

A bispecific EpCAM/CD133-targeted toxin is effective against carcinoma

Nate N. Waldron · Sanford H. Barsky ·
Phillip R. Dougherty · Daniel A. Vallera

Received: 14 March 2013 / Accepted: 21 June 2013 / Published online: 31 July 2013
© Springer-Verlag France 2013

Abstract The discovery of chemoresistant cancer stem cells (CSCs) in carcinomas has created the need for therapies that specifically target these subpopulations of cells. Here, we characterized a bispecific targeted toxin that is composed of two antibody fragments and a catalytic protein toxin allowing it to bind two CSC markers on the same cell killing this resistant subpopulation. CD133 is a well-known CSC marker and has been successfully targeted and caused regression of head and neck squamous cell carcinoma (HNSCC) *in vivo*. To enable it to bind a broader range of CSCs, an anti-epithelial cell adhesion molecule (EpCAM) scFv was added to create dEpCAMCD133KDEL, a deimmunized bispecific targeted toxin on a single amino acid chain. This bispecific potently inhibited protein translation and proliferation *in vitro* in three different types of carcinoma. Furthermore, in a CSC spheroid model dEpCAMCD133KDEL eliminated Mary-X spheroids, an inflammatory breast carcinoma. Finally, this bispecific also caused tumor regression in an *in vivo* model of HNSCC. This represents the first bispecific CSC-targeted toxin and warrants further development as a possible therapy for carcinoma.

Keywords Cancer stem cells · Targeted toxins · Immunotoxins · CD133 · EpCAM · Head and neck squamous cell carcinoma

N. N. Waldron · P. R. Dougherty
Department of Pharmacology, University of Minnesota, 210
Delaware Street Southeast, Minneapolis, MN 55455, USA

S. H. Barsky
Department of Pathology, University of Nevada School of
Medicine, Manville Medical Building, Reno, NV 89557, USA

D. A. Vallera (✉)
Department of Therapeutic Radiology-Radiation Oncology,
Section on Molecular Cancer Therapeutics, University
of Minnesota Masonic Cancer Center, 425 East River Parkway,
Minneapolis, MN 55455, USA
e-mail: valle001@umn.edu

Introduction

Epithelial cell adhesion molecule (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 [1, 2]. 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 [3, 4]. 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) [5, 6]. 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 [7]. Today, it is an established CSC marker in many carcinomas, such as breast, colon, prostate, and HNSCC among others [8, 9]. Like EpCAM, CD133 is involved in the Wnt/ β -catenin signaling pathway [10–12]. We recently synthesized a new anti-CD133 monoclonal antibody (clone 7) that differs from other anti-CD133 antibodies in that it recognizes the extracellular domain and is cross-reactive with mouse CD133 [13]. 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* [14–16]. 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 [17–20].

Because of the efficacy shown by a novel monospecific CD133-directed targeted toxin [14] and the promising results of clinical trials using a monospecific anti-EpCAM TT [21–23], 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 [24]. 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 [14, 17, 19, 25].

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 humanized anti-EpCAM scFv from the antibody MOC31, anti-CD133 scFv from clone 7, and a deimmunized truncated form of PE 38 used previously [14, 17–19]. 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-β-D-thiogalactopyranoside 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 (Minneapolis, MN).

Purification of dEpCAMCD133KDEL

Purification was performed as described previously [26]. Briefly, the protein was expressed and purified from inclusion

bodies using the Novagen pET expression system (Darmstadt, Germany). Then a two-step purification procedure was performed using an ion-exchange fast protein liquid chromatography (Q sepharose Fast Flow, Sigma, Saint Louis, MO) followed by size exclusion chromatography (Hiload Superdex 200, GE Life Sciences, Uppsala, Sweden). The purified protein was then analyzed by Bradford to determine the concentration and by SDS-PAGE stained with Coomassie Brilliant Blue to determine purity. The CD133 scFv from clone 7 and an anti-EpCAM Fc that was used to construct another bispecific TT described previously [20] were also produced and purified using this purification procedure and used in flow cytometric assays to determine the binding of dEpCAMCD133KDEL. DT2219 ARL and 2219 KDEL are anti-B cell targeted toxins used as controls [19].

Cell lines and culturing techniques

UMSCC-11B is an HNSCC line that was derived from larynx tumor following chemotherapy [27]. UMSCC11B-luc was transfected using a luciferase reporter construct and maintained with 10 μg/mL of blastocidin. Cells were transfected using Invitrogen's Lipofectamine Reagent. NA-SCC is another HNSCC line isolated from a tongue tumor [28]. 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-11B 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 (Manassas, VA) 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 FACSCalibur 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 were 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 IC_{50} values. Plates would then be incubated for an additional 48 h. ^3H -thymidine or ^3H -leucine

would then be added for a final 18-h 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 [14]. Briefly, cells were

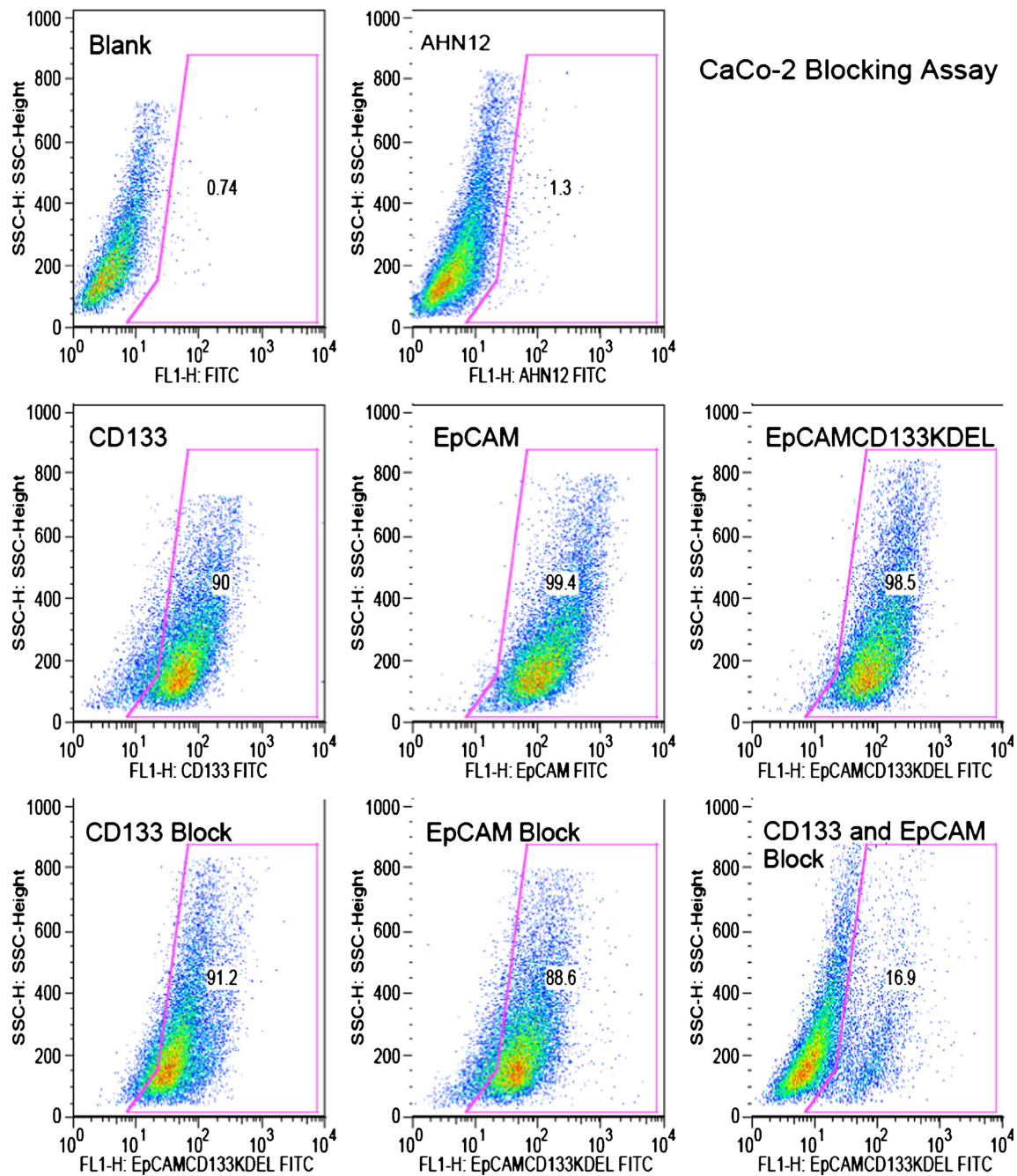


Fig. 1 Flow cytometry was performed on Caco-2 cells to test the binding ability of FITC-labeled dEpCAMCD133KDEL. When either CD133 scFv or EpCAM scFv was added separately with dEpCAM133KDEL-FITC to the cells, the binding of the bispecific drug was not blocked. In contrast, when both were added in combination, 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

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 are 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 UMSSC-11B/luc cells were injected into the right flank of 15 nude mice. Starting on day 7, tumors were treated with 20 $\mu\text{g}/\text{mouse}$ of dEpCAMCD133KDEL, 2219KDEL, or 100 μL of saline. Mice were given four courses of injections where one course was three injections given every other day (MWF) for 1 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 UMSSC-11B/luc cells were injected into the right flanks of nine nude mice. Treatment began on day 12 and continued four times a week (MTWTh) for 7 weeks with five mice receiving 20 μg of dEpCAMCD133KDEL and the remaining four mice receiving 10 μg 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, Cranbury, NJ). Mice were injected with 3 mg of luciferin substrate

10 min 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 per second per cubic meter per steradian.

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 on Fig. 1, FITC-labeled CD133 scFv from clone 7 and a humanized 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 to determine whether it might be a suitable target for dEpCAMCD133KDEL. As seen in Table 1, HT-29 was 98.8 % EpCAM positive and 2.61 % CD133 positive. The table also shows two HNSCCs (UMSSC-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

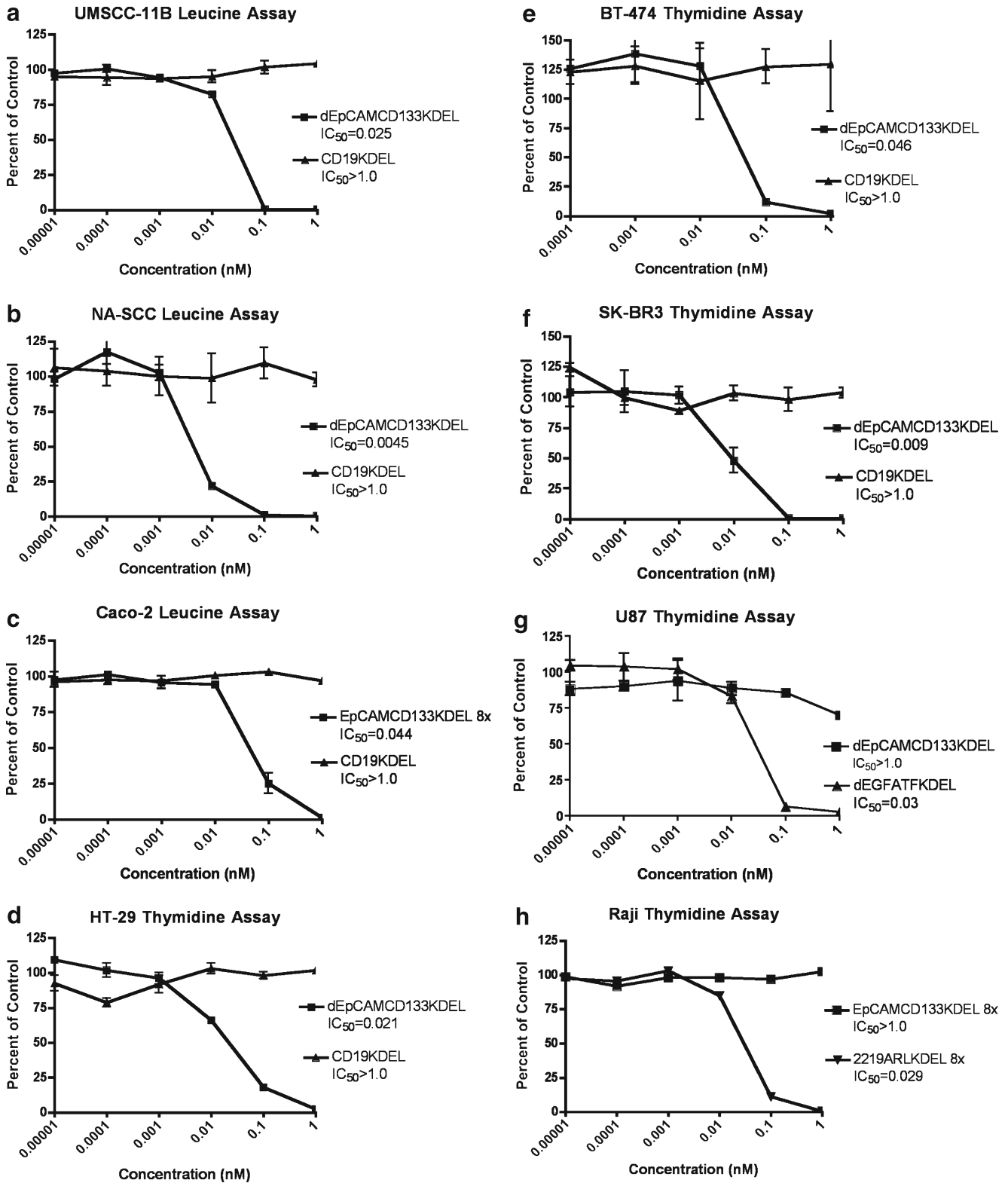
To test the efficacy of dEpCAMCD133KDEL, the HNSCC lines were tested in ^3H -leucine incorporation assays to determine

Table 1 Flow cytometry was performed on a number of cancer lines to determine the level of EpCAM and CD133 positivities

Cell Line	EpCAM+	CD133+	AHN12
Caco-2	99.4	90.0	1.3
HT-29	98.8	2.61	2.18
UMSSC-11B	98.3	5.90	0.50
NA-SCC	97.9	5.88	0.65
BT-474	97.2	4.09	0.47
SK-BR3	96.7	4.40	0.98
U87	1.33	2.38	0.46
Raji	0.27	2.27	91.2

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. A CD45 antibody, AHN-12, was used as a negative control for all the cell lines except Raji, a B cell lymphoma, where it acted as the positive control

Fig. 2 Leucine and thymidine incorporation assays show the ability of dEpCAMCD133KDEL to inhibit the proliferation of carcinoma lines. UMSSC-11B (a) and NA-SCC (b) cells were incubated with dEpCAMCD133KDEL and the 50 % inhibitory concentration (IC_{50}) was determined to be 0.025 and 0.0045 nM, respectively. Two colon carcinoma lines Caco-2 (c) and HT-29 (d) were also potently inhibited by dEpCAMCD133KDEL with IC_{50} values of 0.044 and 0.021 nM, respectively. dEpCAMCD133KDEL was also tested against BT-474 (e) and SK-BR3 (f). Two breast carcinomas and subnanomolar IC_{50} values were 0.046 and 0.009 nM, 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 IC_{50} of 0.03 nM. Likewise, dEpCAMCD133KDEL had no effect on Raji, a B cell lymphoma, but the positive control targeted toxin d2219ARLKDEL inhibited with an IC_{50} value of 0.029 nM



the level of protein translation inhibition. As seen in Fig. 2a, b, dEpCAMCD133KDEL inhibited both UMSCC-11B and NA-SCC with IC₅₀ values of 0.025 and 0.0045 nM, 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 Fig. 2e, f as well. However, as seen in Fig. 2g, h, the two EpCAM negative cell lines were not affected by dEpCAMCD133KDEL at the concentrations tested, while positive controls potently inhibited proliferation.

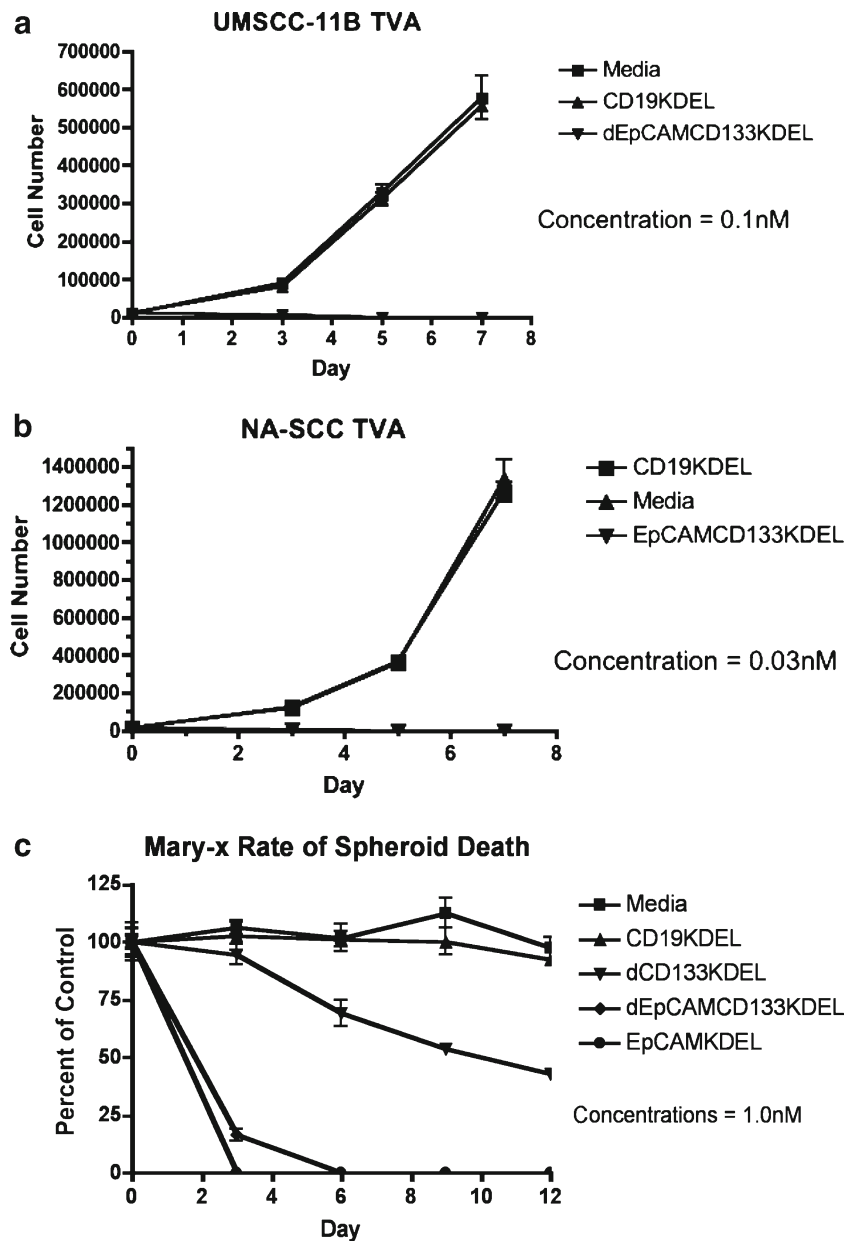
To determine whether the inhibition of protein translation was causing cell death in the cancer lines, time course viability assays were performed. As seen in Fig. 3a, dEpCAMCD133KDEL killed all UMSSC-11B carcinoma cells while a negative control TT, CD19KDEL, did not. NA-SCC cells were killed in the same manner in Fig. 3b. Furthermore, when inflammatory breast carcinoma Mary-X spheroids were tested in a time course spheroid assay in Fig. 3c, dEpCAMCD133KDEL eliminated the

spheroids while the negative control had no effect. The mono-specific 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 UMSSC-11B/luc cells into their right flanks. In the first study (Fig. 4), three groups of mice (five mice/group) were treated intratumorally starting on day 7. Thus, these were smaller tumors at the time the therapy was initiated. Treatment was

Fig. 3 Time course viability assays were performed for the two HNSCC lines, UMSSC-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, were also effective at eliminating tumor spheroids compared with the negative control-targeted toxin, CD19KDEL, and the media control



Study 1

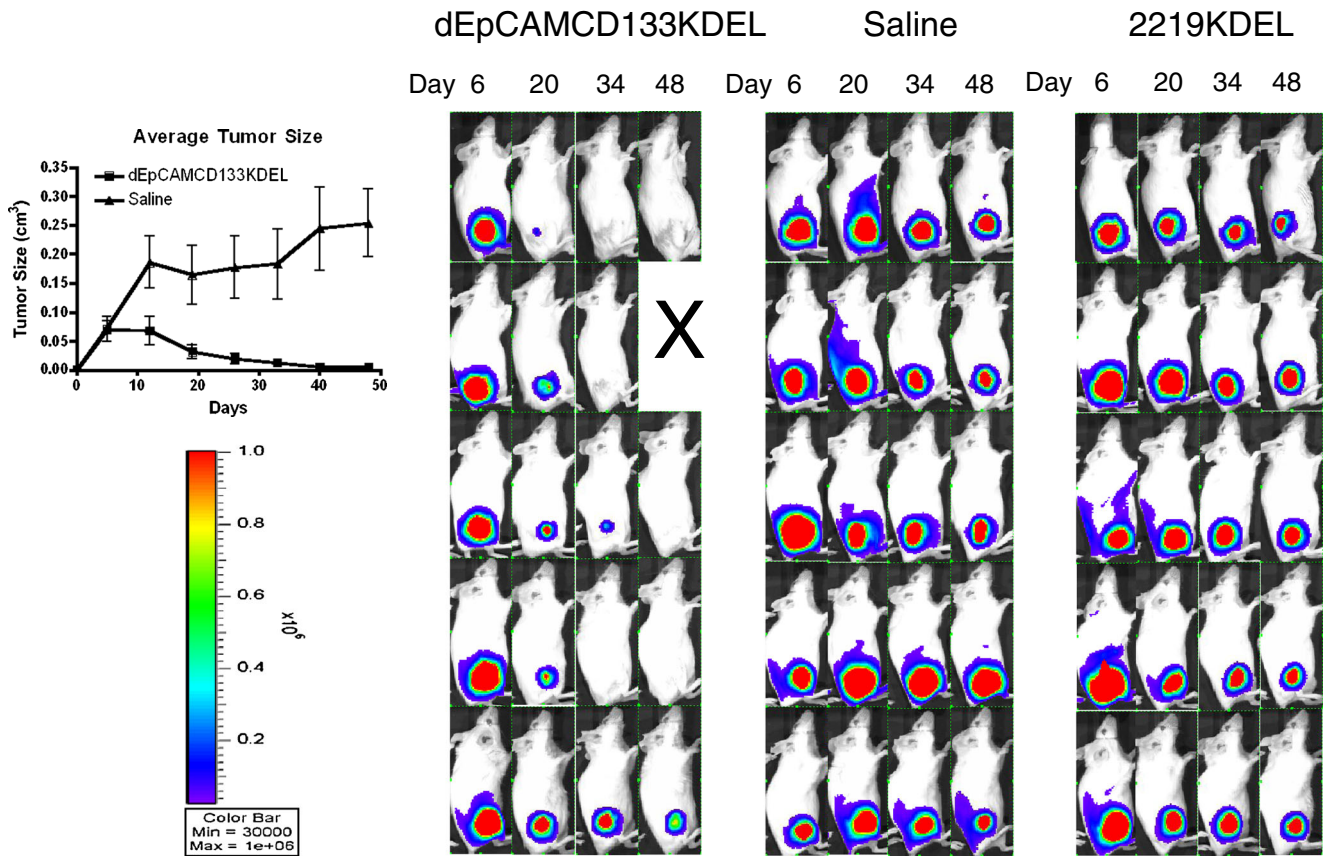


Fig. 4 In experiment 1, UMSSC-11B/luc cells were injected into the right flanks of nude mice and treatment began on day 7. dEpCAMCD133KDEL caused tumor regression in all treated animals, with four of five being tumor free on day 48. Bioluminescence is shown on each picture as a

function of photons/s/cm²/sr. The intensity of the signal is illustrated by the color bar. The average tumor measurement by caliper measurement on the graph corresponds to the imaging data showing tumor regression in the dEpCAMCD133KDEL-treated mice

discontinued on day 40. All five dEpCAMCD133KDEL-treated tumors regressed, with four mice achieving tumor-free status measured by imaging. Caliper data reflected the imaging results. In the second study shown in Fig. 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 five mice was treated with dEpCAMCD133KDEL and another group was treated with the negative control DT2219ARL since there are no B cell markers on UMSSC-11B. Treatment was discontinued on day 63. Figure 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 two of the four 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 Fig. 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 Fig. 3c in that the dEpCAM133KDEL was more effective than monospecific dCD133KDEL. In addition, it was not possible to assess monospecific EpCAMKDEL at this dose since it exceeded the MTD.

Discussion

A common problem in carcinoma therapy is drug refractory relapse. CSCs have been widely implicated as the particular cells

Study 2

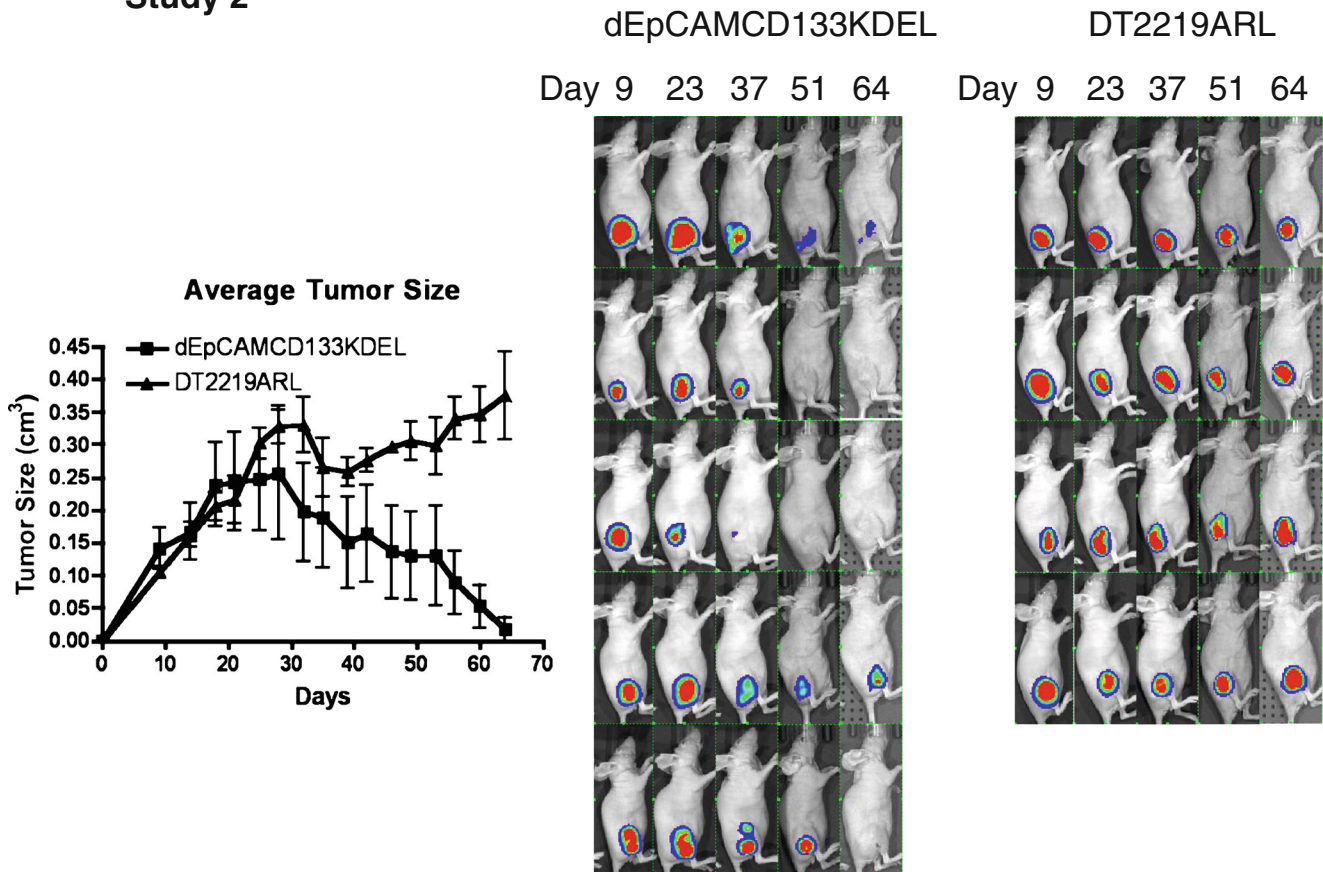


Fig. 5 In experiment 2, UMSCC-11B/luc cells were injected into the right flanks of nude mice and allowed to grow until day 12 when treatment began. The starting average tumor size was almost twice the starting size of the tumors in experiment 1. Imaging and caliper data

show tumor regression in all five dEpCAMCD133KDEL-treated mice, while the negative control, DT2219ARL, had no effect. Three out of five mice were tumor free when the study was discontinued on day 64

responsible for the development of this tumor chemoresistance [8, 29–32]. 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 CSCs.

In these studies, we determined the efficacy of dEpCAMCD133KDEL against head and neck, breast, and colon carcinoma and found that 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 [33]. This is significant because tumor spheroids are enriched with CSCs and show enhanced tumorigenicity and clonogenic and differentiation potential [34]. Furthermore, we have shown the ability of this bispecific TT 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 CSC population within carcinomas via CD133 but it can also bind EpCAM, a commonly overexpressed marker in many carcinomas. EpCAM has recently been recognized as a CSC marker [5, 35, 36]. Furthermore, high EpCAM expression correlates with increased tumorigenicity while little or no EpCAM expression does not [5, 6].

There are potential benefits in simultaneously targeting two independent markers expressed on CSCs. First, not all carcinomas express the same CSC markers [8, 37]. 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 [38]. For instance, markers such as CD133 that are known to be expressed on many CSC populations undergo a high rate of

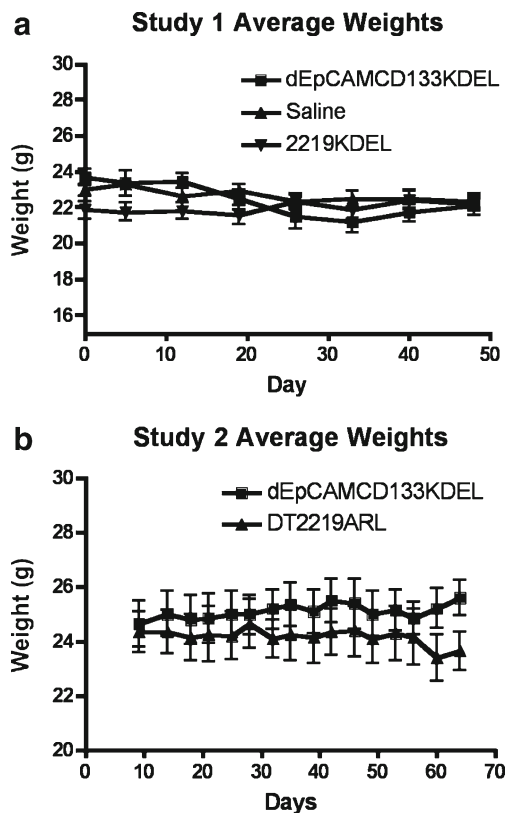


Fig. 6 Average group weights from mouse experiments 1 (a) and 2 (b) are shown. No toxic side effects were visually observed in the mice. Additionally, no significant weight loss occurred despite the high multiple dosing which indicates the absence of significant off-target effects by dEpCAMCD133KDEL

plasticity, a phenomenon in which CD133 expression can arise from the more differentiated CD133 negative cell population [39]. 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 TT *in vivo*. This prevented EpCAMKDEL from being tested at the same dose as dEpCAM133KDEL. The monospecific drugs are also smaller than the bispecific TT and may be more readily filtered into the liver and/or kidney causing these nontarget toxicities. Taken together, these data show that dEpCAMCD133KDEL was more effective compared with dCD133KDEL and was more effective *in vitro* and *in vivo*.

A major limitation of past TTs has been the development of neutralizing antibodies following prolonged treatment. We have addressed this issue by deimmunizing the truncated version of PE 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 [14, 40]. This deimmunized toxin has been used and tested for a number of targeted toxins we have developed [14, 17, 19].

Past studies have shown that current chemotherapy in combination with a TT is more effective than using either therapy alone [41, 42]. 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.

We chose our animal model based on the fact that intratumoral injection is an accepted model for testing drug efficacy in head and neck cancer. There are many examples of head and neck cancer clinical trials whereby drugs are administered directly to the tumor site [21, 43]. Undoubtedly, this relates to the accessibility of the primary tumor nodule. Still, it will be important to understand the activity of the drug when given systemically. Thus, future studies are planned to optimize the dose and dose schedules using intravenous or interperitoneal injections in other models of carcinoma.

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.

Acknowledgments This work was supported in part by the US Public Health Service Grant R01-CA36725 awarded by the NCI and the NIAID, DHHS, the Randy Shaver Foundation, the Atwater Cancer Drug Development Award, and a CETI translational award from the University of Minnesota Masonic Cancer Center.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Trzpis M, McLaughlin PM, de Leij LM, Harmsen MC (2007) Epithelial cell adhesion molecule: More than a carcinoma marker and adhesion molecule. *Am J Pathol* 171:386–395
- Munz M, Baeuerle PA, Gires O (2009) The emerging role of EpCAM in stem cell signaling. *Cancer Res* 69:5627
- Takahashi-Yanaga F, Kahn M (2010) Targeting Wnt signaling: can we safely eradicate cancer stem cells? *Clin Cancer Res* 16:3153–3162
- Moncharmont C, Levy A, Gilormini M, Bertrand G, Chargari C, Alphonse G, Ardail D, Rodriguez-Lafrasse C, Magne N (2012) Targeting a cornerstone of radiation resistance: cancer stem cell. *Cancer Lett* 322:139–147
- Van der Gun BT, Melchers LJ, Ruiters MH, de Leij LF, McLaughlin PM, Rots MG (2010) EpCAM in carcinogenesis: the good, the bad or the ugly. *Carcinogenesis* 31:1913–1921
- Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 8:755–768
- Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW (1997) AC133, A novel marker for human hematopoietic stem and progenitor cells. *Blood* 90:5002–5012
- Boman BM, Wicha MS (2008) Cancer stem cells: a step toward the cure. *J Clin Oncol* 26:2795–2799
- Ferrandina G, Petrillo M, Bonanno G, Scambia G (2009) Targeting CD133 antigen in cancer. *Expert Opin Ther Targets* 13:823–837
- Rappa G, Fodstad O, Lorico A (2008) The stem cell-associated antigen CD133 (Prominin-1) is a molecular therapeutic target for metastatic melanoma. *Stem Cells* 26:3008–3017
- Mak AB, Nixon AM, Kittanakom S, Stewart JM, Chen GI, Curak J, Gingras AC, Mazitschek R, Neel BG, Stagljar I, Moffat J (2012) Regulation of CD133 by HDAC6 promotes β -catenin signaling to suppress cancer cell differentiation. *Cell Rep* 2:951–963
- Takenobu H, Shimozato O, Nakamura T, Ochiai H, Yamaguchi Y, Ohira M, Nakagawara A, Kamijo T (2011) CD133 suppresses neuroblastoma cell differentiation via signal pathway modification. *Oncogene* 30:97–105
- Swaminathan SK, Niu L, Waldron NN, Kalscheuer S, Zellmer D, Olin MR, Ohlfest JR, Vallera DA, Panyam J (2013) Identification and characterization of a novel scFv recognizing human and mouse CD133. *Drug Deliv Transl Res*. doi:10.1007/s13346-012-0099-6
- Waldron NN, Kaufman DS, Oh S, Inde Z, Hexum MK, Ohlfest JR, Vallera DA (2011) Targeting tumor-initiating cancer cells with dCD133KDEL shows impressive tumor reductions in a xenotransplant model of human head and neck cancer. *Mol Cancer Ther* 10:1829–1838
- Ohlfest JR, Zellmer D, Panyam J, Swaminathan SK, Oh S, Waldron NN, Toma S, Vallera DA (2012) Immunotoxin targeting CD133+ breast carcinoma cells. *Drug Deliv Transl Res*. doi:10.1007/s13346-012-0066-2
- Skubitz APN, Taras EP, Boylan KLM, Waldron NN, Oh S, Panoskaltis-Mortari A, Vallera DA (2013) Targeting CD133 in an in vivo ovarian cancer model reduces ovarian cancer progression. *Gynecol Oncol*. doi:10.1016/j.ygyno.2013.05.027
- Stish BJ, Oh S, Chen H, Dudek AZ, Kratzke RA, Vallera DA (2009) Design and modification of EGF4KDEL 7mut, a novel bispecific ligand-directed toxin, with decreased immunogenicity and potent anti-mesothelioma activity. *Br J Cancer* 101:1114–1123
- Tsai AK, Oh S, Chen H, Shu Y, Ohlfest JR, Vallera DA (2011) A novel bispecific ligand-directed toxin designed to simultaneously target EGFR on human glioblastoma cells and uPAR on tumor neovasculature. *J Neurooncol* 103:255–266
- Vallera DA, Oh S, Chen H, Shu Y, Frankel AE (2010) Bioengineering a unique deimmunized bispecific targeted toxin that simultaneously recognizes human CD22 and CD19 receptors in a mouse model of B-cell metastases. *Mol Cancer Ther* 9:1872–1883
- Stish BJ, Chen H, Shu Y, Panoskaltis-Mortari A, Vallera DA (2007) Increasing anticarcinoma activity of an anti-erbB2 recombinant immunotoxin by the addition of an anti-EpCAM sFv. *Clin Cancer Res* 13:3058–3067
- MacDonald GC, Rasamoeliso M, Entwistle J, Cizeau J, Bosc D, Cuthbert W, Kowalski M, Spearman M, Glover N (2009) A phase I clinical study of VB4-845: Weekly intratumoral administration of an anti-EpCAM recombinant fusion protein in patients with squamous cell carcinoma of the head and neck. *Drug Des Devel Ther* 2:105–114
- Kowalski M, Entwistle J, Cizeau J, Niforos D, Loewen S, Chapman W, MacDonald GC (2010) A phase I study of an intravesically administered immunotoxin targeting EpCAM for the treatment of nonmuscle-invasive bladder cancer in BCG-refractory and BCG-intolerant patients. *Drug Des Devel Ther* 4:313–320
- Kowalski M, Guindon J, Brazas L, Moore C, Entwistle J, Cizeau J, Jewett MA, MacDonald GC (2012) A phase II study of oportuzumab monatox: an immunotoxin therapy for patients with noninvasive urothelial carcinoma in situ previously treated with Bacillus Calmette–Guérin. *J Urol* 188:1712–1718
- Fitzgerald D, Pastan I (1989) Targeted toxin therapy for the treatment of cancer. *J Natl Cancer Inst* 81:1455–1463
- Kreitman RJ, Pastan I (1995) Importance of the glutamate residue of KDEL in increasing the cytotoxicity of Pseudomonas exotoxin derivatives and for increased binding to the KDEL receptor. *Biochem J* 307(697):29–37
- Vallera DA, Chen H, Sicheneder AR, Panoskaltis-Mortari A, Taras EP (2009) Genetic alteration of a bispecific ligand directed toxin targeting human CD19 and CD22 receptors resulting in improved efficacy against systemic. *Leuk Res* 33:1233–1242
- Worsham MJ, Chen KM, Meduri V, Nygren AO, Errami A, Schouten JP, Benninger MS (2006) Epigenetic events of disease progression in head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 132:668–677
- Abu-Ali S, Fotovati A, Shirasuna K (2008) Tyrosine-kinase inhibition results in EGFR clustering at focal adhesions and consequent exocytosis in uPAR down-regulated cells of head and neck cancers. *Mol Cancer* 7:47
- Eyler CE, Rich JN (2008) Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Onc* 26:2839–2845
- Fabrizi E, di Martino S, Pelacchi F, Ricci-Vitiani L (2010) Therapeutic implications of colon cancer stem cells. *World J Gastroenterol* 16:3871–3877
- Dylla SJ, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL (2008) Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One* 3:e2428
- Dallas NA, Xia L, Fan F, Gray MJ, Gaur P, van Buren G 2nd, Samuel S, Kim MP, Lim SJ, Ellis LM (2009) Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. *Cancer Res* 69:1951–1957
- Alpaugh ML, Tomlinson JS, Shao ZM, Barsky SH (1999) A novel human xenograft model of inflammatory breast cancer. *Cancer Res* 59:5079–5084
- Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA (2010) Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 148:3–15
- Imrich S, Hachmeister M, Gires O (2012) EpCAM and its potential role in tumor-initiating cells. *Cell Adh Migr* 6:30–38
- Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, Jia H, Ye Q, Qin L, Wauthier E, Reid LM, Minato H, Honda M, Kaneko S,

- Tang ZY, Wang XW (2009) EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* 136:1012–1024
37. Rutella S, Bonanno G, Marone M, De Ritis D, Mariotti A, Voso MT, Scambia G, Mancuso S, Leone G, Pierelli L (2003) Identification of a novel subpopulation of human cord blood CD34⁺CD133⁺CD7⁺CD45b⁺ lineage-cells capable of lymphoid/NK cell differentiation after in vitro exposure to IL-15. *J Immunol* 171:2977–2988
 38. Floor S, van Staveren WC, Larsimont D, Dumont JE, Maenhaut C (2011) Cancer cells in epithelial-to-mesenchymal transition and tumor-propagating-cancer stem cells: distinct, overlapping or same populations. *Oncogene* 30:4609–4621
 39. Suuronen EJ, Wong S, Kapila V, Waghay G, Whitman SC, Mesana TG, Ruel M (2006) Generation of CD133⁺ cells from CD133⁺ peripheral blood mononuclear cells and their properties. *Cardiovasc Res* 70:126–135
 40. Onda M, Beers R, Xiang L, Nagata S, Wang QC, Pastan I (2008) An immunotoxin with greatly reduced immunogenicity by identification and removal of B-cell epitopes. *Proc Natl Acad Sci U S A* 105(701):11311–11316
 41. Hassan R, Broaddus VC, Wilson S, Liewehr DJ, Zhang J (2007) Anti-mesothelin immunotoxin SS1P in combination with Gemcitabine results in increased activity against mesothelin-expressing tumor xenografts. *Clin Cancer Res* 13:7166–7171
 42. Pearson JW, Sivam G, Manger R, Wilttrout RH, Morgan AC Jr, Longo DL (1989) Enhanced therapeutic efficacy of an immunotoxin in combination with chemotherapy against an intraperitoneal human tumor xenograft in athymic mice. *Cancer Res* 49:4990–4995
 43. Fujiwara S, Wada H, Miyata H, Kawada J, Kawabata R, Nishikawa H, Gnjatic S, Sedrak C, Sato E, Nakamura Y, Sakakibara M, Kanto T, Shimosegawa E, Hatazawa J, Takahashi T, Kurokawa Y, Yamasaki M, Nakajima K, Takiguchi S, Nakayama E, Mori M, Doki Y (2012) Clinical trial of the intratumoral administration of labeled DC combined with systemic chemotherapy for esophageal cancer. *J Immunother* 35(6):513–521