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14. ABSTRACT The overall objective of our work on human ovarian carcinoma cells is to apply our previous molecular and cellular findings on the role of hyaluronan-CD44-CD147 interactions in cancer stem cell properties, especially drug resistance, to improvement of therapy for malignant ovarian carcinoma. In this grant we have shown that: a) drug-resistant human ovarian carcinoma cell lines contain CD133-positive/ CD147-positive/ CD44-positive cancer stem-like cells in similar proportion to that in human patient ascites-derived ovarian carcinoma cells, thus documenting their suitability for our studies; b) small hyaluronan oligosaccharides sensitize drug-resistant human ovarian carcinoma cells to various chemotherapeutic agents in culture and in vivo; c) CD147 silencing, via delivery of CD147 siRNA in liposomes, sensitizes cisplatin-resistant human ovarian carcinoma cells to cisplatin treatment in vivo; d) CD147 silencing decreases rates of metastases of human ovarian carcinoma cells in vivo. These results form the basis of promising new approaches to therapy in patients with recurrent, drug-resistant ovarian carcinoma.					
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INTRODUCTION

Our previous studies have shown that hyaluronan, the hyaluronan receptors CD44 or LYVE-1, and the Ig superfamily member CD147 act cooperatively to promote malignant and drug-resistant properties. This most likely occurs through assembly and/or stabilization of plasma membrane signaling complexes containing CD44 or LYVE-1 in association with CD147, receptor tyrosine kinases and transporters implicated in malignancy and resistance to therapies (Ghatak et al., 2005; Grass et al., 2013; Qin et al., 2011; Slomiany et al., 2009a; Slomiany et al., 2009b; Slomiany et al., 2009c) [reviewed in (Grass et al., 2014; Toole, 2009)]. CD147 (emmprin; basigin) is a cell surface member of the Ig superfamily that induces expression of hyaluronan and matrix metalloproteinases, and promotes cell invasiveness, anchorage independent growth, drug resistance, and tumor growth and metastasis *in vivo* (Caudroy et al., 2002; Dai et al., 2013; Grass et al., 2012; Marieb et al., 2004; Zucker et al., 2001)[reviewed in (Grass et al., 2014)]. We have shown recently that sub-populations of ovarian carcinoma cells and other cancer cell types with high expression of cell surface CD147 have similar properties to cancer stem cells, including enhanced levels of anchorage-independent growth, drug resistance and invasiveness (Dai et al., 2013).

Many of the activities of CD147 in cancer cells are dependent on hyaluronan-CD44 or -LYVE-1 signaling (Ghatak et al., 2005; Grass et al., 2013; Marieb et al., 2004; Misra et al., 2003; Qin et al., 2011), and CD44 is one of the most common markers for carcinoma cancer stem cells (Zoller, 2011). The overall objectives of our work are to determine the mechanisms whereby hyaluronan-CD44-CD147 interactions influence malignant cell behavior and therapy resistance, and to apply our findings to the improvement of therapy, in particular in recurrent ovarian carcinoma. For example, we have found that multivalent interactions of hyaluronan polymer with CD44 are necessary for stabilizing CD44-CD147 signaling complexes, and that small, monovalent, hyaluronan oligosaccharides antagonize hyaluronan-receptor signaling by disrupting constitutive hyaluronan polymer-receptor interaction, thus leading to inhibition of oncogenic signaling pathways, chemoresistance and malignant characteristics (Ghatak et al., 2002; Ghatak et al., 2005; Gilg et al., 2008; Grass et al., 2013; Misra et al., 2006; Qin et al., 2011; Slomiany et al., 2009a; Slomiany et al., 2009b; Slomiany et al., 2009c). In particular we have found that treatment with small hyaluronan oligosaccharides is effective in sensitizing various types of drug-resistant cancer cells to chemotherapeutic agents (Gilg et al., 2008; Misra et al., 2005; Misra et al., 2003; Qin et al., 2011; Slomiany et al., 2009a). Most notably, these oligosaccharides inhibit tumor growth by drug-resistant cancer stem cell sub-populations obtained from human patient-derived ovarian carcinoma cells (Slomiany et al., 2009b).

Our aims for this grant are to establish efficacy for small hyaluronan oligosaccharides as chemo-sensitizing agents in xenograft models of human ovarian carcinoma cells, to determine whether siRNA directed against CD147 or CD44 affect ovarian carcinoma chemoresistance and metastasis, and to explore mechanisms for increasing efficiency of delivery of these agents together with chemotherapeutic agents.

KEYWORDS

Ovarian carcinoma; hyaluronan; CD147; CD44; drug resistance

OVERALL PROJECT SUMMARY

The overall objective of our work on human ovarian carcinoma cells is to apply our findings on the role of hyaluronan-CD44-CD147 interactions in drug resistance to improvement of therapy for malignant ovarian carcinoma. The specific Aims of this grant were: 1. to establish efficacy for small hyaluronan oligosaccharides as a chemo-sensitizing agent in xenograft models of human ovarian carcinoma cells, and to explore mechanisms for increasing efficiency of delivery of hyaluronan oligosaccharides; 2. to determine whether CD44-CD147 antagonists are potential inhibitors of human ovarian tumor progression.

During the course of this grant we have: a) shown that drug-resistant human ovarian carcinoma cells contain CD133-positive/ CD147-positive/ CD44-positive cancer stem-like cells in similar proportions to those found in human patient ascites-derived ovarian carcinoma cells, thus documenting their suitability for our studies; b) demonstrated that small hyaluronan oligosaccharides sensitize drug-resistant human ovarian carcinoma cells to various chemotherapeutic agents in culture and in vivo; c) completed preparation and testing of hyaluronan oligosaccharide conjugated to the chemotherapeutic agent, docetaxel; unfortunately this conjugate did not show the expected increase in efficacy in vivo; d) shown that siRNAs against the hyaluronan receptor, CD44, and the regulator of hyaluronan synthesis, CD147, sensitize drug-resistant ovarian carcinoma cells to cisplatin in culture; e) identified a highly effective siRNA against CD147 that has been loaded into liposomes for higher efficacy; testing in vivo showed that CD147 silencing decreases resistance to cisplatin and formation of metastases.

a) Fluorescence-activated cell sorting (FACS) analyses of human ovarian carcinoma cells

We analyzed SKOV3 human ovarian carcinoma cells, cisplatin-resistant A2780cp20 human ovarian carcinoma cells, multidrug-resistant Hey-A8-MDR human ovarian carcinoma cells, and patient ascites-derived primary human ovarian carcinoma cells by FACS. In the case of the primary ascites-derived cells, we first separated the cells from ascites fluid by low speed centrifugation and then removed red blood cells. We then incubated the remaining cells in tissue culture dishes for a short period – the rapidly attaching cells, mainly fibroblasts, were discarded. The suspended cells were re-plated for 24 hours during which the cancer cells attached but most leucocytes did not. The attached cells were washed and used for FACS analysis as described previously (Slomiany et al., 2009b).

SKOV3 cells, A2780cp20 cells, Hey-A8-MDR cells, and primary cells derived from human ascites as described above were subject to FACS analysis in the same manner, as follows. We first separated live cells from dead cells, and then separated CD45-negative cells from CD45-positive putative leucocytes. The CD45-negative cells were then split into CD133-negative and CD133-positive sub-populations. From past data from this (Slomiany et al.,

2009b) and other laboratories (Silva et al., 2011), we expect the CD133-positive cells to be enriched in cancer stem-like cells. For all three cell types, approximately 5% of the CD45-negative cells were CD133-positive (Fig. 1). Since CD44 and CD147 are also associated with cancer stem cells, we then analyzed the distribution of CD44 and CD147 in the CD133-positive sub-population. For the SKOV3, A2780cp29 and Hey-A8-MDR cells, 80-100% of the CD133-positive, cancer stem-like cells were also positive for CD44 and CD147 (Fig. 1B-D). In the case of the ascites-derived cells, ~70% were CD44-positive and CD147-positive, with most of the remaining cells being CD44-positive/CD147-negative (Fig. 1A).

These results show clearly that all three human ovarian carcinoma cell lines, as well as the primary ascites cells, include a sub-population of cells with similar cancer stem-like markers. Since our analyses of primary cells and cell lines exhibited similar cancer stem-like cell populations, and since we were unable to obtain sufficient numbers of cells in the cancer stem-like cell fractions for detailed analyses, we carried out subsequent analyses with drug-resistant human ovarian carcinoma cell lines.

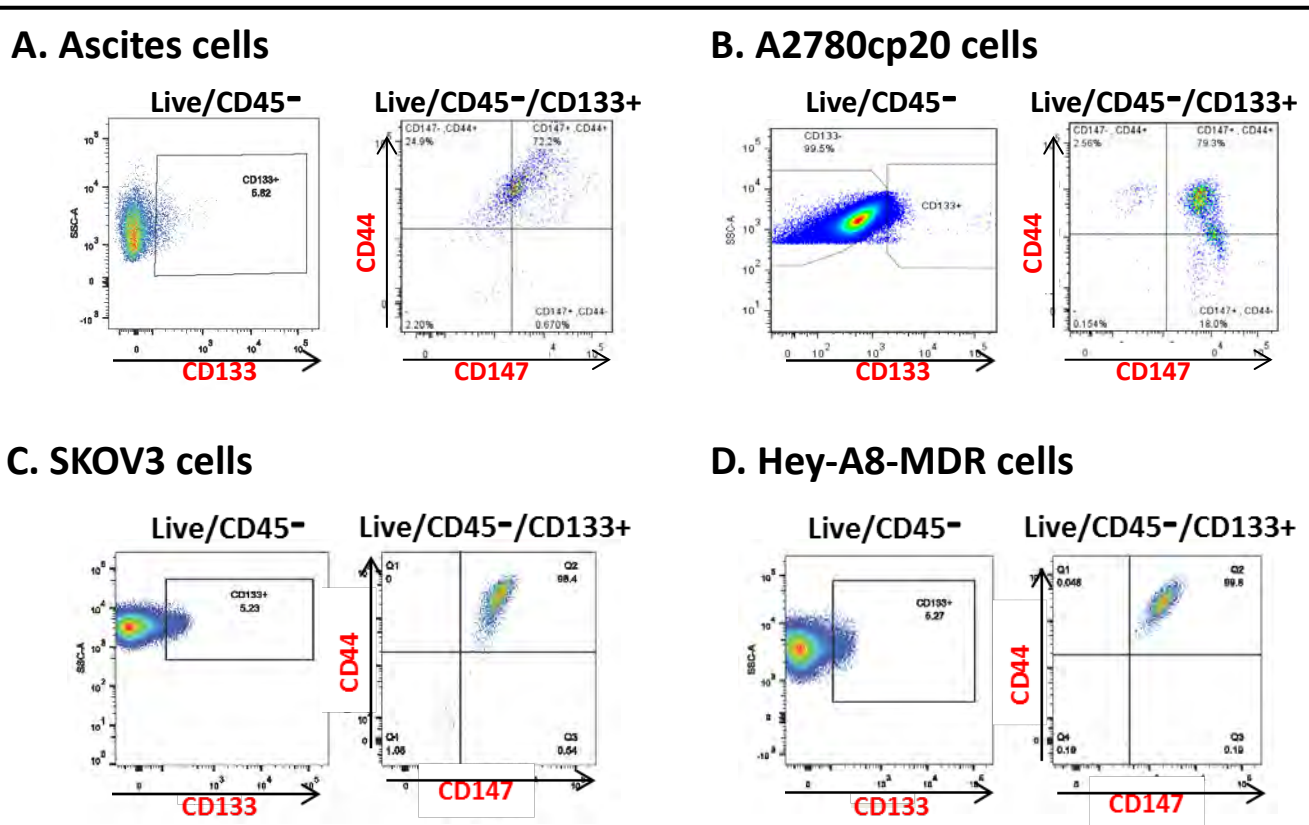
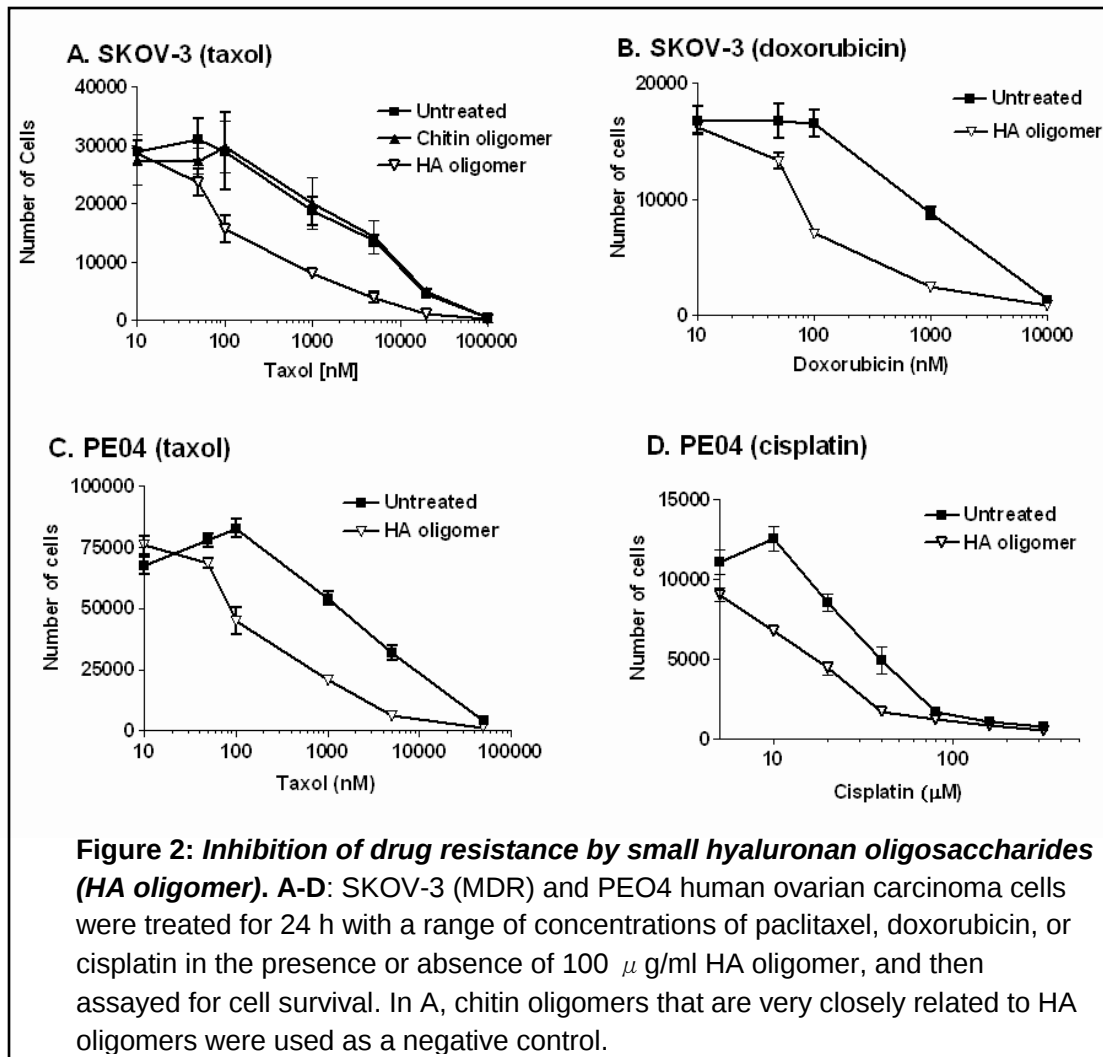


Figure 1: FACS analyses of ovarian carcinoma cells. A. Patient-derived ascites cells; B. A2780cp20 cells; C. SKOV3 cells; D. Hey-A8-MDR cells. In each case, dead cells and CD45-positive cells were eliminated from the analyses. CD133-positive cells were analyzed within the live cell/CD45-negative population, and then the CD133-positive cells were analyzed for CD147 and CD44. In each case, ~5% of the live/CD45-negative population was CD133-positive. ~80-100% of the CD133-positive A2780cp20, SKOV3 or Hey-A8-MDR cell population was CD147-positive and CD44-positive. Likewise, ~70% of the CD133-positive ascites cell population was CD147-positive and CD44-positive.

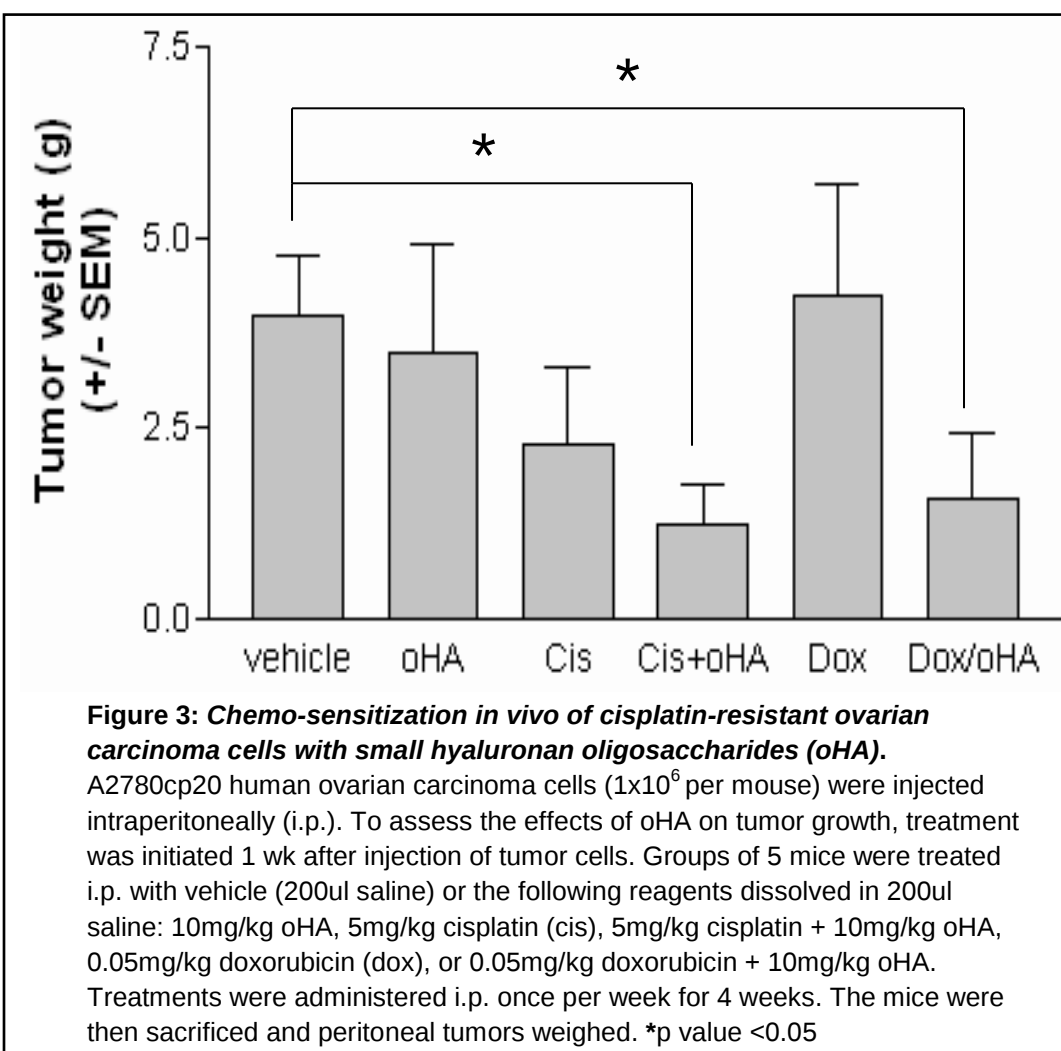
b) Chemo-sensitization by small hyaluronan oligosaccharides *in vitro*

We have shown that co-treatment with small hyaluronan oligosaccharides sensitizes human ovarian carcinoma cells (a drug-resistant line of SKOV-3 cells, and PEO4 cells) to various chemotherapeutic agents, i.e. taxol, doxorubicin and cisplatin, in cell culture (Figure 2).



c) Chemo-sensitization by small hyaluronan oligosaccharides *in vivo*

Using intraperitoneal xenografts of cisplatin-resistant A2780cp20 human ovarian carcinoma cells, we have now shown that co-treatment with small hyaluronan oligosaccharides sensitizes these cells to treatment with cisplatin *in vivo* (Figure 3). The oligosaccharides also sensitized these cells to treatment with doxorubicin *in vivo* (Figure 3). Doses of cisplatin and doxorubicin were chosen to give sub-optimal responses when used alone. Co-treatment with the hyaluronan oligosaccharides plus either drug caused large decreases in tumor growth, as compared to drug treatment alone (Figure 3).

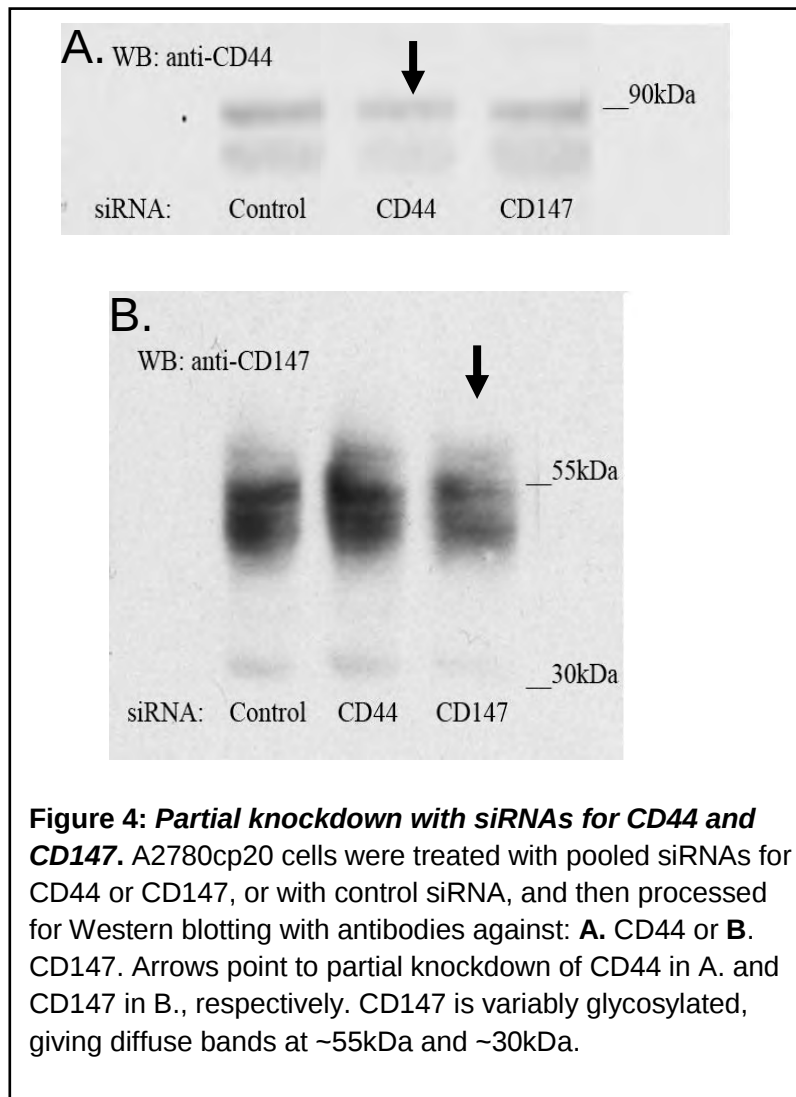


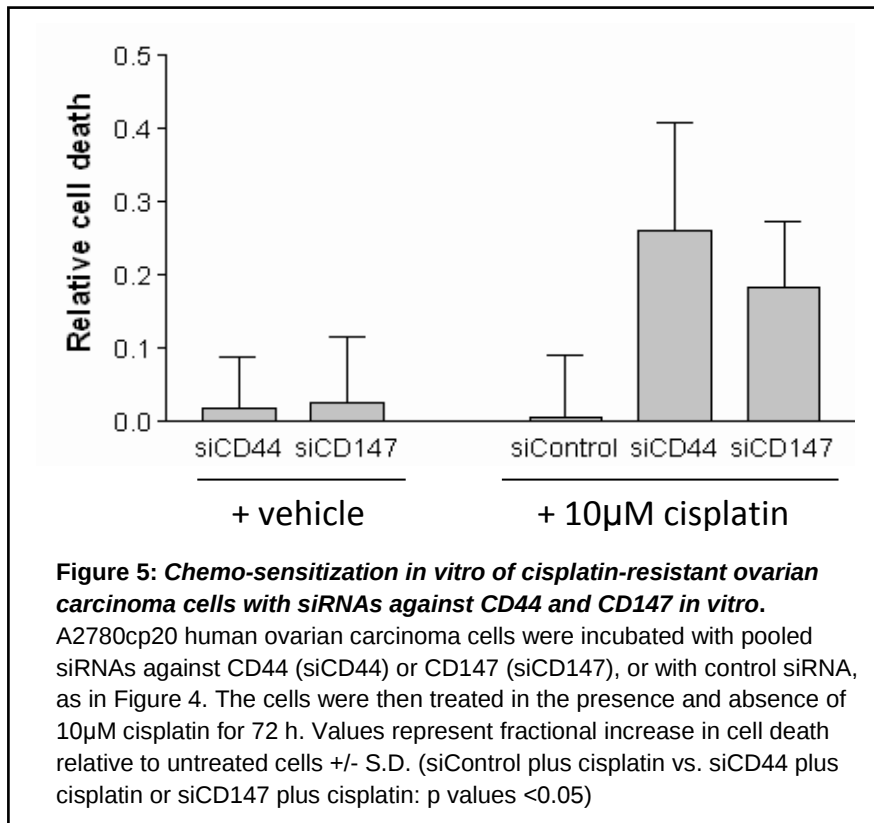
d) *Hyaluronan oligosaccharide-conjugated docetaxel*

In collaboration with Drs. Dahai Jiang, Selanere Mangala and Anil Sood (MD Anderson Cancer Center), we have prepared docetaxel conjugated with small hyaluronan oligosaccharides in a similar manner as performed previously with polymeric hyaluronan (Lee et al., 2012). After several preliminary experiments to obtain appropriate composition and effectiveness in culture, we prepared a large scale batch of these conjugates for use in xenograft experiments. We anticipated that these conjugates would show greater efficacy in vivo than simple mixtures of docetaxel and hyaluronan oligosaccharide, as used previously, since the hyaluronan oligosaccharide should bind CD44 in such a manner as to inhibit the activity of endogenous hyaluronan-CD44-CD147-receptor tyrosine kinases/ transporter complexes (Ghatak et al., 2005; Grass et al., 2012; Grass et al., 2013; Qin et al., 2011; Slomiany et al., 2009a; Slomiany et al., 2009b; Slomiany et al., 2009c), and induce internalization of the docetaxel conjugate along with CD44 (Lee et al., 2012; Thankamony and Knudson, 2006). Unfortunately the conjugate did not show efficacy in vivo, either alone or with chemotherapeutic agents.

e) Chemo-sensitization by siRNAs against CD44 and CD147

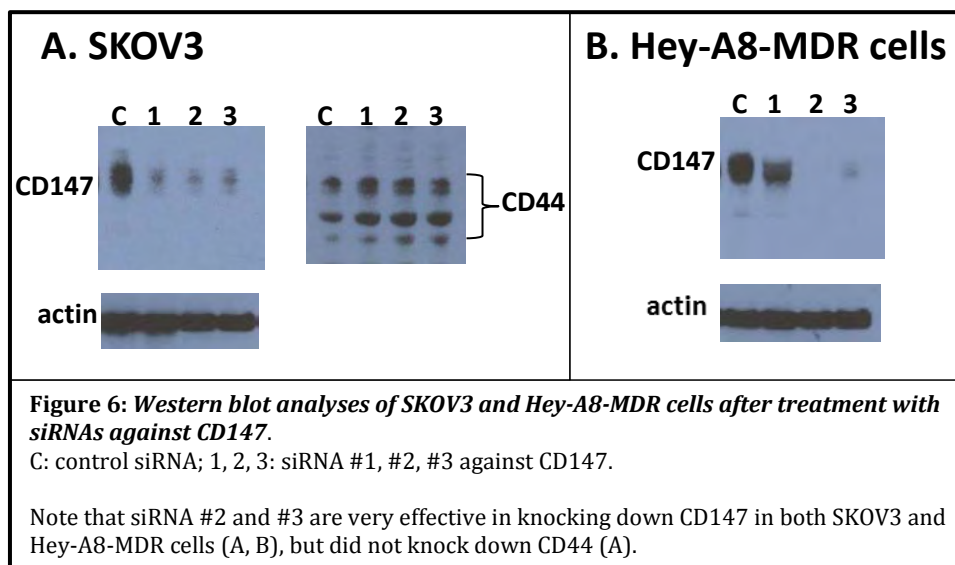
Despite previous success in obtaining efficient knockdown in various cancer cell types with siRNAs against CD44 and CD147 (Ghatak et al., 2005; Grass et al., 2012; Misra et al., 2005; Qin et al., 2011; Slomiany et al., 2009c), we have had difficulties obtaining efficient knockdown in A2780cp20 cisplatin-resistant ovarian carcinoma cells. However, we succeeded in obtaining partial but significant knockdown (Figure 4). Using these conditions we have also shown corresponding partial effects of these siRNAs on cisplatin resistance in the A2780cp20 cells in culture (Figure 5).





f) Chemo-sensitization in vivo: CD147 siRNA-loaded liposomes

Also in collaboration with Drs. Jiang, Mangala and Sood, we have prepared liposomes containing siRNA against CD147. We anticipated that these liposome particles will increase the efficacy of action of siRNA against CD147 in similar manner to the approach previously used by the Sood laboratory (Landen et al., 2010; Mangala et al., 2009; Spannuth et al., 2011). Despite earlier difficulties with obtaining efficient knockdown, we have now identified siRNAs against CD147 that are very efficient (Fig. 6). The siRNA #2 (Fig. 6), has now been used to manufacture the liposomal delivery nanoparticles for use in xenograft experiments.



We have completed testing of CD147 silencing, using liposomal delivery of CD147 siRNA. In this experiment, we compared the effects of liposome-encapsulated control siRNA (control siRNA-DOPC) alone with liposome-encapsulated control siRNA plus cisplatin, liposome-encapsulated CD147 siRNA (CD147 siRNA-DOPC), and liposome-encapsulated CD147 siRNA plus cisplatin. The effects of these treatments on tumor growth in vivo are summarized in Figure 7, which shows that CD147 silencing had a strong effect on sensitization of cisplatin resistant A2780cp20 cells to cisplatin treatment (Fig. 7).

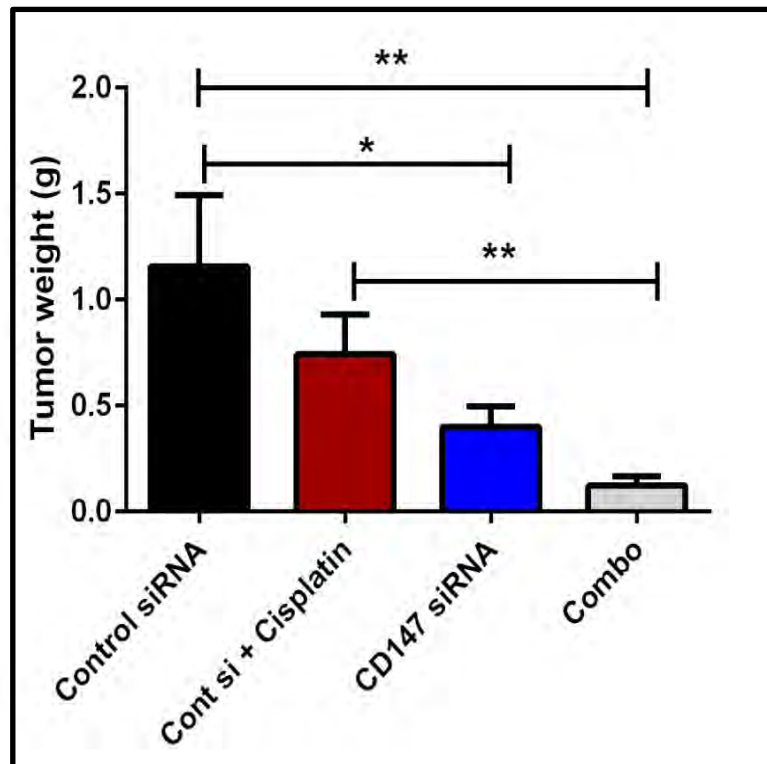
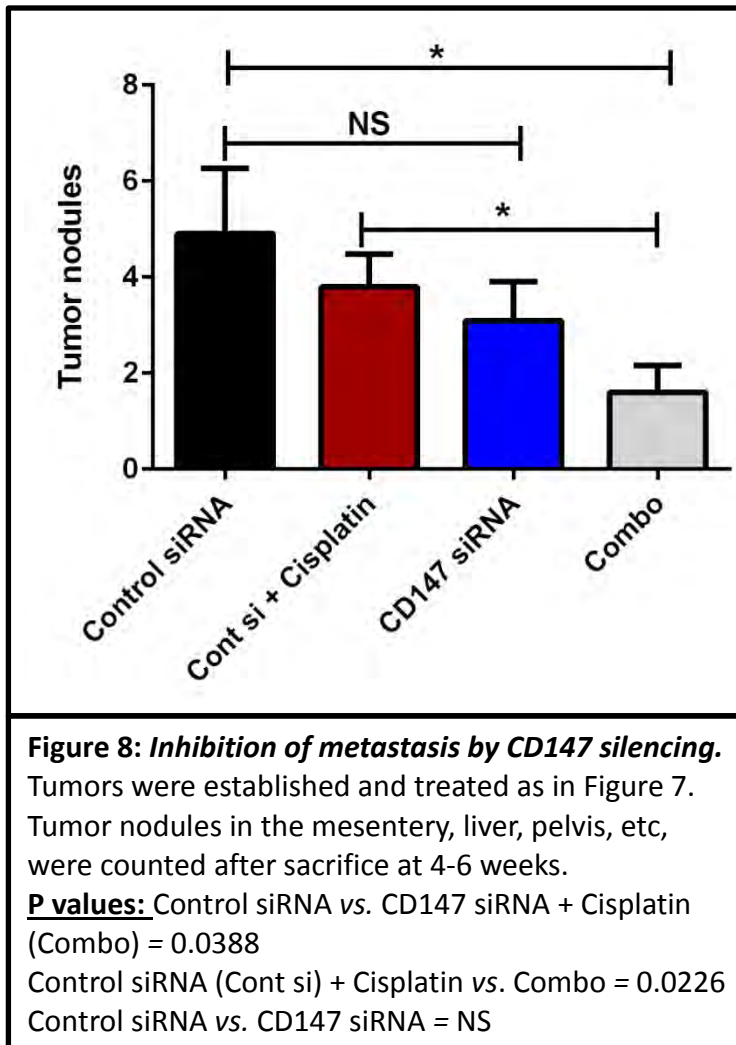


Figure 7: Chemo-sensitization of cisplatin-resistant cells in vivo by CD147 silencing. Tumors were established by i.p. injection of 1.0×10^6 A2780cp20 cells. Once established, this tumor model has been shown to reflect the growth pattern of advanced ovarian cancer (Landen et al., 2010; Mangala et al., 2009; Spannuth et al., 2011). To assess the effects of siRNA therapy on tumor growth, treatment was initiated 1 wk after i.p. injection of tumor cells. Mice were divided into five groups (n = 10 mice per group): (a) control siRNA-DOPC (150 μ g/kg i.p. twice weekly), (b) control siRNA-DOPC (Cont si) (150 μ g/kg i.p. twice weekly) + cisplatin (160 μ g/mouse i.p. weekly), (c) CD147 siRNA-DOPC (150 μ g/kg i.p. twice weekly), and (d) CD147 siRNA-DOPC + cisplatin (Combo: doses same as individual treatments). Treatment was continued until control mice became moribund (typically 4-6 wk following tumor cell injection). Tumor weights were measured after sacrifice.

P values: Control siRNA vs. Combo = 0.0068; Control siRNA + Cisplatin vs. Combo = 0.0047; Control siRNA vs. CD147 siRNA = 0.0440.

g) Inhibition of metastasis by CD147 silencing

In addition to the effects on drug resistance described above, it was observed that CD147 silencing in combination with cisplatin treatment led to a significant decrease in formation of metastatic nodules compared to controls or to the effect of cisplatin alone (Fig. 8). No significant effects were observed in the weights of mice after any of the treatments, suggesting that no significant toxic effects took place.



KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that drug-resistant human ovarian carcinoma cells contain CD133-positive/ CD147-positive/ CD44-positive cancer stem-like cells in similar proportions to those found in human patient ascites-derived ovarian carcinoma cells
- Demonstration that small hyaluronan oligosaccharides sensitize drug-resistant human ovarian carcinoma cells to various chemotherapeutic agents in culture and in vivo
- Demonstration that siRNAs against the hyaluronan receptor, CD44, and the regulator of hyaluronan synthesis, CD147, sensitize drug-resistant ovarian carcinoma cells to cisplatin in culture
- Demonstration that CD147 silencing, via delivery of CD147 siRNA in liposomes, sensitizes cisplatin-resistant human ovarian carcinoma cells to cisplatin treatment in vivo
- Demonstration that CD147 silencing, via delivery of CD147 siRNA in liposomes, decreases rates of metastases of human ovarian carcinoma cells in vivo.

CONCLUSIONS

Human ovarian carcinoma cell lines contain a sub-population of drug-resistant cancer stem-like cells, which is characterized by high levels of CD133, CD147 and CD44 and which is found in similar proportions in primary human ovarian carcinoma cells. Small hyaluronan oligosaccharides sensitize drug-resistant human ovarian carcinoma cells to cisplatin and doxorubicin treatment *in vivo*. CD44 and CD147 RNAi constructs also sensitize cisplatin-resistant human ovarian carcinoma cells to cisplatin in culture. We have prepared liposomes loaded with an effective siRNA against CD147 that are designed to inhibit the previously demonstrated hyaluronan-CD44-CD147 axis more efficiently than soluble reagent. Liposome-encapsulated CD147 siRNA sensitized cisplatin-resistant ovarian carcinoma cells to cisplatin and decreased rates of metastasis in vivo. These results form the basis of promising new approaches to therapy in patients with recurrent, drug-resistant ovarian carcinoma.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Manuscript in preparation on use of small hyaluronan oligosaccharides and CD147 silencing for inhibition of ovarian carcinoma progression

INVENTIONS, PATENTS AND LICENSES

Nothing to report

REPORTABLE OUTCOMES

- Potential use of small hyaluronan oligosaccharides as adjunct treatment of chemoresistant ovarian carcinoma
- Potential use of CD147 silencing as adjunct treatment of chemoresistant ovarian carcinoma
- Potential use of CD147 silencing in treatment of metastatic ovarian carcinoma

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