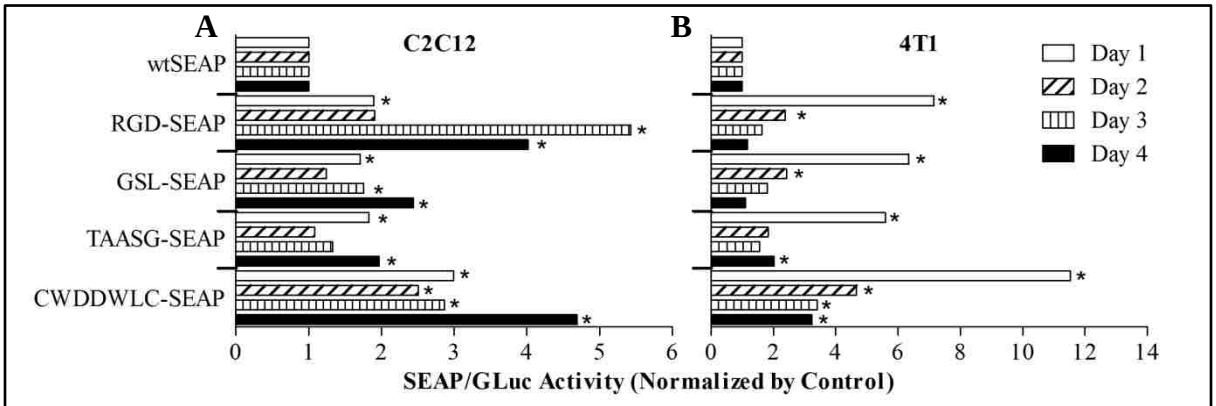


Figure 2.3. Increase in SEAP activity by inserting the selected peptides from murine muscle and tumor cells over time. Peptide-SEAP activity of the murine muscle cell line C2C12 (**a**) and adenocarcinoma cell line 4T1 (**b**) after transfection with peptide-SEAP constructs at each time point. The abbreviations are the same as described in the Figure 2.2 legend. * represents $p<0.05$ compared to the WT-SEAP activity.

Transfected murine tumor cells also produce increases in peptide-SEAP activities but to a much higher degree compared to the other tested cell types. When murine adenocarcinoma cells (4T1) were transfected in the same manner as the EC40 and C2C12 cells, all peptide-SEAP fusion protein reached greater activity with TAASG-, GSL-, and RGD-SEAP activities ranging from approximately 5- to 7-fold increases and CWDDWLC-SEAP having a greater than 11-fold increase in activity compared to the WT-SEAP on day 1 **(Fig. 2.3B**). On days 2, 3, and 4 the peptide-SEAP activity continued to be higher but to a lesser degree compared to day 1 with only CWDDWLC- and TAASG-SEAP activities being significantly higher compared to the WT-SEAP on day 4. These results confirm that inserting peptide-encoding-sequences to the SEAP

encoding sequence in the same open reading frame can have drastic effects on the resulting enzyme, and CWDDWLC is consistently the peptide that creates the highest elevation of SEAP activity.

Modification of the CWDDWLC Sequence Significantly Alters the Activity of the Resulting Enzyme. To further analyze whether the increase of SEAP activity by integrating the CWDDWLC-encoding DNA is dependent on the peptide sequence, we generated a construct containing an almost identical peptide with the insertion of one amino acid, yielding a sequence of CWDDGWLC-SEAP. The only difference in the peptides is a Gly residue located between the second Asp and Trp residues of the CWDDWLC peptide. We compared the activity of the new construct CWDDGWLC- with CWDDWLC- and WT-SEAP transfected into human cell lines.

Firstly, transfection of CWDDWLC-SEAP pDNA into human T (Jurkat), colon tumor (HT29), and kidney tumor (HEK293) cells resulted in elevated SEAP activity compared to the WT-SEAP similar to that seen in the murine cell lines (**Fig. 2.4**). For both HT29 cells and

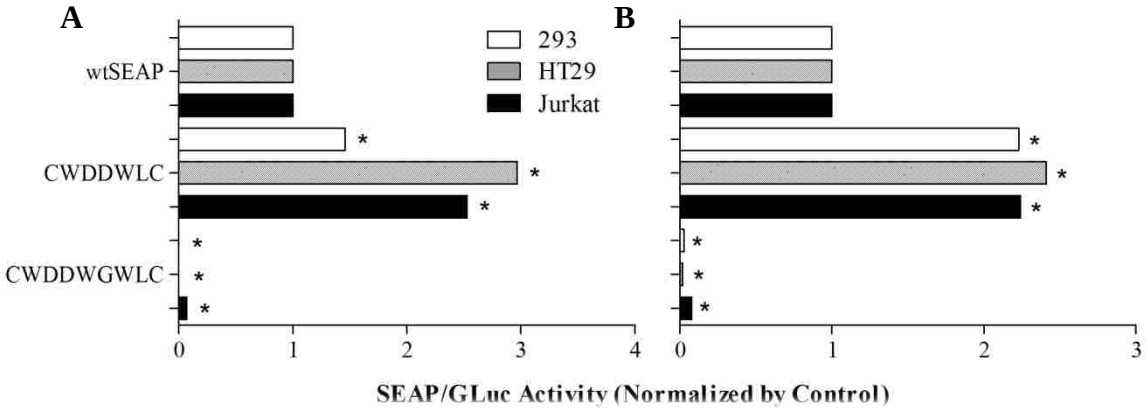


Figure 2.4. Increase in SEAP activity by inserting the peptide in human cell lines is amino-acid sequence specific. SEAP activity in Human T (Jurkat), colon tumor (HT29), and kidney (HEK293) cell lines on days 1 (**a**), and 4 (**b**). The abbreviations are the same as described in the Figure 2 legend. CWDDWLC, the selected peptide that maximizes the SEAP activity as was shown in Figure 3. CWDDGWLC, the sequence containing one Gly residue inserted in the CWDDWLC peptide to illustrate the sequence specificity. * represents $p<0.05$ compared to the WT-SEAP activity.

HEK293 cells, the medium was collected the same as the murine cell lines so these data show that this phenomenon is not species specific and could translate into a clinical setting. Also, the Jurkat cell line is not adherent so instead of collecting and replacing the medium, 50 μL of medium was collected and assayed at every time point. The increase in CWDDWLC-SEAP activity occurs in all human cell lines derived from different tissues (**Fig. 2.4**).

Secondly, the addition of the Gly residue dramatically and significantly diminished the detectable activity of the resulting SEAP enzyme (**Fig. 2.4**). When the CWDDGWLC-SEAP DNA was transfected into all three human cell lines, the activity of the enzyme was at least10-fold less than the WT-SEAP activity and at least 20-fold less than the CWDDWLC-SEAP activity. So, the increase in activity is dependent on the structure and sequence of the CWDDWLC peptide.

Similar to the single amino acid insertion, we also tested amino acid deletion. Removal of the Cys residues that flank the peptide completely abolished the effect of the peptide on the SEAP activity (**Fig. 2.5**). In both C2C12 and HT29 cell lines, transfection with the CWDDWLC-

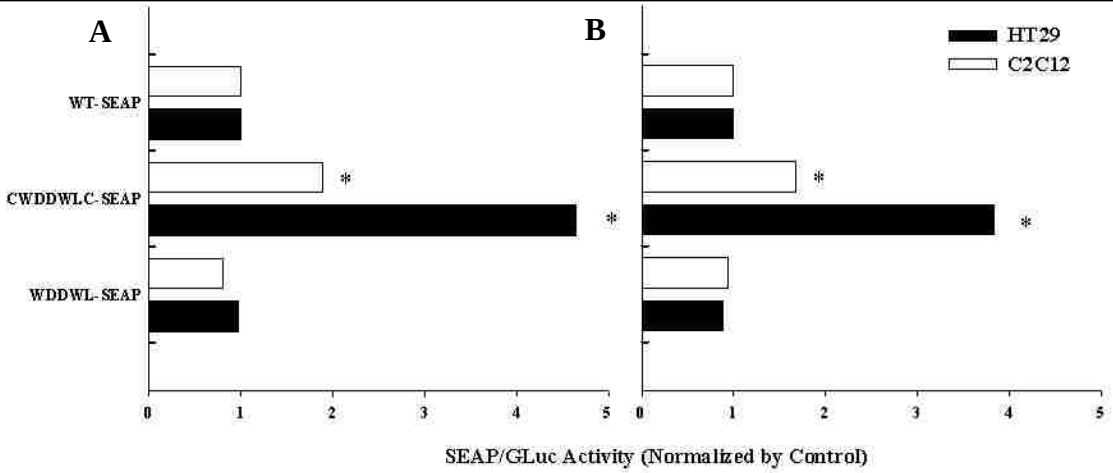


Figure 2.5. Removal of the flanking Cys residues reduces the SEAP activity to the WT-SEAP level. SEAP activity in both human (HT29) and murine (C2C12) cell lines on days 1 (**A**) and 4 (**B**). CWDDWLC-SEAP, the reporter gene modified with the selected peptide coding sequence that maximizes SEAP activity. WDDWL-SEAP, the same selected peptide without the flanking Cys residues. * represents $p<0.05$ compared to the WT-SEAP activity.

SEAP plasmid DNA repeatedly showed increased SEAP activity compared to the WT-SEAP transfected cells; however, Cys-less WDDWL-SEAP encoding plasmid DNA transfected cells produced levels of SEAP activity that were not significantly different than transfection with WT-SEAP encoding DNA (**Fig. 2.5**).

The Same Trends in Activity are Seen with *In Vivo* Transfection but with No Difference in the Level of SEAP Protein. After demonstrating that the increased activity seen by conjugating CWDDWLC to SEAP plasmid DNA occurs in all tested cell lines, the next step was to see if this effect also occurs *in vivo*, which determines the ultimate application value for reporting purposes. To determine this effect *in vivo*, mice were injected with 5 µg of peptide-SEAP plasmid DNA or WT-SEAP followed by electroporation in each rear tibialis muscle.

The same as seen *in vitro*, the CWWDWLC-SEAP activity was near 6-fold higher than that of the WT-SEAP activity on days 1 and 5 (**Fig. 2.6**). Interestingly, the WDDWL-SEAP activity did

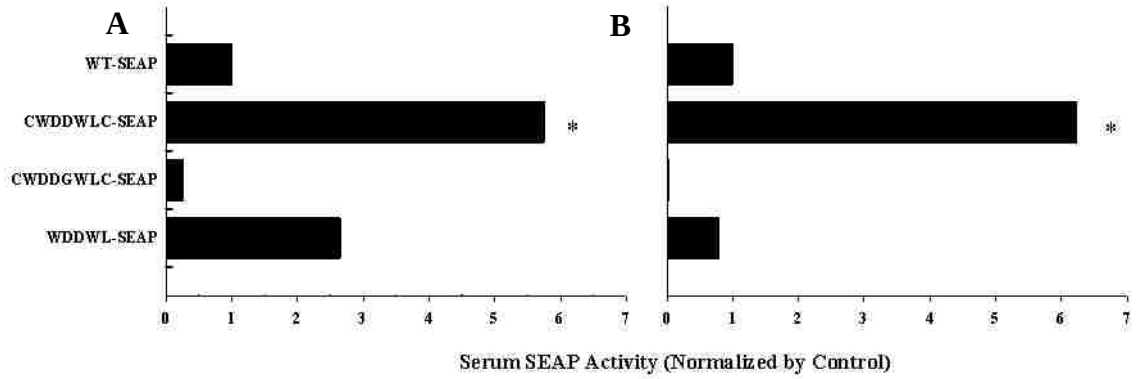


Figure 2.6. Changes in peptide-SEAP activity from *in vivo* transfections. Mice were injected with 5 µg of plasmid DNA into each rear tibialis muscle followed by electroporation, and serum was collected and analyzed for SEAP activity on days 1 (**A**) and 5 (**B**). * represents $p<0.05$ compared to the WT-SEAP activity.

appear to be increased on day 1, but this increase was not statistically significant, and by day 5 it was relatively the same as the WT-SEAP activity. Likewise, the CWDDGWLC-SEAP activity, while still significantly lower than the WT-SEAP, was only about 4-fold less than the WT-SEAP as compared to 10-fold less as seen in the *in vitro* data; however, by day 5 the CWDDGWLC-

SEAP activity was undetectable in the serum. A similar experiment performed using nude mice treated with either CWDDWLC-SEAP or WT-SEAP plasmid DNA revealed similar results with differences in SEAP activity in the serum (data not shown).

To determine if the changes in activity result from differences in the concentration of the SEAP protein, serum samples from each group on days 1 and 5 were analyzed via western blot using an antibody that is specific for the SEAP protein and does not bind endogenous mouse alkaline phosphatases. Astonishingly, the western blot revealed that there is no correlation between the changes in activity from the peptide-SEAP constructs and actual levels of the SEAP protein on any of the days tested (**Fig. 2.7**). Even on day 5 when there was an almost undetectable level of CWDDGWLC-SEAP activity, there is no difference among the amounts of SEAP protein present in any of the groups.

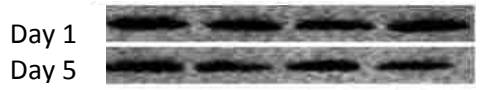


Figure 2.7. Serum SEAP activity levels do not correspond to changes in the amount of SEAP protein present in the serum. The same serum samples assayed in Figure 6 were analyzed via western blot, and there were no differences in the amount of the SEAP protein present for WT-SEAP (**A**), CWDDWLC-SEAP (**B**), CWDDGWLC-SEAP (**C**), and WDDWL-SEAP (**D**).

Discussion

For the first time we show the dramatic effects that small peptides can have on the activity of SEAP due to integration of mini-coding-peptide sequences into the SEAP coding sequence in the same open reading frame. These peptides were believed to have tumor- and tissue-targeting abilities [103], but the majority failed to show any targeting abilities in experiments performed in our lab (unpublished data). This result does not mean that these peptides do not have such tumor-targeted property, but instead it suggests that the peptides have to be meticulously used for the purpose of tumor targeting. Genetically engineering these peptides with this reporter gene seems to not be the best approach to exhibit the tumor-targeted

property of these peptides, but when some of these peptides are fused with other viral vector genes, tumor-tropism was improved [92, 106, 107].

Interestingly, we found that the peptides had extreme effects on the activity of SEAP reporter gene to which they were fused in the same open reading frame (**Figs. 2.2-2.5**). Several methods are currently employed to increase the levels of gene products from the *in vitro* and *in vivo* delivery of DNA. Increasing the level of gene delivery is one area of focus for increasing the products of gene therapy. To accomplish this feat, several different modalities are used including the modification of viral vectors to elongate circulation times, reduce immunogenicity, and increase the accumulation of viral particles at target areas [89, 91, 92]. Also, the level of gene transfection with plasmid DNA can be increased by using several different techniques such as gene-gun, electroporation, ultrasound, and several others [90]. Likewise, increasing the level and duration of transgene expression can be accomplished by other specific methods: intuitive promoter selection [82, 93-96], decreased unmethylated CpG levels [80, 97], optimized codon use [98, 99], and decreased size of transfected DNA [100]. Another manner is to increase the activity per molecule of gene products which has been accomplished via DNA shuffling for green fluorescent protein [108]. DNA shuffling and the new method reported here for increasing the activity of gene products has the potential to work in concert with these other widely used methods to further increase the efficacy of gene therapy. This concept should be tested in the future since it may improve the sensitivity of this reporter gene.

The increase in activity by insertion of the discovered peptide occurs in a gene-specific manner because the same peptide that enhances SEAP activity only slightly increases the expression of MHCI, a hallmark indicator of IFNa biological activity [109], by CWDDWLC-IFNα compared to wild-type IFNa (data not shown). The increase was neither statistically

significant nor therapeutically relevant. So, the large increase in activity seen in SEAP by the CWDDWLC peptide is not universal; however, other peptides may increase the biological activity of different therapeutic proteins. Screening several different peptides for individual proteins is necessary to discover the most effective peptide-protein combinations, which is the central concept for this MS. This concept is also different from the DNA shuffling technique in that the recombinant protein from DNA shuffling is generated through using variants of the same gene from the same and/or different species [110] while our concept is to insert a peptide at the N or C terminal to increase the protein function.

One possible explanation for these dramatic differences would be that the peptides are interacting with cell surface ligands. Several of these peptides were thought to be targeted to the upregulation of certain proteins on the surface of cells during neovascularization due to tumor development [78, 79, 111-113]. Another explanation could be that the peptides somehow affected the secretion signal in the SEAP protein so the SEAP remained in the cell. However, these differences were not a result of peptide interactions with cell surface motifs or a loss of a secretion signal because the cell lysates did not show any significant differences in SEAP activity between the WT-SEAP and any peptide-SEAP groups (**Fig. 2.2B**). Also, this change in activity was not dependent on the type of cell because the same differences were seen when murine muscle cells (C2C12) and adenocarcinoma cells (4T1) were transfected with the same SEAP constructs (**Fig. 2.3A**,**B**, respectively). Likewise, this effect is also not restricted to murine cells because the same effects are evident in human immune (Jurkat), colon tumor (HT29), and kidney tumor (HEK293) cell lines (**Fig. 2.4**). The varying activity levels of SEAP seen in the different cell types can be attributed to the cell-dependent activity of the CMV promoter. While CMV is a broadly used promoter for different cell lines and animal models, it is not always the

best choice for all cell types [82, 93, 95, 96], and modulation of the promoter based on the tissue to be transfected could lead to higher expression levels which will further increase the level of activity of the CWDDWLC-SEAP. So, the effects that the peptides have on the SEAP activity levels are not restricted to a certain cell type nor are they specific to a certain species. These facts are very important for the translation of the use of these highly sensitive reporter genes into other models such as large animals.

The sequence of the CWDDWLC peptide is very important for the increased activity effect to occur. Modification of the sequence by introducing a Gly residue between the second Asp and Trp residues rendered the resulting enzyme almost inactive (**Fig. 2.4**). Further modification of the sequence by removing the flanking Cys residues also had serious consequences to the activity of the resulting SEAP enzyme. Interestingly, the loss of the Cys residues reduced the activity of the SEAP to almost identical the activity of the WT-SEAP enzyme (**Fig. 2.5**). Interestingly, peptides with flanking Cys residues typically form loop secondary structures resulting from a disulfide bond between the two residues [114]. So, one possible explanation for the loss of the increase in activity could be due to the loss of the loop structure.

Another important aspect of this phenomenon is that it also occurs *in vivo*. As seen by measuring the SEAP activity in the serum of mice treated with the WT-SEAP, CWDDWLC-SEAP, and both modified peptide-SEAP constructs, the increase in CWDDWLC-SEAP activity as compared to the WT-SEAP continues for at least 5 days in both Balb/c (**Fig. 2.6**) and nude mice. By days 10 and 15, the SEAP activity in all groups is reduced to insignificant levels (data not shown), which is most likely the result of an immune response against SEAP which is an enzyme of human origin. Amazingly the level of SEAP protein present in the serum of these

mice is the same regardless of the activity of the different SEAP enzymes (**Fig. 2.7**). So, the differences in the detectable levels of the SEAP enzymes are due to changes in the activity of the enzymes and not differences in the amounts of SEAP produced.

One of the main restraints which continues to hinder gene therapy is the level of gene expression, and several methods attempt to break these chains that bind gene therapy. There continues to be progress in this field, and the simultaneous use of multiple methods to increase the efficacy of gene therapy is important for gene therapy to become a major element in biomedical research and therapeutics. The use of peptides to increase the activity of proteins to which they are conjugated has the potential to be another method to synergistically increase the efficacy of gene therapy.

References

75. Meng Y, *et al.* (2005). Continuous, noninvasive monitoring of local microscopic inflammation using a genetically engineered cell-based biosensor. *Lab Invest* **85**: 1429-1439.

76. Li L, *et al.* (2008). Functional imaging of interleukin 1 beta expression in inflammatory process using bioluminescence imaging in transgenic mice. *BMC Immunol* **9**: 49.

77. Hauck ES, Zou S, Scarfo K, Nantz MH, Hecker JG (2008). Whole Animal In Vivo Imaging After Transient, Nonviral Gene delivery to the Rat Central Nervous System. *Mol Ther*.

78. Balza E, *et al.* (2006). Targeted Delivery of Tumor Necrosis Factor-alpha to Tumor Vessels Induces a Therapeutic T Cell-Mediated Immune Response that Protects the Host Against Syngeneic Tumors of Different Histologic Origin. *Clinical Cancer Research* **8**: 2575-2582.

79. Enback J, Laakkonen P (2007). Tumour-homing peptides: tools for targeting, imaging and destruction. *Biochem Soc Trans* **35**: 780-783.

80. Kawano H, *et al.* (2007). Improved anti-cancer effect of interferon gene transfer by sustained expression using CpG-reduced plasmid DNA. *Int J Cancer* **121**: 401-406.

81. Li CY, Huang Q, Kung HF (2005). Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol* **2**: 81-91.

82. Nathwani AC, Davidoff A, Hanawa H, Zhou J-F, Vanin EF, Nienhuis AW (2001). Factors influencing in vivo transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. *Blood* **97**: 1258-1265.

83. Nienhuis AW (2008). Development of gene therapy for blood disorders. *Blood* **111**: 4431-4444.

84. Meager A (2002). Biological assays for interferons. *J Immunol Methods* **261**: 21-36.

85. Abdel-Razaq W, Bates TE, Kendall DA (2007). The effects of antidepressants on cyclic AMP-response element-driven gene transcription in a model cell system. *Biochem Pharmacol* **73**: 1995-2003.

86. Badr CE, Hewett JW, Breakefield XO, Tannous BA (2007). A highly sensitive assay for monitoring the secretory pathway and ER stress. *PLoS ONE* **2**: e571.

87. Hiramatsu N, Kasai A, Hayakawa K, Yao J, Kitamura M (2006). Real-time detection and continuous monitoring of ER stress in vitro and in vivo by ES-TRAP: evidence for systemic, transient ER stress during endotoxemia. *Nucl Acids Res* **34**: e93-.

88. Hiramatsu N, Kasai A, Meng Y, Hayakawa K, Yao J, Kitamura M (2005). Alkaline phosphatase vs luciferase as secreted reporter molecules in vivo. *Anal Biochem* **339**: 249-256.

89. Eto Y, Yoshioka Y, Mukai Y, Okada N, Nakagawa S (2008). Development of PEGylated adenovirus vector with targeting ligand. *International Journal of Pharmaceutics* **354**: 3-8.

90. Gao X, Kim KS, Liu D (2007). Nonviral gene delivery: what we know and what is next. *AAPS J* **9**: E92-104.

91. Mahasreshti PJ, *et al.* (2006). Ovarian cancer targeted adenoviral-mediated mda-7/IL-24 gene therapy. *Gynecol Oncol* **100**: 521-532.

92. Okada Y, *et al.* (2004). Optimization of antitumor efficacy and safety of in vivo cytokine gene therapy using RGD fiber-mutant adenovirus vector for preexisting murine melanoma. *Biochim Biophys Acta* **1670**: 172-180.

93. Yew NS (2005). Controlling the kinetics of transgene expression by plasmid design. *Advanced Drug Delivery Reviews* **57**: 769-780.

94. Fabre EE, Bigey P, Orsini C, Scherman D (2006). Comparison of promoter region constructs for in vivo intramuscular expression. *J Gene Med* **8**: 636-645.

95. Edelman GM, Meech R, Owens GC, Jones FS (2000). Synthetic promoter elements obtained by nucleotide sequence variation and selection for activity. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 3038-3043.

96. Li X, Eastman EM, Schwartz RJ, Draghia-Akli R (1999). Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nat Biotechnol* **17**: 241-245.

97. Yew NS, *et al.* (2002). CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. *Mol Ther* **5**: 731-738.

98. Angov E, Hillier CJ, Kincaid RL, Lyon JA (2008). Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host. *PLoS ONE* **3**: e2189.

99. Burgess-Brown NA, Sharma S, Sobott F, Loenarz C, Oppermann U, Gileadi O (2008). Codon optimization can improve expression of human genes in Escherichia coli: A multi-gene study. *Protein Expression and Purification* **59**: 94-102.

100. Hirata K, Nishikawa M, Kobayashi N, Takahashi Y, Takakura Y (2007). Design of PCR-amplified DNA fragments for in vivo gene delivery: size-dependency on stability and transgene expression. *J Pharm Sci* **96**: 2251-2261.

101. Craig R, Cutrera J, Zhu S, Xia X, Lee YH, Li S (2008). Administering plasmid DNA encoding tumor vessel-anchored IFN-alpha for localizing gene product within or into tumors. *Mol Ther* **16**: 901-906.

102. Li S, Zhang X, Xia X (2002). Regression of Tumor Growth and Induction of Long-Term Antitumor Memory by Interleukin 12 Electro-Gene Therapy. *Journal of the National Cancer Institute* **94**: 762-768.

103. Craig R, Li S (2006). Function and Molecular Mechanism of Tumor-Targeted Peptides for Delivering Therapeutic Genes and Chemical Drugs. *Mini-Reviews in Medicinal Chemistry* **6**: 109-120.

104. Pasqualini R, Ruoslahti E (1996). Organ targeting in vivo using phage display peptide libraries. *Nature* **380**: 364-366.

105. Verhaegent M, Christopoulos TK (2002). Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization. *Anal Chem* **74**: 4378-4385.

106. Lamfers MLM, *et al.* (2002). Potential of the Conditionally Replicative Adenovirus Ad5-{Delta}24RGD in the Treatment of Malignant Gliomas and Its Enhanced Effect with Radiotherapy. *Cancer Res* **62**: 5736-5742.

107. Witlox AM, *et al.* (2004). Conditionally Replicative Adenovirus with Tropism Expanded towards Integrins Inhibits Osteosarcoma Tumor Growth in Vitro and in Vivo. *Clin Cancer Res* **10**: 61-67.

108. Crameri A, Whitehorn EA, Tate E, Stemmer WP (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat Biotechnol* **14**: 315-319.

109. Brassard DL, Grace MJ, Bordens RW (2002). Interferon-alpha as an immunotherapeutic protein. *J Leukoc Biol* **71**: 565-581.

110. Minshull J, Stemmer WP (1999). Protein evolution by molecular breeding. *Curr Opin Chem Biol* **3**: 284-290.

111. Arap W, Pasqualini R, Ruoslahti E (1998). Cancer Treatment by Targeted Drug Delivery to Tumor Vasculature in a Mouse Model. *Science Magazine* **279**: 377-380.

112. Bazan-Peregrino M, Seymour L, Harris A (2007). Gene therapy targeting to tumor endothelium. *Cancer Gene Therapy* **14**: 117-127.

113. Work LM, *et al.* (2006). Vascular Bed-Targeted *in Vivo* Gene Delivery Using Tropism-Modified Adeno-associated Viruses. *Molecular Therapy* **13**: 638-693.

114. Colombo G, *et al.* (2002). Structure-activity relationships of linear and cyclic peptides containing the NGR tumor-homing motif. *J Biol Chem* **277**: 47891-47897.

Chapter 3

Discovery of a Linear Peptide for Improving Tumor Targeting of Gene Products and Treatment of Distal Tumors by IL12 Gene Therapy

Introduction

Interleukin 12 (IL12), discovered by Giorgio Trinchieri in 1989 [115], bridges the innate and adaptive immune responses by inducing IFNγ production primarily from natural killer and T cells. Cancer therapy with IL-12 exploits its natural immune functions to polarize T cells to the T_h1 phenotype, boost effector T cells, downregulate angiogenesis, remodel the extracellular matrix, and alter the levels of immune suppressive cytokines [116]. Due to these activities, IL12 is one of the most promising cytokines for immunomodulatory cancer therapy.

The initial clinical trials with IL12 resulted in grave toxicities including deaths, which severely downgraded the reputation and potential application of this effective cytokine. In reality, most anticancer drugs or biological modalities are associated with systemic toxicity. It is imperative to solve this problem for effectively and safely treating the extremely high numbers of cancer patients [116].

A popular strategy for sequestering the effects of cytokine therapies in the tumor environment is targeting cellular markers that are upregulated exclusively in the tumor cells or the tumor microenvironment. Indeed, conjugating IL12 to tumor-specific antibodies, such as L19 [117] and HER2 [118], and tumor vasculature-specific peptides, such as RGD [119] and CNGRC [120], improves the efficacy of treatments; however, frequent administrations of recombinant cytokines are needed which increase the immunogenicity, toxicity, and cost. A gene therapy approach would reduce these limitations.

Intratumoral IL12 gene therapy is able to eradicate 40% of tumors in an SCCVII model while systemic delivery via intramuscular administration fails to eradicate any tumors [121]; however, direct injection into tumor sites is rarely noninvasively or post-surgically available. Several methods have been developed to target the IL12 effect to the tumor after systemic

delivery. For example, modifying viral vectors with tissue specific gene promoters such as the CALC-I promoter [122], capsid-expressed tumor-specific peptides [123], and polyethylene glycol or other nanoparticles [124, 125] increases tumor specific expression and decreases systemic expression; however, the fenestrated vasculature of the tumor environment allows for the gene products to leak out of the tumor environment leading to systemic toxicities [126]. So, a gene product that can interact with and remain in the tumor environment will increase the level of therapeutic efficacy and decrease systemic toxicity.

To this end, we used an *in vivo* reporter gene mediated screening strategy [127] to identify a new tumor targeting peptide, VNTANST [128]. A DNA fragment encoding VNTANST was inserted directly before the stop codon of the IL-12 encoding sequence in plasmid DNA. Transfection of this plasmid DNA via intramuscular (i.m.) electroporation (EP) into muscle tissue distal from the tumor site inhibited tumor growth and extended survival in multiple tumor models and two mouse strains and reduced lung metastasis in a spontaneous metastatic model. Due to this broad targeting nature and to simplify the description, the peptide VNTANST was renamed the Comprehensive Carcinoma Homing Peptide (CHP). Also, we identified the receptor for CHP as vimentin, which is upregulated in several tumor types. Vimentin expression in tumors is associated with the epithelial to mesenchymal transition (EMT) and increased malignancy and metastasis in tumors [129-133]. Lastly, this gene product-targeted approach minimized the risk of IL12-induced toxicity.

Materials

Plasmid DNA Preparation. All SEAP gene constructs were generated via direct PCR as previously described [127]. The wild type IL12 gene construct (wtIL12) was obtained from Valentis, Inc. (San Francisco, CA) [134], and gene sequences encoding the peptide sequences

were inserted directly prior to the stop codon of the IL12 p40 subunit encoding region using the primer sequences listed in Table 3.1. The IL12 plasmid includes both the p35 and p40 subunits. The control plasmid DNA (Control) consisted of a deletion of the IL12 gene from the IL12 construct. All plasmid DNAs were manufactured with the Qiagen EndoFree plasmid preparation kit (Alameda, CA).

Table 3.1 Primers for PCR.

SEAP Constructs		
Peptide	Forward Primer 5'-3'	Reverse Primer 5'-3'
CGFELETC	CCAGGATCCTAAAAGGGCAG	TTATCACTCGAGGCAAGTCTCTAGCTCGAATCCACATG TCTGCTCGAAGCGGCC
NGYEIEWYSWVTHGM Y	CCAGGATCCTAAAAGGGCAG	TTATCAGTACATACCGTGAGTAACCCAGGAGTACCAC TCGATCTCGTAACCGTTTGTCTGCTCGAAGCGGCCGG
TAASGVRSMH	CCAGGATCCTAAAAGGGCAG	TTATCAATGCATACTACGGACACCACTAGCAGCAGTT GTCTGCTCGAAGCGGCCGG
ATWLPPA	CCAGGATCCTAAAAGGGCAG	TTA TCAAGCTGGA GGGAGCCACG TAGCTGTCTG CTCGAAGCGG CCGG
CNGRC	CCAGGATCCTAAAAGGGCAG	TTATCAACAACGACCGTTACATGTCTGCTCGAAGCGG CCGG
HTMYYHHYQHHL	CCAGGATCCTAAAAGGGCAG	TTATCAAAGGTGATGCTGATAGTGATGGTAATACATA GTGTGTGTCTGCTCGAAGCGGCCGG
GSL	CCAGGATCCTAAAAGGGCAG	TCGTCTAGATTATCACAGACTTCCACCCGGGTGCGCG GCGTCG
NSSRGLG	CCAGGATCCTAAAAGGGCAG	TTATCAACCGAGATCCCTACTGCTGTTTGTCTGCTCGA AGCGGCC
RGD4C	CCAGGATCCTAAAAGGGCAG	TTATCAGCAGAAACAATCACCGCGGCAATCACA

IL12 Constructs		
Peptide	Forward Primer 5'-3'	Reverse Primer 5'-3'
VNTANST	GTCGACCCCGCCCAAGAAC TTGCAG	ACTAGTTTATCAAAGCTTTGTCGAGTTAGCCGTGTTGAC GGATCGGACCCTGCAGGGA
CDGRC	GTTCGAATCTGCGATGGAA GATGCCAGCGCAAGAAAGA AAAG	GAACAAAAGCTGGTACCGG

Cell lines, *In Vitro* Gene Transfer, and IFNγ Induction. CT26, SCCVII, 4T1, EMT6, and B16F10 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA), the AT84 cell line was a generous gift from Dr. Edward Shillitoe (State University of New York Upstate Medical School), and MCF7 cells were provided by Dr. Bolin Liu (University of Colorado Denver School of Medicine). All cell lines were maintained in Dulbecco's modified

Eagle's medium containing 10% fetal bovine serum (DMEM) (Life Technologies, Carlsbad, CA) at 37 °C and 5% CO_2.

For *in vitro* transfections, 4T1 cells were suspended at a concentration of $1x10^7$ cells/mL Opti-mem medium (Life Technologies), and 100 µL of this suspension were transferred to individual electroporation cuvettes and 2 µg of Control, wtIL12, CDGRC-IL12, or CHP-IL12 plasmid DNA was added (n=3). Each cuvette was pulsed with one 75-ms pulse of 150 V, and the suspensions were transferred to individual wells of a 6-well plate containing 900 µL DMEM. The next day, 900 µL of medium was collected, placed on ice, and analyzed for the presence of IL12p70 using an IL12p70 ELISA (eBiosciences, San Diego, CA) as per the manufacturer's instructions. The spleen from a naïve Balb/c mouse was placed in serum-free RPMI-1640 containing Pen/Strep/Glu (RPMI), splenocytes were filtered through a 70 µm cell strainer, and suspended in 10 mL RPMI. After the cell suspension was centrifuged for 10 min at 1,000 rpm, the supernatant was removed, cells resuspended in 10 mL RBC lysis solution, centrifuged again, and then resuspended in RPMI at a concentration of $2x10^6$ cells per 100 µL. $2x10^6$ cells were placed into wells of a 6-well plate. Condition medium from the plasmid DNA-transfected cells containing 150 pg/mL IL12 was transferred to these wells and the volume was adjusted to 1 mL with DMEM. The next day, the mediums were collected and assayed for the presence of IFNγ using an IFNγ ELISA (eBiosciences) as per the manufacturer's instructions.

Animal Models, Tumor Inoculations, *In Vivo* Gene Transfer, Protein Extraction, and Therapeutic Analyses. All animals used in this study were maintained under and animal protocols were performed following National Institutes of Health guidelines, approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University. Balb/c mice were obtained from the in-house breeding colony, and C3H, Nude, and $wtIL12^{-/-}$ mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were six to eight weeks

old upon initiation of experiments. Tumor models were initiated via subcutaneous inoculations of 30 µL cell suspensions containing $1x10^5$ 4T1 cells or $2x10^5$ cells for all other cell lines in 1x PBS. Orthotopic EMT6 tumors were initiated by inoculating $1x10^5$ cells in the mammary fatpads of female Balb/c mice.

For *in vivo* i.m gene transfections, plasmid DNA was diluted in 0.45% NaCl to a concentration of 5 µg/30 µL was injected into each rear tibialis muscle, and the muscles were immediately subjected to EP as previously described [135]. When 4T1 tumors were 3 to 4 mm in diameter or all other tumor models were 4.0 to 4.5 mm in diameter, the first treatment was performed, and a second identical treatment was performed 10 days later. Tumor volumes were determined as previously described [127]. To determine the distribution of the fusion gene products peptide-SEAP and peptide-IL12, the treatments were performed when tumors reached 6-7 mm in diameter; 72 h after treatment, mice were sacrificed via CO_2 asphyxiation, and then tissues were collected, wrapped in foil, and flash-frozen in liquid nitrogen (LN). To extract proteins, the frozen tissues were smashed with a hammer, placed in 1x lysis buffer (Promega, Madison, WI), beaten for 1 minute with a mini-beadbeater 8 (Biospec, Bartlesville, OK), and spun at 16,000 x *g* for 5 min. The supernatant was transferred to a new tube. Serum was collected by extracting blood from the left ventricle, transferring it to Serum Separator Tubes (BD, Franklin Lakes, NJ), and spinning at 5,000 x g for 5 min. The serum was then transferred to a new 1.5 mL tube.

India ink inflation was performed to determine the level of lung metastasis. After CO_2 asphyxiation, the thoracic cavity was opened, the trachea exposed, and the trachea clipped with a hemostat. 1.5 mL 15% India ink was injected into the lung which was then transferred into 20

mL Fekete's solution and incubated overnight. The next day, white metastatic nodules were counted using a dissecting microscope.

For FACS analyses, tumor infiltrating cells were isolated by extracting the tumors, cutting them into pieces, and resuspending the mixture in sterile PBS (without Ca^{2+} and Mg^{2+}) containing a mixture of collagenase IV, hyaluronidase V (Sigma-Aldrich, St. Louis, MO), and DNase II (Fisher, Pittsburgh, PA). The tissue suspension was placed in a shaker at 37 ºC for 1-2 hours, and then poured through a 70 µm cell strainer, followed by washing twice in PBS with Ca^{2+} and Mg^{2+}. The isolated cells were stained with the FITC conjugated anti-CD11c (AbD Serotec, Raleigh, NC) and goat anti-mouse CD80 (R&D, Minneapolis, MN) for 30 min at 4˚C , washed with PBS, and then stained with PE conjugated anti-goat IgG (Cedarlane Laboratories, Burlington, NC) . The expression of the proteins was analyzed on FACS Calibur (BD Biosciences, San Jose, CA) and analyzed with FCS Express 3 (De Novo Software, Los Angeles, CA). Splenocytes were also isolated from Balb/c mice bearing orthotopic EMT6 tumors, and a CTL assay was performed as described previously [127]. Serum was collected from 4T1-tumor bearing mice 3 days after treatments with Control, wtIL12, and CHP-IL12 plasmid DNA as described above. The serum was analyzed for the presence of IFNγ as described above.

Peptide-biotin Distribution, Vimentin Depletion, and Tissue Staining. CHP-biotin was synthesized by United Biochemical Research, Inc. (Seattle, WA) at >95% purity, resuspended in H_2O with 5% glycerol, and stored at -80 °C. The peptide sequence is NH_2-VNTANSTGG-biotin. Control-biotin was created by conjugating a non-specific peptide (CTSTSPLPPPSHSTSKKG, Alpha Diagnostics, San Antonio, TX) to EZ-Link Amine-PEG2-Biotin via 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) cross-linking (Pierce, Rockford, IL) following the manufacturer's instructions. The peptide-biotin conjugates (10 µg/100 µL normal saline) were

injected into the tail vein of C3H mice bearing SCCVII tumors with 6-7 mm diameters. For vimentin depletion studies, goat polyclonal anti-vimentin (Millipore, Billerica, MA) was purified via protein-G antibody purification (Pierce), and 150 µg was added to the peptide-biotin conjugate solutions for administration. One hour after i.v. administration, mice were perfused via injection of 10 mL 1x PBS into the left ventricle after cutting the right atrium. The tissues were immediately removed, wrapped in foil, and flash frozen in LN. Four- to five-micron sections were placed on poly-L-lysine coated slides.

The sections were fixed in ice-cold acetone, non-specific interactions were blocked with 1% BSA in PBS, and endogenous peroxidase activity was suppressed with Stable peroxidase suppressor (Pierce). Since the peptides already contained biotin, the peptides were incubated with the avidin-HRP reagent Vectastain ABC (Vector Biolabs, Philadelphia, PA) for 45 min, washed in PBS, and then incubated with 1x Metal Enhanced DAB (Pierce). The sections were then counter-stained with Meyer's hematoxylin (blue) for 2 min or eosin (pink) for 20 s.

Isolation of Cell Surface Proteins, Identification of CHP Receptor, and Western Blot. Cell surface proteins were isolated from SCCVII cells using the Cell Surface Protein Isolation Kit (Pierce) and following the manufacturer's instructions. A streptavidin agarose column (Peirce) was loaded with CHP-biotin (3 mg/mL in PBS for 10 min), and the cell surface protein suspension was incubated on the column overnight at RT. Next, five fractions were eluted with 8 M Guanidine-HCl, pH 1.5, and then 1 M Na_2HPO_4 was added to the fractions at a 1:10 ratio. From these fractions, volumes containing 40 µg of protein were mixed with 2x SDS loading buffer and added to wells of a 12% polyacrylamide gel and an electric field was applied. The gel was then incubated with Coomassie Brilliant Blue R250 followed by destaining solution (10%

Acetic Acid and 20% MeOH). Images were captured with a VersaDoc Model 1000 and Quantity One Version 4.4.1 software (BioRad, Hercules, CA).

To identify the protein from fraction two, the protein in the gel was extracted using the Trypsin Profile IGD Kit (Sigma) with the ProteoPrep Reduction and Alkylation Kit (Sigma) following the manufacturer's instructions. Liquid chromatography electrospray tandem mass spectrometry (LC MS/MS) was used to analyze the peptide mixture extracted from gel spots. Tryptic digests of gel spots (~6 µl) were diluted with 0.1% formic acid (10 µl) and 10 µl injected by microplate autosampler (Famos, Dionex Corporation, Sunnyvale, CA) onto a 0.3 x 1 mm trapping column (PepMap C18, Dionex Corporation) using a nano LC system equipped with Switchos and Ultimate 2000 pumps (Dionex Corporation), at a flow rate of 10 µl/min. The switchos valve was set on loading position prior to sample loading. After sample loading trapping column was washed with 0.1% formic acid at flow rate of 5ul/min for additional 5 min and then switchos valve was switched to inject position. Peptides were then eluted at 200 nl/min and chromatographed on a 75 µm x 15 cm Biobasic C18 column (Vydac HPLC Columns, Grace Davison, IL), with a gradient of 5-40% acetonitrile over 60 min followed by 80% acetonitrile for 5 min. The eluent was directed into a quadrupole time-of-flight mass spectrometer (Q-Star, Applied Biosystems MDS Sciex) and ionized immediately using electrospray source (Nano spray II, Applied Biosystems MDS Sciex) at high voltage of 2.5 kv with nebulizer gas at level 2. The mass spectrometer was operated in IDA (information dependent acquisition) mode with the three most intense ions in each survey scan subjected to MS/MS analysis using collision energies ranging from 20 eV to 50 eV. MS/MS data obtained from Q-Star was processed for database search using Mascot search engine (Matrix science, UK). A Mascot search was performed using the following parameters: Type of search, MS/MS ion search; Database, nrNCBI; Taxonomy,

all; Enzyme, trypsin; Fixed modification, carbamidomethyl (C); Mass values, monoisotopic; Protein mass, unrestricted; Peptide mass tolerance, ± 0.2 Da; Fragment mass tolerance, ± 0.2 Da; and Maximum miss cleavage, 1. The results and matched peptides from the Mascot search are listed in Appendix A.

A cell-free assay was developed to confirm that vimentin interacts with CHP. Wells of a microtiter plate were coated with 50 µL of 100 mM $NaHCO_3$ (coating buffer) or 5 µg/mL of either vimentin-GST or GST (ProSpec) in coating buffer and incubated at 4°C overnight. After 2 washes with PBS, non-specific binding was blocked by incubating the wells with 100 µL 1x BSA for 2 h at room temperature (RT). After another wash (twice), 100 µL of PBS containing 10 ng CHP-biotin was added to each well (n=6 for each coat), incubated for 2 h at RT, and then washed 4 times with PBS. Avidin-HRP (100 µL, eBiosciences) was added to each well, incubated for 30 min, and the wells were washed 7 times with PBS. Lastly, 100 µL TMB substrate (eBiosciences) was added to each well for 15 min followed by 50 µL Stop solution (eBiosciences), and the absorbance at 450 nm was read using a SpectraCount and PlateReader Version 3.0 software (PerkinElmer, Waltham, MA).

To prepare cells for western blot analysis of cellular expression of vimentin, when SCCVII, CT26, 4T1, and B16F10 cells were 95% confluent in individual wells of 6-well plates, the cells were directly lysed with 60 µL Laemmli sample buffer. For preparation of ex vivo samples, tissues and tumors were processed as described previously. Volumes of the tissue lysates containing 40 µg of protein were mixed with 2x SDS loading buffer. Twenty microliter volumes of the cell lysates or tissue lysates were added to a 12% polyacrylamide gel and subjected to SDS-PAGE and then transferred to a TransBlot Transfer Medium nitrocellulose membrane (Bio-Rad Laboratories). Immunoblotting of the membrane was performed with a

1:100 dilution of polyclonal Goat anti- vimentin (Millipore) and a 1:5,000 dilution of the secondary horseradish peroxidase conjugated rabbit anti-goat IgG. The peroxidase signal was generated with the Western Lightning ECL (PerkinElmer) and visualized with a Kodak Image Station 440CF using the 1D Image Analysis Software v3.6 (PerkinElmer).

Analysis of Toxicity Induced by Gene Therapy Treatments. SCCVII tumors were induced in C3H mice as described above, and allowed to grow to a volume of 300 mm^3. Groups of four mice for each treatment at each time point were treated with either wild-type IL-12 or CHP-IL-12 as described above at a dose of 1 μg, 2 μg, and 10 μg plasmid DNA; a fourth set of mice received 3 treatments of 2 μg. Mice were sacrificed on days 1, 3, and 30 after the second treatment, blood was collected in serum separator tubes, and livers were fixed in 10% neutral-buffered formalin.

Serum chemistry profiles were analyzed by a private GLP-certified diagnostic laboratory (Antech Diagnostics, Memphis, Tennessee). Formalin-fixed tissue was cut-in, embedded in paraffin, and sectioned into 5 μm sections. Sections were mounted on glass slides and stained with hematoxylin and eosin prior to microscopic examination by a pathologist. A liver toxicity scoring system based on the number of characteristic liver lesions (foci of hepatocellular necrosis with Kupffer cell hyperplasia) per 200x field. Sections were scored blindly and recorded for analysis.

Statistical Analyses. All statistical analyses were performed with GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA). One-way ANOVA with Bonferroni's post-hoc test was used to analyze the following data: ratios of Tissue/Serum (T/S) SEAP levels, production of fusion gene products from *in vitro* transfected 4T1 cells, inhibition of metastasis, IFNγ serum levels, and CHP/vimentin interaction. Tumor versus normal tissue distributions of

exogenous IL12 or CHP-IL12 gene products in $IL12^{-/-}$ mice and CTL data were analyzed via one-tailed unpaired T tests. All tumor growth experiments were analyzed via two-way ANOVA plus Bonferroni's post-hoc test. Mantel-Cox tests were used to analyze differences in survival of mice. Liver toxicity was first analyzed using blind pathological scores of the liver tissues, but no differences were seen among time points, so the data was pooled to create a larger sample size and then analyzed with one-sided Fisher's exact tests comparing the number of mice having lesions from CHP-IL12 and wtIL12 fusion plasmid DNA treated mice.

Results

CHP Increases Accumulation of the Fusion Reporter Gene Product and Biotin-CHP Conjugate into Tumors. Several fusion gene constructs were cloned by inserting peptide encoding DNAsequences directly prior to the stop codon in a secreted alkaline phosphatase (SEAP) reporter plasmid DNA (**Fig. 3.1A**) [127]. These peptide-SEAP fusion gene constructs were delivered via i.m. EP of the anterior tibialis muscles in mice bearing tumors located 1 cm craniodorsal of the tail. After 72 h, tumors and serum were collected and analyzed for SEAP distribution. It has been shown that inserting peptides into the SEAP plasmid can alter SEAP activity but not protein production [135]. To compensate for the altered SEAP activity, we used the ratio of the SEAP activity between tumors and serum (T/S SEAP).

CHP, a linear peptide, repeatedly increased the T/S SEAP levels in several tumor models compared to wtSEAP. In Balb/c mice bearing colon carcinomas (CT26), CHP showed the greatest increase in T/S SEAP (orange; **Fig. 3.1B**). To identify the peptides with potential for targeting multiple tumor models, some of these peptides were also tested in other models. In two squamous cell carcinoma models (SCCVII and AT84) in C3H mice, 5- and 7-fold increases in T/S SEAP were seen, respectively, for CHP-SEAP (red and blue, respectively; **Fig. 3.1B**). In a

breast adenocarcinoma model (4T1) in Balb/c mice, T/S SEAP was increased 15-fold for CHP-SEAP compared to wtSEAP (green; **Fig. 3.1B**). Importantly, the gene product targeting property of CHP was also confirmed in a xenogeneic human breast cancer model (MCF7) with a 4-fold

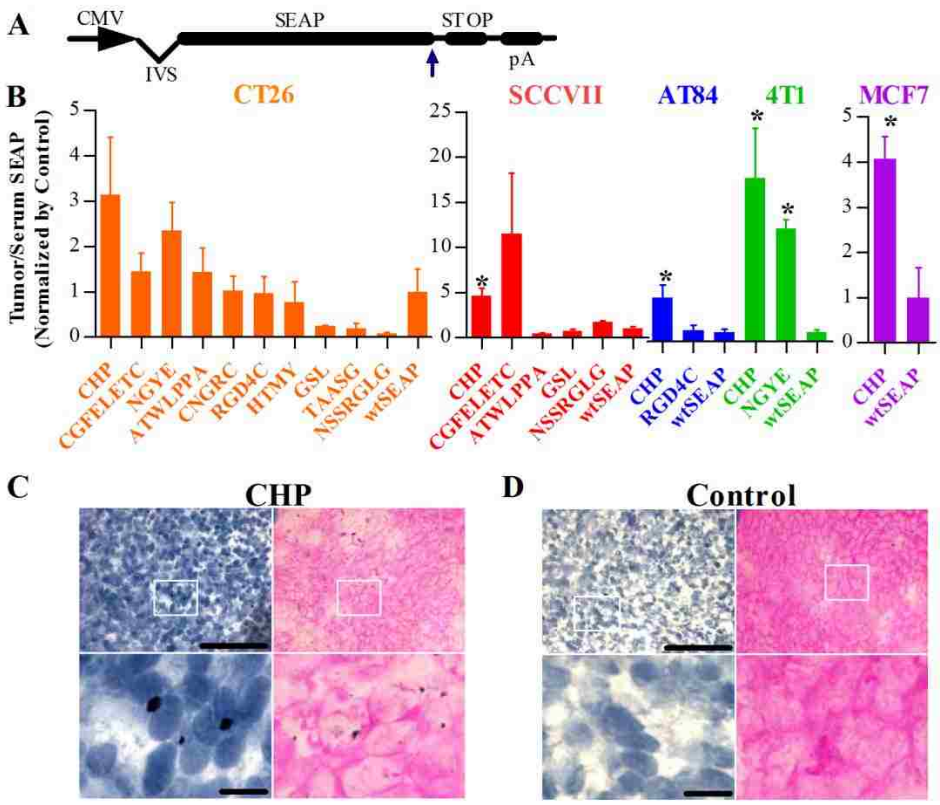


Figure 3.1. Accumulation of peptide-SEAP reporter gene products and CHP-biotin in tumors. (**A**) The peptide-SEAP constructs with insertion of the peptide-coding sequence directly before the stop codon (blue arrow). (**B**) T/S SEAP levels 72 h after intramuscular EP of peptide-SEAP plasmid DNA in syngeneic CT26 (orange, n=3), SCCVII (red, n=4), AT84 (blue, n=4), and 4T1 (green, n=4) tumor-bearing mice as well as xenogeneic MCF7 tumor-bearing mice (purple, n=4). Columns represent the ratio of the control-normalized SEAP/protein (pg/mg) in tumor to SEAP (pg/mL) in the serum and error bars represent s.e.m. (* represent $p<0.05$ compared to wtSEAP). (**C** and **D**) DAB staining of tumor tissues from CHP-biotin and Control-peptide-biotin treated mice counterstained with either hematoxylin (blue) or eosin (pink). The bottom images are larger versions of the areas within the white squares. Scale bars represent 100 µm in the top panels and 200 µm in the bottom panels. EP, electroporation; CMV, Cytolmegalovirus promoter; IVS, intron; SEAP, SEAP-coding sequence; STOP. Stop codon; pA, bovine growth hormone polyadenylation signal.

increase in T/S SEAP (purple; **Fig. 3.1B**), which suggests this peptide has potential application for human tumors.

In addition to the quantitative T/S SEAP data above, we were interested in visualizing the CHP distribution in the tumors and throughout the body. To easily detect the localization of this targeted peptide, synthetic CHP-biotin conjugate or a control-peptide-biotin conjugate (Control-biotin) was injected into the tail vein of SCCVII tumor bearing C3H mice. CHP preferentially accumulated deep into the tumor environment, and, as seen in slides counterstained with either hematoxylin (**Fig. 3.1C**, top and bottom left) or eosin (**Fig. 3.1C**, top and bottom right), the CHP-biotin localized in the tumor tissue (19.6 ± 1.3 positive per field, n=5 fields). In contrast, Control-biotin was not able to penetrate deep into the tumor tissues (1.8 ± 0.37 positive per field, n=5 fields, $p<0.0001$ compared to CHP-biotin) (**Fig. 3.1D**). Negligible levels of biotin accumulated in the hearts, lungs, livers, and kidneys of mice treated with either CHP- or Control-biotin; however, similar levels of CHP-biotin and Control-biotin were detected in the spleens (**Appendix B**), most likely due to non-specific uptake by the efficient mononuclear phagocytes bounding splenic red pulp sinuses.

CHP-IL12 Gene Product Maintains Targeting and Biological Functions. Peptide-IL12 fusion gene constructs were generated by inserting the peptide coding sequences directly before the stop codon of the p40 subunit in an IL12 plasmid DNA (**Fig. 3.2A**) [135]. CHP-IL12, CDGRC-IL12, wtIL12, or empty vector plasmid DNA were transfected into 4T1 cells. After 24 h, equivalent levels, ~175 pg/μL, of the IL12p70 heterodimer were detected in the medium of all three IL12 gene plasmid DNA transfected cells, and negligible IL12p70 was detected in the control wells (**Fig. 3.2B**). Transferring the IL12 containing medium to splenocytes induced similar levels of IFNγ, a hallmark of IL12 function (**Fig. 3.2C**) indicating that these fusion IL12

proteins possess the same biological function as wtIL12.The distribution of CHP-IL12 in the tumor, kidney, liver, and serum was determined via IL12p70 ELISA 72 hours after treating CT26 tumor-bearing IL12 knockout Balb/c ($IL12^{-/-}$) mice with the CHP-IL12 and wtIL12 plasmid DNA. The CHP-IL12 protein localized in the tumor environment as seen by the 4-fold increase in T/S IL12 ratio compared to wtIL12 (orange, **Fig. 3.2D**). Likewise, CHP-IL12 increased the Tumor/Kidney and Tumor/Liver IL12 ratios compared to wtIL12 (green and blue, respectively; **Fig. 3.2D**). So, a single copy of the CHP peptide is capable of targeting each IL12 molecule to the tumor microenvironment.

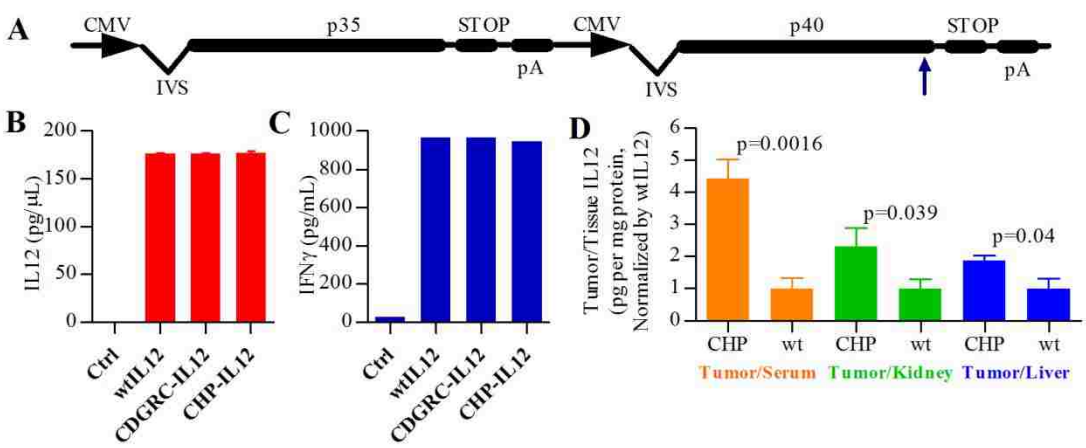


Figure 3.2. Biological activity and targeting ability of peptide-IL12 fusion gene products. (**A**) The peptide-IL12 constructs with insertion of the peptide-coding sequence directly before the stop codon in the p40 coding region (blue arrow). (**B**) Expression of IL12 after *in vitro* transfection of 4T1 cells with control, wtIL12, and peptide-IL12 (n=3) and (**C**) induction of IFNγ from splenocytes after transfer of condition medium containing Control, wtIL-12, or peptide-IL12 gene products. (**D**) IL12 accumulation in tumor-bearing $IL12^{-/-}$ mice treated with CHP-IL12 or wtIL12 determined via an IL12p70 ELISA. Columns represent the wtIL12-normalized level of IL12/protein (pg/mg) in tumor per IL12/protein (pg/mg) in kidneys (green) and livers (blue) and IL12 pg/mL serum (orange, n=4). Error bars represent s.e.m. (* represent $p<0.05$ compared to all groups). CMV, Cytomegalovirus promoter; IVS, intron; SEAP, SEAP-coding sequence; STOP,

CHP-IL12 Fusion Gene Therapy Increases Inhibition of Primary and Metastatic Tumor Growth and Extends Survival. Balb/c mice bearing 4T1 or CT26 tumors and C3H mice bearing SCCVII tumors were treated via i.m. EP with empty (Control), wild type IL-12 (wtIL12), and CHP-IL12 (CHP-IL12) fusion gene plasmid DNA. The treatment was repeated 10 days later. In the highly aggressive syngeneic 4T1 model, CHP-IL12 gene therapy, compared to wtIL12 gene therapy, slowed tumor growth by 33%, while both wtIL12 and CHP-IL12 treated tumors were less voluminous than control DNA treated mice (**Fig. 3.3A**). Likewise, CHP-IL12

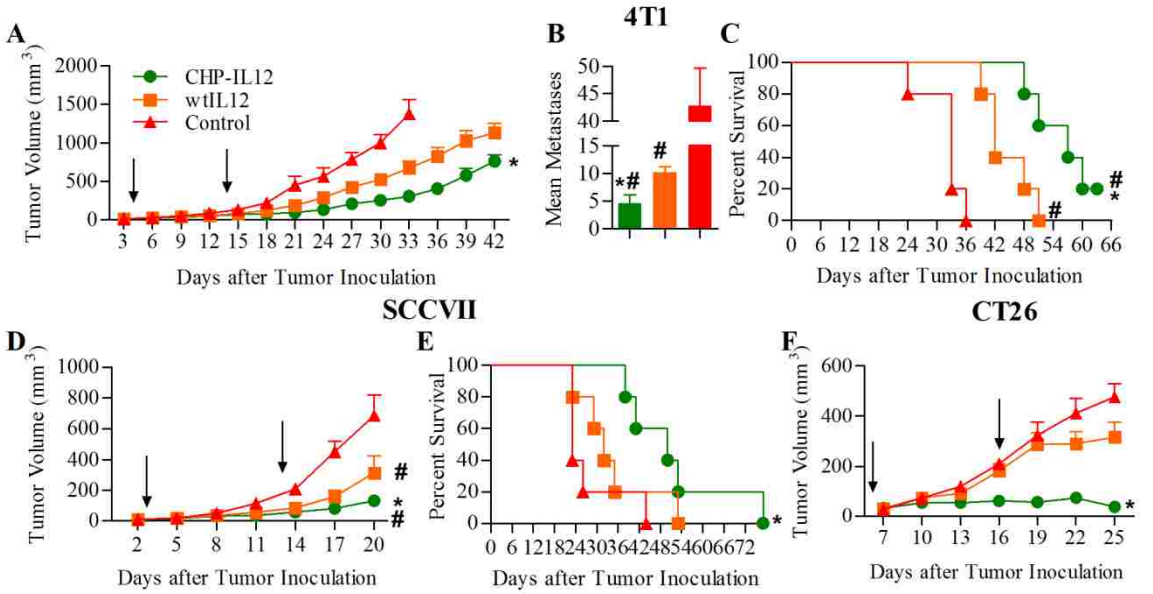


Figure 3.3. Inhibition or regression of primary tumor growth, decrease in metastatic tumor incidence, and increase in survival time by systemic delivery of CHP-IL12 plasmid DNA. (**A**) Tumor growth following treatments with CHP-IL12, wtIL12, and control plasmid DNA in 4T1 tumor-bearing balb/c mice (n=5; * represents $p<0.05$ at day 30 and $p<0.001$ from day 33 until day 42 compared to wtIL12 plasmid DNA and $p<0.01$ at day 21 and $p<0.001$ from day 24 to day 33 compared to control plasmid DNA). (**B**) Metastatic nodules in the lungs of mice (n=5) treated as in **A** and sacrificed 17 days after the second treatment (* represents $p<0.05$ compared to wtIL12 plasmid DNA; # represents $p<0.001$ compared to control plasmid DNA). (**C**) Kaplan-Meier survival analysis of the same mice treated in **A** (* represents $p<0.05$ compared to wtIL12 plasmid DNA; # represents $p<0.001$ compared to control plasmid DNA). (**D**) Tumor growth following treatments as in **A** in SCCVII tumor-bearing C3H mice (n=5; * represents $p<0.05$ on days 17 and 20 compared to wtIL12 plasmid DNA and control plasmid DNA). (**E**) Kaplan-Meier survival analysis of the same mice treated in **D** (* represents $p<0.05$ compared to wtIL12 and control plasmid DNA). (**F**) Treatments as in **A** in CT26 tumor-bearing balb/c mice (n=5; * represents $p<0.05$ compared to wtIL12 plasmid DNA, n=4, on day 25, and control plasmid DNA, n=3, on days 19 through 25). Black arrows represent treatments, and error bars represent s.e.m.

treatments extended survival further than wtIL12 and Control (**Fig. 3.3C**). In the same tumor model and treatment regimen, CHP-IL12 gene therapy reduced by half the number of spontaneous metastatic nodules in the lungs compared to wtIL12 (**Fig. 3.3B**). Similarly, in the SCCVII model CHP-IL12 improved tumor growth inhibition by 50% compared to wtIL12 (**Fig. 3.3D**) and extended survival of mice compared to both Control and wtIL12 (**Fig. 3.3E**).

In a third syngeneic model, CT26, CHP-IL12 treatments inhibited tumor volumes starting only a few days after one treatment, and tumors began to regress after the second treatment (**Fig. 3.3F**). After day 25, tumors in both wtIL12 and CHP-IL12 treated mice began to be eradicated. By day 55, 100% of mice treated with CHP-IL12 were tumor-free while only 75% of wtIL12 treated mice were tumor-free.

The CHP-IL12 treatments increased the immune response to the tumor cells. To understand the mechanism by which CHP-IL12 boosts inhibition of tumor growth as compared to wtIL12, both CTL activity and tumor microenvironment immune cell profiling were analyzed. The rationale is that intratumoral injection, associated with a high level of IL12 in tumors, boosted anti-tumor immune responses as compared to i.m. injection of IL12 plasmid DNA, which is associated with a very low level of IL12 in tumors [121]. Treatment with CHP-IL12 increased the number of the tumor-infiltrating mature dendritic cells (DC) in the tumor environment as determined by FACS for $CD11c^{+}/CD80^{hi}$ expression (**Fig. 3.4A**). Tumors from wtIL12 treated mice contained 76.9% mature DC, an increase from 73.7% in control treated mice. This population in CHP-IL12 treated mice was even higher at 82.3% (**Fig. 3.4A**). In agreement with this increase in mature DC in tumors, tumor-specific CTL activity was increased with CHP-IL12 treated mice compared to wtIL12 treated mice (**Fig. 3.4B**). Furthermore, CHP-IL12 treatments did not cause any further increase in serum IFNγ levels, so these immune

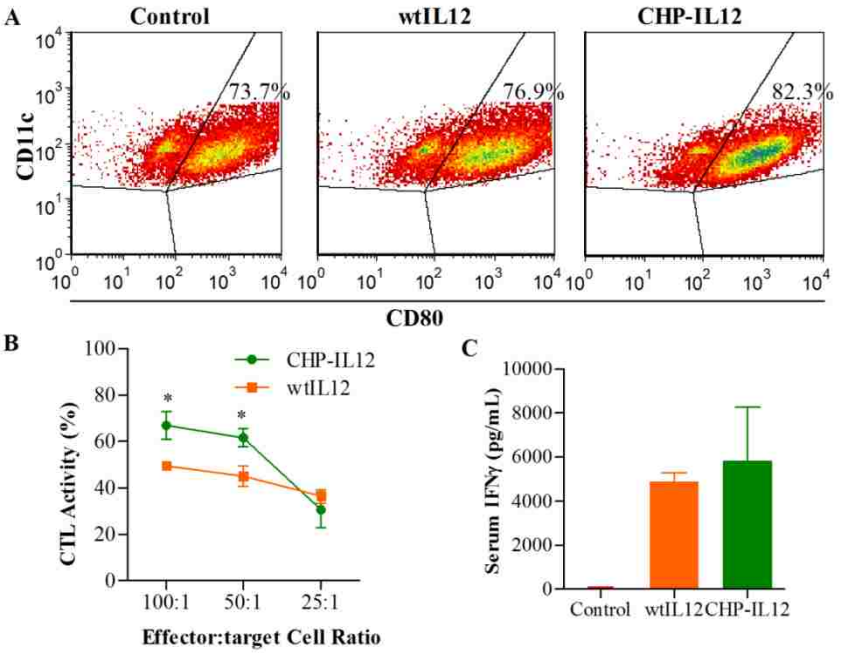


Figure 3.4. Increased antitumor immune response by CHP-IL12 fusion gene treatments. (**a**) FACS analysis of tumor infiltrating cells isolated from SCCVII tumors from C3H mice treated as described in Fig. 5 collected 7 days after the second treatment. The top right quadrant of the colored dot plot representation of cells gated for $CD11c^{+}$ represents activated DC ($CD80^{hi}$). (**b**) Tumor-specific CTL activity from wtIL12 and CHP-IL12 fusion gene plasmid DNA treated mice bearing orthotopic EMT6 tumors collected (* represents $p<0.05$). (**c**) Serum IFNγ levels from 4T1-tumor bearing Balb/c 3 days after treatments with CHP-IL12, wtIL12, and control plasmid. Error bars represent s.e.m.

responses are not the result of widespread IL12 activity (**Fig. 3.4C**). These results suggest that CHP-IL12 improves the anti-tumor immune response of effector cells in the tumor microenvironment.

CHP Homes to Vimentin Expressed in the Tumor Environment. CHP-biotin was used to isolate the CHP receptor from a pool of cell-surface receptor proteins isolated from SCCVII cells. Mass spectrometry of the isolated protein (**Fig. 3.5A**, white arrow) identified this protein as vimentin. To validate this receptor, CHP-biotin was added to wells of a polystyrene plate that were coated with coating buffer only, GST, or recombinant Vimentin-GST. Indeed, CHP

interacts with vimentin as the vimentin-coated wells retained a significantly higher level of HRP activity (**Fig. 3.5B**).

To determine the levels of vimentin expression in normal tissues versus tumors, tissue lysates from an SCCVII tumor, heart, lung, liver, kidney, spleen, and serum from a C3H mouse were probed for vimentin expression via western blot analysis (WB). Very low levels of

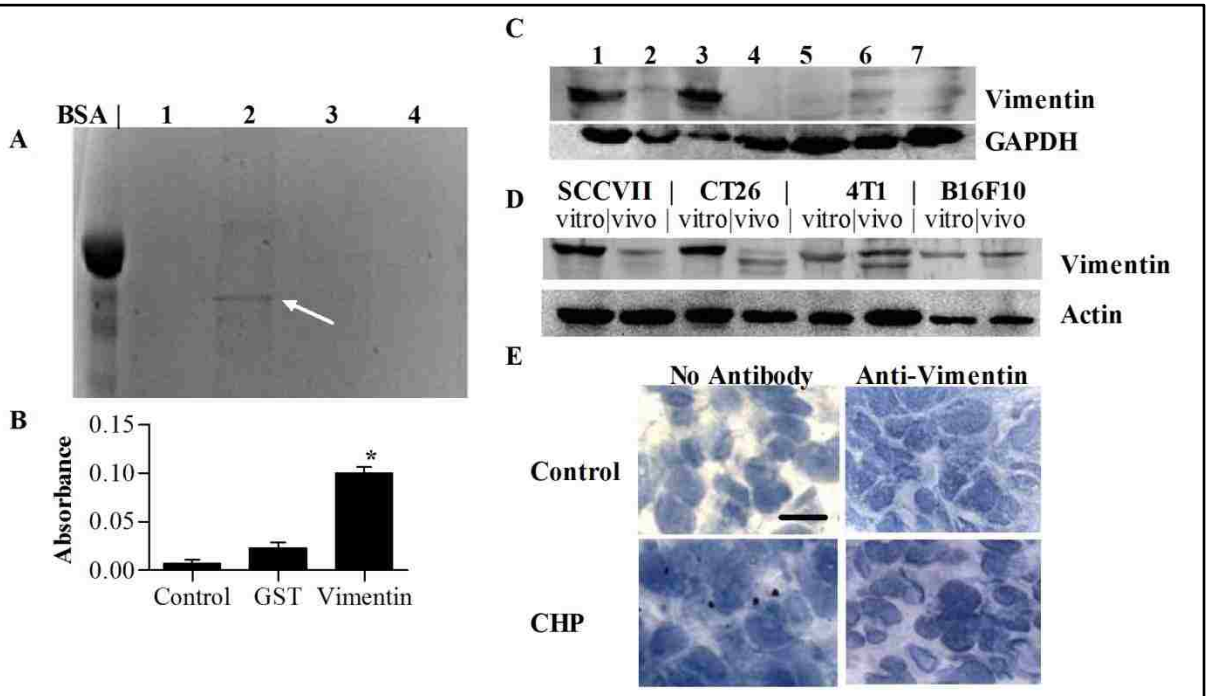


Figure 3.5. Identification of CHP interacting protein: vimentin. (**A**) SDS-PAGE analysis of potential receptors for CHP isolated via affinity chromatography of a pool of cell-surface proteins isolated from SCCVII cells. The only distinct band (white arrow) was located in the second fraction, and mass spectrometry identified this band as vimentin. (**B**) Interaction of CHP-biotin with recombinant vimentin-GST (Vimentin), GST, and coating buffer only (Control) coated wells of a polystyrene plate (n=6; * represents $p<0.001$ compared to both GST and Control, errors bars represent s.e.m.). (**C**) Western blot analysis of vimentin expression in an SCCVII tumor (1) and heart (2), lung (3), liver (4), kidney (5), spleen (6), and serum (7) from SCCVII-tumor bearing C3H mice. (**D**) Western blot analysis of vimentin expression in *in vitro* and *ex vivo* tumor samples. (**E**) Accumulation of peptide-biotin in syngeneic SCCVII tumor bearing C3H mice following i.v. injection of either Control-biotin (top left and right) or CHP-biotin (bottom left and right) without (top and bottom left) or with (top and bottom right) depletion of vimentin with a co-injection of purified polyclonal goat anti-vimentin (100 µg) in the same i.v. injection as the peptide-biotin.

vimentin were detected in the heart, liver, kidney, spleen, and serum (**Fig. 3.5C**, lanes 2 and 4 through 7) while high levels of vimentin were detected in the tumor and lung (**Fig. 3.5C**, lanes 1 and 3). Similarly, analysis of vimentin expression in SCCVII, CT26, 4T1, and B16F10 (melanoma cell line derived from C57Bl/6 mice) tumor cell lines and *ex vivo* tumor tissues shows that all these tumor cell lines and their respective tumor models express vimentin (**Fig. 3.5D**), which explains the universal tumor homing property as illustrated in **Fig. 3.1**.

To determine that vimentin is the receptor protein interacting with CHP which is responsible for tumor homing, we performed tail vein injections of Control-biotin and CHP-biotin with or without blocking vimentin using purified polyclonal goat anti-vimentin in SCCVII tumor-bearing C3H mice. As expected, injection of Control-biotin with or without anti-vimentin did not result in any accumulation of peptide in the tumor (**Fig. 5e**, top left and top right, 1.8 ± 0.37 with anti-vimentin and 2.6 ± 0.51 without anti-vimentin, n=5). However, CHP-biotin did accrue in the tumor environment (**Fig. 5e**, bottom left, 19.6 ± 1.3 positive per field, n=5, $p<0.0001$ compared to all other groups), but co-administration with anti-vimentin almost completely inhibited the tumor targeting ability of CHP (**Fig. 5e**, bottom right, 1.2 ± 0.58 positive per field, n=5).

CHP-IL12 Reduces the Level of Toxic Lesions in the Liver. IL12 induces liver toxicity (unpublished data) and it was our expectation that the tumor-homing CHP-IL12 may reduce the toxicity. To test this hypothesis, SCCVII tumor-bearing C3H mice were treated with two treatments of 1 μg (2 x 1 μg), 2 μg (2 x 2 μg), or 10 μg (2 x 10 μg) or three treatments of 2 μg (3 x 2 μg) of wtIL12 or CHP-IL12, and mice were sacrificed on days 1, 3, and 30 after the final treatment. At low levels of plasmid DNA administration, 2 x 1 μg and 2 x 2 μg, there were no differences between wtIL-12 and CHP-IL12 treatments; however, at the therapeutic level, 2 x 10

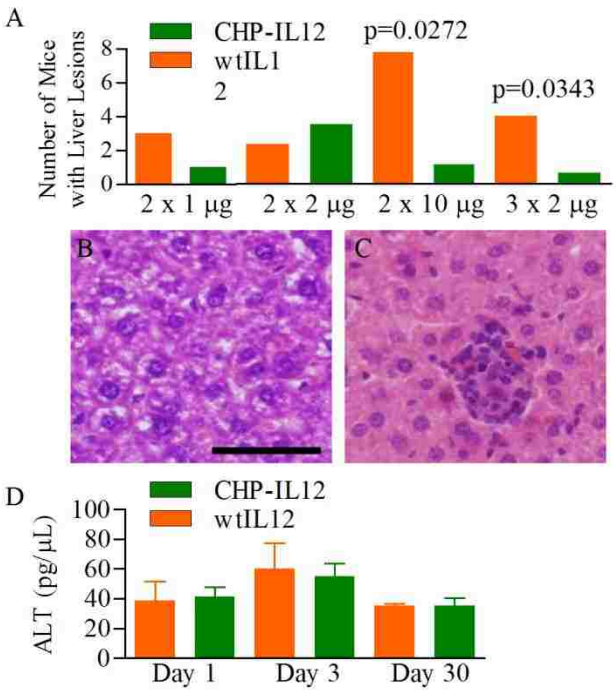


Figure 3.6. Decreased liver toxicity of IL12 treatments with CHP-IL12. (**A**) Number of SCCVII tumor-bearing C3H mice with toxic lesions on the liver following two treatments of 1 µg (2 x 1 µg), 2 µg (2 x 2 µg), or 10 µg (2 x 10 µg) or three treatments of 2 µg (3 x 2 µg) of wtIL12 or CHP-IL12 (n=12). (**B**) Representative image of a normal liver area. (**C**) Representative image of a toxic lesion. Scale bar represents 50 µm in **B** and **C**. (**D**) Levels of Alanine Transaminase (ALT), a key indicator of liver function, for both plasmid DNA treatments at all DNA levels and time points.

µg, and the triple treatment, 3 x 2 µg, CHP-IL12 treatments caused toxic lesions in only one mouse while wtIL12 treatments had significantly higher numbers (**Fig. 3.6A**). Serum chemistry profiles of these mice revealed that there were no differences between any treatment, regimen, or time points, and all levels for the hallmarks of toxicity, such as Alanine Transaminase, were normal (**Fig. 3.6D**).

Discussion

Tumor targeting can be achieved via the screening of various libraries to select tumor-targeted peptides, DNA/RNA aptamers, antibodies, etc; however, the only mechanism that can be used for homing gene products from systemically injected genes will be tumor-targeted mini-peptide encoding DNA. These peptides are tiny which eliminates the concern of immunogenicity (**Appendix C**) and reduces the effect on the biological function of the gene product, though some minipeptides may boost or inhibit gene function [135]. The tiny peptide encoding DNA sequences can be easily fused with any therapeutic gene. Finally, these peptides can complement

existing tumor targeting approaches such as transcriptional targeting [122], translational targeting [136], and targeted delivery [117-120].

In this study, we have identified a 7 amino acid targeted peptide, CHP. In this gene therapy method, CHP is superior to the known cyclic tumor-homing peptides such as CNGRC and RGD4C (**Fig. 3.1**), which rely on disulfide bonds to maintain the structure of the targeting peptides [137, 138]. This linear structure may decrease the effect of the peptide on the activity of the fusion protein and vice versa.

Other tumor targeting peptides can deliver small molecules with only one copy for each small-molecule payload but require multiple copies of the peptide to target larger molecules such as a full length cytokine [139]. In this study, we demonstrate that fusion of a single copy of CHP encoding DNA can boost the accumulation of IL12 in tumors, suggesting one copy of CHP is sufficient to carry one copy of IL12 to the tumor site. Of course, increasing the number of CHP peptides per molecule may also increase the targeting efficiency.

Currently, most tumor-targeting strategies are based on extremely specific interactions, and the ability to target the tumor environment is constrained to a single cell type or specific type of tumor. We have shown that CHP can increase the efficacy of IL12 gene therapy to inhibit tumor growth in the three tumor cell lines, breast adenocarcinoma, squamous cell carcinoma, and colon carcinoma, and in two different mouse strains (**Fig. 3.1**). Subsequently, CHP-IL12 extends survival more than wtIL12 treatments in both the breast adenocarcinoma and squamous cell carcinoma (**Fig. 3.3C,E**). Similarly, CHP-IL12 treatments inhibit the development of spontaneous lung metastasis, which is the primary killer of cancer patients (**Fig. 3.3B**). This increase in antitumor response is associated with increases in both tumor-specific CTL activity (**Fig. 3.4**) and IL12 accumulation in tumors (**Fig. 3.2D**). This result is in agreement with the

result that intratumoral delivery of IL12 yields better anti-tumor efficacy than systemic delivery [121]. The discovery of CHP permits us to use systemic delivery to target IL12 to tumors without the need of intratumoral delivery, which is not realistic for treating internal tumors, metastatic tumors, and residual tumor cells after standard therapy. In this regard, this peptide will be extremely valuable, and further study is warranted.

We were able to identify a receptor for CHP as vimentin (**Fig. 3.5**), an intermediate filament protein conventionally regarded as an intracellular structural protein in cells of mesenchymal origin such as fibroblasts, chondrocytes, and macrophages [129]. Vimentin expression has been reported to be increased in several tumor models, including human prostate, colon [131], hepatocellular [130], and gemcitabine-resistant pancreatic cancers [133], and the tumor stromal cells in human colorectal tumors [132]. The upregulation of vimentin is associated with the epithelial-to-mesenchymal transition (EMT), which is important for motility as well as metastasis in several tumors.

Though vimentin is upregulated in tumors, two remaining questions are how the CHP targets an intracellular receptor and how the peptide avoids targeting lungs where vimentin is also expressed. These questions were perfectly addressed recently by the discovery that vimentin is expressed on the cell surface of tumor cells [140] and epithelial cells during angiogenesis [141]. Additionally, some human tumor-initiating cells remaining after treatment overexpress vimentin on the tumor cell surface [142]. Another important aspect of vimentin is the conserved sequences among mouse, rat, dog, and humans [143]. This information along with the T/S SEAP accumulation in the xenogeneic human tumor model (**Fig. 3.1B**) strongly suggests that CHP targeting will crossover to human treatments.

We also confirmed that vimentin is expressed at very low levels in the heart, liver, kidney, spleen, and serum of C3H mice, yet it is highly expressed in lung tissue. Since most general expression of vimentin is intracellular [129, 144, 145], this expression should not be a target of CHP. As seen in Appendix B, there was no accumulation of CHP-biotin in the lung sections which supports this notion. Conversely, vimentin is highly expressed in SCCVII tumors in C3H mice, and CHP-biotin did accumulate in the SCCVII tumors (**Fig. 3.2**). Likewise, the tumor cells and corresponding syngeneic tumors both express detectable levels of vimentin.

A potential problem with using CHP for targeting immunomodulatory agents is the cellular fate of the surface-bound peptide and its payload. Ise et al. have recently described the internalization of vimentin-bound ligands which could block the ability of the IL12 to stimulate an anti-tumor immune response [146]. According to their studies, the vimentin-bound ligands are still located on the cell surface after 2 h. The half-life of circulating IL-12 is between 2.5 and 3.3 h [147]; therefore, internalization would not affect the IL-12 activity. Further, if CHP is eventually internalized by the tumor cells, then CHP would also be a potent targeting modality for using intracellular antitumor agents, which is an area that requires further investigation.

We have developed a fully functional tumor targeting IL12 gene construct that can be delivered systemically for treating distally located neoplastic diseases. Inserting peptide-encoding sequences directly prior to the stop codon in the p40 gene of an IL12 plasmid did not interfere with transcription, translation, post-translational modifications, or therapeutic functionality of the IL12 gene product (**Fig. 3.2**). Also, CHP maintains its tumor-targeting ability as seen in $IL12^{-/-}$ mice (**Fig. 3.2**) and can increase the therapeutic efficacy of systemic IL12 gene-therapy treatments (**Fig. 3.3**) while decreasing liver toxicity (**Fig. 3.6**). Future studies will include investigating the fate of vimentin-bound CHP, methods to increase the targeting

efficiency, the potential for CHP to target other modalities, and the efficacy of other CHP-antitumor fusion gene constructs.

References

115. Kobayashi M, *et al.* (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* **170**: 827-845.

116. Del Vecchio M, *et al.* (2007). Interleukin-12: biological properties and clinical application. *Clin Cancer Res* **13**: 4677-4685.

117. Halin C, *et al.* (2002). Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. *Nat Biotechnol* **20**: 264-269.

118. Dela Cruz JS, Trinh KR, Morrison SL, Penichet ML (2000). Recombinant anti-human HER2/neu IgG3-(GM-CSF) fusion protein retains antigen specificity and cytokine function and demonstrates antitumor activity. *J Immunol* **165**: 5112-5121.

119. Dickerson EB, *et al.* (2004). Enhancement of the Antiangiogenic Activity of Interleukin-12 by Peptide Targeted Delivery of the Cytokine to {alpha}v{beta}3 Integrin. *Mol Cancer Res* **2**: 663-673.

120. Colombo G, *et al.* (2002). Structure-activity relationships of linear and cyclic peptides containing the NGR tumor-homing motif. *J Biol Chem* **277**: 47891-47897.

121. Li S, Zhang L, Torrero M, Cannon M, Barret R (2005). Administration route- and immune cell activation-dependent tumor eradication by IL12 electrotransfer. *Mol Ther* **12**: 942-949.

122. Yamazaki M, *et al.* (2002). Effective gene therapy for medullary thyroid carcinoma using recombinant adenovirus inducing tumor-specific expression of interleukin-12. *Gene Ther* **9**: 64-74.

123. Okada Y, *et al.* (2004). Optimization of antitumor efficacy and safety of in vivo cytokine gene therapy using RGD fiber-mutant adenovirus vector for preexisting murine melanoma. *Biochim Biophys Acta* **1670**: 172-180.

124. Gao JQ, *et al.* (2007). Effective tumor targeted gene transfer using PEGylated adenovirus vector via systemic administration. *J Control Release* **122**: 102-110.

125. Wang H, *et al.* (2008). Integrin-targeted imaging and therapy with RGD4C-TNF fusion protein. *Mol Cancer Ther* **7**: 1044-1053.

126. Maeda H, Fang J, Inutsuka T, Kitamoto Y (2003). Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications. *Int Immunopharmacol* **3**: 319-328.

127. Craig R, Cutrera J, Zhu S, Xia X, Lee YH, Li S (2008). Administering plasmid DNA encoding tumor vessel-anchored IFN-alpha for localizing gene product within or into tumors. *Mol Ther* **16**: 901-906.

128. Work LM, *et al.* (2006). Vascular Bed-Targeted *in Vivo* Gene Delivery Using Tropism-Modified Adeno-associated Viruses. *Molecular Therapy* **13**: 638-693.

129. Dandachi N, *et al.* (2001). Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors, and oncoproteins. *J Pathol* **193**: 181-189.

130. Matos JM, Witzmann FA, Cummings OW, Schmidt CM (2009). A pilot study of proteomic profiles of human hepatocellular carcinoma in the United States. *J Surg Res* **155**: 237-243.

131. Moisan E, Girard D (2006). Cell surface expression of intermediate filament proteins vimentin and lamin B1 in human neutrophil spontaneous apoptosis. *J Leukoc Biol* **79**: 489-498.

132. Ngan CY, *et al.* (2007). Quantitative evaluation of vimentin expression in tumour stroma of colorectal cancer. *Br J Cancer* **96**: 986-992.

133. Wang Z, *et al.* (2009). Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway. *Cancer Res* **69**: 2400-2407.

134. Zhu S, Lee DA, Li S (2010). IL-12 and IL-27 sequential gene therapy via intramuscular electroporation delivery for eliminating distal aggressive tumors. *J Immunol* **184**: 2348-2354.

135. Cutrera J, Dibra D, Xia X, Li S (2010). Enhancement of reporter gene detection sensitivity by insertion of specific mini-peptide-coding sequences. *Cancer Gene Ther* **17**: 131-140.

136. Stoff-Khalili MA, *et al.* (2008). Cancer-specific targeting of a conditionally replicative adenovirus using mRNA translational control. *Breast Cancer Res Treat* **108**: 43-55.

137. Corti A, Curnis F, Arap W, Pasqualini R (2008). The neovasculature homing motif NGR: more than meets the eye. *Blood* **112**: 2628-2635.

138. Temming K, Schiffelers RM, Molema G, Kok RJ (2005). RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. *Drug Resist Updat* **8**: 381-402.

139. Garanger E, Boturyn D, Jin Z, Dumy P, Favrot MC, Coll JL (2005). New multifunctional molecular conjugate vector for targeting, imaging, and therapy of tumors. *Mol Ther* **12**: 1168-1175.

140. Huet D, *et al.* (2006). SC5 mAb represents a unique tool for the detection of extracellular vimentin as a specific marker of Sezary cells. *J Immunol* **176**: 652-659.

141. Bhattacharya R, *et al.* (2009). Recruitment of vimentin to the cell surface by beta3 integrin and plectin mediates adhesion strength. *J Cell Sci* **122**: 1390-1400.

142. Creighton CJ, *et al.* (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A* **106**: 13820-13825.

143. Thiery JP (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2**: 442-454.

144. Gilles C, *et al.* (1999). Vimentin contributes to human mammary epithelial cell migration. *J Cell Sci* **112 (Pt 24)**: 4615-4625.

145. Nieminen M, Henttinen T, Merinen M, Marttila-Ichihara F, Eriksson JE, Jalkanen S (2006). Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol* **8**: 156-162.

146. Ise H, *et al*. (2010). Vimentin and desmin possess GlcNAc-binding lectin-like properties on cell surfaces. *Glycobiology* **20**: 843-864.

147. Trinchieri G, Scott P (1994). The role of interleukin 12 in the immune response, disease and therapy. *Immunol Today* **15**: 460-463.

Chapter 4

Concluding Remarks

Overall Summary of Findings

As discussed in Chapter 1, tumor-targeted cytokine therapies are poised to be a major breakthrough for immunotherapies as they have the potential to increase the level of intratumoral cytokines while lowering the systemic levels leading to higher efficacy and lower toxicity [148-151]. The major problems that face these therapies are the methods of administration and the availability of targets. Specifically, metastatic and residual tumor cells are nearly impossible to treat directly until they are established and invasive, thereby necessitating invasive procedures or systemic treatments. To solve these problems, new targeted-cytokine treatment strategies are needed.

In Chapter 2, we examined the effects of inserting mini-peptide coding sequences into a secreted alkaline phosphatase (SEAP) reporter gene plasmid DNA. *In vitro* transfection of several mini-peptide SEAP plasmid DNAs in several different normal and cancer cell lines revealed dramatic changes in the activity of the gene products which were dependent on the peptide and not the cell type. Specifically, we showed that the peptide CWDDWLC significantly improved the activity of the SEAP enzyme. Removing the flanking Cys residues on this peptide ablated this activity increase, and inserting a Gly residue into the sequence (CWDDGWLC) reduced the SEAP activity to nearly undetectable levels. The increased activity of the CWDDWLC-SEAP enzyme also occurs following intramuscular transfection in a mouse model. Surprisingly, western blot analyses showed that the increased SEAP activity was not due to an increase in the amount of SEAP protein produced. These studies revealed the drastic effects that inserting peptides could have on the activity of the SEAP enzyme, but the production of the gene product was not altered. So, this peptide-SEAP system could be used to screen peptides for

tumor-targeting capabilities, but the only way to correct for the activity modifications is to use the ratio of activities seen in the different tissues.

As described in Chapter 3, we used the peptide-SEAP screening strategy to identify the best peptide which can be used to target an *in vivo* produced product to the tumor environment. In multiple tumor models including a human xenogeneic model, the peptide VNTANST (referred to as the Comprehensive Carcinoma Homing Peptide, CHP) significantly increased accumulation of SEAP in the tumor environment as evidence by the increased Tumor/Serum SEAP levels. Then, we created a tumor-targeted IL12 fusion gene construct (CHP-IL12) which maintained both the biological activity of the IL12 and the targeting ability of CHP. Systemic treatments with CHP-IL12 plasmid DNA in three syngeneic tumor models resulted in inhibition of primary tumor growth, and survival was extended in two of the models compared to wtIL12 gene treatments. Additionally, CHP-IL12 treatments decreased the development of metastasis. The improved efficacy of these treatments resulted from an increase in the level of mature DC in the tumor environment and elicited more potent tumor-specific cytotoxicity by CTL. Additionally, these treatments reduced systemic toxicity and resulted in significantly less lesions in the livers compared to wtIL12 plasmid DNA treatments. Affinity chromatography and mass spectrometry identified the target for CHP as vimentin. These results show the potential for using these mini-peptides for targeting *in vivo* produced gene products to tumors.

Significance of Research

Improving cancer treatments is necessary to combat the incessant presence of neoplastic diseases and to improve the quality of life of patients undergoing these treatments. Immunomodulatory therapy is an encouraging field that can offer increased efficacy, decreased toxicity, and improved quality of life, and cytokines hold great potential for these therapies. To

date, IL-2 and IFNα are the only cytokines approved for cancer therapies, but IL12 and GM-CSF are on the precipice of becoming the next possibilities. Unfortunately, the toxicities that are associated with these treatments, especially IL-12, continue to hinder their translation into clinical applications [152]. The keys to improving IL12 therapy and reducing these toxicities are increasing the level of intratumoral IL12 and decreasing systemic IL12 [150]. Recombinant versions of IL-12, such as mrIL12vp [148] and L19-IL12 [149], and IL-12 plasmid loaded viral vectors, such as AdRGD-IL12 [151], have been developed to target to the tumor, yet none have been able to overcome the low therapeutic efficacy and high toxicity. A novel method to reach these goals is a non-viral tumor targeted cytokine therapy.

Vital to making this therapy work is identifying a modality that can be produced *in vivo* from plasmid DNA. Of the current tumor-targeting modalities, mini-peptides which specifically home to receptors uniquely located in the tumor environment are the only method that can be used for this purpose; however, until now no peptides have been shown to have the ability to be transduced and produced at a site distal from the tumor and then target to the tumor environment. To this end, the reporter-gene mediated screening strategy developed as described in Chapter 2 was used to identify CHP, a peptide with these characteristics.

Also, using this strategy to serially screen peptides in multiple tumors can identify peptides that target tumor-specific modalities in several types of tumors. In this case, vimentin is the target which we were able to exploit. Other methods, such as Witheferin A and anti-vimentin antibodies have used vimentin as a target for therapy [153, 154]; however, this CHP-mediated tumor targeting gene therapy is the first non-recombinant protein method used. Vimentin is an especially appropriate target to use for tumor-homing strategies because it is expressed on the cell surface of several human tumors [155-158], chemotherapy-resistant tumors [159], tumor-

initiating cells [160], and epithelial cells in angiogenic vessels [161]. Additionally, the results seen in these studies are important because the sequence of vimentin is conserved between mice and humans [162]. So, using CHP as a targeting peptide for clinical applications is promising.

Using CHP, we have shown that systemic delivery of a tumor-targeted cytokine gene can be used for treating tumors. After systemic delivery, the gene product homes to the tumor environment, increases the anti-tumor immune response, and decreases the toxic side effects. Further, these results were seen with only two treatments separated by 10 days. The recombinant targeted and viral gene treatments require several treatments, usually administered daily or every other day for up to 90 days to see similar responses [163, 164]. In addition to the improved quality of life for patients undergoing these treatments, the cost of producing microgram levels of plasmid DNA versus the milligram levels of recombinant proteins will be beneficial to the patients, insurance companies, and tax-payers.

Future Directions

The potential for using CHP to target gene products to tumors is clearly evidenced by the results presented here; however, the capability of CHP to target other payloads is still unknown. The CHP-biotin experiments described in Chapter 3 (**Fig. 3.2**) prove that the targeting capacity of CHP is not strictly for gene products. So, testing the size and biological limits of what CHP can carry is necessary. Conjugating CHP to chemotherapeutic drugs, tyrosine kinase inhibitors, siRNA, and any other anticancer medication can be potentially beneficial in the same manner that improved the efficacy and reduced toxicity of systemic IL12 gene treatments. Truly, this is an exciting peptide which demands further investigation.

Similarly, the mini-peptide tumor-targeted cytokine approach has many avenues that need to be explored. CHP was a peptide identified out of a relatively small subset of known

tissue- and tumor-targeting peptides [165]; therefore, the possibility of identifying peptides with similar multi-tumor targeting abilities that can target *in vivo* produced gene products is high. Alternatively, identifying which cytokines can be used in this method is necessary. GM-CSF, IL15 [153], and other cytokines that are already being explored for cancer treatments can potentially be used, and their efficacy in these treatments will be tested. Furthermore, promising results from combinatorial cytokine and chemotherapeutic treatments [166] could also be improved by potentially using peptide-cytokine gene therapies in combination with peptide-chemotherapy systemic treatments. In conclusion, the results described in this work are merely the beginning of what seems to be a promising new approach for improving cancer treatments.

References

148. Dickerson EB, *et al.* (2004). Enhancement of the Antiangiogenic Activity of Interleukin-12 by Peptide Targeted Delivery of the Cytokine to {alpha}v{beta}3 Integrin. *Mol Cancer Res* **2**: 663-673.

149. Halin C, *et al.* (2002). Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. *Nat Biotechnol* **20**: 264-269.

150. Li S, Zhang L, Torrero M, Cannon M, Barret R (2005). Administration route- and immune cell activation-dependent tumor eradication by IL12 electrotransfer. *Mol Ther* **12**: 942-949.

151. Okada Y, *et al.* (2004). Optimization of antitumor efficacy and safety of in vivo cytokine gene therapy using RGD fiber-mutant adenovirus vector for preexisting murine melanoma. *Biochim Biophys Acta* **1670**: 172-180.

152. Atkins MB, *et al.* (1997). Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. *Clin Cancer Res* **3**: 409-417.

153. Lahat G, *et al.* (2010). Vimentin is a novel anti-cancer therapeutic target; insights from in vitro and in vivo mice xenograft studies. *PLoS One* **5**: e10105.

154. van Beijnum JR, *et al.* (2006). Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood* **108**: 2339-2348.

155. Huet D, *et al.* (2006). SC5 mAb represents a unique tool for the detection of extracellular vimentin as a specific marker of Sezary cells. *J Immunol* **176**: 652-659.

156. Matos JM, Witzmann FA, Cummings OW, Schmidt CM (2009). A pilot study of proteomic profiles of human hepatocellular carcinoma in the United States. *J Surg Res* **155**: 237-243.

157. Moisan E, Girard D (2006). Cell surface expression of intermediate filament proteins vimentin and lamin B1 in human neutrophil spontaneous apoptosis. *J Leukoc Biol* **79**: 489-498.

158. Ngan CY, *et al.* (2007). Quantitative evaluation of vimentin expression in tumour stroma of colorectal cancer. *Br J Cancer* **96**: 986-992.

159. Wang Z, *et al.* (2009). Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway. *Cancer Res* **69**: 2400-2407.

160. Creighton CJ, *et al.* (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A* **106**: 13820-13825.

161. Bhattacharya R, *et al.* (2009). Recruitment of vimentin to the cell surface by beta3 integrin and plectin mediates adhesion strength. *J Cell Sci* **122**: 1390-1400.

162. Thiery JP (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2**: 442-454.

163. Ise H, *et al.* (2010). Vimentin and desmin possess GlcNAc-binding lectin-like properties on cell surfaces. *Glycobiology* **20**: 843-864.

164. Meng J, *et al.* (2008). Preclinical safety evaluation of IFN[alpha]2a-NGR. *Regulatory Toxicology and Pharmacology* **50**: 294-302.

165. Craig R, Li S (2006). Function and Molecular Mechanism of Tumor-Targeted Peptides for Delivering herapeutic Genes and Chemical Drugs. *Mini-Reviews in Medicinal Chemistry* **6**: 109-120.

166. Sacchi A, Gasparri A, Gallo-Stampino C, Toma S, Curnis F, Corti A (2006). Synergistic Antitumor Activity of Cisplatin, Paclitaxel, and Gemcitabine with Tumor Vasculature-Targeted Tumor Necrosis Factor-{alpha}. *Clin Cancer Res* **12**: 175-182.

Appendix A.

Mascot Search Results from Mass Spectrometry Analysis

Mascot Search Results

User : Azeem Hasan
Email : azeem@lsu.edu
Search title : D:\Data\Jeffry\32309\Data32309_8247.wiff (sample number 2)
MS data file : C:\DOCUME~1\AZEEMH~1\LOCALS~1\Temp\mas4E01.tmp
Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp : 25 Mar 2009 at 20:24:36 GMT
Protein hits : VIME MOUSE Vimentin.- Mus musculus (Mouse).

Peptide Summary Report

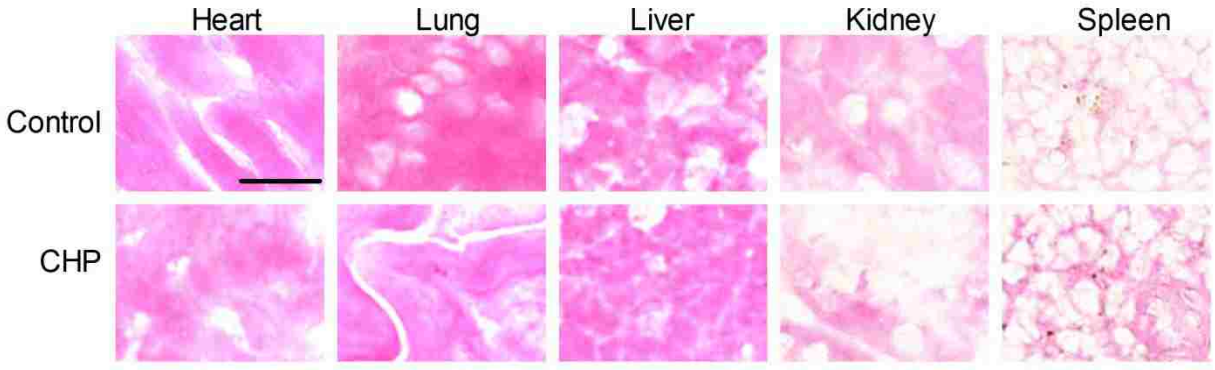
Format As: Peptide Summary — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red
Select All | Select None | Search Selected | Error tolerant | Archive Report

1. VIME MOUSE **Mass:** 53581 **Score:** 784 **Queries matched:** 18 **emPAI:** 1.05
Vimentin.- Mus musculus (Mouse).
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
73	538.79	1075.57	1075.50	0.08	0	39	0.61	1	R.DNLAEDIMR.L
74	547.30	1092.59	1092.52	0.07	0	65	0.0012	1	K.FADLSEAANR.N
76	558.32	1114.62	1114.56	0.06	0	58	0.0071	1	K.VELQELNDR.F
77	561.32	1120.63	1120.58	0.05	0	22	26	1	R.EYQDLLNVK.M
80	585.39	1168.77	1168.71	0.06	0	44	0.17	1	K.ILLAELEQLK.G
83	627.83	1253.64	1253.56	0.08	0	69	0.00044	1	R.LGDLYEEEMR.E
84	648.84	1295.67	1295.60	0.07	0	49	0.049	1	R.EEAESTLQSFR.Q
85	655.35	1308.68	1308.60	0.08	0	48	0.073	1	K.NLQEAEEWYK.S
88	722.91	1443.80	1443.70	0.10	0	45	0.11	1	R.SLYSSSPGGAYVTR.S
89	499.30	1494.87	1494.78	0.10	0	21	20	1	R.TYSLGSALRPSTSR.S
91	511.98	1532.93	1532.84	0.09	1	57	0.0056	1	R.KVESLQEEIAFLK.K
92	514.01	1539.00	1538.90	0.10	1	32	0.72	1	K.ILLAELEQLKGQGK.S
93	779.50	1556.99	1556.89	0.09	0	50	0.019	1	R.ISLPLPTFSSLNLR.E
95	529.97	1586.89	1586.79	0.10	1	50	0.03	1	R.TNEKVELQELNDR.F
98	561.66	1681.97	1681.85	0.11	0	27	5.2	1	R.ETNLESLPLVDTHSK.R
99	563.65	1687.94	1687.82	0.12	1	40	0.29	1	R.VEVERDNLAEDIMR.L
100	578.99	1733.94	1733.81	0.13	1	49	0.036	1	R.LQDEIQNMKEEMAR.H
102	612.98	1835.91	1835.79	0.12	0	21	20	1	R.DGQVINETSQHHDDLE.-

Appendix B.

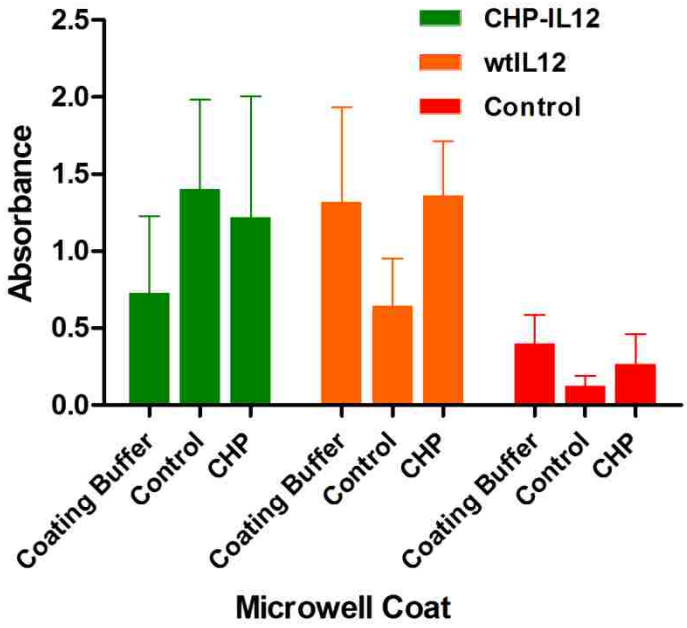
Lack of CHP-biotin Localization in Tissues



Sections from the hearts, lungs, livers, kidneys, and spleens from the same mice in **Figure 3.2** were counterstained with eosin only. Scale bar represents 100 µm.

Appendix C.

Lack of Immunogenecity of CHP



The level of CHP-specific IgG from EMT6-tumor bearing Balb/c mice treated with wtIL12 or CHP-IL12 gene therapy as determined via binding to wells of a microwell plate coated with coating buffer only, control peptide or CHP peptide (n=3). Error bars represent s.e.m.

Appendix D.

Letters of Permission

Springer
the language of science

Title: Passive and Active Tumor Homing Cytokine Therapy
Author: Jeffry Cutrera
Publication: Springer eBook
Publisher: Springer
Date: Jan 1, 2009
Copyright © 2009, Springer

Logged in as:
Jeffry Cutrera
Account #:
3000342450
LOGOUT

Order Completed

Thank you very much for your order.

This is a License Agreement between Jeffry J Cutrera ("You") and Springer ("Springer"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

License number	Reference confirmation email for license number
License date	Sep 17, 2010
Licensed content publisher	Springer
Licensed content publication	Springer eBook
Licensed content title	Passive and Active Tumor Homing Cytokine Therapy
Licensed content author	Jeffry Cutrera
Licensed content date	Jan 1, 2009
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	1
Author of this Springer article	Yes and you are the sole author of the new work
Title of your thesis / dissertation	Identification a Novel Tumor-Targeting-Peptide and Development of a Tumor-Targeted-Cytokine Vector for Systemic Treatment of Primary and Metastatic Malignant Disease
Expected completion date	Dec 2010
Estimated size(pages)	100
Total	0.00 USD

CLOSE WINDOW

SPRINGER LICENSE
TERMS AND CONDITIONS

Sep 21, 2010

This is a License Agreement between Jeffry J Cutrera ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2513770652494
License date	Sep 21, 2010
Licensed content publisher	Springer
Licensed content publication	Springer eBook
Licensed content title	Passive and Active Tumor Homing Cytokine Therapy
Licensed content author	Jeffry Cutrera
Licensed content date	Jan 1, 2009
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	1
Author of this Springer article	Yes and you are the sole author of the new work
Order reference number	
Title of your thesis / dissertation	Identification a Novel Tumor-Targeting-Peptide and Development of a Tumor-Targeted-Cytokine Vector for Systemic Treatment of Primary and Metastatic Malignant Disease
Expected completion date	Dec 2010
Estimated size(pages)	100
Total	0.00 USD
Terms and Conditions	

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License

With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided it is password protected or on the university's intranet, destined to microfilming by UMI and University repository. For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well). Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
However figures and illustrations may be altered minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice:
Please include the following copyright citation referencing the publication in which the material was originally published. Where wording is within brackets, please include verbatim.
"With kind permission from Springer Science+Business Media: <book/journal title, chapter/article title, volume, year of publication, page, name(s) of author(s), figure number(s), and any original (first) copyright notice displayed with material>."

Warranties: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Indemnity
You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by the country's law in which the work was originally published.

Other terms and conditions:

v1.2

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10852694.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2513770652494 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Title: Enhancement of reporter gene detection sensitivity by insertion of specific mini-peptide-coding sequences
Author: J Cutrera, D Dibra, X Xia and S Li
Publication: Cancer Gene Therapy
Publisher: Nature Publishing Group
Date: Aug 28, 2009

Author Request

If you are the author of this content (or his/her designated agent) please read the following. If you are not the author of this content, please click the Back button and select an alternative Requestor Type to obtain a quick price or to place an order.

Ownership of copyright in the article remains with the Authors, and provided that, when reproducing the Contribution or extracts from it, the Authors acknowledge first and reference publication in the Journal, the Authors retain the following non-exclusive rights:

a) To reproduce the Contribution in whole or in part in any printed volume (book or thesis) of which they are the author(s).

b) They and any academic institution where they work at the time may reproduce the Contribution for the purpose of course teaching.

c) To reuse figures or tables created by them and contained in the Contribution in other works created by them.

d) To post a copy of the Contribution as accepted for publication after peer review (in Word or Tex format) on the Author's own web site, or the Author's institutional repository, or the Author's funding body's archive, six months after publication of the printed or online edition of the Journal, provided that they also link to the Journal article on NPG's web site (eg through the DOI).

NPG encourages the self-archiving of the accepted version of your manuscript in your funding agency's or institution's repository, six months after publication. This policy complements the recently announced policies of the US National Instutes of Health, Wellcome Trust and other research funding bodies around the world. NPG recognises the efforts of funding bodies to increase access to the research they fund, and we strongly encourage authors to participate in such efforts.

Authors wishing to use the published version of their article for promotional use or on a web site must request in the normal way.

If you require further assistance please read NPG's online author reuse guidelines.

BACK

Vita

Jeffry Cutrera was born in Baton Rouge, Louisiana. He attended Catholic High School in Baton Rouge, and graduated in the top 10% of his class in May of 2000. During his studies at Louisiana State University, he became interested in a future in scientific research while working in a microbiology lab in the Department of Biological Sciences. After receiving his Bachelor of Science degree in December of 2004, he discovered the research which was taking place in the Department of Comparative Biomedical Sciences at the LSU School of Veterinary Medicine. In the Fall of 2005, he began his studies in the doctoral program under the mentorship of Dr. Shulin Li. He will receive his Doctor of Philosophy degree in December of 2010.