

## A Cancer Gene Therapy Approach that Targets Tumor-associated Hyaluronan

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**Abstract:** The extracellular matrix glycosaminoglycan hyaluronan plays a key role in the development and pathogenesis of malignant disease. Reflecting its functional importance, the molecule is expressed at greatly elevated levels within many solid tumors. Although little explored, differences in the level of hyaluronan present in normal and malignant tissues could potentially be exploited to more effectively target gene therapy to tumor sites *in vivo*. As a first step toward this goal, we describe here a family of chimeric proteins in which the extracellular ligand-binding domain of the hyaluronan receptor CD44 is fused in-frame to the cytoplasmic “death domain” of the pro-apoptotic protein Fas. Although these chimeric proteins can be stably expressed on the surface of transduced tumor cells in the absence of hyaluronan, upon interaction with the ligand, apoptosis is rapidly induced. Both exogenous and endogenous tumor-produced hyaluronan can function as triggers, dramatically reducing clonogenic potential. Together, these studies help validate a broadly applicable gene therapy approach in which the presence of particular multivalent ligands within the tumor microenvironment can be exploited for therapeutic gain.

**Keywords:** hyaluronan, CD44, Fas, gene therapy, apoptosis

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*Cancer Growth and Metastasis* 2009;2 29–43

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## Introduction

It has long been appreciated that the glycosaminoglycan hyaluronan (HA) is selectively enriched within solid tumors, most notably in peritumoral regions.<sup>1-3</sup> The potential importance of this finding with respect to the development and/or pathogenesis of malignant disease is emphasized by the key role played by HA in the regulation of various cell migration, proliferation and differentiation events, particularly during embryonic development.<sup>4</sup> Specifically, HA is highly hydroscopic and can adopt a pseudo-random coil-like configuration that occupies large volumes in solution such that, when present at high concentrations, the molecule can disrupt the organized architecture of tissues, opening spaces through which cells can migrate more easily.<sup>5,6</sup> Conversely, a reduction in HA expression often correlates with the cessation of cell movement and the induction of differentiation. In adults, temporal alterations in the level of HA are seen in the course of tissue injury and repair,<sup>7</sup> while in the case of malignant disease, correlations have been established between HA levels and both local invasion and metastasis.<sup>8</sup> An association between HA levels and disease recurrence and prognosis has also been described in a number of studies.<sup>1,3,9,10</sup> While the mechanisms by which HA mediates these diverse effects on tumor growth remain to be fully defined, it is evident that the molecule functions in both a direct and indirect fashion, altering tumor cell proliferation and differentiation while also enhancing the recruitment of stromal cells and stimulating angiogenesis.<sup>6,11,12</sup>

The biological effects triggered by the presence of HA are mediated by a number of cell surface and soluble HA-binding proteins.<sup>13-19</sup> Among these, members of the CD44 family appear to be particularly important with regard to the development and pathogenesis of malignant disease.<sup>20-23</sup> The CD44 isoform present on most resting and non-transformed cells, designated CD44H or CD44s, has a molecular weight in the range of 80–90 kD. However, alternative splicing events involving a contiguous series of 10 or more exons (v1–v10) present within the single copy CD44 gene can generate a large number of higher molecular weight isoforms that are differentially expressed and may be present at elevated levels on certain activated and/or malignant cell types.<sup>24</sup> Site-directed mutagenesis studies have suggested that

a motif containing two basic amino acids separated by a stretch of 7 non-acidic amino acids (B[X<sub>7</sub>]B) facilitates binding to HA.<sup>25,26</sup> This motif is found in all HA-binding proteins that have been examined to date and occurs twice within the extracellular domain of CD44 in a region shared by all alternatively spliced isoforms.<sup>25,27</sup> An additional B[X<sub>7</sub>]B motif is also present within the sequence encoded by the alternatively spliced exon v10 and plays a key role in mediating adhesive interactions between v10 containing CD44 isoforms.<sup>27</sup>

The upregulation of HA within malignant tissues raises the possibility that differential expression of the molecule could perhaps be exploited in the development of tumor targeted therapy. The approach we have taken in this regard is to generate a family of chimeric proteins in which the extracellular ligand-binding domain of the HA receptor CD44 is fused in-frame to the intracellular “death-domain” of the pro-apoptotic protein Fas (CD44/Fas). It was hypothesized that rather than triggering the signal transduction events normally induced upon the binding of HA by CD44, when expressed in target cells, interaction of the chimeric CD44/Fas protein with HA would instead induce apoptotic death.

The results obtained confirm the potential utility of this approach. Specifically, chimeric CD44/Fas proteins proved effective at binding HA and in triggering apoptosis in transduced target cells upon interaction with ligand. It is proposed that chimeric proteins of this type may prove useful in the context of cancer gene therapy.

## Materials and Methods

### Cell lines

The tumor cell lines K562 (erythroleukemia), T24 (transitional bladder carcinoma), MCF7 (mammary adenocarcinoma) and PC3 (metastatic prostate adenocarcinoma) were obtained from the American Type Culture Collection (Manassas, VA). 293 cells (adenovirus 5 transformed human embryonic kidney cells) were obtained from Qbiogene (Carlsbad, CA). All cell lines were incubated at 37 °C in an atmosphere containing 5% CO<sub>2</sub> in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate (DMEM + 10% FBS).

## Monoclonal antibodies

Two mouse monoclonal antibodies (mAbs) directed against human Fas were used in the course of this study. The first, designated DX2 (BD Biosciences, San Jose, CA), is directed against an epitope present on the extracellular domain of Fas and thus recognizes endogenous Fas but not the CD44/Fas chimeras described below that contain only the transmembrane and/or cytoplasmic domain of the molecule. The second, mAb 3D5 (Alexis Biochemicals, San Diego, CA), defines an epitope encoded within the death domain of Fas and thus recognizes both endogenous Fas and the CD44/Fas chimeras.

The generation and characterization of the anti-CD44 mAb 4A4 has been described in detail elsewhere.<sup>28,29</sup> The phycoerythrin-conjugated anti-CD44 mAb G44-26 (CD44-PE) was purchased from BD Biosciences.

## Expression vectors

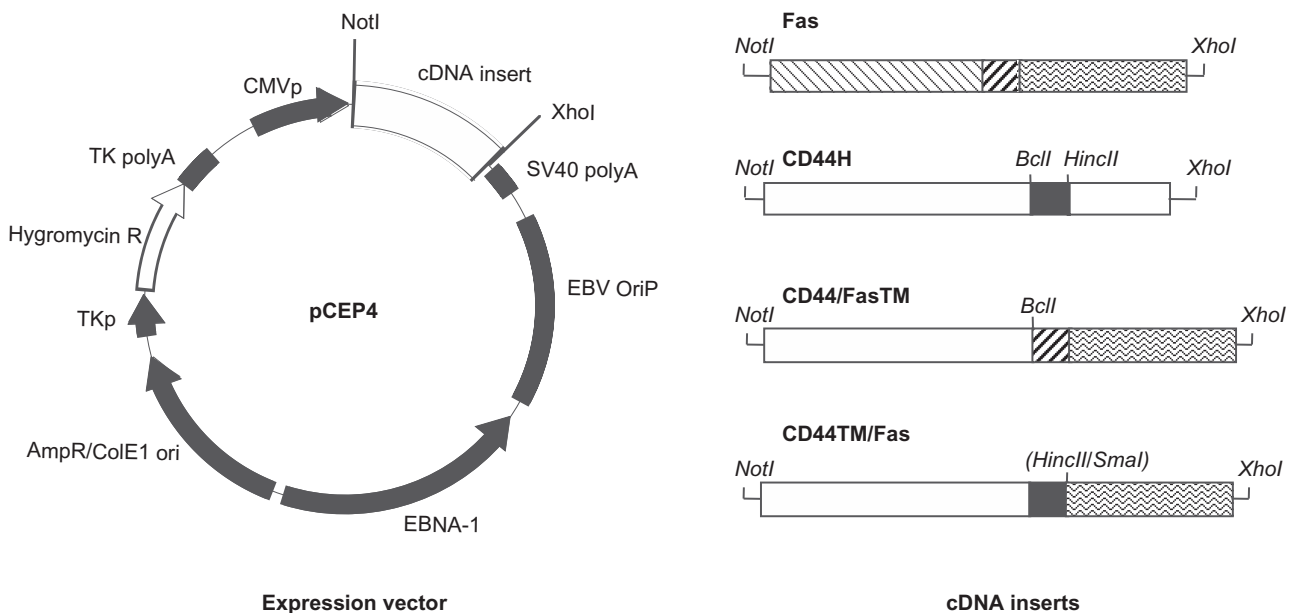
The EBV-based episomal expression vector pCEP4 was purchased from BD Biosciences. The major features of both pCEP4 and the cDNA constructs used in this study are shown in Figure 1.

Two chimeric proteins designated CD44/Fas<sup>TM</sup> and CD44<sup>TM</sup>/Fas were generated for use in this study. As shown in Figure 1, both contain the extracellular

HA-binding domain of CD44 and the cytoplasmic domain of Fas that includes the so-called “death domain” that triggers an apoptotic response upon receptor ligation. Where the two constructs differ is with respect to the sequences used to join these two functionally important regions. Thus, CD44/Fas<sup>TM</sup> incorporates the membrane spanning domain of Fas, while in CD44<sup>TM</sup>/Fas the membrane spanning domain of CD44 is instead used (Fig. 1).

To generate the vector pCEP4.CD44H, a fragment containing a full-length cDNA encoding the 90 kD “hemopoietic” or “standard” isoform of CD44 (CD44H/CD44s) was released from the plasmid pCDM8.CD44 clone #2.7<sup>30</sup> by digestion with HindIII and NotI and ligated into the HindIII-NotI sites of pCEP4.

To construct pCEP4.Fas, a full length human Fas cDNA (~1.5 kb) was first generated by PCR using Jurkat cDNA as a template and the following primer pair: 5' Fas UTR (5'-GCGGAATTCAGGGGC-GGGCACTGGCAC-3') and 3' Fas UTR (5'-GGCTCGAGAATCTTTTCAAACACTAATTGC-3') containing respectively engineered 5' EcoRI and XhoI sites. After 40 cycles (95 °C for 30s, 60 °C for 30s, 72 °C for 2.5 min), the resultant PCR products were separated by gel electrophoresis and a species



**Figure 1. Major features of the pCEP4 vector and cDNA constructs used in this study.** The two CD44/Fas chimeras differ from one another only with respect to the origin of their membrane spanning domains. Thus the membrane spanning domain present in CD44/Fas<sup>TM</sup> is derived from Fas, while that present in CD44<sup>TM</sup>/Fas is derived from CD44H.



of the appropriate size isolated, digested with EcoR1 and Xho1 and cloned into EcoR1-Xho1 digested pBluescript II (KS+) generating vector pBS.Fas. The insert was subsequently sequenced and proved to be identical to the published Fas sequence with the exception of an A to G substitution at position 1117 resulting in a single amino acid change at residue 297, that converts a Glu residue within the cytoplasmic domain of the mature protein to Gly. Finally, the full-length Fas cDNA was isolated from pBS.Fas by digestion with Not1 and Xho1 and the resultant fragment cloned into the Not1-Xho1 sites of pCEP4.

pCEP4 vectors encoding the CD44/Fas chimeras CD44/Fas<sup>TM</sup> and CD44<sup>TM</sup>/Fas (Fig. 1) were generated as follows:

#### Construction of the CD44<sup>TM</sup>/Fas chimera

In order to acquire the restriction sites needed to facilitate subsequent cloning steps, a fragment containing the full-length CD44H cDNA was first released from the pCEP4.CD44H vector described above by digestion with Xho1. The ends were blunted/filled-in using T4 DNA polymerase and the fragment cloned into the EcoRV site of plasmid pZErO2 (Invitrogen, Carlsbad, CA). After checking for orientation, the full-length CD44H cDNA was once again isolated by digestion with Not1-EcoR1 and cloned into Not1-EcoR1 digested pBluescript II (KS+) generating plasmid pBS.CD44H. Finally, the full-length CD44H cDNA was again released, this time by digestion with Not1 and Xho1, gel purified, then partially digested with HincII generating a Not1-HincII (blunt) fragment that contains the extracellular and membrane spanning domains of CD44H (CD44<sup>TM</sup>).

A cDNA encoding the cytoplasmic domain of human Fas was generated by PCR using the pCEP4.Fas plasmid described above as a template and the following primer pair: 5'-Fas CYTO2 (5'-GCCCGGGT-GAAGAGAAAGGAAGTAGAG-3') and 3'-Fas UTR (5'-GGCTCGAGAATCTTTTCAAACACTA-ATTGC-3') containing respectively engineered 5' Sma1 and Xho1 sites. After 35 cycles (95 °C for 30s, 60 °C for 30s, 72 for 1 min), the resultant PCR products were separated by gel electrophoresis and a fragment of the appropriate size isolated. The ends were repaired/filled-in with T4 DNA polymerase and the fragment cloned into the EcoRV site of pZErO2. After checking for appropriate (i.e. reverse) orientation, the

resultant vector was sequentially digested with Not1 then with Sma1 and the linearized plasmid ligated with the Not1-HincII fragment containing the extracellular and membrane spanning domains of CD44H generated as described above, producing the full-length CD44<sup>TM</sup>/Fas chimera with a Not1 site at its 5' end and a Xho1 site at its 3' end. For expression studies, the chimeric cDNA was isolated from the pZErO2 vector by digestion with Not1 and Xho1 and cloned into the Not1-Xho1 sites of pCEP4.

#### Construction of the CD44/Fas<sup>TM</sup> chimera

A cDNA encoding the membrane spanning and cytoplasmic domains of human Fas was generated by PCR using pCEP4.Fas plasmid DNA as a template and the following primer pair: 5'-Fas<sup>TM</sup> (5'-AACGTGAT-CATCCTTGTCTTCTTCTTTG-3') and 3'-Fas UTR (5'-GGCTCGAGAATCTTTTCAAACACTAATTGC-3') containing respectively engineered 5' Bcl1 and Xho1 sites. After 35 cycles (95 °C for 30s, 60 °C for 30s, 72 °C for 1 min), the resultant PCR products were separated by gel electrophoresis and a fragment of the appropriate size isolated. The ends were repaired/filled-in with T4 DNA polymerase and the fragment cloned into the EcoRV site of pZErO2. After identifying a clone in which the insert is present in the appropriate (i.e. reverse) orientation, a fragment containing the membrane spanning and cytoplasmic domain of Fas (Fas<sup>TM</sup>) was isolated by digestion with Bcl1-Xho1. The pBS.CD44H vector generated as described above was also digested with Bcl1 and Xho1 thereby removing sequences encoding the membrane spanning and cytoplasmic domains of CD44H. The resultant vector was ligated with the Bcl1-Xho1 Fas<sup>TM</sup> fragment producing the full-length CD44/Fas<sup>TM</sup> chimera with a Not1 site at its 5' end and a Xho1 site at its 3' end. The first 5 amino acids in the transmembrane domain of the CD44/Fas<sup>TM</sup> chimera correspond to CD44H while the remainder of the membrane spanning domain and the entire cytoplasmic domain correspond to Fas. Finally, the chimeric cDNA was isolated from the pBS vector by digestion with Not1 and Xho1 and cloned into the Not1-Xho1 sites of pCEP4.

#### Transfection of K562 cells

An electroporation technique was used to introduce pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/



Fas<sup>TM</sup> or pCEP4.CD44<sup>TM</sup>/Fas plasmid DNA into K562 cells. Briefly, log-phase K562 cells were harvested, washed several times in phosphate buffered saline (PBS) before being resuspended in PBS at  $1-2 \times 10^7$  cells/ml. Twenty  $\mu\text{g}$  of each plasmid DNA were added to  $4 \times 10^6$  cells and the cell suspensions transferred to 2 mm gap cuvettes and electroporated using the BTX ECM 600 Electroporator System (BTX, San Diego, CA). The settings were 280 V, capacitance 500  $\mu\text{F}$ , and resistance 48  $\Omega$ . The time constants obtained generally ranged from 3.3 to 3.5 msec. Immediately after electroporation, the cells were diluted with 35 ml DMEM + 10% FBS and plated in 15 cm Integrid tissue culture dishes (Falcon). Hygromycin B (Sigma) was added 24 h later at a final concentration of 250  $\mu\text{g}/\text{ml}$  to select for cells that had taken up and expressed plasmid DNA.

### FACS analysis

K562 cells transfected with pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/Fas<sup>TM</sup> or pCEP4.CD44<sup>TM</sup>/Fas plasmid DNA and selected and maintained in hygromycin (250  $\mu\text{g}/\text{ml}$ ) were examined for CD44 and Fas expression by FACS analysis. Briefly, approximately  $2 \times 10^5$  cells were incubated on ice for 30 min with either the anti-Fas mAb DX2 or the anti-CD44 mAb 4A4 at a final concentration of approximately 2  $\mu\text{g}/\text{ml}$ . Following extensive washing in Hank's balanced salt solution (HBSS) containing 2% FBS (HBSS + 2% FBS), the cells were incubated for a further 30 min on ice with FITC-conjugated rabbit anti-mouse Ig (2  $\mu\text{g}/\text{ml}$ ) (BD Biosciences). After additional washing, the cells were resuspended in HBSS + 2% FBS containing 1  $\mu\text{g}/\text{ml}$  propidium iodide to facilitate the identification and exclusion of dead cells and analyzed on a FACSCalibur (BD Biosciences, Immunocytometry Systems, San Jose, CA).

### HA binding assay

The HA-binding activity of K562 cells transfected with pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/Fas<sup>TM</sup> or pCEP4.CD44<sup>TM</sup>/Fas was determined by FACS analysis. For each line, approximately  $2 \times 10^5$  cells were resuspended in 0.5 ml of HBSS + 2% FBS, a 1:100 dilution of anti-CD44 mAb PE-G44-26 (BD Pharmingen), and a 1:1000 dilution of fluorescein isothiocyanate-conjugated HA (FITC-HA,

$\sim 50 \mu\text{g}/\text{ml}$  final), and the mixture was incubated for 1 h on ice. Following repeated washing with HBSS + 2% FBS, cells were resuspended in HBSS + 2% FBS containing 1  $\mu\text{g}/\text{ml}$  propidium iodide and analyzed on a FACSCalibur (BD Biosciences Immunocytometry Systems).

### Induction and measurement of apoptosis

Ten cm non-tissue culture Petri dishes (Falcon) were coated with HA by adding 5 ml of a solution of potassium hyaluronate purified from human umbilical cord (Sigma) (5 mg/ml in PBS) and incubating overnight at 4 °C. Unbound HA was removed by extensive washing and dishes carefully drained. Approximately,  $1 \times 10^7$  K562 cells transfected with either pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/Fas<sup>TM</sup> or pCEP4.CD44<sup>TM</sup>/Fas in a final volume of 3 ml HBSS were added to each HA-coated plate and the cells allowed to settle and interact with the immobilized HA for approximately 30 min. All of the cells, whether adherent or not, were then harvested by gentle pipetting, washed 3 times with PBS, resuspended in 50 ml DMEM + 10% FBS and the cell suspension added to a 125 cm<sup>2</sup> tissue culture flask (Falcon). Flasks incubated at 37 °C and at various time points aliquots of cells were removed, washed with PBS and the cell pellets gently resuspended in 5 ml 70% ethanol. Samples were stored at -20 °C until all time points had been collected. The cells were then spun down and the percentage of hypodiploid apoptotic cells determined after staining in a PBS solution containing propidium iodide and RNase A.

### Western blot analysis

293 cells were electroporated with pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/Fas<sup>TM</sup> or pCEP4.CD44<sup>TM</sup>/Fas plasmid DNA, harvested 2 days later by incubation with PBS containing 3 mM EDTA, and the expression of both CD44 and Fas determinants examined by Western blot. Briefly, cells were washed in PBS and their membranes solubilized by incubating the pellets for 15 min on ice in 10 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40 at  $\sim 2 \times 10^7$  cells/ml lysis buffer. Nuclei and detergent insoluble material were removed by centrifugation and the lysates stored at -80 °C until required. Thawed samples were added to an equal volume



of non-reducing sample buffer (125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8) and incubated at 100 °C for 5 min. Total cellular proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The filters were incubated overnight at 4 °C in PBS containing 5% (w/v) milk protein then probed with either mAb 4A4, which recognizes an epitope present in the extracellular domain of CD44, or mAb 3D5, directed against the death domain of Fas (2 µg/ml in HBSS + 2% FBS). After extensive washing, filters were incubated for a further 1–2 hr with a 1:100 dilution of horse radish peroxidase (HRP)-conjugated rabbit anti-mouse Ig secondary antibody. After additional washing, the filters were developed in ECL solution (Amersham Pharmacia Biotech) and protein products imaged on photographic film (Amersham Life Sciences, Buckinghamshire, UK).

### Clonogenic survival assay

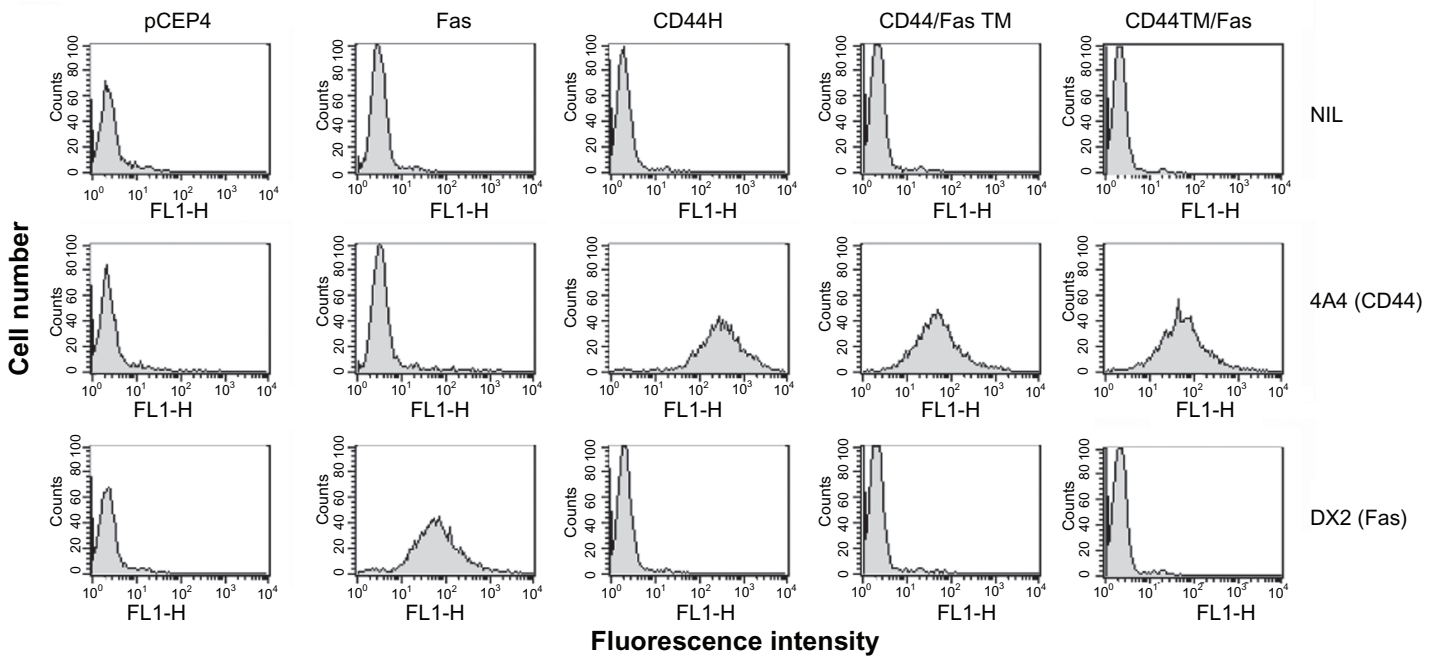
The effect of CD44/Fas<sup>TM</sup> and CD44<sup>TM</sup>/Fas expression on the long-term survival and proliferation of tumor cell lines was determined using a standard clonogenic assay. pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/Fas<sup>TM</sup> or pCEP4.CD44<sup>TM</sup>/Fas plasmid DNA was introduced into 293, T24, PC3 and MCF7 cells by electroporation as follows. Tumor cells were harvested by trypsinization and resuspended in PBS at  $1\text{--}2 \times 10^7$  cells/ml. Twenty µg of plasmid DNA were added to  $4 \times 10^6$  cells and the cell suspensions transferred to 2 mm gap cuvettes and electroporated using the BTX ECM 600 Electroporator System (BTX, San Diego, CA). For 293 and MCF7 cells a voltage of 270 V, capacitance of 400 µF and resistance setting of 72 Ω were used. For T24 and PC3 cells 280 V and a resistance of 48 Ω were employed with capacitance settings of 400 µF and 300 µF respectively. Time constants generally ranged from around 3.0 to 3.5 msec for 293, MCF7 and T24, and 2.0–2.2 msec for PC3. Immediately after electroporation, the transfected cells were transferred into 35 ml DMEM + 10% FBS and plated in 15 cm Integrid tissue culture dishes (Falcon). Hygromycin B (Sigma) was added 24 h later to a final concentration of either 200 µg/ml (293, PC3 and MCF7) or 250 µg/ml (T24) to select for cells that had taken up and expressed plasmid DNA. Around 10–14 days later, plates were fixed and stained with

a methylene blue/methanol solution and the number of hygromycin-resistant colonies determined.

### Results

For the chimeras described in this study to be of use in cancer therapy, it is important that the encoded proteins are stable and can be expressed on the surface of transduced cells at a level sufficient to produce a biological effect upon binding of ligand. In order to demonstrate that this was indeed the case, both the CD44/Fas<sup>TM</sup> and CD44<sup>TM</sup>/Fas cDNA constructs were cloned into the EBV-based episomal plasmid vector pCEP4 (Fig. 1) and introduced into CD44-negative K562 cells by electroporation. Transfectants were selected in the presence of hygromycin and surface expression of the corresponding chimeric proteins determined by FACS analysis using an antibody directed against the extracellular domain of CD44. Full length CD44H and Fas cDNAs were used as controls. As shown in Figure 2, in the absence of added ligand, K562 cells expressing high levels of CD44/Fas<sup>TM</sup> or CD44<sup>TM</sup>/Fas could be readily established and maintained for extended periods of time *in vitro* without apparent loss of viability. Interestingly, although the two Fas chimeras were expressed at a similar level on the surface of transduced K562 cells (as determined by mean fluorescence intensity), the level of wild-type CD44 was generally higher, even though the same vector system and transfection procedures were used.

To ensure that the chimeric proteins are processed in an appropriate manner, pCEP4 vectors encoding the CD44/Fas<sup>TM</sup> and CD44<sup>TM</sup>/Fas cDNAs were introduced into 293 cells by electroporation and 24 hr later the transfected cells were harvested, membrane lysates prepared and Western blots run and probed with mAbs directed against either CD44 (mAb 4A4) or the the death domain of Fas (mAb 3D5). 293 cells were specifically chosen for these studies as they are not only CD44-positive but express primarily CD44H (MW 80–90 kD) and not higher molecular weight isoforms, allowing the proteins encoded by the two transgenes to be compared in size and relative expression to endogenous CD44H. As shown in Figure 3, the results obtained confirm the presence within transfected cells of proteins with an apparent molecular weight of around 80–90 kD that react with both the anti-CD44 and anti-Fas death domain antibodies.

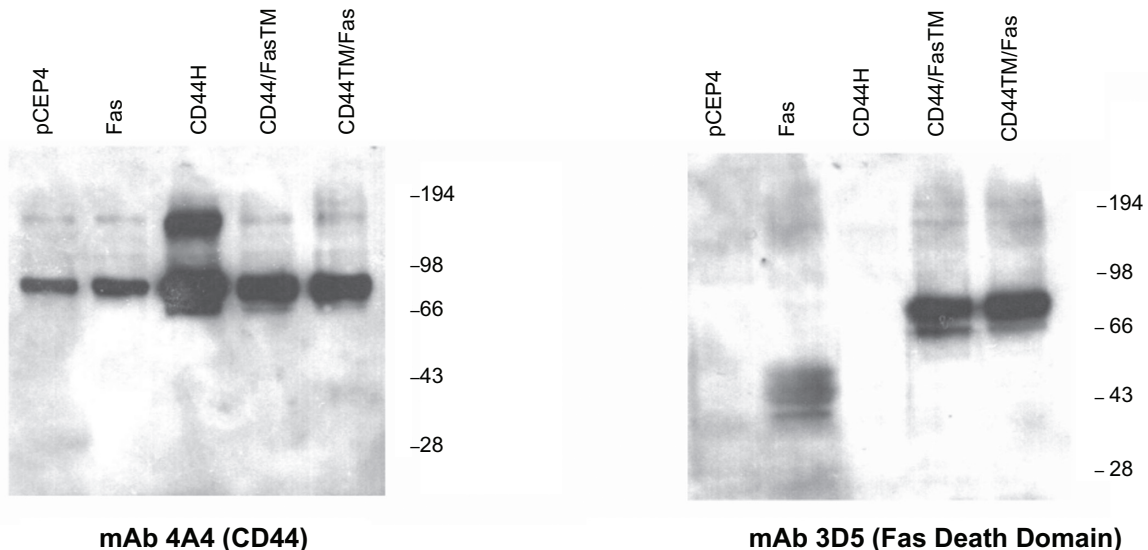


**Figure 2. Expression of chimeric CD44/Fas proteins on the surface of transduced tumor cells.** pCEP4.CD44/FasTM or pCEP4.CD44TM/Fas plasmid DNAs were introduced into CD44-negative K562 tumor cells by electroporation. pCEP4, pCEP4.Fas and pCEP4.CD44H plasmids were used as controls. Transfected cells were selected in the presence of Hygromycin B and the expression of CD44 and Fas on the surface of established lines determined by FACS analysis after staining respectively with mAb 4A4 or mAb DX2.

Low levels of a higher molecular weight species of around 160–180 kD corresponding to CD44 homodimers are evident on blots probed with the CD44 mAb 4A4 (Fig. 3). Expression of this species is dramatically upregulated in cells transfected with CD44H but

remains low in cells transfected with CD44/FasTM or CD44TM/Fas (Fig. 3).

Monomeric Fas itself has a molecular weight of between 37–50 kD depending on the nature and extent of post-translational modification. While a diffuse



**Figure 3. Western blot analysis of CD44/Fas expression in transduced tumor cells.** 293 cells were electroporated with pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/FasTM or pCEP4.CD44TM/Fas plasmid DNA, harvested 2 days later, and the expression of both CD44 determinants and Fas determined by Western blot analysis. Lysates were run on a 7.5% SDS-PAGE gel ( $2 \times 10^5$  cell equivalents per lane), transferred to nitrocellulose, and probed with mAbs directed against the extracellular domain of CD44 (mAb 4A4) or the death domain of Fas (mAb 3D5). After extensive washing, filters were incubated with and appropriate HRP-conjugated secondary antibody, developed in an ECL solution and protein products imaged on photographic film.

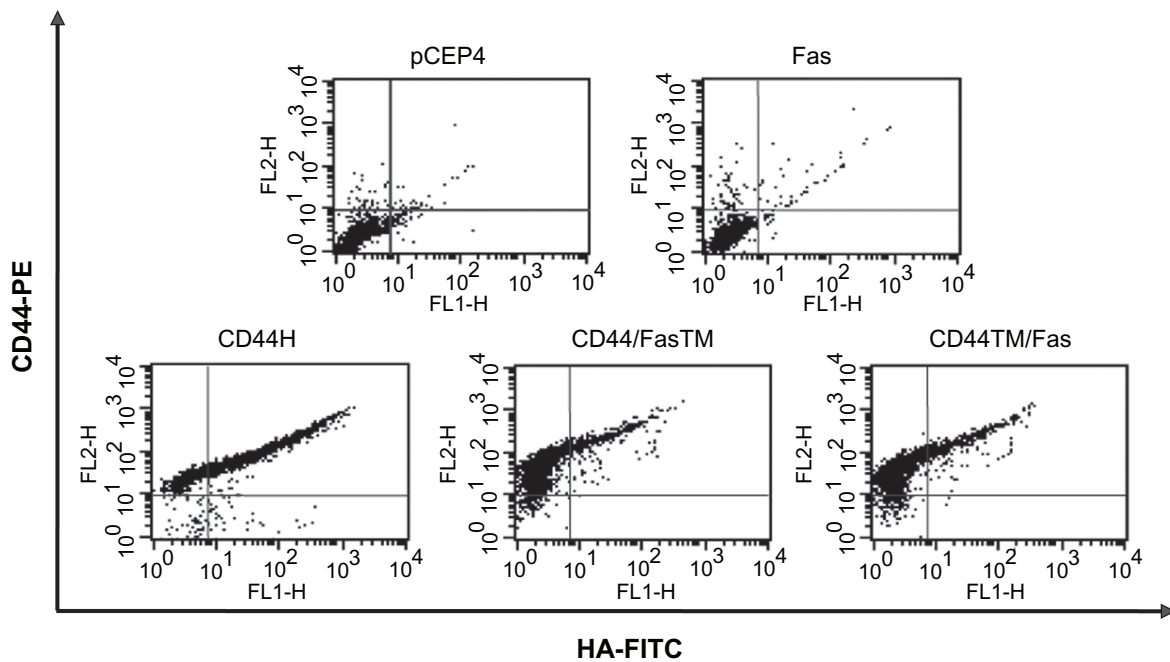
species of this size is present in cells transfected with pCEP4.Fas, little if any expression is evident in control 293 cells or in 293 cells transfected with pCEP4.CD44H, pCEP4.CD44/FasTM or pCEP4.CD44TM/Fas (Fig. 3).

The CD44/Fas chimeras were next tested for their HA-binding ability. Transfected K562 cells expressing wild type CD44H or either CD44/FasTM or CD44TM/Fas were double-labeled with anti-CD44-PE and FITC-HA allowing the relationship between CD44 levels and the amount of HA bound to be determined. The results obtained indicate that cells need to express a certain critical threshold level of CD44 before HA binding can occur. Above this threshold there is linear relationship between CD44 expression and the amount of HA bound (Fig. 4). Importantly, although the threshold level needed to permit HA binding may be slightly lower for CD44H than for either CD44/FasTM or CD44TM/Fas, both chimeras are able to bind HA when expressed at modest levels (Fig. 4).

To determine whether the CD44/Fas chimeras can trigger an apoptotic response upon binding of ligand, K562 cells expressing CD44H or either CD44/FasTM or CD44TM/Fas were allowed to adhere to plastic surfaces coated with HA and the percentage apoptotic

(i.e. hypodiploid) cells determined at various time points thereafter. As shown in Figure 5, although CD44H expressing cells bind avidly to HA, they remain largely viable during a 12 hour period of observation. In contrast, a large proportion of K562 cells expressing either of the two CD44/Fas chimeras undergo apoptosis upon binding to HA. Although the ultimate magnitude of the response is similar for both CD44/FasTM and CD44TM/Fas, the rate of apoptosis was consistently greater for cells expressing CD44/FasTM, with near maximal cell death evident as early as 1.5 hours after binding to HA (Fig. 5). By 3 hours, however, the percentage of apoptotic cells was more or less equivalent for both CD44/FasTM- and CD44TM/Fas.

Finally, the CD44/Fas chimeras were tested for their ability to kill various epithelial tumor cell types that constitutively produce HA. As shown in Figure 6, both CD44/Fas chimeras produced dramatic reductions in the clonogenic potential of the tumor cell lines tested when introduced and expressed by electroporation. There was no significant difference in their relative efficacy as determined using this particular assay system. In agreement with previous studies, wild-type Fas was also effective at killing tumor cells that constitutively express Fas-ligand



**Figure 4. HA-binding activity of chimeric CD44/Fas proteins.** K562 cells transfected with pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/FasTM or pCEP4.CD44TM/Fas were incubated for 1 hr on ice in a solution of HBSS + 2% FBS containing a 1:100 dilution of the anti-CD44 mAb PE-G44-26 (BD Pharmingen) and a 1:1000 dilution of FITC-HA (~50 µg/ml final). Following repeated washing with HBSS + 2% FBS, cells were resuspended in HBSS + 2% FBS containing 1 µg/ml propidium iodide and analyzed on a FACScalibur (BD Biosciences, Immunocytometry Systems, San Jose, CA).



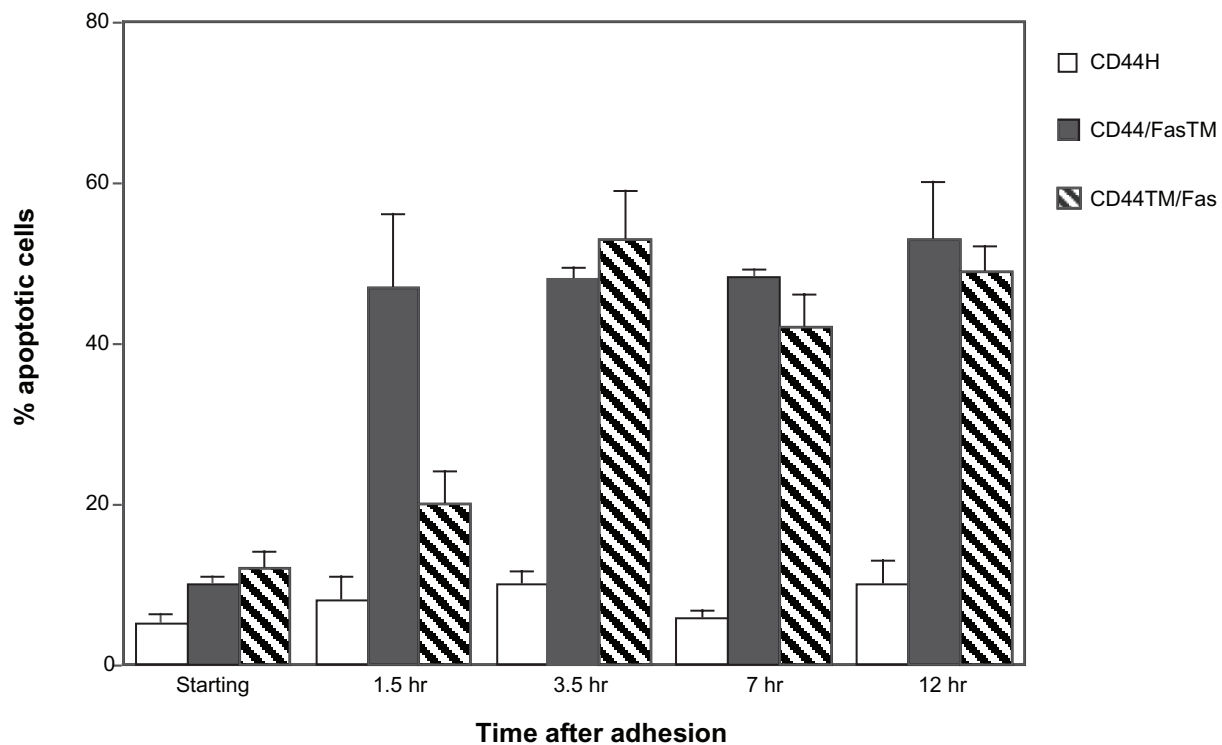
(FasL) (e.g. 293, MCF-7 and PC-3) but had no effect on the survival and proliferation of the FasL-negative cell line T24 (Fig. 6). Interesting, overexpression of CD44H, although far less effective than the corresponding CD44/Fas chimeras, had a modest inhibitory effect on the clonogenic potential of all four of the tumor lines.

## Discussion

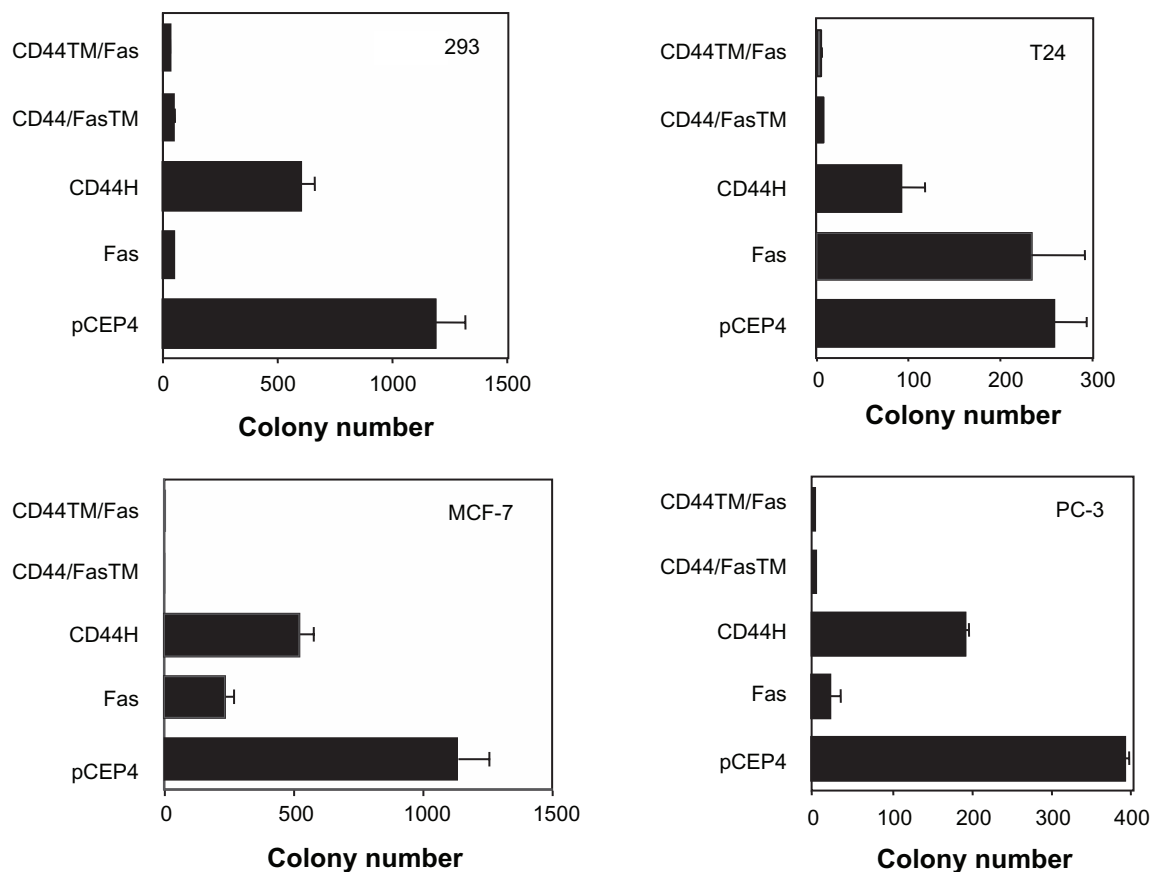
Selective targeting of tumor tissues is key to the success of cancer gene therapy, resulting in increased efficacy while minimizing toxicity. While considerable progress has been made with respect to gene targeting through the use of viral or other vectors with restricted tropism and/or promoter or other regulatory elements that can direct gene expression to desired cell types, there remains considerable room for improvement. Efficient *in vivo* targeting in particular has proven problematic. Recently we have advocated a novel functional targeting strategy in which the differential presence of various cytokines or extracellular matrix proteins within the tumor

microenvironment can be exploited to trigger a therapeutic effect such as cytotoxicity or immune stimulation. The basic approach involves the use of chimeric proteins which, in their simplest form, incorporate both a receptor domain that can recognize and bind a differentially expressed ligand and an effector domain that can translate such binding into a biological effect. In the present manuscript we describe one such family of chimeric proteins in which the extracellular domain of the adhesion protein CD44 is fused in frame to the death domain of the pro-apoptotic protein Fas. These molecules were designed to induce the death of transduced cells upon interaction with the CD44 ligand HA.

Although by no means restricted solely to malignant tissues, HA levels are often greatly elevated within solid tumors.<sup>23,31–33</sup> This association is in agreement with a large body of data suggesting an important role for HA in regulating the proliferation, differentiation and migration of both normal and malignant cells.<sup>5,34</sup> Increasing HA production by over expression of the HA synthase enzyme, HAS2,



**Figure 5. Induction of apoptosis upon interaction of CD44/Fas chimeras with HA.** K562 cells transfected with either pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/FasTM or pCEP4.CD44TM/Fas were added to HA-coated dishes and allowed to settle and interact with the immobilized ligand for approximately 30 min. Both adherent and non-adherent cells were then harvested, resuspended in DMEM + 10% FBS and cultured. At various time points aliquots were removed and the percentage of hypodiploid apoptotic cells determined by FACS analysis after fixation in 70% ethanol and staining in a solution of propidium iodide and RNase A. The data shown represents the mean  $\pm$  SEM of triplicate cultures.



**Figure 6. Effect of CD44/Fas expression on the clonogenic survival of tumor cells.** 293, MCF-7, PC3 and T24 tumor cells were electroporated with either pCEP4, pCEP4.Fas, pCEP4.CD44H, pCEP4.CD44/FasTM or pCEP4.CD44TM/Fas plasmid DNA and plated in 15 cm Integrid tissue culture dishes. Cells that had taken up plasmid DNA were selected in the presence of Hygromycin B and the ability of transfectants to survive and generate colonies determined on day 10–14 after plates were fixed and stained in a solution of methanol/methylene blue. The colony count data shown represents the mean  $\pm$  SEM of triplicate cultures.

enhanced the growth of fibrosarcoma and breast cancer xenografts in nu/nu mice.<sup>35,36</sup> Similar results were obtained when the HA synthase HAS3 was overexpressed in human prostate tumor cells,<sup>37</sup> while transfection of antisense Has2 and Has3 reduced the growth and vascularization of prostate tumors *in vivo*.<sup>38</sup> In keeping with this finding, studies have shown that metastatic colon carcinoma cells express higher levels of HAS3 and produce more HA than equivalent cells isolated from a primary tumor.<sup>39</sup> Later work from the same group confirmed that pericellular HA is necessary for colon carcinoma cell invasion and that this process is critically dependent upon the interaction of HA with CD44.<sup>40</sup>

In addition to direct effects on tumor growth and metastasis, signals transduced upon the interaction of CD44 with HA also enhance the resistance of tumor cells to various chemotherapeutic agents including doxorubicin, taxol, vincristine, and methotrexate.<sup>23</sup>

This potentially important mechanism may help explain the differential ability of CD44-positive cancer stem cells to survive drug treatment.<sup>23</sup> In support of this conclusion, a number of studies have shown that exposure to hyaluronidase can improve the efficacy of cancer drug treatment.<sup>41–43</sup> Blocking the HA-binding activity of CD44 is also effective.<sup>23,44,45</sup> Although the mechanisms responsible for this effect remain to be fully elucidated, it has been reported that the interaction between CD44 and HA can regulate the expression and/or function of a number of transporter proteins that mediate drug efflux including MRP2,<sup>46,47</sup> P-glycoprotein<sup>46</sup> and BCRP.<sup>48</sup> Interestingly, CD44 co-localizes with P-glycoprotein to defined plasma membrane lipid microdomains (lipid rafts).<sup>49</sup> Moreover, in drug resistant cell lines, CD44 and P-glycoprotein co-immunoprecipitate.<sup>50</sup> More provocatively, agents that interfere with the function of P-glycoprotein affect the membrane



distribution of CD44 and interfere with various CD44-dependent activities including cell motility and invasion.<sup>50</sup> Further emphasizing the complex nature of the relationship between HA production and both drug resistance and the development and pathogenesis of solid tumors, there is also evidence that certain ABC transporter proteins involved in multi-drug resistance may also play a role in the export of HA from cells.<sup>51,52</sup> ABC-C (MRP5) appears to be particularly important in this regard.<sup>51,52</sup> As one might expect, blocking the activity of MRP5 with the specific cGMP phosphodiesterase inhibitor zaprinast not only enhanced drug sensitivity but also reduced the migration and metastasis of human melanoma xenografts in SCID mice.<sup>53</sup>

In addition to its differential expression within the tumor microenvironment and involvement in tumor development and pathogenesis, the physiochemical nature of HA makes it an attractive target for the therapeutic approach described in this study. Specifically, triggering of Fas-induced apoptosis is critically dependent upon receptor oligomerization and the natural ligands that bind to and activate Fas and other members of the TNF-receptor superfamily, including FasL, are all trimers.<sup>54,55</sup> HA is a simple molecule composed of 2,000–25,000 repeating disaccharides of glucuronic acid and N-acetyl-glycosamine forming a linear structure with a molecule weight ranging from  $10^5$ – $10^7$  Da that adopts a pseudo random coil configuration in aqueous solution.<sup>34</sup> Studies suggest that HA oligomers containing 6–18 sugar residues bind to CD44 in a monovalent fashion while larger polymers are multivalent, have a higher affinity and efficiently crosslink CD44 inducing aggregation of the molecule in the plane of the plasma membrane.<sup>56,57</sup> Since the minimum motif recognized by CD44 is repeated many thousands of times within a large molecular weight HA molecule, it was hypothesized that HA would be particularly efficient at binding to and crosslinking the CD44/Fas chimeras thereby inducing the degree of oligomerization necessary to trigger the desired apoptotic response. The results of the present study confirm that this is indeed the case. With regard to the safety of the proposed therapeutic approach, it is reassuring that both CD44/FasTM and CD44TM/Fas exhibit minimum toxicity in the absence of HA (Fig. 2) and only induce significant levels of cell death when it is added (Fig. 5).

For the CD44/Fas chimeras to be effective in cancer gene therapy, it is necessary not only that the corresponding ligand be present but that the receptor is active in the desired cell type. Differential activation of ligand binding within the tumor microenvironment would clearly be an advantage with respect to targeting specificity. In this regard, it is well established that the activation status of CD44 varies depending on the cellular context in which the molecule is expressed. Thus, while resting hemopoietic cells are mostly CD44-positive, they do not bind HA unless appropriately stimulated.<sup>58–61</sup> Fortunately, when present on tumor cells, CD44 is generally constitutively active.<sup>59,62</sup> While the mechanisms that regulate the affinity of CD44 for HA remain to be fully elucidated, various postranslational modifications including glycosylation,<sup>63–68</sup> sulfation,<sup>69,70</sup> phosphorylation<sup>71,72</sup> and acylation<sup>73</sup> have been implicated in the process. The level of CD44 expressed by a cell is also clearly important. In keeping with this suggestion, the data shown in Figure 4 indicates that in transfected K562 cells, HA is only bound when CD44 or the CD44/Fas chimeras are expressed above a certain critical threshold.

In cells expressing a particular level of CD44, avidity for ligand can be further increased by clustering, which produces locally high concentrations of the receptor. This conclusion is supported by studies in which HA-binding can be induced by antibody-mediated crosslinking of CD44.<sup>64,74</sup> There is also evidence that CD44 can form covalently linked homodimers following appropriate stimulation.<sup>75</sup> As evidenced by the Western blot data shown in Figure 3, spontaneous dimerization of wild-type CD44 clearly occurs in transduced 293 cells. Although it has been suggested that covalent dimerization of CD44 in lymphoid cells is critically dependent upon a cysteine residue (Cys286) present in the membrane spanning domain of the molecule,<sup>75,76</sup> the fact that neither CD44TM/Fas nor CD44/FasTM exhibited significant evidence of dimerization when expressed in 293 cells (Fig. 3) argues that the cytoplasmic domain of CD44 may be more important, at least in this particular cell line. The limited dimerization of CD44 that is seen in untransfected 293 cells is in keeping with previous data suggesting that a certain threshold level of expression must be reached in order for CD44 to dimerize and/or form clusters.<sup>77</sup> Thus some



stimuli that induce CD44 clustering and activation of HA-binding may do so simply by upregulating overall CD44 expression.<sup>77</sup> In any event, dimerization of CD44 is obviously not essential for HA binding as both CD44/Fas transgenes clearly possess this activity if expressed at a high enough level.

Although far less effective than the corresponding CD44/Fas chimeras, the results shown in Figure 6 demonstrate that CD44H alone can inhibit the survival and/or proliferation of tumor cells when introduced and expressed at a high level. Despite the fact that the interaction between CD44 and HA clearly promotes the proliferation of many diverse tumor cell types, there is much evidence that in some circumstances growth inhibition can result. Cross-linking of CD44 has, for example, been shown to induce a number of myeloid cell lines to arrest in G0/G1 and undergo terminal differentiation.<sup>78</sup> This effect appears to involve reduced expression and phosphorylation of c-jun, increased expression of p21 and reduced phosphorylation of Rb with a resultant decrease in the kinase activity of Cdk2 and Cdk4.<sup>79,80</sup> Crosslinking of CD44 by immobilized antibodies was similarly shown to enhance the apoptotic response of lymphomas treated with dexamethasone.<sup>81</sup> In this case, the mechanism involves both downregulation of the anti-apoptotic protein Bclx(L) and upregulation of the pro-apoptotic protein Bax.<sup>81</sup> With regard to primary cells, ligation of CD44 can cause both dendritic cells and neutrophils to undergo apoptosis.<sup>82,83</sup> Interestingly, the effect on dendritic cells appears to be mediated, at least in part, via HA-stimulated production of inducible nitric oxide synthase (iNOS).<sup>82</sup> Thus, while no clear consensus has emerged as to how CD44 can transduce both growth promoting and growth inhibiting signals upon antibody-mediated crosslinking or binding of ligand, the findings of these and other studies suggest that both the level of CD44 expressed by a cell, as well as the presence of particular alternatively spliced CD44 isoforms, are likely to prove important. Cellular context is also key, reflecting the expression and functional activity of various molecules that interact directly or indirectly with CD44 and/or transduce signals mediated via the receptor. Suffice to say, that until the mechanisms controlling the growth promoting and growth inhibiting activity of CD44 are better understood, the use of the wild-type

molecule in cancer gene therapy is likely to prove problematic.

A potential problem with using Fas-induced apoptosis as an effector mechanism in cancer gene therapy is that defects in apoptosis are not uncommon in malignant cells.<sup>84–89</sup> Fortunately, these are normally relative rather than absolute. Thus, while certain tumor cell lines, including the bladder line T24 used in this study, are resistant to conventional Fas agonists, such as the anti-Fas mAb C-11, apoptotic death could be induced upon infection with an adenoviral vector encoding FasL.<sup>90</sup> Clearly, the magnitude of the stimulus is a key component and resistance can be overcome if the trigger used is sufficiently active or present at a high enough level.

In summary, chimeric receptor proteins of the type described in this study provide a potentially powerful means of exploiting the differential presence of various multivalent ligands within the tumor micro-environment in order to induce targeted tumor cell death. Since the signals that activate the receptor have a restricted distribution, off-target effects are likely to be minimized as, even if the transgene is inappropriately expressed in non-targeted tissues, a response will not occur unless the required triggering signal is also present. That a certain threshold level of ligand is needed for apoptosis to be induced is another inherent advantage of this particular system allowing some “leakiness” in the activity the transcriptional or other elements used to target gene expression to tumor cells to be tolerated. As efficient induction of tumor cell death will only occur in tissue sites where both the chimeric receptor and corresponding ligand are highly expressed and active, it is hoped that systemic toxicity will be minimized. *In vivo* studies to test this hypothesis are currently underway.

Although early results are encouraging, it is evident that a potential limitation of the pro-apoptotic gene therapy approach described herein is that only cells that directly take up and express the chimeric construct will be killed. Since it is unlikely that significant bystander killing will occur, success is thus critically dependent upon achieving efficient *in vivo* gene transfer. It is for this reason that we have chosen to focus our efforts on targeting, not tumor cells themselves, but instead the vascular endothelial cells that line the blood vessels upon which the survival and growth of a tumor depends.<sup>91</sup> The vasculature



is an attractive target as even limited damage to a tumor-associated blood vessel may result in occlusion and the cessation of blood flow thereby preventing the delivery of oxygen and nutrients to many thousands of tumor cells. It thus follows that even the relatively poor transduction efficiencies that can realistically be obtained using currently available delivery systems is likely to translate into greatly amplified “bystander” killing of malignant cells. Results obtained with small molecule “vascular disrupting agents” (VDA) such as Combretastatin A4 and ZD6126 serve to validate this strategy.<sup>92–95</sup>

## Acknowledgements

These studies were supported by grants from the National Cancer Institute (CA100004), the Arizona Biomedical Research Commission (Contract 0706) and the University of Arizona, Women’s Cancers Better Than Ever Program. We gratefully acknowledge the technical support and other input of Dr. Carmine Carpenito.

## Disclosures

The authors report no conflicts of interest.

## References

1. Auvinen P, Tammi R, Parkkinen J, et al. Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. *Am J Pathol.* 2000;156:529–36.
2. Stern R. Association between cancer and “acid mucopolysaccharides”: an old concept comes of age, finally. *Semin Cancer Biol.* 2008;18:238–43.
3. Tammi RH, Kultti A, Kosma VM, Pirinen R, Auvinen P, Tammi MI. Hyaluronan in human tumors: pathobiological and prognostic messages from cell-associated and stromal hyaluronan. *Semin Cancer Biol.* 2008;18:288–95.
4. Fenderson BA, Stamenkovic I, Aruffo A. Localization of hyaluronan in mouse embryos during implantation, gastrulation and organogenesis. *Differentiation.* 1993;54:85–98.
5. Toole BP. Hyaluronan in morphogenesis. *J Intern Med.* 1997;242:35–40.
6. Toole BP. Hyaluronan promotes the malignant phenotype. *Glycobiology.* 2002;12:37R–42R.
7. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol.* 2007;23:435–61.
8. Zhang L, Underhill CB, Chen L. Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. *Cancer Res.* 1995;55:428–33.
9. Aaltomaa S, Lipponen P, Tammi R, et al. Strong Stromal Hyaluronan Expression Is Associated with PSA Recurrence in Local Prostate Cancer. *Urol Int.* 2002;69:266–72.
10. Bohm J, Niskanen L, Tammi R, et al. Hyaluronan expression in differentiated thyroid carcinoma. *J Pathol.* 2002;196:180–5.
11. Slevin M, Krupinski J, Gaffney J, et al. Hyaluronan-mediated angiogenesis in vascular disease: uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol.* 2007;26:58–68.
12. Itano N, Kimata K. Altered hyaluronan biosynthesis in cancer progression. *Semin Cancer Biol.* 2008;18:268–74.
13. Knudson CB, Knudson W. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J.* 1993;7:1233–41.
14. Neame PJ, Barry FP. The link proteins. *EXS.* 1994;70:53–72.
15. Pilarski LM, Masellis-Smith A, Belch AR, Yang B, Savani RC, Turley EA. RHAMM, a receptor for hyaluronan-mediated motility, on normal human lymphocytes, thymocytes and malignant B cells: a mediator in B cell malignancy? *Leuk Lymphoma.* 1994;14:363–74.
16. Lesley J, Hyman R. CD44 structure and function. *Front Biosci.* 1998;3:d616–30.
17. Evanko SP, Tammi MI, Tammi RH, Wight TN. Hyaluronan-dependent pericellular matrix. *Adv Drug Deliv Rev.* 2007;59:1351–65.
18. Heino J, Kapyla J. Cellular receptors of extracellular matrix molecules. *Curr Pharm Des.* 2009;15:1309–17.
19. Pure E, Assoian RK. Rheostatic signaling by CD44 and hyaluronan. *Cell Signal.* 2009;21:651–5.
20. Tuszyński GP, Wang TN, Berger D. Adhesive proteins and the hematogenous spread of cancer. *Acta Haematol.* 1997;97:29–39.
21. Knudson W. The role of CD44 as a cell surface hyaluronan receptor during tumor invasion of connective tissue. *Front Biosci.* 1998;3:d604–15.
22. Hill A, McFarlane S, Johnston PG, Waugh DJ. The emerging role of CD44 in regulating skeletal micrometastasis. *Cancer Lett.* 2006;237:1–9.
23. Toole BP, Slomiany MG. Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells. *Semin Cancer Biol.* 2008;18:244–50.
24. Cooper DL, Dougherty GJ. To metastasize or not? Selection of CD44 splice sites. *Nat Med.* 1995;1:635–7.
25. Yang B, Yang BL, Savani RC, Turley EA. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. *EMBO J.* 1994;13:286–96.
26. Wang C, Entwistle J, Hou G, Li Q, Turley EA. The characterization of a human RHAMM cDNA: conservation of the hyaluronan-binding domains. *Gene.* 1996;174:299–306.
27. Hayes GM, Chiu R, Carpenito C, Dougherty ST, Dougherty GJ. Identification of sequence motifs responsible for the adhesive interaction between exon v10-containing CD44 isoforms. *J Biol Chem.* 2002;277:50529–34.
28. Dougherty GJ, Cooper DL, Memory JF, Chiu RK. Ligand binding specificity of alternatively spliced CD44 isoforms. Recognition and binding of hyaluronan by CD44R1. *J Biol Chem.* 1994;269:9074–8.
29. Droll A, Dougherty ST, Chiu RK, et al. Adhesive interactions between alternatively spliced CD44 isoforms. *J Biol Chem.* 1995;270:11567–73.
30. Dougherty GJ, Landorp PM, Cooper DL, Humphries RK. Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte “homing” receptor expressed by hemopoietic cells. *J Exp Med.* 1991;174:1–5.
31. Knudson W, Biswas C, Li XQ, Nemecek RE, Toole BP. The role and regulation of tumour-associated hyaluronan. *Ciba Found Symp.* 1989;143:150–9; discussion 159–69, 281–5.
32. Toole BP, Wight TN, Tammi MI. Hyaluronan-cell interactions in cancer and vascular disease. *J Biol Chem.* 2002;277:4593–6.
33. Heldin P, Karousou E, Bernert B, Porsch H, Nishitsuka K, Skandalis SS. Importance of hyaluronan-CD44 interactions in inflammation and tumorigenesis. *Connect Tissue Res.* 2008;49:215–8.
34. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer.* 2004;4:528–39.
35. Kosaki R, Watanabe K, Yamaguchi Y. Overproduction of hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenicity. *Cancer Res.* 1999;59:1141–5.
36. Koyama H, Hibi T, Isogai Z, et al. Hyperproduction of hyaluronan in neu-induced mammary tumor accelerates angiogenesis through stromal cell recruitment: possible involvement of versican/PG-M. *Am J Pathol.* 2007;170:1086–99.
37. Liu N, Gao F, Han Z, Xu X, Underhill CB, Zhang L. Hyaluronan synthase 3 overexpression promotes the growth of TSU prostate cancer cells. *Cancer Res.* 2001;61:5207–14.
38. Simpson MA, Wilson CM, McCarthy JB. Inhibition of prostate tumor cell hyaluronan synthesis impairs subcutaneous growth and vascularization in immunocompromised mice. *Am J Pathol.* 2002;161:849–57.
39. Bullard KM, Kim HR, Wheeler MA, et al. Hyaluronan synthase-3 is upregulated in metastatic colon carcinoma cells and manipulation of expression alters matrix retention and cellular growth. *Int J Cancer.* 2003;107:739–46.



40. Kim HR, Wheeler MA, Wilson CM, et al. Hyaluronan facilitates invasion of colon carcinoma cells in vitro via interaction with CD44. *Cancer Res.* 2004;64:4569–76.
41. Kerbel RS, Rak J, Kobayashi H, Man MS, St. Croix B, Graham CH. Multicellular resistance: a new paradigm to explain aspects of acquired drug resistance of solid tumors. *Cold Spring Harb Symp Quant Biol.* 1994;59:661–72.
42. Baumgartner G, Gomar-Hoss C, Sakr L, Ulsperger E, Wogritsch C. The impact of extracellular matrix on the chemoresistance of solid tumors—experimental and clinical results of hyaluronidase as additive to cytostatic chemotherapy. *Cancer Lett.* 1998;131:85–99.
43. Misra S, Ghatak S, Zoltan-Jones A, Toole BP. Regulation of multidrug resistance in cancer cells by hyaluronan. *J Biol Chem.* 2003;278:25285–8.
44. Toole BP, Ghatak S, Misra S. Hyaluronan oligosaccharides as a potential anticancer therapeutic. *Curr Pharm Biotechnol.* 2008;9:249–52.
45. Toole BP, Slomiany MG. Hyaluronan, CD44 and Emmprin: partners in cancer cell chemoresistance. *Drug Resist Updat.* 2008;11:110–21.
46. Misra S, Ghatak S, Toole BP. Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. *J Biol Chem.* 2005;280:20310–5.
47. Ohashi R, Takahashi F, Cui R, et al. Interaction between CD44 and hyaluronate induces chemoresistance in non-small cell lung cancer cell. *Cancer Lett.* 2007;252:225–34.
48. Gilg AG, Tye SL, Tolliver LB, et al. Targeting hyaluronan interactions in malignant gliomas and their drug-resistant multipotent progenitors. *Clin Cancer Res.* 2008;14:1804–13.
49. Bacsó Z, Nagy H, Goda K, et al. Raft and cytoskeleton associations of an ABC transporter: P-glycoprotein. *Cytometry A.* 2004;61:105–16.
50. Miletto-Gonzalez KE, Chen S, Muthukumaran N, et al. The CD44 receptor interacts with P-glycoprotein to promote cell migration and invasion in cancer. *Cancer Res.* 2005;65:6660–7.
51. Prehm P, Schumacher U. Inhibition of hyaluronan export from human fibroblasts by inhibitors of multidrug resistance transporters. *Biochem Pharmacol.* 2004;68:1401–10.
52. Schulz T, Schumacher U, Prehm P. Hyaluronan export by the ABC transporter MRP5 and its modulation by intracellular cGMP. *J Biol Chem.* 2007;282:20999–21004.
53. Monz K, Maas-Kuck K, Schumacher U, et al. Inhibition of hyaluronan export attenuates cell migration and metastasis of human melanoma. *J Cell Biochem.* 2008;105:1260–6.
54. Cremesti A, Paris F, Grassme H, et al. Ceramide enables fas to cap and kill. *J Biol Chem.* 2001;276:23954–61.
55. Fanzo JC, Lynch MP, Phee H, et al. CD95 rapidly clusters in cells of diverse origins. *Cancer Biol Ther.* 2003;2:392–5.
56. Underhill CB, Chi-Rosso G, Toole BP. Effects of detergent solubilization on the hyaluronate-binding protein from membranes of simian virus 40-transformed 3T3 cells. *J Biol Chem.* 1983;258:8086–91.
57. Lesley J, Hascall VC, Tammi M, Hyman R. Hyaluronan binding by cell surface CD44. *J Biol Chem.* 2000;275:26967–75.
58. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. *Adv Immunol.* 1993;54:271–335.
59. Smadja-Joffe F, Legras S, Girard N, et al. CD44 and hyaluronan binding by human myeloid cells. *Leuk Lymphoma.* 1996;21:407–20, color plates following 528.
60. DeGrendele HC, Kosfisz M, Estess P, Siegelman MH. CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. *J Immunol.* 1997;159:2549–53.
61. Levesque MC, Haynes BF. Activated T lymphocytes regulate hyaluronan binding to monocyte CD44 via production of IL-2 and IFN-gamma. *J Immunol.* 2001;166:188–96.
62. Marhaba R, Zoller M. CD44 in cancer progression: adhesion, migration and growth regulation. *J Mol Histol.* 2004;35:211–31.
63. Bennett KL, Modrell B, Greenfield B, et al. Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. *J Cell Biol.* 1995;131:1623–33.
64. Lesley J, English N, Perschl A, Gregoroff J, Hyman R. Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. *J Exp Med.* 1995;182:431–7.
65. Bartolazzi A, Nocks A, Aruffo A, Spring F, Stamenkovic I. Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. *J Cell Biol.* 1996;132:1199–208.
66. English NM, Lesley JF, Hyman R. Site-specific de-N-glycosylation of CD44 can activate hyaluronan binding, and CD44 activation states show distinct threshold densities for hyaluronan binding. *Cancer Res.* 1998;58:3736–42.
67. Esford LE, Maiti A, Bader SA, Tufaro F, Johnson P. Analysis of CD44 interactions with hyaluronan in murine L cell fibroblasts deficient in glycosaminoglycan synthesis: a role for chondroitin sulfate. *J Cell Sci.* 1998;111:1021–9.
68. Skelton TP, Zeng C, Nocks A, Stamenkovic I. Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan. *J Cell Biol.* 1998;140:431–46.
69. Brown KL, Maiti A, Johnson P. Role of sulfation in CD44-mediated hyaluronan binding induced by inflammatory mediators in human CD14(+) peripheral blood monocytes. *J Immunol.* 2001;167:5367–74.
70. Delcommenne M, Kannagi R, Johnson P. TNF-alpha increases the carbohydrate sulfation of CD44: induction of 6-sulfo N-acetyl lactosamine on N- and O-linked glycans. *Glycobiology.* 2002;12:613–22.
71. Uff CR, Neame SJ, Isacke CM. Hyaluronan binding by CD44 is regulated by a phosphorylation-independent mechanism. *Eur J Immunol.* 1995;25:1883–7.
72. Formby B, Stern R. Phosphorylation stabilizes alternatively spliced CD44 mRNA transcripts in breast cancer cells: inhibition by antisense complementary to casein kinase II mRNA. *Mol Cell Biochem.* 1998;187:23–31.
73. Thankamony SP, Knudson W. Acylation of CD44 and its association with lipid rafts are required for receptor and hyaluronan endocytosis. *J Biol Chem.* 2006;281:34601–9.
74. Lesley J, Kincade PW, Hyman R. Antibody-induced activation of the hyaluronan receptor function of CD44 requires multivalent binding by antibody. *Eur J Immunol.* 1993;23:1902–9.
75. Liu D, Sy MS. Phorbol myristate acetate stimulates the dimerization of CD44 involving a cysteine in the transmembrane domain. *J Immunol.* 1997;159:2702–11.
76. Liu D, Sy MS. A cysteine residue located in the transmembrane domain of CD44 is important in binding of CD44 to hyaluronic acid. *J Exp Med.* 1996;183:1987–94.
77. Liu D, Liu T, Li R, Sy MS. Mechanisms regulating the binding activity of CD44 to hyaluronic acid. *Front Biosci.* 1998;3:d631–6.
78. Gadhoun Z, Delaunay J, Maquarre E, et al. The effect of anti-CD44 monoclonal antibodies on differentiation and proliferation of human acute myeloid leukemia cells. *Leuk Lymphoma.* 2004;45:1501–10.
79. Charrad RS, Gadhoun Z, Qi J, et al. Effects of anti-CD44 monoclonal antibodies on differentiation and apoptosis of human myeloid leukemia cell lines. *Blood.* 2002;99:290–9.
80. Zada AA, Singh SM, Reddy VA, et al. Downregulation of c-Jun expression and cell cycle regulatory molecules in acute myeloid leukemia cells upon CD44 ligation. *Oncogene.* 2003;22:2296–308.
81. Guy R, Yefenof E, Naor D, Dorogin A, Zilberman Y. CD44 co-stimulates apoptosis in thymic lymphomas and T cell hybridomas. *Cell Immunol.* 2002;216:82–92.
82. Yang T, Witham TF, Villa L, et al. Glioma-associated hyaluronan induces apoptosis in dendritic cells via inducible nitric oxide synthase: implications for the use of dendritic cells for therapy of gliomas. *Cancer Res.* 2002;62:2583–91.
83. Takazoe K, Tesch GH, Hill PA, et al. CD44-mediated neutrophil apoptosis in the rat. *Kidney Int.* 2000;58:1920–30.
84. Shin EC, Shin JS, Park JH, Kim JJ, Kim H, Kim SJ. Expression of Fas-related genes in human hepatocellular carcinomas. *Cancer Lett.* 1998;134:155–62.
85. Ramenghi U, Bonisconi S, Migliaretti G, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations is a familial trait predisposing to development of autoimmune diseases and cancer. *Blood.* 2000;95:3176–82.
86. Raisova M, Bektas M, Wieder T, et al. Resistance to CD95/Fas-induced and ceramide-mediated apoptosis of human melanoma cells is caused by a defective mitochondrial cytochrome c release. *FEBS Lett.* 2000;473:27–32.



87. Snow AL, Chen LJ, Nepomuceno RR, Krams SM, Esquivel CO, Martinez OM. Resistance to Fas-mediated apoptosis in EBV-infected B cell lymphomas is due to defects in the proximal Fas signaling pathway. *J Immunol.* 2001;167:5404–11.
88. Badillo-Almaraz I, Badillo-Salas C, Villalobos R, Avalos-Diaz E, Herrera-Esparza R. Defective expression of FasL and Bax in human lung cancer. *Clin Exp Med.* 2003;3:106–12.
89. Blomberg J, Ruuth K, Jacobsson M, Hoglund A, Nilsson JA, Lundgren E. Reduced FAS transcription in clones of U937 cells that have acquired resistance to Fas-induced apoptosis. *FEBS J.* 2009;276:497–508.
90. Sudarshan S, Holman DH, Hyer ML, Voelkel-Johnson C, Dong JY, Norris JS. In vitro efficacy of Fas ligand gene therapy for the treatment of bladder cancer. *Cancer Gene Ther.* 2005;12:12–8.
91. Chaplin DJ, Dougherty GJ. Tumour vasculature as a target for cancer therapy. *Br J Cancer.* 1999;80 Suppl 1:57–64.
92. Chaplin DJ, Hill SA. Selective induction of tumor ischemia: development of vascular targeting agents for cancer therapy. *Curr Opin Investig Drugs.* 2002;3:1381–4.
93. Davis PD, Dougherty GJ, Blakey DC, et al. ZD6126: a novel vascular-targeting agent that causes selective destruction of tumor vasculature. *Cancer Res.* 2002;62:7247–53.
94. Siemann DW, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. *Cancer.* 2004;100:2491–9.
95. Siemann DW, Chaplin DJ, Walicke PA. A review and update of the current status of the vasculature-disabling agent combretastatin-A4 phosphate (CA4P). *Expert Opin Investig Drugs.* 2009;18:189–97.

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