DEVELOPMENT AND CHARACTERIZATION OF CD133
POSITIVE CANCER STEM CELL TARGETED TOXINS
FOR USE IN CARCINOMA THERAPY

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To all my friends that I’ve laughed with, complained to, studied with, have depended on, and helped get me to where I am today, thank you. I hope I have and can be as much help to all of you as you have been to me.
DEDICATION

I dedicate this work to my baby girl Nyah. I hope that finishing this work will enable me to better provide and care for you. It is my prayer that you will find your true purpose in this world and pursue it with all your heart with God as your vow. If you do, you will be eternally happy.
ABSTRACT

Cancer Stem cells (CSC) have been shown to play an important role in a number of carcinomas. Although representing a subpopulation of many cancers, CSC are extremely important because they are more drug resistant than the more differentiated cancer cells which make up the bulk of most solid tumors. High numbers of CSC is an indicator of poor clinical outcome and have been shown to be a cause in drug refractory relapse, which is the single most urgent problem in carcinoma therapy. CD133 is a cell surface receptor that has been identified as a CSC marker in breast, brain, colon, pancreatic, and recently in Head and Neck (HN) carcinoma. Our laboratory bioengineered a targeted toxin called dCD133KDEL consisting of the scFv portion of a novel anti-CD133 monoclonal antibody on the same molecule as truncated pseudomonas exotoxin. Binding of dCD133KDEL was demonstrated on a variety of carcinoma lines and we verified the ability of the anti-CD133 scFv to sort tumor initiating cells. Since enhanced tumor initiation is a hallmark of CSC, we demonstrated that dCD133KDEL was able to prevent tumor initiation. Importantly, even though CD133 was expressed only on a subpopulation of cells, dCD133KDEL prevented cell proliferation in vitro and had powerful anti-cancer effects in vivo in xenograft mouse models of head and neck cancer. The therapeutic potential of dCD133KDEL was further investigated in xenograft models of human breast and ovarian cancer where it was effective when administered systemically as well. To further study therapeutic potential, we assessed the reactivity of this drug on normal human progenitor cells since CD133 is a known progenitor cell marker. dCD133KDEL did not kill normal human CD133+ stem cells at the same concentrations as it did for carcinoma cells, indicating a therapeutic window exists. Drug safety studies were
performed in mice and the maximum tolerated dose of dCD133KDEL was established. Liver damage was shown to be the dose limiting toxicity.

Since CD133 positive cells can develop from CD133 negative cell populations, a phenomenon known as stem cell plasticity, we developed a bispecific targeted toxin (dEpCAMCD133KDEL) capable of targeting both CD133 and the epithelial cell adhesion molecule, EpCAM, an overexpressed marker on most carcinomas. dEpCAMCD133KDEL potently inhibited cell proliferation of a number of carcinoma lines in vitro and was also effective at eliminating tumor spheroids, which have been shown to be enriched for CSCs. This bispecific agent was also effective at causing tumor regression in a model of HN cancer in vivo.

Because of the preliminary effectiveness of dCD133KDEL and dEpCAMCD133KDEL in preclinical evaluation, these drugs warrant further development for possible use in carcinoma therapy. These two CSC targeted toxins could be extremely useful in situations where our current drugs are failing because of the progression of a critical drug resistant CSC population. Since, targeted toxins work synergistically with current chemotherapy, these two targeted toxins could be used as an adjunct to current therapy to target the CSC population specifically, while traditional chemotherapy and radiation can still be used to target the rapidly dividing bulk of the tumor. We believe that future success in cancer treatment must include approaches to target CSC as well as the majority of less differentiated cancer cells that comprise a tumor.
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Chapter I

Background of Head and Neck Squamous Cell Carcinoma
Head and Neck Cancer Epidemiology

Carcinoma is the most common type of cancer in the world and arises from epithelial tissue that line exterior and interior surfaces of the body. Head and neck cancer includes malignancies that develop in the oral cavity, pharynx, larynx, and trachea with approximately 550,000-650,000 people diagnosed worldwide annually. Squamous cell carcinoma makes up 90% of head and neck cancer and head and neck squamous cell carcinoma (HNSCC) is currently the 6th most common form of cancer worldwide [1-3]. Use of tobacco products, heavy alcohol consumption, and chewing betel quid are three of the most common risk factors for HNSCC. However, HPV, particularly type 16, was recently identified as a causal factor as well. Current trends show only HPV as causing an increasing incidence of HNSCC, while all other incidence factors are decreasing in prevalence [1-3]. It has yet to be shown whether HPV vaccines used for cervical cancer prevention could also be used to prevent HPV mediated HNSCC.

Current Treatment

The current standard of care for patients with HNSCC depends largely on the stage of the disease. Staging is determined based on the size of the tumor, involvement of locoregional lymph nodes, and whether there are distant metastases. Stage I and II do not have any lymph node involvement or metastases, while stage III and IV do [2]. Depending on the primary tumor site, surgery and/or radiotherapy is currently the first line treatment for patients diagnosed with stage I or II [1,2]. Stage III and IV typically requires concurrent chemotherapy in addition to radiotherapy for any unresectable
tumors. However, induction chemotherapy has also been shown to be effective as well [4,5]. About two-thirds of patients with HNSCC present with logoregional advanced disease [1,2]. The current chemotherapy regimen for these patients is docetaxel, Cisplatin, and fluorouracil in combination followed by radiotherapy [4]. Many other platinum and taxol reagents have also been used. Recently, many chemotherapy alternatives have been tested as well including Gefitinib, Cetuximab, and Erlotinib. However, these anti-epithelial growth factor receptor (EGFR) agents have shown surprisingly low single agent activity when considering the high level of EGFR expression on most HNSCCs [2,6]. Sorafinib, a Raf kinase and vascular endothelial growth factor receptor (VEGFR) inhibitor has shown good preclinical activity in combination with chemotherapy, but the clinical benefit for these agents thus far has been very underwhelming [7,8].

Despite the dozens of clinical trials testing new agents for HNSCC therapy over the past several decades, improvements in long-term survival of patients with stage III and IV disease have been minimal. Overall 5-year survival for all stages is 60%. However, the median survival of patients with advanced logoregional disease in recent clinical trials is only 7.6 to 9.2 months [1,6]. Furthermore, over 50% of patients with stage III or IV disease that are responding to treatment, will relapse within two years [1]. Sadly, patients with HNSCC also have a higher suicide rate compared to the overall cancer patient population because of the effect this cancer can have on physical appearance and quality of life [9]. A new approach is desperately needed in order to effectively treat these patients and increase life expectancy and quality.
Cancer Stem Cell Hypothesis

Recently, much research has been done validating a new hierarchical theory of cancer development and progression in carcinomas that may explain the high rate of drug refractory relapse in HNSCC patients. The cancer stem cell (CSC) theory states that there exists a subpopulation of cells within solid tumors that have the capability, much like normal stem cells, to self-renew and divide asymmetrically. This allows for the propagation and differentiation of CSCs into the entire heterogeneous cell types commonly found in tumors [10-15]. This dynamic model contrasts with the clonal evolution model that suggests all cells within a tumor are equally tumorigenic and that tumors can arise from any cell type through a series of genetic mutations and this is responsible for the heterogeneity present in solid tumors [10,14,16]. However, this clonal model does not adequately explain the unique ability of certain subpopulations of cancer cells to form tumors at log fold lower concentrations than other cancer cell populations or their ability to differentiate into all the cell types that existed in the parent tumor. Furthermore, these cells are more chemoresistant and radioresistant than the bulk of cells that make up the tumor [10-15]. This provides an answer as to why so many HNSCC patients suffer from drug resistant relapse. Many patients initially respond to treatment, but current therapies are mostly effective at killing the non-CSC populations, which allows the CSC population to survive and repopulate the tumor in many cases. New adjunct therapies are needed to target the CSC subpopulation of cells to complement traditional treatment options and prevent the all too common problem of drug resistant relapse.
Preface

This chapter has been published:

Chapter II

An Old Idea Tackling a New Problem: Targeted Toxins Specific for Cancer Stem Cells
1. Introduction

The idea of specifically targeting and killing cells responsible for disease is not a new one. Paul Ehrlich discovered the first targeted therapy, Arsphenamine, in 1909. This work inspired scientists in a range of disciplines over the next 100 years to continue working and developing new therapeutics that specifically destroy target cells. Immunotoxins, hereafter referred to more generally as targeted toxins, descended from this early work and have been used in cancer therapy for decades [17,18].

Targeted toxins (TTs) are biological drugs consisting of a ligand linked to a protein toxin. Some of the most commonly used toxins are pseudomonas exotoxin (PE), diptheria toxin (DT), ricin, saporin, bouganin, and gelonin. These toxins act catalytically on target cells to induce apoptosis using a range of mechanisms from inhibiting protein translation via ADP-ribosylation of EF-2 (PE and DT) to inactivating ribosomes (ricin, saporin, bouganin, gelonin) [19]. While the mechanism of action may vary, the one constant is that the TT must reach the target cells and the ligand portion of the molecule must bind its specific receptor and then be internalized before it is able to induce apoptosis. These ligands are typically antibodies, antibody fragments, cytokines, or growth factors, all specific to their target receptor [19,20].

The past decade has witnessed a paradigm shift within the cancer field. There has been overwhelming evidence recently presented in a wide range of tumor types that there exists a typically small subpopulation of cells within the cancer that have been termed cancer stem cells (CSCs). These cells can enhance tumor initiation, self-propagation, and differentiation into all the phenotypically diverse cells found within the tumor population.
Furthermore, CSCs have been shown to be more chemotherapy and radiation resistant than non-CSCs [21,22]. This explains the single most difficult problem in treating cancer patients, drug-refractory relapse. Thus, new therapeutic agents are necessary to target these cells in particular in order to prevent drug-resistant relapse. A logical approach to eliminate CSCs is to target their unique cell surface markers.

A number of markers have been identified that allow for the separation and ultimately targeting of CSCs. In 1997, Bonnet and Dick reported the first CSCs in acute myeloid leukemia. These cells were characterized as being CD34+/CD38− [23]. Since then a number of markers have been used to successfully identify CSCs in a broad range of tumor types. However, only cell surface markers, not intracellular markers, are useful when it comes to treatment using TTs. Thus, this review will focus only on TTs specific for these markers. Table 2-1 shows the CSC TTs discussed herein and their phase of development.

2. Cancer Stem Cell Targeted Toxins

2.1. CD123

CD123, also known as IL-3Rα, is the alpha subunit of interleukin-3 and is expressed on leukemic stem cells (LSCs) in Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML), Myelodysplastic Syndrome (MDS), and Systemic Mastocytosis [24]. An anti-IL3 Diphtheria toxin fusion protein (DT38IL3) recently completed a phase I clinical trial [25]. Out of 45 patients, three had complete or partial responses with four more having minimal responses. Transaminasemia, vascular leak syndrome, and fever were the main toxicities in this study. These toxicities point to the need to improve
selectivity and potency in order to increase efficacy. A TT called 26292(Fv)-PE38-KDEL was developed to target LSCs that shows a promising increase in selectivity and potency compared to DT388IL3. This TT combines a single chain variable fragment (scFv) with a truncated form of pseudomonas exotoxin, which was mutated to increase activity and has shown good activity against several leukemia lines [26]. The activity appears to be dependent on a threshold level of CD123 expression on the leukemia cells, which can vary greatly from patient-to-patient. Additional studies are needed to further assess efficacy and toxicity of this TT.

2.2. CD44

CD44 has become a useful marker for identifying CSCs in AML, colon, head and neck squamous cell carcinoma (HNSCC), and other cancers as well [12,27]. CD44 is a member of a cell adhesion molecule (CAM) family of proteins involved with regulating growth, differentiation, survival, and migration. Bivatuzumab is a humanized monoclonal antibody specific for the anti-CD44v6 isoform of CD44. This isoform in particular correlates with poor prognosis in a number of cancers, including gastric, breast, and colorectal cancer [28,29].

Researchers have recently tried targeting CD44 with Bivatuzumab conjugates with some promising results [30,31,32]. One of these conjugates is a targeted toxin named Bivatusumab mertansine (or BIWI 1), and it has recently been tested clinically to treat head and neck squamous cell carcinoma (HNSCC). BIWI 1 consists of a monoclonal antibody conjugated to a potent maytansine derivative. In the phase 1 study of patients with advanced multi-drug-refractory HNSCC, three patients out of 31 showed significant
improvement in the study with visible tumor regression [31,32]. However, the study was terminated prior to completion following the death of a patient in a parallel study due to toxic epidermal necrolysis. While this study’s objective was to determine toxicity and safety, the fact that some efficacy was observed suggests potential of targeting CD44. However, more work is needed to reduce the limiting toxicities.

2.3. EpCAM

Epithelial cell adhesion molecule (EpCAM, also known as CD326 and ESA) is a well-known overexpressed marker on many carcinomas. Initially thought to simply be a cell-cell adhesion molecule, more recently it has been discovered that EpCAM has diverse roles within cancer cells that range from cell signaling and migration to proliferation and differentiation [33]. Even more interesting are the findings that EpCAM is expressed at an even higher level on CSCs and correlates with increased tumorigenesis versus non-EpCAM positive cells in breast, pancreatic, hepatocellular, HNSCC, and other carcinomas as well [34,35,36]. This finding may not be completely surprising based on the recent evidence showing EpCAM is a direct target in the Wnt/β-catenin signaling pathway, which is a critical pathway in both normal adult stem cells and CSCs [22,37]. Furthermore, EpCAM is an attractive receptor for TTs because it efficiently internalizes when bound by an antibody or an scFv enabling toxin bearing molecules easy access to the interior of the target cells where they can act to induce apoptosis [38]. Recently, a number of anti-EpCAM TTs have been developed and show great promise in both pre-clinical testing and in recent clinical trials.
One of these EpCAM TTs was created by conjugating α-amanitin (a mushroom toxin) to the anti-EpCAM chimerized monoclonal antibody chiHEA125. This targeted toxin called chiHEA125-Ama was then tested against pancreatic carcinoma *in vitro* and in a mouse xenograft model with significant efficacy. Six of ten and nine of ten in the highest concentration groups (50 and 100 µg/kg) showed no visible tumors at day 16 after only two injections of chiHEA125-Ama. It was also found to be potently active against other EpCAM positive carcinoma lines *in vitro* as well. At the doses tested, no observable toxicity was detected in an analysis of blood liver enzymes levels [39]. Pancreatic cancer is the fourth leading cause of death in the United States and with extremely limited therapeutic options available chiHEA125-Ama warrants further development as a potential anticancer agent for pancreatic (and other EpCAM expressing) cancer [40].

An interesting TT was recently developed that combines the same truncated form of pseudomonas exotoxin with an anti-EpCAM DARPin and is named Ec4-ETA [41]. DARPins, or designed ankyrin repeat proteins, are a new class of binding molecules that can be engineered to be highly specific and have very high affinity for a given receptor. They are very stable, easily manipulated, and produce high yields when expressed in *Escherichia coli*. Ec4-ETA is the first known example of a DARPin targeted toxin. Ec4-ETA was tested *in vitro* against a number of EpCAM positive carcinomas and showed IC$_{50}$ values in the sub-picomolar range. It also efficiently localized to colon carcinoma xenografts when given systemically and significantly inhibited tumor growth.

Furthermore, Ec4-ETA was well tolerated and showed no detectable liver toxicity in the mice. This study shows that DARPins could be used in place of antibodies or antibody fragments in TTs as successful, high affinity targeting ligands.
Oportuzumab Monatox, also known as VB4-485, is an anti-EpCAM TT produced by Viventia Biotechnologies that has recently completed three clinical trials [42,43,44]. Oportuzumab Monatox (OM) was produced by fusing a truncated form of Pseudomonas exotoxin A (ETA) to an scFv of the humanized anti-EpCAM antibody MOC31. The first two clinical trials were in patients with grade 2 or 3 stage drug-refractory transitional cell carcinoma of the bladder. In the phase one study, 64 patients were enrolled in dose escalating cohorts where the highest dose given was 30.16 mg. Patients received intravesically administered OM once a week for six consecutive weeks, and were followed for an additional 4–6 weeks and then reassessed. During the study no dose-limiting toxicity was identified and so a maximum tolerated dose (MTD) was not determined. They did find that 77% of the patients did develop human anti-toxin antibodies by the end of the study, with 16% developing human anti-human antibodies. However, upon reassessment at the 12-week time point 24 of the patients (39%) achieved a complete response defined as a nonpositive urinary cytology with either a normal cystoscopy or an abnormal cystoscopy with a negative biopsy [42]. This is impressive especially since the doses in this study were not optimized for efficacy. In the phase two study 46 patients received either 6 or 12 weekly 30 mg treatments. At the end of the study 20 patients (44%) achieved a complete response and 7 patients (16%) were still disease free 18–25 months following the end of the study. Furthermore, no patients had to discontinue treatment due to an adverse effect [43]. OM may prove to be a valuable agent for bladder carcinoma in monotherapy or possibly in combination with other therapies.
In another phase one clinical trial in twenty patients with advanced recurrent HNSCC, OM was administered intratumorally weekly for four weeks. The MTD was determined to be 930 µg with elevated liver enzymes being the dose limiting toxicity. All patients at the end of the study had detectable anti-toxin antibodies and neutralizing antibodies developed in seven of the patients. However, there was a positive response in 87.5% of EpCAM-positive patients. Four patients showed tumor growth stabilization, while 10 others had a notable regression. Another four patients exhibited complete responses to the treatment [44].

Because neutralizing antibodies are a problem for OM, Viventia has developed a new variation of this drug with reduced immunogenicity. This new variant, VB6-845 uses the same anti-EpCAM scFv, but the pseudomonas exotoxin fragment is swapped out for a deimmunized version of Bouganin, a plant toxin that acts through deadenylation of rRNA thus blocking protein translation. Preclinical testing has shown good efficacy and safety and it will be interesting to see how this drug performs in upcoming clinical trials [45]. Overall, OM has exhibited very promising results and may be an effective therapeutic in the treatment of HNSCC as well as transitional cell carcinoma of the bladder.

Our laboratory has developed a bispecific TT that targets both EpCAM and erbB2, the gene product of Her2 that is overexpressed on 30–40% of ovarian and breast cancers [38]. This bispecific, called DTEpCAM23, showed potent picomolar activity in vitro against a range of carcinomas including breast, colon, ovarian, lung, and prostate cancer. DTEpCAM was more effective than either monospecific TT alone or in combination. Furthermore, in two tumor models of colon cancer, DTEpCAM23 significantly inhibited
tumor growth. Bispecific TTs may prove to be very useful moving forward because they can target both CSC and the more differentiated tumor cell populations as well.

2.4. CD133

Another major cancer stem cell marker that has been targeted is CD133, also known as Prominin-1. CD133 is a pentaspan membrane glycoprotein and has been shown to be a marker of the CSC populations in many carcinomas including breast, colon, prostate, liver, pancreatic, lung and HNSCC [12,46]. It is known to be associated with the Wnt signaling pathway because down-regulation of CD133 results in corresponding degradation of β-catenin and decreased proliferation in vitro and in vivo [47,48,49]. However, the specific function of this cell surface receptor is still unknown.

The first known TT that selectively inhibits the CD133+ cell population is called CdtA^{C149A,C178A}BC-CD133MAb. This TT uses the anti-CD133 antibody AC133 conjugated to cytolethal distending toxin (Cdt). Cdt acts as a nuclease and damages host DNA leading to growth arrest and subsequent cell death. In this study, the proliferation of CD133+ HNSCC cells was arrested thus providing a necessary proof of concept showing that CD133 is internalized (a necessary step in TT function) and can be specifically targeted to inhibit cell growth [50].

Our group has recently developed a deimmunized anti-CD133 TT called dCD133KDEL. It combines an scFv from a novel monoclonal antibody (clone 7) with a deimmunized truncated form of pseudomonas exotoxin A [51]. This new monoclonal antibody is unique in that it binds all isoforms of CD133 thus avoiding the controversy surrounding AC133 resulting from its binding to only some undifferentiated epitopes and restricting
its use [52]. By mutating immunogenic epitopes on the pseudomonas exotoxin, we significantly reduced the risk of dCD133KDEL having the same problem many other TTs have had in the clinic: that is the development of neutralizing antibodies against the toxin portion of the protein. In our first paper on dCD133KDEL, we showed its ability to inhibit the proliferation of two HNSCC cell lines in vitro, suppress tumor initiation, and cause significant tumor regression in vivo while only killing the small subpopulation of CD133+ cells. A therapeutic window was demonstrated between dCD133KDEL’s killing of CD133+ cancer stem cells in our time course viability assay and killing of normal CD133+ hematopoietic stem cells in a colony formation assay [51]. In a second paper we showed dCD133KDEL is effective against breast carcinoma in vitro and in a systemic mouse model as well [53]. We have also tested this TT against the ovarian cancer line OVCAR5 in vitro and in vivo with very good preliminary results [54]. It also has an excellent safety profile that makes it possible to give multiple courses of 20 µg injection per nude mouse, where one course is 3 weekly injections given Monday, Wednesday, and Friday. The MTD in athymic nude mice was determined to be 2.0 mg/kg (5 times the dose used in vivo) and the limiting toxicity at this dose is liver toxicity indicated by elevated Alanine Transaminase levels (unpublished data). Table 2-2 summarizes the various tumors and model systems we have investigated to date using dCD133KDEL. This new CSC specific TT shows significant potential as a possible therapeutic for carcinomas where CD133 has been shown to be a marker for CSCs.

3. Conclusions
CSCs have been shown to be more resistant to chemotherapy and irradiation than more differentiated cancer cells that make up the bulk of the tumor. CSC then may be at the root of our most serious problem in cancer, drug refractory tumor relapse. Thus, it would be perilous to ignore them as therapeutic targets. TTs may prove to be uniquely qualified to fill this role because of their extremely high potency and exquisite selectivity.

A major concern of drugs that target CSC surface markers is that they would also kill normal adult progenitor cells that also express these same markers. In our work on dCD133KDEL, we partially addressed this concern by testing umbilical cord blood cells that were 76% CD133+. At a concentration 100 times greater than used to inhibit the proliferation of HNSCC cells \textit{in vitro}, the normal human hematopoietic stem cells were not inhibited [51]. There are several hypotheses that could explain this therapeutic window. First, the CD133+ cells could have been killed and then replaced by the CD133- cell population. Two different groups have demonstrated this type of plasticity in progenitor cells recently [55,56]. Second, normal hematopoietic progenitors may be more quiescent and have a slower endocytic uptake than CSC and thus not be as effected by the toxin. Finally, the CSC may express CD133 at a higher level then the normal progenitor cells, which is true in colorectal, pancreatic, gastric, and hepatocellular carcinomas [57]. Each of these separately or in combination could explain the ability for dCD133KDEL to specifically kill CSC and not normal hematopoietic progenitors.

In the broader picture, each target will have a different therapeutic window, but our work shows significant promise that CSCs can be selectively eliminated. However, several strategies exist to limit the toxicity of systemically administered drugs. We described a method called ToxBloc where an intraperitoneal pre-dose of the ligand without toxin was
given prior to injection with the targeted
toxin. This allowed us to give doses 15-fold higher than the maximal tolerated dose [58].
Other possible methods include photochemical internalization and ultrasound triggered
drug delivery via microbubbles [59,60].

Since many tumors are phenotypically diverse, the use of multiple drugs in combination
may be necessary to successfully increase the percentage of tumor regressions in patients.
It is already established that TTs work synergistically with chemotherapy [61,62]. So,
new TTs that selectively attack CSCs could be an important weapon to combat drug
refractory relapse.
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<td>Phase I</td>
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Table 2-1 Targeted Toxins directed against CSC markers currently under investigation.

Notes: Several laboratories are now investigating a range of different approaches and toxins targeting cancer stem cell associated markers. In several of the preclinical studies, more than one cancer type was investigated.
<table>
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**Table 2-2** A summary of the cancer and model types used to date by our group in evaluating the efficacy of dCD133KDEL.

Notes: Our group has published independent reports using 3 different xenograft models to assess the efficacy of dCD133KDEL in immunodeficient mice.
Preface

This chapter has been published:

Chapter III

Targeting Tumor-Initiating Cancer Cells with dCD133KDEL Shows
Impressive Tumor Reductions in a Xenotransplant Model of Human
Head and Neck Cancer
Introduction

Evidence has mounted over the last decade that cancers contain a small subset of their own stem-like cells termed cancer stem cells (CSC). These CSC are known to enhance tumor initiation, self-renew, and also differentiate into phenotypically diverse cancer cells with more limited proliferative potential [63-65]. A point of controversy regarding CSC is the existence of plasticity between stem cells and their more differentiated derivatives causing some to believe that more differentiated cancer cells can become reprogrammed and revert back to CSC [66]. CSC are more resistant to current chemotherapy agents and have distinct cell surface markers [12,21]. Because of this, it is important to develop new therapeutics to specifically target CSC. CD133 (prominin-1) is a 5-transmembrane glycoprotein whose function remains unknown. It is expressed on hematopoietic, endothelial, and neuronal stem cells [67-69] and has recently been identified as a CSC marker in a variety of carcinomas as well [12,13,70-72].

Targeted toxins (TT) are a class of biological drug consisting of a catalytic protein toxin chemically or genetically linked to a ligand recognizing a specific marker expressed on cancer cells [73]. The catalytic destruction of the target cell is dependent on the internalization of the target receptor. A recent study has shown that antibodies targeting the CD133 receptor are efficiently internalized [74]. Other studies have shown that CD133+ cells possess a very strong ability to initiate tumors, but CD133- cells do not [75,76]. Thus, we reasoned that a targeted toxin directed against CSC via CD133 might prove highly disruptive to the dynamic process of tumor initiation and progression.

A novel targeted toxin called deimmunized CD133KDEL (dCD133KDEL) was synthesized using an anti-CD133 scFv reactive against loop two of the extracellular
domain of CD133. This scFv was taken from the monoclonal antibody that has been shown to recognize both the glycosylated and unglycosylated forms of CD133 [52]. This scFv was then cloned onto the same molecule containing a truncated form of pseudomonas exotoxin A (PE38) that has been successfully established as a clinically useful toxin [77]. Studies show that fewer than 1000 molecules of PE38 delivered to the cytosol are sufficient to bring about cell death [78]. In addition, we added a Lys-Asp-Glu-Leu (KDEL) C-terminus signal to our drug that provides enhanced tumor killing by preventing luminal ER protein secretion [79]. Also, genetic engineering was used to address a major shortcoming of targeted toxins, their immunogenicity. To accomplish this, the PE toxin was mutated to remove immunogenic epitopes that were recently mapped [80] Studies show that three separate TT made with this deimmunized variant have highly reduced anti-toxin levels in mice despite multiple treatments with the drug compared to the non-mutated parental form [81-83].

In this paper, we show that dCD133KDEL specifically kills CD133+ tumor initiating cells and can arrest the proliferation of head and neck carcinoma cells in vitro and in vivo. dCD133KDEL is a novel deimmunized TT that target tumor initiating cells and is effective at preventing tumorigenesis and treating established tumors. This represents a significant and highly selective tool that can be utilized to study stem cell populations and as a possible clinical adjunct to chemotherapy.

Materials and Methods

Construction of dCD133KDEL
dCD133KDEL was synthesized from the fusion of DNA fragments encoding the scFv portion (anti-CD133scFv) of clone 7 [52] and a deimmunized, truncated form of pseudomonas exotoxin 38 [81-83]. The assembled fusion gene contained (5’ to 3’) an NcoI restriction site, the ATG initiation codon, the gene for CD133 scFv, the DNA sequence encoding a seven amino-acid EASGGPE linker, the gene encoding for the first 362 amino acids of truncated deimmunized with the DNA sequence for KDEL replacing the REDLK at the c-terminus, followed by a NotI restriction site at the 3’ end of the fusion gene. The resulting 1846 base pair gene was spliced into the pET28c bacterial expression vector containing an inducible isopropyl-b-D-thiogalactopyranoside T7 promoter and a kanamycin selection gene (Figure 3-1A). To verify that the dCD133KDEL gene had been cloned correctly and in frame, DNA sequence analysis was performed at the University of Minnesota BioMedical Genomics Center. The CD133scFv was separately cloned into the pET28c bacterial expression vector and produced to determine CD133 expression of various cell lines in flow cytometry studies.

**Purification of CD133scFv and dCD133KDEL**

Purification of CD133 scFv and dCD133KDEL was performed as described previously [84]. Briefly, each protein was expressed and purified from inclusion bodies using a Novagen pET expression system (Novagen, Madison WI) followed by a 2-step purification consisting of ion exchange fast protein liquid chromatography (Q sepharose Fast Flow, Sigma) and size exclusion chromatography (Hiload Superdex 200, Pharmacia). The purified protein was then analyzed by SDS-PAGE and stained with Commasie Brilliant Blue to determine purity.
Cell Lines and Culturing Technique

UMSCC-11B is a squamous cell carcinoma cell line that was derived from larynx tumor following chemotherapy [85]. UMSCC11B-luc were transfected using a luciferase reporter construct, and were maintained under 10ug/ml of blastocidin. Cells were transfected using Invitrogen’s Lipofectamine™ Reagent. NA-SCC is another squamous cell carcinoma line isolated from a tongue tumor [86]. Dr. Frank Ondrey (University of Minnesota) graciously supplied these two lines. Caco-2 (a colorectal carcinoma) and HEK293 (a human embryonic kidney cell line) were obtained from ATCC. Only cells that were greater than 90% viable were used for experimentation.

Flow Cytometry and CD133+ Cell Enrichment

Flow cytometry was performed using a FACS Caliber at the University of Minnesota’s Flow Cytometry Core Facility. FITC-labeled antibodies were used and results were analyzed using FLOWJO. Sorting was performed using a magnetic bead selection kit following manufacturer’s instructions (Stem Cell Technologies, Tukwila, WA). Briefly, cells were concentrated to 2 x 10^8 cells/mL. FITC-labeled CD133scFv was added to cells at a concentration of 1.0ug/mL and incubated for 15 minutes. The EasySep FITC Selection Cocktail® was then added followed by the EasySep® Magnetic Nanoparticles after a 15 minute incubation. The cells were mixed and then placed within the magnet. Unbound cells were eluted and bound cells collected.

Time Course Viability Assays
Trypan blue viability assays were performed by plating 10,000 cells/well into 24 well plates. Toxin and media were replaced daily at 0.01nM for NA-SCC cells or 0.03nM for UMSCC-11B cells. Wells were harvested every other day using trypsin digestion and counted on a hemocytometer via trypan blue staining. Untreated wells typically became confluent around day 8. For the time course viability assays on CD133 negative sorted cells, UMSCC-11B cells were incubated with CD133 scFv-FITC and sorted using a FACS ARIA at the University of Minnesota’s Flow Cytometry Core Facility. 20,000 cells/well were sorted into 24 well plates. The rest of the time course viability assay was performed as described above.

**Hematopoietic Colony-Forming Unit Assays**

Two types of assays were performed: short-term (2 week) hematopoietic colony-forming assays (CFC) and long-term (5 week) culture assays (LT-CIC) [87]. For CFC assays, progenitor cells were sorted from human umbilical cord blood (UCB). CD34+ cells were sorted using magnetic selection, and collected in MethoCult GF+ H4435 (StemCell Technologies, Vancouver, BC, Canada; Cat. No. 04435) consisting of 1% methylcellulose, 30% FBS, 1% BSA, 50ng/ml stem cell factor, 20ng/ml granulocyte–macrophage colony-stimulating factor, 20ng/ml interleukin (IL)-3, 20ng/ml, IL-6, 20ng/ml granulocyte colony stimulating factor, and 3U/ml erythropoietin. Targeted toxin was added at the desired dosages and cultures were incubated at 37°C, 5% CO2 for 2 weeks and then scored for colony-forming units according to standard criteria after 2 weeks. CFU-GEMM, CFU-GM, and BFU-E were counted individually. For LT-CIC
assays, were cultured in commercial Myelocult media from StemCell Technologies with M2-10B4 stromal cells. Colonies were quantitated after 5 weeks.

**Tumor Initiation Experiment**

For the tumor initiation study, UMSCC-11B squamous carcinoma cells were injected into the right flank of nude mice at a concentration of 300,000 cells/mouse. Tumor size was measured using digital calipers and tumor volume (width x length x height) was calculated. Prior to injection cells were treated with 0.03nM of either dCD133KDEL or the non-specific toxin, CD22KDEL. A third group of cells was left untreated and unsorted. For the sorted group, cells were sorted for CD133 expression with a magnetic bead kit as previously described, plated overnight, harvested, and injected the following day.

**Tumor Treatment Study and Imaging**

For experiment 1, nude mice were injected with 3 million UMSCC11B-luc cells into the right flank. Tumors were treated with dCD133KDEL, CD3CD3KDEL, or PBS starting on day 8 when they had become palpable. A single course of treatment consisted of an injection of 20ug of drug given every other day (MWF) and mice were given a total of 8 injections. Controls were treated with PBS. Tumor volume was measured with calipers over time. For experiment 2, nude mice were injected with 6 million UMSCC-11B-luc cells in the right flank. Tumors were treated starting on day 10 post-inoculation. Treatment was done as in experiment 1, except mice received 16 injections. Also, an anti-B cell control, CD19KDEL, was used. Tumor volume was calculated prior to treatment.
Since tumors were marked with a luciferase reporter gene, mice were imaged to determine their bioluminescent activity as described previously (81). Briefly, mice were injected with 100ul of 30mg/ml luciferin substrate 10 minutes prior to imaging and then anesthetized via inhalation of isoflurane gas. The mice were then imaged using the Xenogen Ivis 100 imaging system and analyzed with Living Image 2.5 software (Xenogen Corporation, Hopkington MA). Five-minute exposures were made and units for the regions of interest (ROI) were expressed as photons/sec/cm$^2$/sr. For experiment 1, tumor treatment was stopped on day 24. For experiment 2, treatment was stopped on day 45.

**Histology**

Suspected tumor free survivors were sacrificed and for histology evaluation at the termination of the experiment. Liver, Kidney, and skin (from the tumor site) samples were taken. These samples were embedded in OCT compound (Miles, Elkhark, IN), snap frozen in liquid nitrogen, and stored at -80°C until sectioning. Sections of the tissues were cut, thawed, and mounted on glass slides. Slices were fixed for 5 minutes in acetone and then stained with Hematoxylin and Eosin (H&E).

**Determining immunogenicity of deimmunized CD133KDEL**

To determine whether mutated CD133KDEL elicited less of an anti-toxin response than non-mutated parental toxin, female Balb/c (n=5/group) were injected intraperitoneally once weekly with 0.25 μg of either deimmunized CD133KDEL or non-mutated parental PE38KDEL toxin. Each week, five days after injection, the mice were bled via face vein.
Serum was isolated using centrifugation, frozen, and the level of anti-PE38KDEL IgG in each serum sample was measured by ELISA. Briefly, PE38KDEL was bound 96-well microtiter plates at a concentration of 5μg/ml overnight at 4°C. Unbound protein was washed away with PBS-T and blocking was performed for 1 h with 5% milk/PBS-T. Serum samples were diluted appropriately and 100 μl of each was added to wells in triplicate. After 3 hours, each well was washed with PBS-T. Peroxidase-conjugated rabbit anti-mouse IgG (Sigma) was added to each well. After 2 hours samples were washed and o-Phenylenediamine dihydrochloride substrate was added. After 30 min, the absorbance at 490 nm was measured using a microplate reader. Quantification of actual anti-PE38KDEL IgG present in each sample was determined by comparing the absorbance values in each well to a standard curve prepared using M40-1 monoclonal anti-PE38KDEL antibody from Dr. Robert Kreitman (NIH, Bethesda, MD).

Results

Anti-CD133 scFv construct binds selectively

SDS-PAGE analysis showed that following ion exchange and size exclusion chromatography, dCD133KDEL was greater than 95% pure (Figure 3-1B). To establish that the anti-CD133 scFv construct recognized the CD133 receptor, HEK293 cells were transfected with a gene encoding the CD133 receptor. Figure 3-1C is a histogram plot showing a high degree of reactivity of the anti-CD133-FITC with the transfected cells. Anti-CD22-FITC, however, did not bind the transfected cells. Interestingly, it appears that there is a high intensity and a low intensity peak indicating populations with high and low antigen densities. Table 3-1 shows the expression of
CD133 on various cell populations measured by flow cytometry. UMSCC-11B and NA-SCC cell lines were 4.0% and 5.9% CD133+, respectively. These cells were tested with anti-CD19-FITC as a negative control and were less than 1.2% CD19+. Caco-2, a human colorectal cell line, was tested as positive control and was 79% positive for CD133 expression. These numbers are from representative experiments that have been duplicated at least 3 times each. Together, these findings showed that the CD133 scFv is specific to the CD133 receptor that is selectively expressed on a small subpopulation of cancer cells.

**Time Course Viability Assays Show Proliferation Inhibition**

If CD133 is a marker for CSC, then selective killing of this subpopulation of cells should be sufficient to inhibit proliferation [65]. To test this, dCD133KDEL was added to cells in a trypan blue viability time course assay to determine the number of viable cells. As seen in Figure 3-2A, the proliferation of UMSCC-11B cells was almost completely ablated, while a non-specific control toxin targeting CD22 did not inhibit cell proliferation. dCD133KDEL had the same effect on NA-SCC cells, another head and neck carcinoma line (Figure 3-2B). To determine whether dCD133KDEL was having off-target effects, we sorted UMSCC-11B cells for CD133- cells and performed a time course viability assay. As seen in Figure 3-2C, the targeted toxin showed no effect on the CD133- sorted cells. These plots show representative experiments that have each been reproduced.

**Effect on Human Progenitor Colony Formation**
CD34+ UCB cells are considered a rich source of normal hematopoietic progenitor cells. To determine the effect of dCD133KDEL on the development of hematopoietic cells derived from CD34+ progenitors, sorted CD34+ cells were treated with drug and evaluated in two standardized assays of hematopoietic stem/progenitor cell survival and function. Notably, the UCB CD34+ cells used for this study were first studied using 2-color flow cytometry and 67% coexpressed CD34 and CD133, twenty-four percent were CD34+CD133- and none of the cells were CD34-CD133+ (data not shown). dCD133KDEL drug concentrations were selected based on the results in Figure 3-2. Initially, studies of hematopoietic colony formation as a measure of hematopoietic progenitor cells were done by incubating the UCB CD34+ cells with dCD133KDEL Media and drug were renewed weekly in each well. After 2 weeks, hematopoietic colonies were counted. Figure 3-3 shows that only the cells cultured in the presence of intact DT toxin underwent significant reductions in colony formation. Even at concentrations of dCD133KDEL that greatly exceeded those used to inhibit cancer stem cells in the trypan blue viability assays, there was minimal effect on CFU-GM, CFU-GEMM, and BFU-E colony development. Additionally, we added dCD133KDEL to 5-week long-term culture initiating cell (LT-CIC) assays that better quantify putative hematopoietic stem/progenitor cells. As with the CFC study, there was minimal effect of dCD133KDEL on human LT-CICs after 5 weeks in culture. These experiments were reproduced with similar results (data not shown). Together, these results suggest that dCD133KDEL is not destructive to normal human hematopoietic stem/progenitor cells that may express CD133.
CD133+ Cells Efficiently Initiate Tumors

CD133+ CSC possess the unique ability to initiate tumors [75,76]. Thus, a tumor initiation study was performed to determine whether cells pretreated with dCD133KDEL would form tumors in vivo (Figure 3-4). UMSCC-11B cells were pretreated with 0.03nM of dCD133KDEL for 6 days prior to inoculation in the flanks of nude mice. Control mice were injected with either untreated cells or cells treated with a non-specific toxin targeting CD3. UMSCC-11B cells were also enriched for CD133+ cells using magnetic bead separation prior to inoculation. When cells were sorted using the magnetic bead separation and run using flow cytometry, the expression of CD133+ cells increased 4 fold (data not shown). As seen in Figure 3-4, cells treated with dCD133KDEL prior to injection had the lowest incidence of tumor formation (1 of 4). The single tumor that formed was markedly smaller than tumors formed by the control or CD133 enriched cells. CD133+ enriched cells formed tumors at the highest incidence, formed earlier and grew much larger than control tumors. These results further indicate that we are killing CSC and support the fact that CD133 is a CSC marker in head and neck carcinomas.

Tumor Treatment Studies Show Impressive Tumor Regression

In experiment 1, animals given intratumoral injections of 20ug/injection of dCD133KDEL regressed and were gone by day 24 when treatment was stopped (Figure 3-5). In contrast, control tumors given either PBS (M1-M3) or treated in an identical fashion with anti-T cell CD3CD3KDEL (M4, M5) did not regress. Real time imaging data are shown and correlated with caliper data shown in the plot. Again, no tumor was detected by day 24 in treated mice, confirming the caliper measurements. In experiment
2, animals were given twice the number of tumor cells, so tumors grew faster, and treatment was delayed until day 10. Although tumors grew more aggressively, all mice treated with dCD133KDEL responded to treatment. Mice treated with control CD19KDEL did not. Together, the two experiments showed that by killing the CD133+ subpopulation of cells, the tumors were unable to generate more cells and eventually regressed.

Histological analysis was performed on tumors from experiment 1. Figure 3-5B shows a skin section from a mouse, taken on day 59, in which an epithelial-like tumor is prominent. The tumor is robust and vascularized. Other sections revealed necrosis. Also shown is healthy skin from a mouse that was treated intratumorally with dCD133KDEL, which prevented tumor growth and eventually led to complete tumor elimination. The skin pathology looks mostly normal with intact hair follicles, although some evidence of inflammation is still present. The section is part of the histological analysis that confirms the animal was tumor free. The livers and kidneys of both treated and non-treated animals showed no evidence of tumor indicating that it did not metastasize to these organs.

**Anti-toxin Levels Greatly Reduced in dCD133KDEL Immunized Mice**

To ensure that we had effectively deimmunized dCD133KDEL, BALB/c mice were immunized with either mutated dCD133KDEL or parental non-mutated PE38KDEL. Mice (n=5/group) were immunized intraperitoneally once a week and were bled on day 63. Despite 9 weekly immunizations with drug (0.25 ug/injection), the dCD133KDEL group had significantly lower serum anti-toxin levels than the animals immunized with
parental toxin (Figure 3-6). Generally, deimmunization resulted in an average 90% reduction in anti-toxin levels.

**Discussion**

The major contributions of this manuscript are the development of a novel targeted toxin, dCD133KDEL, which targets CD133 tumor-initiating cells and its efficacy against two head and neck carcinoma lines. dCD133KDEL is a new and powerful reagent for three main reasons. The drug 1) targets only a small subpopulation of the total tumor, 2) has been successfully deimmunized bypassing a major clinical problem with targeted toxins, and 3) has a mechanism of action unlike typical chemotherapy agents. Targeted toxins function by binding to cell surface receptors, internalizing, and enzymatically inhibiting protein synthesis [88]. CD133 is readily internalized rendering it an excellent marker for a targeted toxin [73]. The fact that we observed heterogenous peaks in CD133 expression in transfected HEK cells in Figure 3-1C supports the argument that CD133 cycles and internalizes.

Bonnet and Dick reported the first CSC subpopulation in leukemia in 1997 [23]. Since then investigators have validated the presence of CSC subpopulations not only in leukemia, but in many carcinomas as well. CD133 in particular has been identified as a CSC marker in these carcinomas [12, 13, 70-72]. Wei et al showed in their work that CD133 is a CSC marker for the head and neck laryngeal cancer line HEP-2. In their study, they showed that CD133+ sorted cells initiated tumors and uniquely possessed clonogenic capacity when compared with CD133- cells [75]. We were able to confirm CD133 as a marker for tumor initiation cells in the current study with a different head and neck cell line UMSCC-11B.
Also unique to our study is the use of a new monoclonal antibody (clone 7) that binds all forms of the CD133 receptor [52]. This is important because commercial antibodies currently available recognize an epitope that can be masked upon differentiation [89]. In contrast, the scFv from clone 7 was used in our construction of dCD133KDEL recognizes only the CD133 peptide backbone, avoiding the issue of epitope masking or differential glycosylation. Specificity was determined by showing that our anti-CD133 scFv recognized cells transfected with DNA encoding the CD133 receptor. Furthermore, two head and neck cell lines contained subpopulations of CD133 expressing cells at similar levels to other head and neck cell lines described in the literature [75, 76, 90]. When dCD133KDEL was tested in vitro, we discovered that it selectively inhibited cancer cell expansion in both cell lines. We found that about 5% of the UMSCC-11B head and neck cancer cells used in the study were Annexin V positive following CD133KDEL treatment indicating apoptotic death. Since flow studies also showed that about 5% of the UMSCC-11B population were CD133+, this suggests an apoptotic death for CD133 cells. We also attempted dual staining to confirm that the same population that was Annexin positive was CD133 positive. However, these mechanistic studies proved complicated since earlier treatment with dCD133KDEL interfered with our ability to later recognize and quantitate CD133+ cells. We attributed this difficulty to either blocking interference or the high modulation rate of CD133.

Since tumor initiation is a hallmark of CSC, UMSCC-11B tumor cells were pretreated with dCD133KDEL and transplanted into nude mice. These cells formed the lowest incidence of tumors when compared with controls, while cells enriched for CD133+ expression formed the largest tumors at the fastest rate. Furthermore, in two
separate studies tumor cells were injected into the flanks of nude mice and when palpable were directly treated with dCD133KDEL. All treated tumors regressed over time, unlike control tumors and all treated animals were impressively tumor free after 79 days. We favor the explanation that dCD133KDEL acts to inhibit the self-renewing ability of CD133+ CSC. However, it is possible that dCD133KDEL has a bystander killing effect but this was not supported by our in vitro studies showing that dCD133KDEL did not kill CD133 negative cancer cells.

Several studies suggest the existence of another CSC population in some tumors that is CD133- [91,92]. For example, Chen et al discovered CD133- cells within glioblastoma primary tumors that had the capability to self-renew and support long-term growth in vitro and initiate tumors in vivo [92]. dCD133KDEL may be a helpful biological tool in validating the existence of these cells and in helping to develop more inclusive therapies that may target all CSC and progenitors simultaneously.

The development of a novel TT that selectively targets CSC may have unique implications for the ubiquitous problem of carcinoma drug resistance and subsequent relapse. Several studies have shown that CSC are resistant to current chemotherapeutic agents [12,13,21,93,94]. Since TT’s work by a different mechanism and because it selectively kills CSC, dCD133KDEL may possess the unique ability to target the very cells responsible for drug resistance. Adding a TT as an adjunct to chemotherapy has been shown to be more effective than using either therapy alone [61,62]. Studies are underway to determine whether dCD133KDEL may be a useful adjunct to chemotherapy.

One of the major limitations of TT in clinical therapy is immunogenicity. Patients will start developing antibodies to the toxin and thus the number of treatments is limited.
To address this problem, we mutated 7 immunogenic epitopes that account for the majority of antibodies produced against this form of PE toxin. We then used this mutated construct to create our fusion protein. As a result, we showed that even after 9 immunizations there is very little antibody produced against the toxin moiety of dCD133KDEL and 40% of the animals had no antibody response at all.

Drug safety issues regarding the effects of dCD133KDEL on normal human hematopoietic progenitor cells are important and need to be addressed prior to any therapeutic consideration. Therefore, we isolated CD34+ cells from UCB an established source of normal human hematopoietic stem cells, cultured them with dCD133KDEL and then determined their ability to form various hematopoietic colonies in established CFU (colony-forming unit) assays. To be sure, we changed media containing drug weekly and cultured the cells in long-term colony initiation assays for 5 weeks. Only toxin alone inhibited survival of hematopoietic progenitor cells. These results indicate that although the UCB CD34+ cells co-express CD133, these cells are resistant to treatment with dCD133KDEL. These findings could be explained by multiple normal stem cell populations that are CD133- since 24% of the starting stem cells were CD34+CD133-.

Rutella et al discovered a stem cell population in human cord blood that was CD133- and still capable of differentiating into multiple cell types [55]. Also, Surronen et al showed that CD133+ cells can be generated from normal CD133- cells [56]. Perhaps dCD133KDEL destroyed normal CD133+ cells and replacements emerged from the CD133- fraction. Alternatively, CD34+CD133+ cells may be metabolically quiescent enough or have other drug resistance mechanism to prevent significant killing by dCD133KDEL. Finally, the expression level of CD133 on tumor cells may be greater
than the expression level on normal progenitors [57]. This could allow for the observed selective killing of CSC over normal stem cells. Sehl et al, used mathematical modeling to conclude that in order for a treatment to be safe it must be highly selective and able to target quiescent cancer stem cells [95] and since dCD133KDEL is selective and because cells do not have to be actively cycling for targeted toxins to be taken up and effectively cause apoptosis, dCD133KDEL warrants further investigation.

Several cell populations in the body express CD133 and therefore may be potential targets for the toxin, causing either short-term or long-term health effects. Thus, extensive toxicity studies will be necessary to validate a CD133 targeted drug for clinical use. These studies will not be trivial. CD133 expression on normal cells has proven somewhat complicated since CD133 has been shown to undergo post-translational modulation such that mRNA and internal protein expression does not correlate with the surface expression [96]. Also, the commercial antibodies such as AC133 only bind certain forms of the receptor [52,89]. Furthermore, studies in an “on target” model closer to humans than rodents may be necessary.

In summary, we developed a novel deimmunized TT that selectively binds the cancer stem cell marker CD133. dCD133KDEL impressively inhibited cell proliferation in vitro, decreased tumor initiation, does not kill normal human hematopoietic progenitor cells, and caused complete tumor regression in a an accepted model of human head and neck cancer which is an xenotransplant flank model. This work represents the development of a new cancer therapeutic that function by selectively targeting the minority cancer stem cell subpopulation within the tumor. We believe dCD133KDEL
warrants further study as a possible solution for drug resistant relapse in human carcinoma.
Figures

A. Targeted toxin construction
- anti-CD133
- PE38KDEL 7mut
- dCD133KDEL, pET28c

B. SDS-PAGE

C. Transfected HEK293 cells
Figure 3-1 A, plasmid map for dCD133KDEL shows the gene position. B, a large single peak of protein detected at an absorbance of 280 nm was collected and then analyzed by SDS-PAGE under nonreducing conditions. The gel lanes from left to right are: lane 1, PE38KDEL 7mut; lane 2, CD133 scFv; lane 3, dCD133KDEL; and lane 4, molecular weight standard. C, flow cytometry study showing that the anti-CD133 scFv recognizes the CD133 receptor. HEK cells were transfected with a gene encoding the CD133 receptor and then reacted with the anti-CD133 scFv labeled with FITC. As controls, cells were also reacted with RFB4 (anti-B cells)-FITC or a no treatment blank. Flow cytometry was done using a FACS caliber and data plotted as a histogram. The number of events is 20,000.
Table 1. Cancer cell lines

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NOTE: CD133 expression was measured on various human carcinoma lines. The anti-CD133scFV was tagged with FITC and then reacted with the head and neck cancer cell lines NA-SCC and UMSCC-11B. The Caco-2 colorectal carcinoma was included as a positive control cell line because it is known to overexpress CD133. Controls included cells reacted with anti-EpCAM-FITC, anti-CD45-FITC, and anti-CD19-FITC. Expression of CD45 and CD19 is mostly restricted to normal malignant hematopoietic cells and thus served as negative controls.

Table 3-1
Figure 3-2 Trypan blue viability studies show that dCD133KDEL selectively inhibits head and neck cancer cells over time. UMSCC-11B (A) or NA-SCC (B) cells were incubated with dCD133KDEL and viability was determined over time by directly counting cells growing in tissue culture wells using a trypan blue vital stain. Live cells exclude the dye and dead cells incorporate it. CD22KDEL, CD19KDEL (anti-B cell), and CD3CD3KDEL (anti-T cell) targeted toxins were included as negative controls. Media and toxin were replenished daily. In C, UMSCC-11B cells were sorted for CD133− cells and immediately incubated with dCD133KDEL to determine nonspecific killing. dCD133KDEL did not inhibit CD133− UMSCC-11B cells treated immediately following sorting. Error bars were calculated for all data points, but are not visible in some instances because error margins were very slight.
**Figure 3-3** dCD133KDEL does not inhibit hematopoietic colony development. CD34+ sorted umbilical cord cells were cultured in the presence of dCD133KDEL under optimized colony growth conditions. Two types of assays were done over the entire course of the culture period. The first was short-term (2 weeks) colony formation assay measuring CFU-GEMM (A), CFU-GM (B), and BFU-E (C). The second was a long-term (5 weeks) LTC-IC assay (D). Colonies were visually scored in a blinded study.
**UMSCC-11B cells**

<table>
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</tr>
<tr>
<td>dCD133KDEL-depleted</td>
<td>1 of 4 (25%)</td>
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<tr>
<td>Controls</td>
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Figure 3-4 Study of the tumor initiation ability of CD133+ cells, a hallmark of tumor stem cells. Groups of nude mice (4–8/group) were injected in the flank with CD133-enriched cells. CD133+ cells were enriched from cultured UMSCC-11B cells using magnetic bead separation. These had the highest tumor incidence and rate of growth. Another group received cells treated with dCD133KDEL before implantation. These had the lowest incidence and lowest growth rate. The controls received unsorted tumor cells that were either untreated or pretreated with an irrelevant control targeted toxin, CD3CD3KDEL. All groups received 300,000 cells. The plot shows the tumor growth rate over time and the table within the figure shows the tumor incidence. The mean tumor volume of the CD133+ sorted group is statistically different than the control group and the dCD133-depleted group using the Student's t tests (P < 0.0045 and 0.0012, respectively).
**Figure 3-5** Tumor treatment studies show tumor regression. In A, tumor size was measured for experiment 1 using digital calipers. A student's paired t test was done on the average tumor size over the course of the experiment between the control and treated groups. The 2 curves are significantly different with a P value of 0.0001. Experiment 1 (B) and experiment 2 (C) are shown with tumor bioluminescent and images showing that dCD133KDEL inhibits the progression of UMSCC-11B-luc nude mice flank tumors. For experiment 1 (B), 3 million cells were injected and tumors were treated with dCD133KDEL (M6–M10) while controls were treated intratumorally with either PBS (M1–M3) or the anti-T CD3CD3KDEL (M4–M5) starting on day 8. This also shows that dCD133KDEL inhibits the progression of flank tumors and correlates with the caliper data. The arrows indicate where treatment (Tx) began and ended. For experiment 2 (C), tumors were induced by injection of 6 million cells. Intratumoral treatment was begun on day 10. Tumors were treated exactly as they were in experiment 1 except mice were given 16 injections instead of 8. Controls were untreated or treated with anti-B cell CD19KDEL. Animals were imaged weekly for both experiments. Bioluminescence intensity was measured. D, histology photomicrograph shows skin tissue taken from the tumor injection site of a representative tumor-free mouse from experiment 1. As a control, a photo is shown of skin tissue taken from a PBS-treated control mouse. Tumor is clearly visualized in controls, but absent in the drug-treated mouse.
Figure 3-6 The immunogenicity of dCD133KDEL. The comparative ability of mutated dCD133KDEL and nonmutated parental toxin PE38KDEL to induce the production of antitoxin antibodies in normal, immunocompetent mice was determined by measuring anti-PE38(KDEL) serum IgG levels on samples of mice immunized weekly with 0.25 μg of either drug (n = 5 group). Animals were bled on day 63 after 9 weekly immunizations. Serum IgG antitoxin levels were measured by indirect ELISA and quantification of antibodies was determined using a standard curve. Differences in the data sets between the PEKDEL-immunized and the dCD133KDEL groups were significant by Student's t test (P < 0.05).
Preface

This chapter has been published:


My contributions to this work were the experiments used as figures 4-3 and 4-4a. All other work contained in this chapter belongs to the other authors of this manuscript.
Chapter IV

Identification and characterization of a novel scFv recognizing human and mouse CD133
Introduction

Human CD133 is a penta-span transmembrane glycoprotein. It is a marker of neural, endothelial, and hematopoietic stem cells [67,68,69,97]. Its expression is also seen in cancer initiating cells in many tumors including that of brain, prostrate, colon, and breast [13,70,72] and is, therefore, used widely as a molecular marker to isolate and characterize these cell phenotypes. Targeting CD133 may also enable the enhanced delivery of cytotoxic therapies to cancer initiating cells [51,53]. Further development of CD133 as a diagnostic marker and/or therapeutic target requires highly sensitive reagents that exclusively recognize CD133. Currently, AC133 and 293C3 antibodies are widely used to study CD133 [98]. These reagents identify glycosylated epitopes of CD133 and not the native protein. Hence, the reliability of these reagents for the accurate detection of CD133 protein levels is a major concern [89,98,99]. To overcome this limitation, we have earlier identified a hybridoma (clone 7) that specifically recognizes CD133 in a range of cell-based assays [52]. Clone 7 recognized both the glycosylated and non-glycosylated epitopes of CD133 protein and, hence, could be used to investigate CD133 expression with greater reliability. For applications such as targeted drug delivery, single-chain variable fragments (scFv) are more convenient and more effective than larger antibodies. In this work, we employed phage display to derive a scFv targeting CD133. Our studies show that the new scFv is capable of recognizing both human and mouse CD133 protein and can be potentially used as a diagnostic tool to characterize CD133 expression.
Materials and Methods

Generation of ScFv library from CD133 hybridoma

The scFv was generated from anti-CD133 hybridoma clone 7 cells [52]. Total RNA was isolated from these cells using an RNeasy micro kit (Qiagen), and first-strand cDNA synthesis was carried out using oligo (dT) 20 primer and Superscript II reverse transcriptase (Invitrogen). Following this, a unique two-step PCR protocol was used to amplify the scFv-encoding gene repertoire. DNA sequences encoding the variable region of light-chain (VL) and heavy-chain (VH) fragments were amplified independently, fused by an overlap extension PCR, and cloned directionally into pCDisplay-4 vector using the rare cutter SfiI enzyme. The digested vector and PCR amplicons were ligated using T4 ligase, precipitated by sodium acetate–ethanol mixture along with glycogen and yeast tRNA carriers, and then transformed into electrocompetent Escherichia coli XLIBLUE. After overnight culture at 37 °C, the amplified recombinant phagemid was extracted from bacteria using a Qiagen kit and stored at −20 °C. When required, E. coli transformed with the library was infected with a VCSM13 helper phage to package the RNA into phage particles.

Library panning against human CD133

Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates were coated with 2 μg of human CD133 protein in 50 μL of 0.1 M NaHCO3 (pH 8.6) and allowed to incubate overnight at 4 °C. The next day, the protein solution was removed and the wells blocked with a blocking buffer containing 1 % bovine serum albumin (BSA) in TBS. The plates were sealed and incubated overnight at 4 °C. The blocking solution was then
removed and replaced with 50 µL of freshly prepared scFv phage library and incubated for 1–2 h at 37 °C. After multiple washes, the bound phage particles were eluted, with a low pH buffer containing 0.1 M glycine–HCl (pH 2.2), and amplified in E. coli for the next round of panning. After three rounds of panning, 30 randomly selected clones were checked for their reactivity against CD133 by phage ELISA. In this assay, phage clones were diluted in 5 % milk solution and incubated with CD133 protein-coated wells for 2 h at 37 °C. Wells were washed five times before incubation with 1:3,000 diluted HRP-conjugated anti-M13 antibody (GE Healthcare) for 1 h at 37 °C. The secondary antibody was then removed, washed five times, and incubated with HRP-substrate TMB (50 µL/well) for 30 min at room temperature. The color development was monitored by measuring absorbance at 450 nm. Plasmid DNA from clones that did bind to the CD133 protein was extracted and sequenced using primers P1: 5’aagacagctatcgcgattgcag3’ and P2: 5’gcccccttattagcgtttgcac3’. The DNA sequences were then analyzed using Lasergene software (DNASTAR Inc., WI, USA).

**Subcloning of CD133 scFv**

The CD133 scFv cDNA was cloned into the pET28c bacterial expression vector as previously described [51]. The sequence of the resulting clone was verified by DNA sequencing analysis performed at the University of Minnesota Biomedical Genomics Center.

**Expression, purification, and characterization**

Purification of the CD133 scFv was performed as described previously [51]. Briefly, the protein was expressed and purified from inclusion bodies using a Novagen pET
expression system (Novagen, Madison WI), followed by a two-step purification process consisting of ion exchange fast protein liquid chromatography (Q sepharose Fast Flow, Sigma) and size exclusion chromatography (Hiload Superdex 200, Pharmacia). About 3 μg of the purified CD133 scFv protein was resolved using 4–15 % SDS PAGE gel (Biorad) and then stained using an E-Zinc Reversible stain kit (Thermo Scientific) to determine purity.

**Cell culture**

All established cell lines used in this work, except ONC94M2, were purchased from the American Type Culture Collection. ONC94M2 is a primary glioma cell line derived from a C57BL/6 mouse where the primary tumor was induced by oncogene transfer using NRAS and SV40LgT, as described in [100]. Caco-2 (a human colorectal adenocarcinoma) and U87 cells (a human glioblastoma) were grown in Minimum Essential Medium containing 20 % fetal bovine serum (FBS), 1 % non-essential amino acids (Sigma), 1 % sodium pyruvate (Sigma), and 1 % penicillin–streptomycin (Invitrogen). 4T1, a mouse mammary tumor cell line, was cultured in RPMI medium supplemented with 10 % FBS and 1 % penicillin–streptomycin. NIH3T3 (a mouse embryo fibroblast cell line) was grown in Dulbecco’s modified Eagle’s medium containing 10 % FBS and 1 % penicillin–streptomycin. All cells were maintained at 37 °C/5 % CO2 in a humidified incubator.

**DNA transfection**
NIH 3T3 cells were seeded in six-well plates. Following attachment, cells were transfected with 1.5 μg mCD133 construct (ATCC catalog no. MGC-25280, image ID 4502359) or empty vector using lipofectamine reagent. After 72 h, cells were trypsinized, immunostained, and then analyzed by flow cytometry.

**Isolation of bone marrow cells**

Bone marrow was obtained from the femur of CD-1 mice. A single cell suspension of the bone marrow was obtained by dilution in cold phosphate-buffered saline (PBS), vigorous pipetting, followed by passage through a 70-μm cell strainer. Collected cells were centrifuged at 1,000 rpm for 7 min, washed once with cold PBS, and counted using a hemocytometer.

**FACS staining protocol**

One million cells were suspended in 100 μL FACS buffer (1× PBS with 1 % BSA and 0.1 % sodium azide) in a flow cytometry tube. The cell suspensions were incubated with 2.5 μg fluorescein isothiocyanate (FITC)-labeled anti-CD133 scFv for 30 min on ice. For the competitive binding assay, 1 million CD-1 mouse bone marrow cells suspended in FACS buffer were pre-incubated with 1 μg Fc Block (BD Pharmingen) on ice for 10 min followed by FITC-labeled anti-CD133 scFv, as described above, and then with 1 μg anti-CD133 Ab-PE (clone 13A4-PE, eBioscience) for another 30 min on ice. Another group received the two reagents in the reverse order. In addition, one group of cells was incubated with both the reagents simultaneously. Finally, cells were washed twice with FACS buffer and resuspended in 500 μL of FACS buffer for flow cytometric analysis.

For immunostaining 4T1 cells with anti-CD133
polyclonal antibody (Abnova), cells were fixed with 2% formaldehyde for 10 min and then permeabilized with ice cold 90% methanol for 30 min. Cells were then incubated with FACS buffer containing 5 μg antibody for 30 min on ice followed by incubation with FITC-labeled anti-rabbit secondary antibody (BD Pharmingen) for 30 min. Cells were then washed twice with FACS buffer before analysis by flow cytometry. The acquired data were analyzed by FLOWJO software (Tree Star Inc., OR, USA).

**Western blot**

ONC94M2 and 4T1 cells were pelleted and lysed using RIPA buffer (Pierce). Protein levels were estimated using a BCA kit (Pierce). Western blotting was done as described in [52]. Anti-human CD133 polyclonal antibody (Abnova) was used as the primary antibody. Anti-rabbit IgG-HRP (Cell Signal) was used as the secondary antibody.

**scFv ELISA**

E. coli TOP10F’ transformed with the scFv clone-11(HA tagged, in pCDisplay-4 vector background) was induced with IPTG (final concentration, 1 mM) and then cultured overnight at 30 °C. Next day, the bacterial culture was pelleted by centrifugation at 15,000 rpm for 30 min at 4 °C and the soluble scFv in the culture supernatant analyzed using ELISA. Briefly, human CD133 protein coated wells were incubated with either scFv, parental monoclonal antibody, or blocking solution (5% non-fat milk in phosphate-buffered saline) for 1 h at 37 °C. The treatments were removed and the wells washed five times with PBS before incubation with HRP conjugated anti-HA (obtained from GenScript USA, 1:2,000 diluted in 5% milk solution) for 1 h at 37 °C. The wells were
washed with PBS and then incubated with the HRP substrate TMB. The color development was analyzed by measuring the absorbance at 450 nm using a plate reader.

**Results**

**Identification of a novel anti-CD133 scFv**

Recombinant anti-CD133 scFv was constructed using VL and VH fragments of the clone 7 anti-CD133 antibody. The VL and VH fragments were amplified by RTPCR. The scFv was constructed using these fragments and a DNA linker that encodes for Ser7Gly10Arg by performing an overlap extension PCR. The resulting PCR products were analyzed by gel electrophoresis. The individual VL and VH components were about 400 bp in size and the assembled scFv found to be 800 bp in size, which was in agreement with the theoretical predictions (Fig. 4-1a). The scFv was then cloned into a pCDisplay-4 vector, transformed into E. coli XL-1 BLUE, and was subsequently infected with the VCSM13 helper phage in order to release the complete phage particles that displayed a library of scFv on their coat surface. The phage library was then screened for reactivity to CD133 by a standard panning process which involved the incubation of the phage particles with CD133 protein-coated plates. Only a fraction of the phage library was found to bind to the plates. These binders were then retrieved, amplified in bacteria, and screened by subsequent panning. After three such rounds, a 394-fold (6.3×10−5/1.6×10−7) enrichment of phage binders was achieved (Fig. 4-1b). At this stage, 30 phage clones were randomly picked to confirm their reactivity against human CD133 by phage ELISA (Fig. 4-1c). In this assay, nine phage clones were found to have high reactivity with.
CD133. The plasmid DNAs of these clones were extracted and then sequenced. Five of them were found to have an identical DNA sequence (data not shown). Phage clone 11 showed a high reactivity. Hence, this clone was selected for subsequent analysis; the plasmid DNA encoding the scFv as a HA-tagged recombinant protein was extracted. This DNA construct has an amber stop codon between the scFv sequence and the vector backbone. Therefore, a non-suppressor E. coli TOP10F’ bacterial strain was used for the scFv protein expression. The scFv was predominantly secreted into bacterial culture media (data not shown). The bacterial culture supernatant containing the secreted scFv protein was diluted and analyzed using ELISA to investigate the specificity of the clone 11 scFv to the human CD133 protein substrate (Fig. 4-1d). In this assay, the parent monoclonal antibody was used as a positive control and showed a higher absorbance (1.4 absorbance units, AU) than the scFv protein (0.9 AU). The background absorbance of the blank treatment was found to be 0.2 AU. These results indicate that the CD133 scFv could recognize and bind to human CD133 protein, however with a lower binding affinity than its parental monoclonal antibody. Further experiments reported in this study were done using anti-CD133– scFv protein without an HA epitope tag. To obtain this, the open reading frame of the scFv was sub-cloned into a PET-28C expression system, bacterially expressed, purified, and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 4-2a). The molecular weight of the anti-CD133 scFv protein was found to be 26 kDa and was in agreement with the theoretical predictions and with our own published results [9]. The amino acid sequence of the recombinant anti-CD133 scFv is shown in Fig. 4-2b.

**Specificity of anti-CD133 scFv**
The specificity of the anti-CD133 scFv was investigated by immunostaining. The scFv was initially conjugated to FITC to enable the direct staining of cells. Anti-CD133 scFv-FITC specifically stained human colon carcinoma Caco-2 cells that express high levels of CD133 [51, 101], but not CD133low/− U87 cells (Fig. 4-2c). Unstained cells and cells stained with a nonspecific antibody (BU-12 FITC) were used as controls. These results established the specificity of anti-CD133 scFv for human CD133 protein.

**Cross-reactivity of anti-CD133 scFv to mouse CD133**

A plasmid encoding the mCD133 gene was transfected into NIH3T3 cells. The cells were then immunostained using anti-CD133 scFv-FITC (Fig. 4-3). About 13 % of the transfected cells were stained by anti-CD133 scFv-FITC. Unstained cells and transfected cells stained with a nonspecific RFB4 (anti-B-cells)-FITC served as controls. Cells transfected with the vector backbone served as an additional control. The results obtained with this study establish the cross-reactivity of the anti-CD133 scFv to mouse CD133 protein. When the transfection experiment was repeated, about 20 % of the transfected cells were found to be immunoreactive with CD133 scFv (data not shown). The low number of cells stained by the scFv may be due to the weak expression of the transfected plasmid and/or due to the cell cycle-associated variations in CD133 surface mobilization affecting its detection by commercial antibodies [102]. To further study the cross-reactivity of anti-CD133 scFv to mouse CD133 protein, immunostaining experiments were done in mouse tumor cells and bone marrow cells. Anti-CD133 scFv-FITC immunostained
ONC49M2 mouse glioblastoma cells (Fig. 4-4a) as well as 4T1 breast carcinoma cells (Fig. 4-4b). CD133 expression in these cell lines was confirmed by Western blotting using a polyclonal anti-mouse CD133 antibody (Fig. 4-4c). CD133 expression in 4T1 cells was further confirmed by indirect immunostaining done with an anti-mouse CD133 polyclonal antibody (Fig. 4-4c). This anti-CD133 polyclonal antibody was raised against the C-terminal region of the CD133 protein, which is localized within the cytosol. Hence, 4T1 cells were fixed and permeabilized before immunostaining. Progenitor cells in the bone marrow are reported to express CD133 [103]. Anti-CD133 scFv-FITC immunostained 4% of the mouse bone marrow cells (data not shown). In order to confirm that anti-CD133 scFv binds to mouse CD133 protein, we co-stained mouse bone marrow cells with a commercially available PE-labeled monoclonal antibody (Fig. 4-5). The order of addition of scFv and the 13A4 clone resulted in distinct immunostaining profiles. When clone 13A4 was added first, around 40% of the CD133 scFv-FITC-stained fraction was co-stained by 13A4. However, when anti-CD133 scFv-FITC was added first or when added along with the 13A4-PE clone, this fraction decreased to 25%. This indicates that epitopes recognized by 13A4 and anti-CD133 scFv partially overlap. As clone 13A4 is known to bind to mouse CD133 [104], the data from these results confirm that anti-CD133 scFv binds to the mouse CD133 protein.

**Discussion**

Human CD133 is a five-transmembrane domain protein composed of 865 amino acids with a molecular weight of 120 kDa [105]. Detailed molecular analysis indicates that
CD133 expression is regulated and that it involves transcription from five alternative promoters, promoter hypermethylation, tissue-specific alternative splicing [105,106,107], and lipid raft-associated sorting of the resulting protein to the apical membrane [108]. The CD133 protein undergoes extensive posttranslational glycosylation modification at eight N-linked glycosylation sites found in its extracellular loops [109]. Recently, a set of genes that may contribute to the CD133 glycosylation has been reported [110].

Emerging studies indicate that cellular conditions like stem cell differentiation [89], cell cycle status [102], and differential glycosylation processing [110] can selectively modify CD133 glycosylation levels without affecting the native CD133 protein levels. Hence, reactivity to AC133 and AC141 reagents that bind to the glycosylation epitopes may not accurately reflect the CD133 protein levels present within the cell. To circumvent this problem, we have earlier identified a monoclonal antibody (clone 7) against CD133. Although clone 7 was raised against a non-glycosylated recombinant CD133 protein, it recognized CD133 protein from both tunicamycin-treated and untreated cell lysates by the Western blot assay [52]. This indicated that clone 7 can recognize glycosylated and non-glycosylated CD133 protein. Since the anti-CD133 scFv described in this study is derived from the clone 7 antibody, it is expected to recognize and bind to both glycosylated and non-glycosylated CD133 protein. Also, it will be interesting to determine whether clone 7 and the scFv identify the different isoforms of CD133 as well as the mature and premature forms of the protein.

An important feature of the anti-CD133 scFv is its ability to cross-react with mouse CD133 protein. This observation is not totally surprising given the fact that human and mouse orthologs of CD133 share a 60 % sequence identity. It is worthwhile to note here
that the parental hybridoma, clone 7, was raised against a recombinant protein consisting of immunogenic amino acids of the human CD133 protein. This stretch of amino acids shares a significant sequence similarity with its mouse counterpart (Fig. 4-6).

The scFvs retain target specificity, but are smaller in size compared to their parental monoclonal antibodies and, hence, are often used for targeted drug delivery. Because the CD133 protein is a known marker for cancer stem cells, we expect the newly developed scFv to be of significant value in developing therapies that selectively target and eliminate cancer stem cells. We are currently investigating the use of anti-CD133 scFv for constructing a targeted immunotoxin, dCD133KDEL, by fusing the anti-CD133 scFv with pseudomonas exotoxin A-PE38 and a c-terminal Lys-Asp-Glu-Leu (KDEL) peptide. This toxin was found to be very potent in selectively targeting and killing CD133+ cancer stem cells in metastatic breast carcinoma [53] and head neck carcinoma xenografts in mouse [51]. The expression of CD133 is not restricted to tumor-initiating cells. Therefore, it is possible that this targeted therapy may have side effects on other CD133-expressing cells like the stem cells present in a normal tissue. In our initial studies with dCD133KDEL, we did not observe any cytotoxicity to normal cells. However, for clinical realization of this immunotoxin, an elaborate evaluation of its effect on normal cells must be carried out. As the anti-CD133 scFv reagent can react with both mouse and human CD133 protein, it is uniquely suited for evaluating the safety of CD133-targeted therapy in mouse xenograft tumor models.
Fig 1: Identification of an anti-CD133 scFv

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</table>

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KSSGGGGSGGGGGGGSSRRSLEVKLVESGPELKKPPGETVKISCKASGYTFTDY
SMHWVQAPGKLKWINGWINTETGEPSYADDFFGRFASLETSATAYLQIN
NLKNEATATYFCATDYGDYFDYWQGTTLTVSS
Figure 4-1

Cloning of anti-CD133 scFv. a) Gel electrophoresis of PCR products. Lane 1 Recombinant scFv constructed using VL and VH fragments and a DNA linker encoding Ser7Gly10Arg; Lane 2 Amplification of VL fragment alone; Lane 3 Amplification of VH fragment alone; Lane 4 Molecular weight ladder. b) The recombinant scFvs are cloned into a phagemid, which were then packaged into M13 phage. They were screened by a biopanning assay by incubating phage particles with CD133-coated plates. Three rounds of panning were done. Titers of the phage particles in each round are shown. At the end of three rounds, phage particles bound to CD133-coated wells were significantly enriched. c) Phage ELISA of 30 randomly picked phage clones. Nine positive clones that showed A450>1 are indicated in black. Clone 11 was chosen for further characterization. d) Clone 11 plasmid DNA encodes a HA-tagged anti-CD133 scFv recombinant protein. It was bacterially expressed and the bacterial culture supernatant analyzed by ELISA. Human CD133 protein-coated wells were incubated with either diluted bacterial supernatant, parental monoclonal antibody (positive control), or blocking solution (blank). The treatments were removed and incubated with an HRP-conjugated anti-HA secondary antibody. The HRP reaction was then analyzed by measuring the absorbance at 450 nm after the addition of TMB substrate.
Fig 2: Expression & Specificity of anti-CD133 scFv

B

CD133+ Caco-2 cells

CD133+ U87 cells

A

Mr: Wt

BU-12 non specific ab
Untreated cells
CD133 scFv-FITC

[Graph showing fluorescence intensity against xenogen % for different cell lines]
Figure 4-2

Expression and specificity of anti-CD133 scFv. a) Anti-CD133 scFv clone 11 was sub-cloned into a PET-28c expression cassette, bacterially expressed. This resulted in the expression of HA-tagless anti-CD133 scFv. SDS-PAGE of the scFv under non-reducing conditions. b) Immunostaining of Caco-2 and U87 cells. Cells were stained with anti-CD133 scFv (green). Cells stained with BU-12-FITC (blue) and unstained cells (red) served as the controls. Anti-CD133 scFv stained only Caco-2 cells, but not U87 cells.
Fig 3: Anti-CD133 scFv cross-reacts with mCD133
Figure 4-3

Cross-reactivity of anti-CD133 scFv with mouse CD133. Immunostaining of mCD133 transfected NIH 3T3 cells. Transfected cells were stained with anti-CD133 scFv-FITC. Untransfected cells (FITC blank), cells transfected with the vector alone, or transfected cells stained with RFB4-FITC antibody served as the controls.
Fig 4: Anti-CD133 scFv immunostains murine cell lines

A

Blank  RFBF-FITC  CD133scFv-FITC

B

Unstained cells  Anti CD133 scFv-FITC
Permeabilised Cells  Secondary antibody alone  Anti-CD133/Secondary antibody

C

ONC49M2  4T1

CD133
Figure 4-4

Immunostaining of mouse cells with anti-CD133 scFv. a) Immunostaining of ONC49M2 mouse glioblastoma cells. Unstained cells or cells stained with the RFB4-FITC antibody served as the controls. b) Immunostaining of 4T1 cells. Left Staining with anti-CD133 scFv-FITC. Right Staining with polyclonal anti-CD133 antibody. 4T1 cells were PFA-fixed and methanol-permeabilized before immunostaining. Permeabilized cells with or without anti-rabbit secondary antibody staining served as the controls. c) Western blotting to demonstrate CD133 expression in ONC49M2 and 4T1 cells.
Fig 5: competitive binding between anti-CD133 scFv and anti-CD133 antibodies
Figure 4-5

Immunostaining of mouse bone marrow cells using anti-CD133 scFv. a) Competitive binding assay of anti-CD133 (clone13A4-PE) and anti-CD133 scFv-FITC. One million mouse bone marrow cells suspended in FACS buffer were incubated with anti-CD133 scFv, anti-CD133 scFv followed by anti-CD133 Ab-PE, anti-CD133 Ab-PE followed by anti-CD133 scFv, or both anti-CD133 scFv and anti-CD133 Ab-PE added simultaneously. Cells were washed twice with FACS buffer and subjected to flow cytometric analysis. The scFv stained fraction was gated and then the double-stained population within them analyzed. b) Bar graph of the results obtained in the above study. Data shown are the mean ± SD (n=03).
Figure 4-6 CD133 protein sequence alignment. Amino acid sequences of mouse (accession no. AAH28286.1) and human CD133 (accession no. AAH12089.1) were used as input sequences. The consensus sequences are marked with yellow (high similarity) and blue (weak similarity). Red line indicates amino acids of human CD133 used in the generation of the parental hybridoma (clone 7).
Preface

This chapter has been published:


My contributions to this work include performing the experiments necessary for Figure 5-1, Table 5-1, and supplemental figure 5-1. I also developed the assay used in Figure 5-3, as seen in chapter 3.
Chapter V

Immunotoxin targeting CD133+ breast carcinoma cells
Introduction

Evidence is mounting that cancers contain a small subset of their own stem cell-like cells that can self-renew. Cancer stem cells (CSCs) have the potential to either (a) again renew, generating more CSCs, or (b) differentiate into phenotypically diverse cancer cells with more limited proliferative potential [12,21,64,65]. CSCs are extremely important because they appear to be at the root of drug-resistant relapse in patients [21]. Treatment failure due to drug-resistant relapse is a problem in breast cancer [111]. According to the American Cancer Society statistics, an estimated 178,480 new cases of invasive breast cancer were diagnosed among women in 2007 and over 40,000 were expected to die of drug refractory disease [112]. Thus, alternative drugs effective against relapse are urgently needed.

CD133 is a five-transmembrane glycoprotein that localizes to membrane protrusions and shares 60% homology at the amino acid level between mouse and human [113,114]. Antibodies recognizing CD133 enrich for human hematopoietic, neuronal, and endothelial stem cells [67,68,69]. CD133 has been identified as a marker of CSCs in many tumors [13,70,72]. CD133 is one of several markers that were useful to enrich for CSC in estrogen receptor-negative breast cancer [115], and in murine models of breast cancer [71]. However, multiple studies have documented plentiful CD133–CSCs in tumors that had previously been shown to be enriched for CSCs by CD133-positive selection, suggesting CD133 expression is dynamic [91,116,117]. A major issue surrounding the usefulness of CD133 as a marker of CSCs is the fact that the most common commercial antibody detection of CD133 [AC133, 293C/AC141 monoclonal antibodies (mAbs)] is restricted to undefined glycosylated epitopes, whereas endogenous...
CD133 is differentially glycosylated. Glycosylation is an enzymatic, posttranslational modification whereby glycans are added to specific residues of a protein, usually in the plasma membrane, which alter the tertiary and quaternary structure of the protein. Glycosylation of CD133 has been found to be highly variable during cell cycle phases and upon differentiation [89,98,102].

It is well known that, at any given time, tumor cells are heterogeneous in their cell cycle status. As detected by the AC133 mAb, CD133 immunoreactivity is highest in cells with increased DNA content, indicating these cells were in the S phase of the cell cycle. Conversely, CD133 detection is lowest when cells were in G1–G0 [102]. Detection by commercial mAbs is therefore modulated by cell cycle phase and state of differentiation [89,98,102]. Indeed, Kemper et al. found a correlation between reduced glycosylation and reduced detection of AC133 and 293C/AC141 mAbs upon differentiation. This lack of detection was not due to CD133 downregulation, as determined by Western blot against CD133 by membrane protein isolates from CSCs [74]. Thus,

CD133 epitopes bound by commercial antibodies are not lost, but rather masked, or shielded through changes in the structure of CD133, likely through differential glycosylation [74]. Kemper et al. also showed that commercial CD133 antibodies only recognize epitopes on extracellular loop 2 of CD133, which, although loop 2 was not shown to contain alternatively spliced exons from the seven known splice variants, the epitope could still become inaccessible via conformational change induced through differential glycosylation [74].
Moreover, CD133 is upregulated in response to hypoxia and chemotoxic stress [118,119,120]. These facts, combined with the realization that cancer cell differentiation is likely not unidirectional, have resulted in controversy over the utility of CD133 as a CSC marker, and the relevance of the CSC hypothesis in general. In order to address this controversy in a clinically meaningful way, as well as gauge therapeutic potential, we sought to develop a reagent to selectively kill CD133+ cells in pre-established tumors. Using our previously described anti-CD133 single-chain variable fragment (scFv), which was shown to react with CD133 independent of glycosylation, we circumvented the problems of differential glycosylation in targeting CD133 [52].

Targeted toxins (TT) are a class of biological drug consisting of a ligand recognizing a marker expressed on cancer cells chemically or genetically linked to a catalytic toxin [121]. The destruction of the target cell is dependent on drug internalization, so only internalizable receptors are targetable. Studies indicate that CD133 is efficiently internalized [74]. A novel TT called dCD133KDEL was synthesized consisting of an anti-CD133 scFv reactive against the extracellular domains of CD133 derived from the novel hybridoma called clone 7 [52]. The scFv was cloned upstream of truncated pseudomonas exotoxin (PE) A called PE38 [73]. PE is a catalytic bacterial toxin requiring few internalized molecules to bring about cell death [78]. Already, it has been used extensively in drug development and many patients have received PE-based targeted toxins [79]. In addition to the unique nature of our targeting ligand, we added a Lys-Asp-Glu-Leu (KDEL) C-terminus signal to our drug, which provides enhanced tumor killing by preventing luminal endoplasmic reticulum protein secretion [77]. Genetic engineering was also used to remove immunogenic epitopes on the PE38 molecule that are
recognized by B cells. Independent studies by us [38,122] and others [80,123] prove that targeted toxins made with this mutation reduce neutralizing antibody responses without a loss in toxin activity. Previously we demonstrated that dCD133KDEL had potent activity against human head and neck carcinoma xenografts, including complete durable tumor regressions, following repeated local intratumoral injection [51]. However, it remained unclear what potential toxicity and efficacy dCD133KDEL would have in a metastatic carcinoma model following repeated systemic dosing. In this paper, we demonstrate the tolerability and efficacy of dCD133KDEL following systemic administration against a metastatic breast carcinoma model. Our data suggest that targeting CD133+ cells with immunotoxins has therapeutic potential and may prove a highly useful tool in stem cell biology.

Materials and Methods

Construction and purification of dCD133KDEL and dEGF4KDEL

The construction and purification of dEGF4KDEL [122] and dCD133KDEL [84] were previously described. Briefly, each protein was expressed and purified from inclusion bodies using a Novagen pET expression system (Novagen, Madison WI) and sonicated. Protein was refolded and then purified using Fast protein liquid chromatography ion exchange chromatography (Q sepharose Fast Flow, Sigma) followed by size exclusion chromatography (Hiload Superdex 200, Pharmacia). Protein was then analyzed by SDS-PAGE and stained with Commassie Brilliant Blue to determine purity.
Cell lines

The human breast cancer carcinoma MDA-MB-231 was originally derived from pleural effusion of patients with stage III breast carcinoma [124]. MDA-MB-231 was genetically altered by transfection with a reporter gene encoding firefly luciferase creating the MDA-MB-231-luc cell line for imaging [122]. The line was subcloned using flow cytometric cell sorting in order to obtain stable transfectants that were highly bioluminescent. Other cell lines used for these studies included breast cancer cell lines MDA-MB-468, SK-BR-3, BT474, and Caco-2, a colorectal cancer line. All lines were obtained from American Type Culture Collection (Rockville, MD) and were grown as adherent monolayers in DMEM/10% fetal calf serum. For the studies requiring reduced O2 conditions, cells were either low O2 shocked or underwent a gradual O2 reduction. The shock consisted of an overnight incubation at 1% O2. The gradual O2 reduction consisted of culturing cells at 14% O2 for 3 days and 5% O2 for 3 days, with a final overnight incubation at 1% O2.

Flow cytometry and CD133+ cell enrichment

Flow cytometry was performed using a FACSCaliber at the University of Minnesota Cancer Center Flow Cytometry facility. For the two-color immunofluorescence studies, MDA-MB-231 cells were incubated with unlabeled primary anti-CD133 followed by incubation with rabbit anti-IgG secondary antibody and antiepidermal growth factor receptor (EGFR)-fluorescein isothiocyanate (FITC)-labeled antibodies. Data was analyzed using FLOWJO software (Tree Star Inc., Ashland, OR). For the study of the
other cell lines, cells were incubated with anti-CD133scFv-FITC, anti-EGFR-FITC, anti-CD45-FITC, or anti-CD19-FITC. For experiments requiring CD133 selection, anti-CD133 scFv-FITC was used in combination with magnetic bead selection kit following manufacturer’s instructions (Stem Cell Technologies, Tukwila, WA). Briefly, cells were concentrated to 2×10^8 cells/ml. CD133 scFv-FITC was added followed by the EasySep FITC Selection Cocktail®. Magnetic nanoparticles were then added and magnetic separation used to separate bound from unbound cells.

**In vitro trypan blue viability assay**

For trypan blue viability assay, 10,000 cells/well were plated into 24-well plates. Drug and media were replaced daily at the indicated dilutions. Cells were harvested, stained, and counted on a hematocytometer. Untreated wells typically became confluent around day 8. This assay permitted us to assess the activity of drug over time.

**In vivo efficacy studies**

Male nu/nu mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited, specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. In order to test
the efficacy of EGF4KDEL and dCD133KDEL against metastatic breast cancer, MDAMB-231-luc cells were injected directly into the spleen of anesthetized mice resulting in systemic metastatic breast cancer model. Mice were given an intrasplenic inoculation of two million MDA-MB-231/luc on day 0. They were treated with multiple IP injections of the indicated TT. Mice were imaged in real time, and images were captured using Xenogen IVIS 100 imaging system (Xenogen Corporation, Hopkington, MA) and analyzed using IGOR Pro 4.09a software (WaveMetrics, Inc., Portland, OR). Prior to imaging, mice were anesthetized using isoflurane gas. All mice received 100 μl of a 30-mg/ml D-luciferin aqueous solution (Gold Biotechnology, St. Louis, MO) IP as a substrate for luciferase 10 min before imaging. All images represent a 5-min exposure time, and all regions of interest are expressed in units of photons per second per square centimeter per steridian (sr).

Results

Dynamic CD133 expression in carcinoma cells

We have previously shown that the anti-CD133 scFv selectively binds CD133+ cells [51]. In order to determine if CD133− cells could acquire CD133 expression over time, we sorted CD133− MDA-MB-231-luc cells using magnetic bead separation and fluorescence activated cell sorting (FACS; data not shown). A prominent population of CD133+ cells spontaneously arose after 2 weeks in culture (Fig. 5-1). This observation lends credence to the theory that cancer cells undergo “plasticity” in their differentiation.
and demonstrates that CD133+ cells can arise from a CD133−cell population, revealing dynamic CD133 expression in cancer.

Table 5-1 shows different breast carcinoma cell lines reacted with anti-CD133scFv-FITC: MDA-MB-468, SKBR-3, and BT474. CD133+ cells in these lines ranged from 4 to 5.2%. EGFR was expressed at high levels on the cells, and negative controls CD45 and CD19 (hematopoietic markers) were not. The colorectal cell line Caco-2 is known to express high levels of CD133 and served as positive control, since over 62% of cells expressed CD133 (Table 5-1)[114]. Two-color flow cytometry studies were performed in order to determine if the CD133-expressing cell population overlapped with the EGFR-expressing cell population. In order to gain insight into how expression might change in hypoxic tumor microenvironments, MDA-MB-231 were cultured under normal O2 conditions, e.g., atmospheric ~21%O2 and under varying conditions of O2 deprivation. Interestingly, Table 5-2 shows the existence of a minority (0.5% of total cells) CD133+EGFR− population that increases four- to eightfold under culture conditions of low O2. Additionally, the CD133−EGFR+ cells appear to diminish from 66 to 27–42% of the total cells as oxygen declines, while dual positive CD133+EGFR+ cells concomitantly increase. Apparently low O2 levels, as would be present in tumors in vivo, enhance CD133 expression in EGFR− and EGFR+ cells.

**dCD133KDEL exhibits potent in vitro activity against human breast cancer**

To determine if dCD133KDEL had an effect on breast cancer cells in vitro, MDA-MB-231 cells were cultured with drug for 4 days and then protein synthesis was evaluated
using a tritiated leucine incorporation assay. The drug selectively killed MDA-MB-231 with an IC50 of 0.0035 nM (Fig. 5-2). Control CD3CD3KDEL, which targets T cells and is not reactive with breast cancer cells, did not inhibit activity. dEGF4KDEL 7mut showed higher activity than dCD133KDEL. These findings correlated with our FACS studies in Table 5-2 showing that EGFR was expressed on a higher proportion of MDA-MB-231 cells.

In order to study the ability of dCD133KDEL to inhibit the expansion of MDA-MB-231 tumor cells over time and perhaps cause their death, we devised an assay based on vital staining with trypan blue. Media with or without drug were changed daily. Figure 5-3a shows that 0.1 nM dCD133KDEL delayed tumor cell growth and 1 nM entirely inhibited growth. As control, treatment with 0.1 nM anti-B cell CD22KDEL had no effect on tumor cell expansion. In a different experiment, Fig. 5-3b demonstrates these results were reproducible using a second negative control whereby 10 nM dCD133KDEL entirely inhibited cell growth, while 10 nM of control anti-B cell CD19KDEL did not. Together, these findings indicate that although the CD133 marker is not expressed on the majority of cells at any point in time, CD133KDEL selectively inhibits tumor cell proliferation in vitro.

In vivo efficacy studies define a therapeutic window for dCD133KDEL

To determine whether dCD133KDEL could mediate a systemic anti-breast cancer effect, MBA-MB-231 cells transfected with a luciferase reporter gene were surgically injected into the spleen of nude mice as previously reported [122]. Figure 5-4 shows imaging data
from individual mice indicating that after multiple treatments beginning on day 6, all mice (M5–M9) had a reduction in tumor burden by day 20 compared to the controls M1–M4 (M1, M2, and M3 controls were untreated and M4 was treated with an irrelevant control CD3CD3KDEL). Mice were treated for 4 weeks. Tumor was still undetectable in 60% of the mice on day 52, whereas the remaining animals had a recurrence.

Since flow cytometry studies showed the existence of a CD133−EGFR+ cell population, we performed a combined therapy experiment in which tumor-bearing mice were given both dCD133KDEL and dEGF4KDEL. In experiment 2, treatment schedules and dosages of both drugs were reduced to suboptimal concentrations (based on prior experiments) to test the effect of drug combination. A suboptimal dose of dEGF4KDEL (3 μg/injection) was combined with a suboptimal dose of dCD133KDEL (6 μg/injection). Mice were given five courses of injections MWF beginning on day 6 as in experiment 1. Although there was some variability in tumor growth in control animals, eventually, these tumors all progressed to a saturating size measured by bioluminescent imaging (Fig. 5-4). In contrast, four out of five mice treated with the combination therapy had nearly a complete response by day 41, which was much greater than treatment with the individual drugs (no durable responses). These results demonstrate the superior efficacy of combination therapy in a scenario where the concentration of either single agent is suboptimal, as might be expected in human patients with considerable disease burden.

A dose escalation experiment was also performed to define the maximum tolerated dose of dCD133KDEL. Systemic IP administration of 20 and 40 μg of dCD133KDEL was well tolerated (Table 5-3), suggesting that the single-agent activity of this drug has a wide therapeutic window. Dose limiting toxicity was found to occur at 100 μg given MWF,
which killed four out of five mice. This is likely due to killing of normal CD133+ cells as addressed in the discussion.

**Discussion**

There is evidence that CSCs are more drug resistant than their differentiated progeny in various tumor models [12,21,69,93,94]. Thus, many now believe that drug-resistant CSCs are the root of tumor relapse in patients with solid tumors and hematologic malignancies, and therefore are vital targets for delivery of cytotoxic drugs. CD133 is a marker that can enrich CSCs. However, there is great controversy surrounding the use of CD133 as a marker for CSCs, largely due to a failure of commercial mAbs to recognize differentially glycosylated forms of CD133, dynamic CD133 immunoreactivity, and the plasticity of cancer cell differentiation. To directly assess the effects of pan-CD133 targeting in the treatment of cancer, we generated a TT using a glycosylation-independent antihuman CD133 scFv conjugated to PE (dCD133KDEL). Pan-CD133 targeting led to complete tumor regression in 60% of dCD133KDEL-treated animals in a mouse model of human metastatic breast carcinoma. However, there are several curious observations made in this study that warrant discussion.

In vitro, dCD133KDEL was able to inhibit proliferation of bulk breast carcinoma cultures, despite that fact that only a fraction (4–10%) was immunoreactive with the CD133scFv at any point in time. There are at least four possible explanations for this finding: (1) since CD133–
cells reacquired CD133 expression (Fig. 5-1), it is possible that CD133 immunoreactivity is continuously changing (on/off) in all the cells within the tumor line over time; (2) CD133 may be associated with the cell cycle [102], thus if a cell attempts mitosis it is killed; (3) there is a hierarchy within the tumor culture being studied, meaning that only CD133+ cells can renew the tumor; and (4) in addition, the limit of detection for what we consider CD133+ by flow cytometry is likely below the threshold of CD133 required for CD133KDEL to kill a cell. A counter hypothesis would be that targeting CD133+ cells will not affect tumor growth because CD133− cells can regenerate the tumor. Operating under the assumption that CD133+ cells are not an appreciable source of tumor self-renewal, one would anticipate negligible efficacy of dCD133KDEL in vivo because of limited CD133 expression on MDA-MB-231. In contrast to that assumption, dCD133KDEL had potent in vivo affects when injected systemically, which supports the hypothesis that CD133+ cells are a source of self-renewal in this human breast carcinoma model. We believe this is a reasonable model of human breast carcinoma metastasis because our previous studies using this model showed that the tumor metastasizes to other critical organs by day 40 [122]. Although the current study is the first demonstration of efficacy against breast cancer following systemic administration of dCD133KDEL, we previously reported the effectiveness of dCD133KDEL following intratumoral injection in a flank model of UMSCC-11B head and neck cell carcinoma [51]. Collectively, our prior work and the current study demonstrate that dCD133KDEL is unique in its ability to kill putative CSC and inhibit tumor growth in two different carcinoma models. While we are cautious to avoid over interpretation of experiments
done with cloned cell lines (rather than primary tumor samples), our data implies that the idea of CD133 as a CSC marker and therapeutic target remains viable.

CD133 is expressed in many normal tissues in mice and humans including the colon, liver, pancreas, and kidney [91,99]. Therefore, potential toxicity warrants serious consideration.

In order for our studies to shed any insight on that issue, the CD133 scFv would have to cross-react with mouse CD133 (a.k.a., Prominin-1 [97]). Flow cytometry studies with the CD133 scFv indicate reactivity with mouse leukemia cells (Supplementary Fig. 5-1), revealing a subset-like staining pattern consistent with human cell lines that harbor a CD133+ subpopulation. Detailed binding studies which validate the cross-reactivity of our antihuman CD133scFv with the mouse CD133 are reported in a separate study (Swaminathan et al., submitted). Therefore, we believe the tolerability and dose-limiting toxicity studies described herein are potentially meaningful. The number of AC133 mAb-reactive CD133 molecules on the surface of primary cells and tumor cell lines has been estimated by flow cytometry [57]. Smith et al. reported 0 and 800 CD133 molecules per cell in primary human kidney epithelial and hepatocyte cultures, respectively. In contrast, gastric, hepatic, and pancreatic tumor cell lines expressed between 6,500 and 66,000 AC133 mAb-reactive CD133 molecules per cell, an enrichment of several thousandfold over selected normal cells. These observations were further validated by immunohistochemistry on primary tissues; a high percentage of tumor samples were found to be positive for CD133 in gastric (47–55 %), pancreatic (55–68 %), and cholangiocarcinomas (67 %, biliary type of liver cancer). CD133 expression in corresponding normal tissues was analyzed and included biliary ducts of the liver,
pancreatic acinar and ductal epithelium, and gastric glandular crypt epithelium. Weak apical membranous staining was observed. Generally, it was concluded that CD133 expression levels in normal tissues are lower than in tumors. The relatively higher expression of CD133 in tumors suggests that a “therapeutic window” may exist for dCD133KDEL whereby tumoricidal activity could be achieved without reaching dose-limiting toxicity. We demonstrated this in a murine model of metastatic breast cancer whereby therapeutic efficacy of dCD133KDEL was achieved at a dose five times less than the maximum tolerated dose (Table 3).

All chemotherapeutics used in the treatment of cancer have some degree of toxicity on normal cells. Molecularly targeted drugs minimize this toxicity by selectively killing cells expressing a specific marker. Low levels of target expression on normal cells relative to cancer cells combined with low toxicity at therapeutic doses in mouse models of human cancer are needed to move drugs from bench to bedside. An example of this can be found in the IL13Rα2 TT for recurrent glioblastoma, which moved to a phase III clinical trial, ultimately failing due to lack of efficacy rather than dose-limiting toxicity [125]. The dynamic expression of CD133 and ability of CD133− cells to regenerate normal tissues may also contribute to the therapeutic window we observed. This idea is supported by our finding that treatment of CD34+ human cord blood progenitor cells with dCD133KDEL did not attenuate colony formation [51]. This may relate to the existence of independent, stable CD133+ and CD133− normal progenitor cell populations.

For example, Freund et al. sorted nonoverlapping CD34+CD133− and CD133+ blood stem cells [126]. Their studies showed a significantly higher proportion of erythroid colony-forming progenitor cells concentrated in the CD34-enriched fraction. Such
findings imply that normal hematopoietic progenitors are not limited to CD133+ cells, and even if CD133+ progenitors are damaged, other progenitors that do not express CD133 will regenerate the tissue. Together, lower CD133 expression on normal tissues relative to tumors, a therapeutic window in a murine model, and our observation that dCD133KDEL does not attenuate colony formation using bona fide human hematopoietic progenitor cells indicates the drug may be therapeutically useful. Our data also shed insight with respect to strategies that can be employed to overcome tumor heterogeneity.

An important finding in these studies is that treatment with suboptimal doses of two different targeted toxins had a minimal therapeutic effect in the systemic breast cancer model, while a combination of these same suboptimal dosages had a much greater effect. One explanation for this finding is that dCD133KDEL targets the minority population of CSCs and dEGF4KDEL targets the majority fraction of more differentiated cancer cells.

The two-color flow cytometry studies shown in Table 2 reveal the existence of a CD133+EGFR− population that would not be killed by targeting EGFR. One potential explanation for this is that CD133− cells can give rise to CD133+ cells. Our data in Fig. 5-2 supports this since CD133−-sorted subpopulation of MDA-MB-231 cells gave rise to CD133+ cells after 2 weeks in culture. Thus, it would not be surprising that eliminating both fractions would have superior antitumor activity. An alternative explanation is that a second, independent CSC population is contained in the CD133− cell fraction, and perhaps, dEGF4KDEL is effective in targeting this fraction. Wright et al. studied murine Brca1 mutant breast tumors [71]. Their sorting studies revealed that these tumors also contained two distinct CSC populations, one CD133+ and the other CD133−. Both populations were consistent with a stem cell phenotype, had high expression of stem cell
associated genes (Oct4, Notch1, Alldh1, and Sox1), had tumor initiation properties, and were significantly more drug resistant. Studies are underway to determine whether dEGF4KDEL targets a second nonoverlapping CD133−CSC population. Treatment of breast cancer patients with lapatinib, an EGFR pathway inhibitor, resulted in a decrease in the percentage of putative CSCs (based on immunophenotype) in a study of disassociated tumor cells from breast cancer core biopsies, which indicates that EGFR might be a viable second target [127]. It is also possible that CD133 expression is dynamic and heterogenous on CSCs such that, at any given time, a subset is “CD133+” and thus is targeted by dCD133KDEL. As discussed in the “Introduction” section, CD133 glycosylation varies greatly with cell cycle status and state of differentiation [52,74,121]. Even though anti-CD133 scFv can bind the surface of unglycosylated CD133 in vitro, the state of CD133 glycosylation in vivo could induce conformational changes within extracellular domains such that internalization of ligand (such as dCD133KDEL) is prohibited. Clearly, further studies are needed to better understand the relationship between CD133 glycosylation and the binding ability of CD133 immunoreactive proteins.

In summary dCD133KDEL represents a new biologic tool that can be used to interrogate the clinical significance of eradicating CD133+ cells. We also intend to use it to explore whether CD133+ cells represent a potential reservoir of chemo/radiotherapy-resistant cells in vivo and further investigate whether this drug can be clinically useful. CD133 is enriched in tumors during tumor hypoxia and in conditions of stress such as chemotherapy and irradiation [118,120]. Thus, it logically follows that a drug targeting CD133 would have unique synergy with standard cytotoxic therapies. Although
selectively targeting CSCs is appealing, the plasticity of tumor cell differentiation suggests that simultaneous targeting of the bulk tumor by combination therapies will lead to superior therapeutic outcome.


Figures

**Figure 5-1** CD133 expression is dynamic. Grayscale contour plot showing that after 2 weeks of culturing CD133-negative MDA-MB-231 cells that were selected using magnetic bead separation, a prominent population of CD133-positive cells can be again identified using anti-CD133-FITC. The blank control shows the background with untreated cells.
**Figure 5-2** Dose curve of dCD133KDEL against breast carcinoma cells. Plots of tritiated leucine incorporation assay showing that dCD133KDEL selectively inhibits MDA-MB-231 cells. Cells were incubated with drug for 3 days, pulsed with radioleucine and then harvested with standard harvesting techniques. Cells were treated with CD3CD3KDEL (anti-T cell) targeted toxin as a negative control.
A  
Trypan Blue Viability Assay: Experiment 1

B  
Trypan Blue Viability Assay: Experiment 2
**Figure 5-3** dCD133KDEL inhibits breast cancer in vitro. Plots of trypan blue viability studies showing that dCD133KDEL selectively inhibits MDA-MB-231 breast cancer cells over time. a Experiment 1 and b experiment 2. Cells were incubated with dCD133KDEL and viability was determined over time by directly counting cells growing in tissue culture wells using a trypan blue vital stain. Live cells exclude the dye and dead cells incorporate it. CD22KDEL or CD22KDEL (anti-B cell) TT were included as negative controls. Media and toxin were replenished daily.
Table 1 Flow cytometry studies of CD133 expression on various carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CD133⁺</th>
<th>EGFR⁺</th>
<th>CD45⁺</th>
<th>CD19⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468</td>
<td>5.2</td>
<td>96.1</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td>SKBR3</td>
<td>4.2</td>
<td>99.8</td>
<td>0.7</td>
<td>–</td>
</tr>
<tr>
<td>BT474</td>
<td>4.0</td>
<td>99.9</td>
<td>0.6</td>
<td>–</td>
</tr>
<tr>
<td>Caco-2</td>
<td>62.8</td>
<td>82.7</td>
<td>–</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Table 2  Two-color flow cytometry studies of CD133/EGFR expression on MDA-MB-231 cells

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Normal</th>
<th>Low O₂ shock</th>
<th>Gradual O₂ reduction</th>
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<tr>
<td>CD133⁺EGFR⁻</td>
<td>0.5</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>CD133⁺EGFR⁺</td>
<td>6.4</td>
<td>36.3</td>
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<td>66.0</td>
<td>42.8</td>
<td>27.0</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.1</td>
<td>&lt;0.2</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Controls were cells treated with anti-CD19-FITC
**Figure 5-4** Bioluminescent images showing that dCD133KDEL inhibits the progression of tumors. For experiment 1 (top panel), tumors were induced by systemic surgical intrasplenic injection of two million MDA-MB-231 breast cancer cells. IP treatment was begun on day 6 with single course of treatment consisting of 20 μg of drug given every other day (Monday, Wednesday, and Friday) and mice were treated for 4 weeks. Controls were untreated. Bioluminescence intensity was measured as a function of photons per second per square centimeter per sr. For experiment 2, combination treatment was studied. Systemic MDA-MB-231 tumors were induced in nude mice exactly as in experiment 1. Treatment was begun on day 6 post-tumor inoculation with a suboptimal dose of dCD133KDEL, dEGF4KDEL, or a mixture of both. A single course of treatment consisted of a suboptimal 6 μg of dCD133KDEL or 3 μg EGF4KDEL given every other day (Mondays, Wednesdays, and Fridays) and mice were given five courses of treatment. The last group was treated with both drugs combined. Animals were imaged weekly.
<table>
<thead>
<tr>
<th>CD133KDEL dose (μg)</th>
<th>Surviving mice/total mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>10/10</td>
</tr>
<tr>
<td>40</td>
<td>6/6</td>
</tr>
<tr>
<td>100</td>
<td>1/5</td>
</tr>
</tbody>
</table>

Administered by IP injection on days 0, 2, and 4.
Cross-reactivity of CD133 scFv-FITC with C1498 Mouse Leukemia Cells
Supplemental Figure 5-1 Grayscale contour plot showing that anti-CD133-FITC made with the same scFv used in the construction of dCD133KDEL, recognizes a prominent CD133 positive cell population in cultured C1498 murine leukemia cells.
Preface

This chapter has been submitted for publication:

Waldron NN, Barsky SH, Vallera DA. A bispecific EpCAM/CD133 targeted toxin is effective against carcinoma. Submitted to Targeted Oncology March 2013.
Chapter VI

A bispecific EpCAM/CD133 targeted toxin is effective against carcinoma
**Introduction**

Epithelial cell adhesion molecule, or EpCAM, is an established cancer target for drug therapy and has many diverse roles in cancer cells such as cell signaling, proliferation, differentiation, and migration [33,128]. Recently, interest has intensified since EpCAM has also been shown to be a direct target in the Wnt/β-catenin signaling pathway, a key pathway used by both cancer stem cells (CSCs) and normal adult stem cells that enables cells to self-renew and differentiate into multiple cell types. Mutations or disruptions in this pathway in normal stem cells can lead to excessive proliferation and stem cell self-renewal resulting in tumor formation [22,37]. Unsurprisingly then, it has been shown that high levels of EpCAM expression correlates with increased tumorigenesis in a range of carcinomas including breast, colon, and head and neck squamous cell carcinoma (HNSCC) [35,129]. All of this has made EpCAM an attractive marker for targeted therapy.

CD133 was originally discovered as a pentaspan membrane glycoprotein that was a marker for a population of hematopoietic stem cells [67]. Now it is an established CSC marker in many carcinomas such as breast, colon, prostate, and HNSCC among others [12,46]. Like EpCAM, CD133 is involved in the Wnt/β-catenin signaling pathway [47,48,49]. We recently synthesized a new anti-CD133 monoclonal antibody that differs from other anti-CD133 antibodies in that it recognizes the extracellular domain and is cross-reactive with mouse CD133 [130]. The scFv from this monoclonal antibody was used to construct a deimmunized anti-CD133 targeted toxin, called dCD133KDEL, which showed efficacy against HNSCC, breast, and ovarian carcinoma in vitro and in
vivo [51,53,54]. These findings validated our strategy of targeting CSC, which are critical in cancer cell self-renewal and drug refractory relapse.

Our laboratory has specialized in development of new biological drugs that are best described as bispecific targeted toxins because they consist of two cancer cell-directed ligands on the same molecule as a catalytic toxin [38,88-83]. Because of the efficacy shown by a novel monospecific CD133- directed targeted toxin [51] and the promising results of clinical trials using a monospecific anti-EpCAM TT [23,43,44], we synthesized a novel bispecific TT using both anti-EpCAM and anti-CD133 scFvs. A targeted toxin is a biological drug consisting of a ligand specific for a given cell surface receptor conjugated to a catalytic protein toxin. TTs have been shown to be useful in cancer therapy and provide potent and selective toxicity in targeted cells. The first step in the mechanism of TT action is the binding of the ligand portion of the molecule to the specific receptor. The receptor then must be internalized. The TT then escapes from its vesicle into the cytosol where the toxin portion binds and inhibits its target. Pseudomonas exotoxin (PE) functions by ADP-ribosylation of EF2 thereby inhibiting translation and inducing cell death [73]. In our studies, we used a genetically deimmunized variant of PE that was modified to include the lysosomal retention sequence and C-terminal signal Lys-Asp-Glu-Leu (KDEL) to enhance its potency [79].

In this article, we show that a novel bispecific CSC TT, dEpCAMCD133KDEL, potently inhibits multiple carcinoma lines in vitro, and causes regression in HNSCC tumors in vivo. This represents the first bispecific CSC targeting agent and warrants further development as a possible clinical adjunct for cancer therapy.
Materials and Methods

Construction of dEpCAMCD133KDEL

DNA of dEpCAMCD133KDEL was synthesized using DNA shuffling and DNA ligation techniques resulting in the fusion of genes encoding the anti-EpCAM scFv, anti-CD133 scFV, and a deimmunized truncated form of pseudomonas exotoxin 38 used previously [51,81-83]. The resulting fusion DNA sequence contained (5’-3’) and NcoI restriction site, an ATG initiation codon, the EpCAM scFv, the CD133 scFv, a 7 amino-acid EASGGPE linker, the gene encoding the deimmunized truncated PE38 with a DNA sequence encoding KDEL replacing the REDLK, followed by a NotI restriction site at the 3’ end of the DNA fusion sequence. This gene was then spliced into the pET21d bacterial expression vector containing an inducible isopropyl-b-D-thiogalactopyranoside (IPTG) T7 promoter and a carbenicillin selection gene. The DNA sequence was verified by DNA sequence analysis done at the University of Minnesota BioMedical Genomics Center.

Purification of dEpCAMCD133KDEL

Purification was performed as described previously [84]. Briefly, the protein was expressed and purified from inclusion bodies using the Novagen pET expression system (Novagen). Then a 2-step purification procedure was performed using an ion exchange fast protein liquid chromatography (Q sepharose Fast Flow, Sigma) followed by size exclusion chromatography (Hiload Superdex 200, Pharmacia). The purified protein was
then analyzed by Bradford to determine the concentration and by SDS-PAGE stained with Coomassie Brilliant Blue to determine purity.

**Cell lines and culturing techniques**

UMSCC-11B is an HNSCC line that was derived from larynx tumor following chemotherapy [85]. UMSCC11B-luc was transfected using a luciferase reporter construct, and maintained with 10 mg/mL of blastocidin. Cells were transfected using Invitrogen’s Lipofectamine Reagent. NA-SCC is another HNSCC line isolated from a tongue tumor [86]. Both lines were obtained from Dr. Frank Ondrey (University of Minnesota) who previously obtained them from their originator, Dr. Thomas E. Carey, Department of Otolaryngology-Head and Neck Surgery, University of Michigan in 2009. NA-SCC and UMSCC cell lines were authenticated this year by STR testing done by the Fragment Analysis Facility, John Hopkins University. Caco-2 and HT-29 (colorectal carcinomas), BT-474 and SK-BR3 (breast carcinomas), Raji and Daudi (B-cell lymphomas), and U-87 (glioblastoma) were obtained from ATCC and were positive for the appropriate markers. Only cells that were greater than 90% viable were used for experimentation. Mary-X is an inflammatory breast carcinoma spheroid line and was obtained from Dr. Sanford Barsky (University of Nevada).

**Flow cytometry**
EpCAM and CD133 expression was analyzed using a fluorescence-activated cell sorting (FACS) Caliber at the University of Minnesota Flow Cytometry Core Facility. Antibodies and proteins used in flow cytometry were labeled with fluorescein isothiocyanate (FITC). Analysis of the results was performed using FLOWJO.

**Bioassays to measure cellular proliferation**

To measure the level of proliferation and the effect dEpCAMCD133KDEL was having on the carcinoma lines, 3H-thymidine (or 3H-leucine when cell lines did not take up thymidine) incorporation assays were used. Cells are plated in 96-well flat-bottomed plates and allowed to adhere overnight in appropriate media (leucine-free when 3H-leucine was used). The toxins were then added in triplicate at 10-fold dilutions to determine the IC50 values. Plates would then be incubated for an additional 48 hours. 3H-Thymidine or 3H-leucine would then be added for a final 18-hour incubation. Plates were then frozen to detach cells, and then thawed and harvested onto glass fiber filters, which were then washed, dried, and counted using standard scintillation methods. Trypan blue viability assays were also done to test the activity of dEpCAMCD133KDEL against the HNSCC lines. This was performed as described previously [51]. Briefly, cells were plated in 24 well plates and toxin added daily. Cells were harvested using trypsin and counted using trypan blue. For the Mary-X spheroid assay, Spheroids were plated into 24 well plates and counted to determine the starting number of spheroids per well. Then toxin was added daily and the number of spheroids counted on subsequent days. GraphPad Prism was used to analyze and graph all assay results. Data is reported as the
percentage of control counts in the 3H-Thymidine or 3H-leucine assays and the Mary-X assay, and as cell number in the trypan blue viability assays. All assays were repeated at least three times to ensure reproducibility, and representative figures are shown.

**Tumor treatment studies**

For study one, six million UMSCC-11B/luc cells were injected into the right flank of 15 nude mice. Starting on day 7, tumors were treated with 20ug/mouse of dEpCAMCD133KDEL, 2219KDEL, or 100ul of saline. Mice were given four courses of injections where one course was 3 injections given every other day (MWF) for one week. Mice were weighed weekly and weights were recorded. Image could not be obtained for mouse 2 in the dEpCAMCD133KDEL group for day 48, but was visibly tumor free by caliper measurement.

For study two, 3.5 million UMSCC-11B/luc cells were injected into the right flanks of 9 nude mice. Treatment began on day 12 and continued 4 times a week (MTWTh) for 7 weeks with 5 mice receiving 20ug of dEpCAMCD133KDEL and the remaining 4 mice receiving 10ug of DT2219ARL control, which is its MTD.

For both studies, mice were imaged every other week using the Xenogen Ivis 100 imaging system and analyzed with Living Image 2.5 software (Xenogen Corporation). Mice were injected with 3 mg of luciferin substrate 10 minutes before imaging and anesthetized via isoflurane gas inhalation. Two-minute exposures were performed to capture tumor fluorescence. Units for the regions of interest are expressed as photons/sec/cm2/sr.
Results

dEpCAMCD133KDEL binds selectively

To determine whether both scFv portions of dEpCAMCD133KDEL were functional and bound their respective receptors, flow cytometry was performed on Caco-2 cells, a colon carcinoma. Caco-2 highly expresses both EpCAM and CD133. As can be seen from Figure 6-1, FITC labeled CD133 scFv and EpCAM Fc bound and were 90% and 99.4% positive respectively, while dEpCAMCD133KDEL bound with high affinity as well. When dEpCAMCD133KDEL was blocked with either unlabeled CD133scFv or unlabeled EpCAM separately, the bispecific was still able to bind via its other ligand. But when unlabeled CD133 and EpCAM were both added the binding of dEpCAMCD133KDEL was blocked.

Another colon carcinoma, HT-29, was tested for its level of EpCAM and CD133 expression and determine whether it might be a suitable target for dEpCAMCD133KDEL. As seen in Table 6-1, HT-29 was 98.8% EpCAM positive and 2.61% CD133 positive. The table also shows two HNSCCs (UMSCC-11B and NA-SCC) and two breast carcinomas (BT-474 and SKBR3) which all showed low levels of CD133 expression and very high levels of EpCAM expression. Two negative control cell lines, U87 and Raji, were also tested on flow cytometry and also expressed low levels of CD133, but both had negligible EpCAM expression.
Bioassays show dEpCAMCD133KDEL activity

To test the efficacy of dEpCAMCD133KDEL, the HNSCC lines were tested in 3H-Leucine incorporation assays to determine the level of protein translation inhibition. As seen in Figure 6-2a and b, dEpCAMCD133KDEL inhibited both UMSCC-11B and NA-SCC with IC50 values of 0.025nM and 0.0045nM respectively. Similar IC50 values were obtained when the two colon carcinoma lines were tested. Furthermore, dEpCAMCD133KDEL showed subnanomolar activity against the two breast carcinomas shown in Figure 6-2e and 6-2f as well. However, as seen in Figure 6-2g and 6-2h, the two EpCAM negative cell lines were not affected by dEpCAMCD133KDEL at the concentrations tested, while positive controls potently inhibited proliferation.

To determine whether the inhibition of protein translation was causing cell death in the cancer lines, time course viability assays were performed. As seen in Figure 6-3a, dEpCAMCD133KDEL killed all UMSCC-11B carcinoma cells while a negative control TT, CD19KDEL, did not. NA-SCC cells were killed in the same manner in Figure 6-3b. Furthermore, when inflammatory breast carcinoma Mary-X spheroids were tested in a time course spheroid assay in Figure 6-3c, dEpCAMCD133KDEL eliminated the spheroids while the negative control had no effect. The monospecific targeted toxins were also tested in this assay and both were also able to eliminate tumor spheroids over time.

dEpCAMCD133KDEL effective in HNSCC mouse studies
To determine the ability of dEpCAMCD133KDEL to cause tumor regression in vivo, nude mice were injected with UMSCC-11B/luc cells into their right flanks. In the first study (Figure 6-4), 3 groups of mice (5 mice/group) were treated intratumorally starting on day 7. Thus, these were smaller tumors at the time that therapy was initiated. Treatment was discontinued on day 40. All 5 dEpCAMCD133KDEL treated tumors regressed, with 4 mice achieving tumor-free status. Caliper data reflected the imaging results. In the second study shown in Figure 6-5, tumors were permitted to grow larger and become more established before therapy was begun. Mice were treated intratumorally starting on day 12 instead of day 7. One group of 5 mice were treated with dEpCAMCD133KDEL and another group treated with the negative control DT2219ARL since there are no B cell markers on UMSCC-11B. Treatment was discontinued on day 63. Figure 6-5 shows that all tumors responded to dEpCAMCD133KDEL therapy and three of the tumors completely regressed. The negative control tumors were unaffected. The drop in tumor size in the DT2219ARL group on day 32 occurred because 2 of the 4 tumors ulcerated causing a temporary decrease in tumor size. Interestingly, regressions did take place slowly overtime in keeping with our hypothesis that destruction of CSC prevented self-renewal. We did not see any significant weight loss in either study, which would have been indicative of toxicity. As seen in Figure 6-6, the average weights remained steady throughout each study.

In order to evaluate whether the monospecific drugs were as effective as the bispecific drug, in experiment 2, a third group of mice were treated with monospecific anti-CD133 targeted toxin (not shown). In this case, responses were noted, but not all of the animals completely responded as they did for the bispecific drug. Thus, in vivo
findings correlated with the in vitro Mary-X data shown in Figure 6-3c in that the
dEpCAM133KDEL was more effective than monospecific dCD133KDEL. Also, it was
not possible to assess monospecific EpCAMKDEL at this dose since it exceeded the
MTD and was toxic to the mice. Monospecific drug is smaller than the bispecific drug
and may be more readily filtered into the liver and/or kidney causing these non-target
toxicities. Taken together, these data show that dEpCAMCD133KDEL was more
effective than the monospecifics in the treatment of these carcinomas.

Discussion

A common problem in carcinoma therapy is drug refractory relapse. CSC’s have
been widely implicated as the particular cells responsible for the development of this
tumor chemoresistance [12,13,21,93,94]. Therefore, it is imperative that adjunct therapies
are developed that can target this subpopulation of cells. We have developed and tested
the first known bispecific TT specifically designed to simultaneously bind two
independent markers on CSC’s.

In these studies, we determined the efficacy of dEpCAMCD133KDEL against head and
neck, breast, and colon carcinoma and found it potently inhibited proliferation. We also
showed the ability of dEpCAMCD133KDEL to kill tumor spheroids in an in vitro assay
using the transplantable human inflammatory breast carcinoma, MARY-X [131]. This is
significant because tumor spheroids are enriched with cancer stem cells and show
enhanced tumorigenicity and clonogenic and differentiation potential [132]. Furthermore,
we have shown the ability of this bispecific to cause tumor regression of small tumors
and more established tumors using an in vivo model of HNSCC. This drug is unique because not only does dEpCAMCD133KDEL target the cancer stem cell population within carcinomas via CD133, but it also can bind EpCAM, a commonly overexpressed marker in many carcinomas. EpCAM has recently been recognized as a CSC marker [34-36]. Furthermore, high EpCAM expression correlates with increased tumorigenicity while little or no EpCAM expression does not [35,129].

There are potential benefits to simultaneously targeting two independent markers expressed on CSCs. First, not all carcinomas express the same CSC markers [12,55]. Furthermore, CSC biomarkers are imperfect in that there is a dynamic continuum where certain markers are expressed for varying amounts of time and can be reexpressed from more differentiated non-CSC populations due to back differentiation [133]. For instance, markers such as CD133 that are known to be expressed on many CSC populations undergo a high rate of plasticity, a phenomenon in which CD133 expression can arise from the more differentiated CD133 negative cell population [56]. Thus, using two CSC reactive ligands would target a broader population of CSCs. Targeting a marker such as EpCAM that has more widespread expression would not only target independent populations of CSCs, but also inhibit populations of cancer cells from which CSCs may arise.

The monospecific targeted toxins, EpCAMKDEL and dCD133KDEL, were tested in the in vitro Mary-X spheroid assay to determine their efficacy compared with the bispecific drug. While, EpCAMKDEL was as potent as the bispecific drug in vitro, it was much more toxic than the bispecific drug in vivo. This prevented EpCAMKDEL from being
tested at the same dose as dEpCAM133KDEL. When compared with dCD133KDEL, dEpCAMCD133KDEL was more effective in vitro and in vivo.

A Major limitation of past TT’s has been the development of neutralizing antibodies following prolonged treatment. We have addressed this issue by deimmunizing the truncated version of pseudomonas exotoxin by mutating key immunogenic epitopes on the surface of the molecule. This lowers the immunogenicity of this toxin and significantly reduces the amount of anti-toxin antibodies produced [54,80]. This deimmunized toxin has been used and tested for a number of targeted toxins we have developed [54,81,82,130].

Past studies have shown that current chemotherapy in combination with a TT is more effective than using either therapy alone [61,62]. It will be interesting to test whether this principle is also true when using a CSC targeted therapy in combination with classical chemotherapy. The hypothesis would be that the chemotherapy would target the rapidly dividing bulk of the tumor, while the CSC targeted therapy would eliminate the subpopulation of cells responsible for the development of chemoresistance. This dual approach may be necessary in order to achieve tumor regression and prevent drug refractory relapse.

In summary, we have developed a novel deimmunized bispecific TT that selectively binds both EpCAM and CD133 CSC markers. Targeting these drug resistant CSC offers a potential solution to our most challenging problem in cancer therapy, drug refractory relapse. dEpCAMCD133KDEL potently inhibited cellular proliferation in three different types of carcinoma. Furthermore, it caused tumor regression in in vivo
studies using a HNSCC mouse model. This work represents the first known bispecific CSC TT and we believe dEpCAMCD133KDEL warrants further study as a potential therapy for use in human carcinoma.
Figures
Flow cytometry was performed on Caco-2 cells to test the binding ability of FITC labeled dEpCAMCD133KDEL. When either CD133 scFv or EpCAM scFv were added to dEpCAM133KDEL-FITC, they did not block binding of the bispecific drug. In contrast, when both were added in combination, the binding of dEpCAMCD133KDEL was significantly reduced indicating that both binding domains of dEpCAMCD133KDEL are functional. When one receptor is blocked, the other is still free to bind. However, when both are simultaneously blocked, binding cannot occur.
Table 6-1 Flow cytometry was performed on a number of cancer lines to determine the level EpCAM and CD133 positivity. As shown here, colon carcinoma, HNSCC, and breast carcinoma lines were highly positive for EpCAM, and had a subpopulation of CD133+ cells. Caco-2 was the only line tested that was highly positive for both EpCAM and CD133. U87, a glioblastoma, was mostly negative for EpCAM, but still had a small subpopulation of CD133+ cells. AHN-12, a CD45 antibody, was used as a negative control for all the cell lines except Raji, a B cell lymphoma, where it acted as the positive control.
**Figure 6-2** Leucine and thymidine incorporation assays show the ability of dEpCAMCD133KDEL to inhibit the proliferation of carcinoma lines. UMSCC-11B (A) and NA-SCC (B) cells were incubated with dEpCAMCD133KDEL and the 50% inhibitory concentration (IC50) was determined to be 0.025nM and 0.0045nM respectively. Two colon carcinoma lines Caco-2 (C) and HT-29 (D) also were potently inhibited by dEpCAMCD133KDEL with IC50 values of 0.044nM and 0.02nM respectively. dEpCAMCD133KDEL was also tested against BT-474 (E) and SK-BR3 (F) two breast carcinomas and subnanomolar IC50 values were 0.046nM and 0.009nM respectively. Finally, two negative control lines were tested to test the specificity of dEpCAMCD133KDEL. U87 is a glioblastoma line that is EpCAM negative and has little CD133 expression. dEpCAMCD133KDEL had no effect, while the positive control targeted toxin, dEGFATFKDEL had an IC50 of 0.03nM. Likewise dEpCAMCD133KDEL had no effect on Raji, a B cell lymphoma, but the positive control targeted toxin d2219ARLKDEL inhibited with an IC50 value of 0.029nM.
A. UMSCC-11B TVA

- Media
- CD19KDEL
- dEpCAMCD133KDEL

Concentration = 0.1nM

B. NA-SCC TVA

- CD19KDEL
- Media
- EpCAMCD133KDEL

Concentration = 0.03nM

C. Mary-x Rate of Spheroid Death

- Media
- CD19KDEL
- dCD133KDEL
dEpCAMCD133KDEL
EpCAMKDEL

Concentrations = 1.0nM
Figure 6-3 Time course viability assays were performed for the two HNSCC lines, UMSCC-11B (A) and NA-SCC (B). In both assays, dEpCAMCD133KDEL killed all cells as determined by viability staining using trypan blue. (C) dEpCAMCD133KDEL also eliminated Mary-X spheroids completely by day 5 in a time course spheroid assay. The monospecifics, EpCAMKDEL and dCD133KDEL, also were effective at eliminating tumor spheroids compared to the negative control targeted toxin, CD19KDEL, and the media control.
Figure 6-4 In experiment 1, UMSCC-11B/luc cells were injected into the right flanks of nude mice and treatment began on day 7. dEpCAMCD133KDEL caused tumor regression in all treated animals, with 4 of 5 being tumor free on day 48. The average tumor measurement by caliper measurement corresponds to the imaging data showing tumor regression in the dEpCAMCD133KDEL treated mice.
Figure 6-5 In experiment 2, UMSCC-11B/luc cells were injected into the right flanks of nude mice and allowed to grow until day 12 when treatment began. The starting average tumor size was almost twice the starting size of the tumors in experiment 1. Imaging and caliper data show tumor regression in all 5 dEpCAMCD133KDEL treated mice, while the negative control, DT2219ARL, had no effect. 3 out of 5 mice were tumor free when the study was discontinued on day 64.
Figure 6-6 Average group weights from mouse experiment 1 (A) and experiment 2 (B) are shown. No toxic side effects were visually observed in the mice. Additionally, no significant weight loss occurred despite the high multiple dosing indicating the absence of significant off target effects by dEpCAMCD133KDEL.
Preface

This chapter has been published:

Chapter IX

Bispecific Targeting of EGFR and uPAR in a Mouse Model of Head and Neck Squamous Cell Carcinoma
Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common worldwide form of cancer [134]. While many new therapeutics have been developed over the past 20 years to treat HNSCC, survival rates remain virtually unchanged. A major contributing problem to this in HNSCC and other carcinomas is chemo-resistance [7,16,134,135]. Therefore, new drugs and new drug combinations are urgently needed to overcome the problem of chemoresistance.

Targeting over-expressed tumor markers is a common strategy in HNSCC. Perhaps the most well-known of these over-expressed markers is epithelial growth factor receptor or EGFR [136,137]. EGFR activates cellular pathways responsible for cancer proliferation, invasion, metastasis, angiogenesis, and resistance to apoptotic signals [138]. Thus, new drugs are currently under development to target EGFR in many carcinomas, including HNSCC [139-141].

Urokinase-type plasminogen activator receptor (uPAR) is expressed in a number of solid tumors such as HNSCC. Importantly, uPAR is also expressed on tumor associated stromal cells particularly on the cells that make up the endothelial neovasculature. uPAR normally functions by catalytically converting its ligand pro-uPA into active uPA which causes proteolytic degradation of a number extracellular matrix proteins [142,143]. However, uPAR overexpression in cancer corresponds with poor prognosis because of its pro-invasive, proliferative, and metastatic functions. Thus, uPAR has been an attractive target for anti-cancer therapies [144-146].
Targeted toxins (TTs) are a type of biological drug consisting of a ligand that specifically recognizes a receptor expressed on cancer cells fused to a catalytic protein toxin that are extremely potent. The activity of the TT is dependent on the ligand binding its receptor and becoming internalized. Following internalization the toxin inhibits protein translation within the target cell causing apoptosis [73].

Recently we reported the activity of a deimmunized bispecific TT, dEGFATFKDEL, in glioblastoma [82,147]. This bispecific fusion protein is made up of human EGF and the amino terminal fragment (ATF) of uPA linked to a deimmunized truncated form of Pseudomonas exotoxin A (PE38). This enables the simultaneous targeting of both the overexpressed EGFR on tumor cells and the uPAR on the tumors endothelial neovasculature via enzymatic ADP ribosylation of Elongation Factor-2 [148]. Thus, targeted tumor cells die and the tumor neovasculature is also destroyed thereby starving the tumor. Importantly, this toxin is deimmunized which significantly reduces its ability to elicit neutralizing antibodies [82,147]. Here we studied the efficacy of dEGFATFKDEL for the first time in an intratumoral therapy model of human HNSCC.

Methods

Construction and purification of dEGFATFKDEL

For this study, dEGFATFKDEL was constructed and purified as described previously [82]. Briefly, synthesis of dEGFATFKDEL was accomplished by fusion of the genes encoding human EGF and the amino terminal fragment (ATF) from uPA. These were then genetically linked to a deimmunized, truncated pseudomonas exotoxin 38. This
fusion gene product was then spliced into the Novagen pET28c bacterial expression vector and transfected into competent cells. The bacteria were grown up and protein expression induced using isopropyl-b-D-thiogalactopyranoside (FisherBiotech). Inclusion bodies were isolated and the protein refolded, dialyzed, and purified over a fast protein liquid chromatography ion exchange column (Q Sepharose Fast Flow, Sigma) as well as a size exclusion column (Superdex 200, Pharmacia). The resulting column fractions of the protein peak were pooled and purity was determined by SDS-PAGE stained with Commasie Brilliant Blue.

**Cell lines**

The squamous cell carcinoma line UMSCC-11B was derived from a larynx tumor following chemotherapy treatment at the University of Michigan [85]. Another squamous cell carcinoma, NA-SCC, was isolated from a tongue tumor [86]. Dr. Frank Ondrey (University of Minnesota) obtained these lines from their originator Dr. Thomas E. Carey (Department of Otolaryngology - Head and Neck Surgery, University of Michigan) and supplied us with the cells. STR testing was done at John Hopkins University’s Fragment Analysis Facility to authenticate the UMSCC-11B cell line. MDA-MB-231 cells were originally obtained from pleural effusion of stage III breast carcinoma patients. These cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Raji cells are a B cell line derived from a Burkitt’s Lymphoma and were also obtained from ATCC. The colorectal carcinoma line, Caco-2 was also obtained from
ATCC. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Lonza Group Ltd.).

**Flow cytometry**

Flow cytometry was performed to determine the level of EGF and uPAR expression on the surface of the cell lines. A fluorescence-activated cell sorting (FACS) Caliber was used for all flow experiments at the University of Minnesota’s Flow Cytometry Core Facility. Antibodies were labeled with fluorescein isothiocyanate (FITC) and data analysis was performed using FLOWJO. The percentage of positive cells was determined by gating control cells that were not incubated with antibody.

**Bioassays**

Protein synthesis assays measuring [3H]leucine incorporation were used to determine the effect of dEGFATFKDEL on the cell lines. Proliferation assays were also performed on occasion using [3H]thymidine incorporation. These assays have been described previously [81,82,147]. Briefly, cells are plated in 96-well flat-bottomed plates and allowed to adhere overnight in a 37 °C and 5% CO2 incubator. The targeted toxins were added in triplicate at 10-fold serial dilutions and incubated for 48 h. Wells are then pulsed with either [3H]leucine (protein synthesis assay) or [3H]thymidine (proliferation assay) with 1 μCi per well and allowed to incubate for another 24 h. Plates are then frozen to detach the cells, harvested onto glass fiber filters, washed, dried, and counted using
standard scintillation methods. [3H]leucine assays were done using leucine-free medium. Data are reported as the percentage of control counts.

**In vivo studies**

The in vivo studies were done on male nu/nu mice purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area, and housed in microisolator cages in the pathogen-free AAALAC accredited facility under the care of the Department of Research Animal Resources at the University of Minnesota. All animal research protocols had been approved by the University of Minnesota Institutional Animal Care and Use Committee. For the flank tumor studies, UMSCC-11B and MDA-MB-231 cells were stably transfected with a vector containing a firefly luciferase (luc) gene and a blastocidin resistant gene. This allowed the tumors to be imaged in real time to track the growth or regression of the tumors. For the UMSCC-11B/luc study, four million tumor cells were injected subcutaneously in the left flanks of the mice (5 mice/group). The mice were treated with 2ug’s of dEGFATFKDEL intratumorally MTWTh starting on day 7 post-inoculation and ending on day 35. For the MDA-MB-231/luc study, 3 million cells were injected subcutaneously into the left flanks of the mice. Tumors were injected intratumorally with 3ug of dEGFATFKDEL starting on day 25 and 8 courses of treatment were given where one week of injections (MWF) constitute one course. Tumors for both studies were measured using digital calipers and the volume was calculated as the product of the width, length, and height. The mice were weighed regularly in order to monitor for treatment-related toxicities that typically cause
a drop in body weight. Mice were imaged once a week in real time using Xenogen Ivis imaging system (Xenogen Corporation, Hopkington, MA). Imaging was done as described previously [81,147]. For the imaging, mice were anesthetized using isoflurane gas and then injected with 100 ul of a 30 mg/ml D-luciferin solution (Gold Biotechnology, St. Louis MO) 10 min prior to imaging. Mouse images represent a 5 min exposure time and the units of luminescence is expressed as photons/s/cm2/sr. Prism 4 (Graphpad Software, San Diego, CA) was used for all statistical analysis.

Results

Flow cytometry expression analysis

In order to determine the level of expression of EGFR and uPAR present on the surface of the head and neck carcinoma cell line, UMSCC-11B, flow cytometry was performed. As shown in Fig. 7-1, UMSCC-11B cells are 100% EGFR positive, while only 1.51% uPAR positive. The cells were also probed with the FITC labeled negative control anti-CD19 antibody, HD37, which showed no reactivity against the carcinomas, but did against the B cell lymphoma line Raji. Table 1 shows another head and neck carcinoma, NA-SCC, also highly expresses EGFR at 99%. The breast carcinoma line MDA-MB-231 and colon carcinoma line Caco-2 also highly express EGFR at 91.2% and 98.8% respectively. Human umbilical vein endothelial cells, or HUVECs, were 61% uPAR positive, but had minimal EGFR expression (3.7%). Thus, the head and neck carcinoma lines (UMSCC-11B and NA), as well as the breast carcinoma line (MDA-MB-231) are excellent targets for an EGFR targeted therapy. The primary Human umbilical vein
endothelial cells (HUVEC’s) show that human endothelial cells, which are part of the neovasculature, are uPAR positive and will serve as targets for our bispecific drug.

**In vitro assays**

Based on the results of the flow cytometry studies, [3H]leucine incorporation assays were performed to determine the level of selective protein synthesis inhibition of dEGFATFKDEL on these cell lines. As shown in Fig. 7-2a and b, dEGFATFKDEL selectively inhibited both of the head and neck cell lines, UMSCC-11B, and NA-SCC, with IC50 values at sub-nanomolar concentrations (1.38 e-4 nM and 4.37 e-5 nM respectively). MDA-MB-231, a breast carcinoma line, had a similar IC50 value of 5.01 e-4 nM as seen in Fig. 7-2c. Figure 7-2d shows a Leucine assay graph of a colorectal carcinoma cell line, Caco-2. While dEGFATFKDEL was not quite as potent (IC50=2.19 e-3 nM) against Caco-2, it was still selectively active. The HUVEC’s were tested in Fig. 7-2e to determine whether cells that were uPAR positive, but EGFR negative could still be killed by dEGFATFKDEL. Indeed our bivalent dEGFATFKDEL has an IC50 concentration of 0.03 nM and can therefore bind uPAR-expressing cells, internalize, and inhibit protein synthesis. Thus, both the EGF and ATF portions of our molecule are highly active. Raji cells (a B-cell lymphoma line) were tested to determine whether dEGFATFKDEL could still inhibit protein synthesis even though the line is EGFR and uPAR negative. Fig. 7-2f shows that while a targeted toxin that binds CD22 and CD19 (d2219ARLKDEL) can inhibit protein synthesis, dEGFATFKDEL cannot. Thus, dEGFATFKDEL is a highly specific and bifunctional drug.
**In vivo studies**

To determine the activity of dEGFATFKDEL in vivo, a flank xenograft tumor model was used. For the study, UMSCC-11B cells that had been stably transfected with a gene expressing firefly luciferase (luc) were injected into the left flanks of nude mice. UMSCC-11B/luc tumors were treated intratumorally for 4 weeks starting on day 7 with dEGFATFKDEL. Fig. 7-3a shows a clear inhibition of the tumor growth in treated verse untreated animals. There were 5 mice per group and the results are statistically significant. To show the selective efficacy of dEGFATFKDEL, a negative control group was treated with CD19KDEL (a targeted toxin specific for CD19). Even though the mice were treated aggressively with these targeted toxins, there were no signs of toxicity at the concentrations used based on the unchanging average mouse weights shown in Fig. 7-3b.

Fig. 7-4 shows luminescent images of representative mice from the three groups. The images of the dEGFATFKDEL treated group show a regression over time of the luminescent signal indicating a corresponding tumor regression. Unlike dEGFATFKDEL, CD19KDEL was not effective at causing tumor regression as the CD19KDEL treated mice showed no decrease in luciferase activity.

To confirm the effectiveness of dEGFATFKDEL in another carcinoma, MDA-MBA-231/luc tumors were treated intratumorally for 8 weeks. MDA-MB-231/luc tumors grow slower than UMSCC-11B/luc tumors, thus a modified dose schedule of 3ug every MWF was used instead of the more aggressive MTWTh regimen. As seen in Fig. 7-5, untreated tumors grew normally, while dEGFATFKDEL treated tumors were completely inhibited.
No drug toxicity was seen in the treated mice in the study either (data not shown). These results show that dEGFATFKDEL is effective at inhibiting tumor growth and causing tumor regression in both head and neck carcinoma as well as in a second model of carcinoma.

**Discussion**

This is the first report of a bispecific targeted toxin that simultaneously targets EGFR and the neovasculature in carcinomas. Tumors were chosen that were EGFR positive and uPAR negative. The anti-EGFR moiety of the hybrid protein bound EGFR on the tumors and the anti-uPAR moiety bound on endothelial cells as evidenced by the ability of the drug to kill HUVEC cells. These studies show that dEGFATFKDEL selectively inhibited protein synthesis in both head and neck and breast carcinoma tumors in vitro and caused regression of tumors in vivo. While many therapies have been developed to target either the tumor or the neovasculature, this one simultaneously targets both.

Targeted toxins (TTs) are a type of biological drug consisting of ligands fused to a catalytic protein toxin that specifically bind receptors on cancer cells, internalize, and inhibit protein translation thereby causing apoptosis in the target cells. Historically, one main issue has curtailed the use of targeted toxins in the clinic. The development of antibodies against the toxin portion of the fusion protein has limited the efficacy of targeted toxins because multiple treatments are needed to penetrate solid tumors and cause regression [77]. Pastan and Onda originally showed that mutations made in the immunogenic regions of PE38 allow for repeated treatments using dEGFATFKDEL
without anti-toxin antibodies developing [80]. We used these mutations to develop
dEGFATFKDEL and showed that mice immunized with dEGFATFKDEL had a greatly
reduced capacity for anti-toxin antibody response as compared to unmodified parental
EGFATFKDEL [18]. Indeed, these mutations have allowed us to create several non-
immunogenic targeted toxins that are effective against a range of cancers [51,81,83,147].
Furthermore, TT act synergistically when added as an adjunct to classical chemotherapy
and are more effective in combination than using either therapy separately [61,62].

Targeting the EGF receptor has shown to be effective in cancer therapy because it is over
expressed on many human carcinomas. Several studies using targeted toxins specific for
EGFR have been undertaken with promising results [149-151]. Furthermore, the Food
and Drug Administration (FDA) have approved no less than five EGFR inhibitors for the
treatment of various types of cancer. They include Cetuximab (head and neck and
colorectal cancer), gefitinib (non-small cell lung cancer), erlotinib (non-small cell lung
and pancreatic cancer), panitumumab (colorectal cancer), and lapatinib (breast cancer)
[141,152]. While these drugs have been an important step forward in the treatment of
these cancers, advances have been incremental and there is still much room for
improvement.

Angiogenesis is a critical step in tumorigenesis in which new blood vessels are
formed. Without a sufficient blood supply, tumor growth is limited and the tumor
regresses. Therefore, many anti-angiogenic agents, such as Thalidomide, Bevacizumab,
sunitinib, among many others have been developed and approved by the FDA for the
treatment of many types of cancer [153]. Since uPAR plays a role in neoangiogenesis
within tumors, and is also involved in tumor proliferation, tissue invasion, and metastasis,
it has become an important target [144-146,154]. Thus, we designed dEGFATFKDEL to kill uPAR positive cells in the tumor microenvironment. Interestingly, dEGFATFKDEL is a more potent inhibitor of protein synthesis than the equimolar combination of the two separate TT EGFKDEL and ATFKDEL [147]. One explanation could be that the affinity of the bispecific may be greater than the monospecifics or that the bispecific somehow stabilizes the binding of the scFv’s to their specific receptors. In vivo, the bispecific would also have two different targets in the tumor microenvironment it could bind, thus providing an increased opportunity for the necessary binding and internalization to take place while monospecifics may be able to diffuse out of the tumor more readily. While the mouse model used here does not provide in vivo proof of vascular effects, the in vitro studies clearly show that dEGFATFKDEL is a potent inhibitor of uPAR positive endothelial cells as shown by the HUVEC assays.

Development of bispecific-targeted therapeutics for cancer treatment is on the rise. Many of these function by either retargeting effector molecules or effector cells [155]. One of these is a bispecific antibody developed recently that focuses on inhibiting angiogenesis by targeting PDGFR and VEGFR-A. This bispecific was effective at inhibiting A673 rhabdomyoscarcoma tumors (a typical anti-angiogenesis model) in vivo [156]. However, only targeting the angiogenesis pathway has only been mildly effective in the clinic [157]. Furthermore, the immune system in many cancer patients is compromised either by the cancer itself or by many chemotherapy treatments [158,159]. Bispecific TT, however, do not require immune effector cells to mediate cell death. The catalytic protein toxin enables these molecules to specifically target the tumor cells and tumor microenvironment.
Here we show for the first time that dEGFATFKDEL targets both EGFR positive tumor cells as well as the uPAR positive neovasculature cells in head and neck carcinoma and confirmed its activity in a second carcinoma model as well. This two-pronged approach enables dEGFATFKDEL to potently inhibit protein synthesis in vitro and tumor growth and progression in vivo. Its efficacy, potency, selectivity, and mechanism of action give it an advantage over other biological drugs. Thus, dEGFATFKDEL warrants further study and characterization for the clinical treatment of carcinoma.
Figures

**Figure 7-1** Flow cytometry dot plot of the head and neck carcinoma line UMSCC-11B probed with either FITC conjugated anti-EGFR antibody or anti-uPAR antibody. Cells were also probed with the negative control HD37-FITC (an anti-CD19 antibody).
<table>
<thead>
<tr>
<th></th>
<th>EGFR</th>
<th>uPAR</th>
<th>CD19</th>
</tr>
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<tbody>
<tr>
<td>UMSCC-11B</td>
<td>100.0</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>NA-SCC</td>
<td>99.0</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>91.2</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>HUVEC</td>
<td>3.7</td>
<td>61.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Raji</td>
<td>5.6</td>
<td>7.7</td>
<td>95.8</td>
</tr>
<tr>
<td>Caco-2</td>
<td>98.8</td>
<td>.4</td>
<td>.8</td>
</tr>
</tbody>
</table>

**Table 7-1** Expression levels of EGFR, uPAR, and negative control CD19 was determined by flow cytometry. UMSCC-11B, NA-SCC, MDA-MB-231, and Caco-2 all highly express EGFR, but have negligible uPAR expression. HUVEC contained high levels of uPAR receptor as expected. The negative control antibody, HD-37FITC, bound very highly to Raji cells, a CD19 positive B-cell lymphoma line.
Figure 7-2 The activity of dEGFATFKDEL against the carcinoma lines was determined by Leucine and Thymidine incorporation assays. UMSCC-11B, NA-SCC, MDA-MB-231, and Caco-2 Leucine incorporation assays showed that dEGFATFKDEL was selectively active while the negative control in each case had no effect (a-d). HUVEC primary cells were also tested and were selectively inhibited by dEGFATFKDEL as well. However, dEGFATFKDEL had no effect on the EGF and uPAR negative B cell lymphoma line Raji, while a positive control targeted toxin specific against CD22 and CD19 had activity (f).
A. UMSCC-11B Average Flank Tumor Growth

B. UMSCC-11B Average Body Weights
**Figure 7-3** Mouse tumor model of UMSCC-11B show dEGFATFKDEL is effective at inhibiting tumor growth. As seen in (a) tumor growth was inhibited in dEGFATFKDEL treated mice as compared with the no treatment mice. Student t test analysis was performed for dEGFATFKDEL treated tumors compared with untreated tumors on each day. The tumor size between groups became significantly different on day 23 (p<0.05). Body weight was measured in (b) and shows that treated mice did not have any significant weight loss due to drug toxicity throughout the study.
Representative Mice – Imaging from UMSCC-11B Tumor Study

Figure 7-4 Real time images of representative mice from the UMSCC-11B study are shown. Mice treated with dEGFATFKDEL exhibited tumor reduction over time shown by the corresponding decrease in luminescence. Control treated and untreated mice, however, had continually increasing luminescent levels.
Figure 7-5 A second mouse carcinoma model was used to confirm the results from the UMSCC-11B study. MDA-MB-231 tumors treated with dEGFATFKDEL were inhibited, while untreated tumors grew normally over time. Student t tests were performed on every day. The dEGFATFKDEL treated tumors were statistically different (p<0.05) from the untreated controls on day 35, and on every subsequent day.
Chapter VIII

Conclusion
CSCs are a critical population of cells within carcinomas that possess the unique ability to self-renew, and resist radiation and chemotherapy induced cell death. In order to effectively target this crucial subpopulation of cells, accurate biomarkers are needed. Recently, CD133 was identified as a key marker of CSCs within many types of carcinoma [12,13,70-72]. In the studies described here, we developed a novel targeted toxin that specifically binds the CD133 receptor. The scFv used to create this targeted toxin was taken from a new anti-CD133 monoclonal antibody (clone 7) that is able to bind both glycosylated and non-glycosylated epitopes of the CD133 receptor, improving it over commercial anti-CD133 antibodies. Flow cytometry studies showed that this scFv was able to bind both human and mouse forms of the CD133 receptor using cells transfected with mouse CD133 and by using mouse cell lines [130]. The resulting targeted toxin, dCD133KDEL, was created by combining the scFv with a truncated pseudomonas exotoxin A. PE toxin was mutated and we proved that amino acid substitution resulted in a reduced anti-toxin response in immunocompetent mice confirming our deimmunization protocol. Because only a small portion of the general tumor cell population is CD133+, we developed a time course viability assay that demonstrated the drug’s ability to inhibit tumor cell proliferation in vitro in a variety of carcinoma lines including HNSCC and breast carcinoma lines [51,53]. Carcinoma cell lines that were tested only contained a small subpopulation of CD133 positive cells, with the exception of Caco-2, which was over 79% positive. We also analyzed the ability of dCD133KDEL to prevent tumor initiation in a HNSCC xenograft flank model. This demonstrated that we were indeed selectively killing the CD133 positive CSC subpopulations within HNSCC and thereby inhibiting tumor initiation. We then showed
that dCD133KDEL could be used to achieve tumor regression and generate long-term disease free survivors in the HNSCC model. We discovered that all tumors including larger more established tumors treated intratumorally with dCD133KDEL regressed. Next, we tested the ability of this drug to cause tumor regression in a model of systemic breast cancer. Even when given interperitoneally (IP), dCD133KDEL was able to cause regression alone and in combination with another targeted toxin, dEGF4KDEL[53]. Furthermore, dCD133KDEL was also found to be effective against ovarian carcinoma lines in vitro and in vivo when given IP [skubitz].

Another important aspect of these studies was determining the safety of dCD133KDEL. We found that this drug was very well tolerated in mice compared to other targeted toxins and were able to consistently give twice our typical dose (40ug 3 times a week) with no noticeable side effects [53]. Furthermore, no liver or kidney toxicity was detected except at the LD_{50} dose of 100 ug (Figure 8-1). Since normal progenitor cells can also express CD133, we tested dCD133KDEL against umbilical cord blood progenitor cells that were 67% positive in hematopoietic colony formation assays. Even at concentrations 10-50 times higher than used to inhibit tumor cell proliferation, dCD133KDEL did not have any effect on normal progenitor colony formation [51]. This suggests a therapeutic window exists where dCD133KDEL can kill CD133 positive CSCs and not normal progenitors that also express this marker.

In our studies, we also confirmed findings from other groups that CSC markers are extremely dynamic in nature. CD133- cells were sorted from tumors using flow cytometry and after two weeks in culture CD133 was reexpressed from the negative cells. Furthermore, in the in vitro time course assays, dCD133KDEL did not permanently
inhibit proliferation and negative cells could reexpress CD133. This led us to determine whether therapy could be improved by simultaneously targeting CSC and non-CSC populations, ie, both the CSCs and the populations that can dedifferentiate and give rise to them. Thus, we developed a bispecific targeted toxin, dEpCAMCD133KDEL, which simultaneously targets the overexpressed carcinoma marker EpCAM as well as the CSC marker CD133. This targeted toxin was developed by fusing the DNA for the EpCAM scFv onto the same DNA strand encoding dCD133KDEL. EpCAM was overexpressed on all the carcinoma lines that were tested in flow cytometry and is currently being targeted clinically in several types of cancer [160]. When tested against various carcinoma lines, dEpCAMCD133KDEL potently inhibited proliferation in vitro and was also effective when tested in an in vitro breast cancer spheroid model. Tumor spheroids are enriched for CSC and highly CD133+. Furthermore, dEpCAMCD133KDEL was able to cause tumor regression in a flank tumor model of HNSCC [161].

In a separate study, I studied another bispecific targeted toxin, dEGFATFKDEL, which simultaneously targets EGF receptor, and the urokinase receptor expressed on the tumor vasculature [162]. This bispecific was also potently effective at inhibiting cancer cell proliferation in vitro and causing tumor regression of HNSCC tumors in vivo. We hypothesize that like the combination of dEGF4KDEL and dCD133KDEL, a combination of dEGFATFKDEL and dCD133KDEL would be highly effective. New therapies are urgently needed for the treatment of HNSCC as well as other types of carcinoma. Since drug refractory relapse is the single most serious problem in the cancer field, we developed two novel targeted toxins that can specifically bind and destroy CD133 positive CSCs. Targeted toxins have been shown to work synergistically with
chemotherapy. Thus, we hypothesize that using these CSC targeted toxins in combination with current chemotherapeutic agents also would have synergistic results. Indeed, targeting the cells responsible for chemoresistance while also eliminating chemosensitive cells could result in a comprehensive treatment and improve clinical outcomes for patients with HNSCC. In conclusion, we believe further development of dCD133KDEL and dEpCAMCD133KDEL for clinical testing is warranted.
Figure 8-1 CF1 mice were injected Monday-Thursday with either PBS or dCD133KDEL. Mice were then bled on Friday and blood was analyzed for levels of Alanine transferase (ALT), blood urea nitrogen (BUN), and creatinine. Dotted lines represent normal blood concentrations in mice. At the 20ug dose of dCD133KDEL, no elevated levels of ALT, BUN, or creatinine were detected.
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