CD44 expression level and isoform contributes to pancreatic cancer cell plasticity, invasiveness and response to therapy.

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ABSTRACT

Purpose: A subpopulation of pancreatic adenocarcinoma (PDAC) cells is thought to be inherently resistant to chemotherapy or to give rise to tumor cells that become resistant during treatment. Here we determined the role of CD44 expression and its isoforms as a marker and potential target for tumor cells that give rise to invasive and gemcitabine resistant tumors.

Experimental Design: RT-PCR, Western blotting and DNA sequencing was used to determine CD44 isoform and expression levels. Flow cytometry was used to sort cells on the basis of their CD44 expression level. CD44 expression was knocked down using shRNA. Tumorigenic properties were determined by clonogenic and Matrigel assays, immunohistochemistry, tumor growth in vivo using luciferase imaging and by tumor weight.

Results: We identified an invasive cell population that gives rise to gemcitabine resistant tumors. These cancer cells express a high level of CD44 standard isoform and have an EMT phenotype (CD44s/EMT). In vivo, CD44s/EMT engraft and expand rapidly and give rise to tumors that express high levels of CD44 isoforms that contain multiple exon variants. CD44 low expressing cells show continued sensitivity to gemcitabine in vivo and knockdown of CD44 in CD44s/EMT cells increases sensitivity to gemcitabine and decreases invasiveness.

Conclusion: PDAC cells expressing high levels of CD44s with a mesenchymal-like phenotype were highly invasive and developed gemcitabine resistance in vivo. Thus, initial targeting CD44 or reversing the CD44 high phenotype may improve therapeutic response.
TRANSLATIONAL RELEVANCE

Progression of PDAC following initial response to gemcitabine treatment is attributed to the expansion of a subpopulation of inherently resistant tumor cells. It is important to understand the biology of this resistant subpopulation of PDAC cells and to develop strategies for targeting these cells to prevent tumor regrowth and progression. We found that gemcitabine treatment led to expansion of cells that express CD44. Here we show the separation of a subpopulation of PDAC cells based on high expression level of CD44. This cell population expressed a high level of CD44 standard isoform, was mesenchymal-like, highly invasive and gave rise to gemcitabine resistant tumors that exhibited a CD44 isoform switch with expression of multi-exon isoform variants of CD44. Knockdown of CD44 in CD44 high cell resulted in cells that were more sensitive to gemcitabine and less invasive. These studies suggest that high CD44 expression is a marker for gemcitabine resistance in PDAC and that targeting CD44 high expressing cells may provide a therapeutic strategy for improving survival.

INTRODUCTION

Heterogeneity of cancer cells within tumors and established cancer cell lines is well known. The impact that this heterogeneity on pathobiology and therapy has not been fully investigated. Epithelial to mesenchymal transition (EMT) and acquired cancer stem cell properties are a major cause of cancer cell heterogeneity (1, 2). EMT represents an adaptive plasticity defined as loss of membrane polarity and acquired migratory properties that is characteristic of mesenchymal cells (3). EMT is thought to play an important role in early cancer cell dissemination and metastasis (3, 4).
development of metastasis is believed to require EMT, which enables cancer cells to migrate from their primary tumor sites. However, recolonizing at distant sites may require re-differentiation or a mesenchymal to epithelial transition (MET), which is believed to favor tumor engraftment and growth of cancer cells at metastatic sites (3, 5, 6). The phrase adaptive plasticity explains how cancer cells gain selective growth and survival capabilities through phenotypic changes in response to their environment. During the last few years, the role of epithelial cell plasticity in cancer development has gained more attention, however, the mechanisms that initiates and enables phenotypic changes are not fully defined.

CD44, a non-kinase transmembrane receptor that binds hyaluronan (HA), has been found to play a role in the EMT process (7, 8). CD44 is a widely expressed adhesion molecule, contributes to cell-cell and cell-matrix adhesion, cell growth, differentiation and trafficking, and is highly expressed on stem cells (2, 9). CD44 is encoded by 20 exons and undergoes extensive alternative splicing to generate CD44 standard (CD44s) and CD44 variant (CD44v) forms. CD44s consists the first five exons (exons 1-5) and last five exons (exons 16-20). The CD44 variable exons are typically numbered v1 to v10 (v1 is not encoded in human) corresponding to the genomic exons 6-15 and are alternatively spliced and incorporated between the exon 5 and exon 16, either as a single exon variant or as a combination of several variant exons (7, 10). Functional significance of CD44s and CD44v are not fully understood. Recent studies show aberrant levels and variant forms of CD44 are expressed in a variety of tumor types including PDAC. High level of CD44 is thought to be associated with poor
prognosis of cancer and CD44 v6 and v3 are reported to be related to cancer metastasis (9, 11-17). Studies show that CD44v6 expression is largely restricted to the advanced stages of tumor progression and is more prevalent in metastatic than in non-metastatic colorectal adenocarcinomas (18). Elevated expression of CD44v6 in pancreatic cancer appears to play a functional role in metastasis (13, 19). CD44 isoform switching from a variant form to a standard form during the EMT process has been reported and CD44s is correlated to an EMT phenotype (1, 20, 21). We previously showed that treating a PDAC cell line BxPC-3 with gemcitabine induces cells to undergo an EMT, to become more invasive and to show a phenotypic switch from expressing mainly low levels of CD44v to expressing high levels of CD44s (22). This EMT/CD44s phenotype reverses after multiple passages following removal of gemcitabine, suggesting that PDAC cells respond to chemotherapy by adaptive plasticity and that CD44 may play a role in this process. Isoform switching of CD44 may be a part of tumor progression and appear to play a role in EMT (23).

The expression of CD44s and its isoforms in cancer cells implies that CD44 plays a role in promoting tumorigenesis, suggesting that CD44 may be a molecular target for cancer therapy. Cancer stem cells that express CD44 are reported to be resistant to chemotherapy and to give rise to disease progression during therapy or recurrent disease following therapy (24-28). The role of CD44 in maintenance of the stem cell phenotype is not fully established. In the current study, we demonstrated that CD44 high expressing cells show predominantly a CD44s isoform, display an EMT phenotype, and are highly invasive. CD44 high CFPAC-1 cells showed stem cell properties by
forming tumor spheres *in vitro*. Mice implanted with CD44 high cells developed rapid tumor growth and metastasis. Tumors from CD44 high cells initially responded to gemcitabine but rapidly developed resistance; whereas, tumors from CD44 low cells continued to be responsive to gemcitabine for the 22 week treatment schedule. This suggests that CD44 high cells with predominant expression of CD44s have an initial implantation and growth advantage and give rise to gemcitabine resistant tumors. However, there is an uncoupling of CD44s expression and EMT phenotype during tumor expansion since these tumors show expression of high levels of CD44 variants and E-cadherin. Thus this population of CD44 high expressing cells may show further plasticity by undergoing a CD44 isoform change and a mesenchymal to epithelial (MET) switch. Chemotherapy or other environmental stresses may induce a CD44 high expressing cell population that promotes cell survival and increases invasiveness. Thus, targeting CD44 high expressing cells may be an important strategy for increasing the response to chemotherapy.

**MATERIAL and METHODS**

**Cell Culture and materials**- Human pancreatic ductal adenocarcinomas (PDAC) cell lines BxPC-3, Capan-2, MIA PaCa-2, CFPAC-1, Panc-1, AsPC-1 were from American Type Culture Collection (ATCC, Rock- Ville, MD); UK Pan-1 was established in our laboratory (29); HPNE (hTERT-immortalized human pancreas nestin expressing cell line) was obtained from Michel M. Ouellette (University of Nebraska Medical Center, Omaha, Nebraska), (30). Cells were maintained in medium as recommended and
supplemented with 10% fetal bovine serum in a 37°C, 5% CO₂ incubator. The plasmid shCD44-2 pRRL was a gift from Bob Weinberg (Addgene plasmid # 19123), which targets all isoforms of human CD44 (31). PMMP-luciferase plasmid was from Dr. Ricardo Aguiar (University of Texas Health Science Center, San Antonio, TX). The plasmids were transfected into human embryonic kidney 293T packaging cells (ATCC) using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Cells were then infected with the viral medium from the packaging cells 48 h after transfection. The CD44 knockdown cells were selected by GFP cell sorting (Flow Cytometry Core of University of Texas Health Science Center at San Antonio, TX). The luciferase-expressing clones were selected with 300 μg/ml of G418. CD44 levels were determined by Western blotting analysis and luciferase expression was determined using luciferase assay kit (Promega). Gemcitabine-HCl For Injection was manufactured by Eli Lily (Indianapolis, IN) and was dissolved in sterile saline (0.9% sodium chloride) at a concentration of 20mg/ml.

**Fluorescence-activated cell sorting and ALDHFluor Assay** - Sub-confluent cells were dissociated with trypsin and washed twice in serum-free medium. Cells were labeled with FITC (fluorescein isothiocyanate) conjugated monoclonal anti-CD44 antibody (eBioscience, San Diego, CA) at 4°C for 30 minutes. Brightly and weakly stained cells were separated as gated (Fig 1b) by fluorescence-activated cell sorting and were designated as CD44 high or CD44 low cells respectively. The aldehyde dehydrogenase (ALDH) activity was determined using an ALDEFLUOR assay kit.
(StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocol. Briefly, the cells were stained with ALDHFLUOR reagent (bodipy-aminoacetalddehyde) and/or PE (Phycoerythrin)-conjugated monoclonal CD24 antibody (eBioscience, San Diego, CA) and incubated for 30 minutes at 37°C. A specific inhibitor of ALDH1, DEBA (diemethylamino-benzaldehyde) was used to control background fluorescence. The ALDH activities and positive population and CD24 positive population of the cells were analyzed using the BD LSRII analyzer (BD Biosciences, San Jose, CA, USA)

**Reverse Transcription-PCR (RT-PCR)**- Total RNA from cultured cells or from tumors was extracted by Trizol reagent (Invitrogen, Grand Island, NY) and 1.0 μg of total RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) and 50ng cDNA was used for the following PCR reaction. PCR primers used to amplify CD44 isoforms and CD44s only were previously described (13, 32, 33). The quantitative PCR reaction was done with iQ SYBR Green Supermix (BioRad) according to the manufacturer’s instruction. The CD44s, CD44v3 and CD44v6 primers used for qRT-PCR have been described elsewhere (13, 33). The $2^{-\Delta\Delta CT}$ method was used for calculating the relative mRNA expression.

**DNA sequencing**- PCR products from PDAC cell lines or tumors using CD44 isoform primers were separated by 1.2% agarose gel. DNA fragments of CD44 isoforms were purified by Zymoclean Gel DNA Recovery kit (ZYMO Research, Irvine, CA) according to the manufacturer’s instruction. In brief, DNA fragments of our interests were excised by
clean scalpel under UV light, agarose gel slices were incubated at 55 °C for 5-10 minutes until they are completely dissolved. Melted agarose were applied to a Zymo-spin column and DNA was eluted from the column. At least 150ng of each interested DNA were sent to the DNA Core Facility in the University of Texas Health Science Center for DNA sequencing and were then confirmed using the NCBI blast alignment tool.

**Colony and sphere formation assays**- The tumorigenic properties was determined by colony formation assay and tumor sphere formation assay. For the colony formation, single cell suspension was plated at density of 50, 200, and 500 cells per 60-mm dishes and cultured for 2 weeks. The cells were washed with PBS, then fixed and stained with 0.1% crystal violet/40% methanol and colonies ≥ 1 mm were counted. For the sphere formation, 2000 cells were seeded per well in a 24-well low attachment plate (Corning, Tewksbury MA) and cultured in the serum free media containing 2% B27, 1% N2, 20 ng/ml human bFGF (Invitrogen, Life technologies) and 50ng/ml rhEGF (R&D Systems) for 7-10 days. Spheres ≥ 30 μm were counted under the light microscopy. For serial passages, spheres were disassociated to single cell by trypsin and 2000 cells were replaced per well.

**Western Blotting**- Whole cell lysates were obtained in Laemmilli’s sample buffer and Western blotting analysis was performed using standard techniques as described previously (34). Antibodies against CD44 and ZEB1 were purchased from Cell Signaling (Beverly, MA). E-cadherin and vimentin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
Orthotopic pancreatic cancer mouse model- 4-5 week-old female athymic nude mice were purchased from Harlan Laboratories, Inc. (Indianapolis, Indiana). Mice were housed and maintained in accordance with the standards of Animal Care and Use Committee of Audie Murphy Veterans Administration Hospital (San Antonio, Texas). Cells were grown to 80% confluence, trypsinized and re-suspended in Saline (0.9% sodium chloride). 2.5 x 10^5 cells/50μl were injected into the tail of the pancreas of anesthetized mice. The orthotopic xenograft experiment was repeated to determine gemcitabine response in vivo. Two weeks after implantation, mice were received either gemcitabine (80 mg/kg) or saline by intraperitoneal injection, once a week until the end of experiment. Tumor growth and metastasis were monitored weekly by bioluminescent imaging using the IVIS Imaging system (Caliper LifeSciences, Waltham, MA). Six weeks after implantation, control group mice showing stress signs were euthanized and remaining mice were continually received gemcitabine once a week until stress signs shown. During necropsy, mice were evaluated grossly for evidence of tumor invading and metastasizing into the peritoneum and liver. Liver metastases were also examined by the bioluminescent image during the necropsy.

Histopathology and immunohistochemistry- Paraffin-embedded primary tumor and liver sections were stained with hematoxylin and eosin (H&E) and immunohistochemistry was performed with monoclonal antibodies to CD44 (Cell Signaling Beverly, MA) or to Ki67 (Abcam, Cambridge, MA) in the Histology and Pathology Laboratory (University of Texas Health Science Center, San Antonio, TX). The tumor desmoplasia was evaluated using Sirius Red staining protocol from IHC.
WORLD, LLC (Woodstock, MD) and quantitated using Image J software (NIH). The primary tumor and liver metastasis were examined under the light microscopy.

**Statistical analysis**- Statistical analysis was performed using GraphPad InStat software (GraphPad Software, Inc.). The significance of differences among groups was determined by one-way ANOVA followed by Bonferroni multiple comparison test, t test or Fisher’s exact test accordingly. Statistically significance was considered as p value < 0.05.

**RESULTS**

**Expression of CD44 and EMT makers in PDAC cell lines**

A panel of eight PDAC cell lines and HPNE (30), an immortalized, undifferentiated, non-tumorigenic and nestin positive cell line was examined by Western blotting for the expression of CD44 and markers of epithelial and mesenchymal phenotypes. The cell lines that express a higher level of CD44 also expressed greater levels of the mesenchymal marker vimentin, and the EMT associated transcription factor ZEB-1 and lower levels of E-cadherin an epithelial marker (Fig. 1A). Interestingly, the epithelial-like and mesenchymal-like PDAC cell lines express not only different levels, but also distinct patterns of the CD44 standard isoform (CD44s) and variant isoforms of CD44 (CD44v). PDAC cell lines and the undifferentiated HPNE cell line that possess a mesenchymal phenotype express a high level of CD44s and low levels of single exon or low molecular
size variants of CD44 as determined by RT-PCR (Fig 1C) by DNA sequencing of RT-PCR products and by quantitative RT-PCR (Fig 1D-F). In comparison, PDAC cell lines showing an epithelial-like phenotype expressed CD44v containing combinations of multiple variant exons (Fig.1A-C). Expression of single exon variant CD44v3 and CD44v6 or low molecular size CD44 isoforms possessing limited numbers of variant exons were more common to mesenchymal-like PDAC cell lines (Fig.1B, 1C, primers 2-4). The relative mRNA levels of CD44s were evaluated by quantitative RT-PCR for the eight PDAC cell lines (Fig.1D). In agreement with the Western blot analysis (Fig. 1A) the mRNA levels of CD44s were higher in the four cell lines that exhibit a clear mesenchymal phenotype (MIAPaCa-2, Panc-1 UKPan-1 and AsPC-1) compared to those cells that showed a mixed (CFPAC-1) or an epithelial (Capan-1, Capan-2, BxPC-3) phenotype (Fig. 1D). PDAC cells with an epithelial phenotype showed a relatively lower level of CD44s protein and mRNA expression (Fig. 1A, Fig. 1D-F). Interestingly, the ratio of CD44v (containing v3 and/or v6) to CD44s mRNA expression was higher in cells with an epithelial phenotype and the reverse ratio (higher ratio of CD44s to CD44v) was seen in cells with a mesenchymal phenotype. Correspondingly, ESRP1 was mainly expressed in the epithelial-like phenotype PDAC cell lines (Fig. 1C). Thus, PDAC cells with a mesenchymal phenotype express greater total levels of CD44 with a predominance of CD44s isoform and CD44v expression was restricted to single variant exon or lesser amounts of combination variant isoforms (Fig. 1).

**Isolation of PDAC cells based on expression level of CD44**
The cell lines CFPAC-1 and AsPC-1 showed low levels of expression of both E-cadherin and vimentin and intermediate levels of CD44s with expression of higher molecular weight CD44v isoforms (Fig. 1A), suggesting that these cell lines may represent an intermediate phenotype or contain a mixture of epithelial and mesenchymal phenotypes. To further investigate these possibilities, CD44 high and CD44 low expressing populations of CFPAC-1 and AsPC-1 were separated by flow cytometry (Fig. 2A). The CD44 high and CD44 low populations displayed different morphologies and expressed differential EMT markers; CD44 high cells were more fibroblast like in appearance (Fig. 2B) and expressed high levels of vimentin and low levels of E-cadherin; whereas, CD44 low cells exhibit a more cobblestone like appearance and expressed low levels of vimentin and high levels of E-cadherin (Fig. 2C). CD44 expression levels were confirmed by Western blotting, which showed that CD44 high cells expressed higher levels of CD44 with CD44s as the predominant isoform (Fig. 2C). In comparison with CD44 high cells, CD44 low cells expressed lower level of CD44s (Fig. 2C).

**Oncogenic properties of CD44 high and CD44 low cells**

The oncogenic properties of cells from CD44 high and CD44 low populations were compared by determining their clonogenic, invasive and tumor sphere forming capacities. CD44 high cells isolated from CFPAC-1 and AsPC3 parental cells were more clonogenic and more invasive than the CD44 low cells as determined by colony forming assay and Matrigel invasion assays (Fig. 3A and 3B). CD44 is recognized as a cancer stem cell marker and is widely used to enrich cancer stem cells in vitro in variety
of types of cancers (2, 9). A tumor sphere assay was used to test whether CD44 expression and mesenchymal phenotype were correlated with cancer cell stemness, CD44 high expressing CFPAC-1 cells continuously formed spheres through four subsequent serial passages (Fig. 3C) and cells from these spheres express CD44 and vimentin as determined by immunofluorescent staining (Fig. 3D); whereas, CD44 low CFPAC-1 expressing cells show a decreased capacity to form spheres after serial passages (Fig. 3C). The current understanding of markers for pancreatic cancer stem cells include CD44, CD24 and ALDH1 as reviewed by Vaz et al. and Medema (35, 36). To further assess stem cell properties, CFPAC1/CD44 high and CD44 low cells were compared using FACS analyses to detect CD24 and ALDH positive cells. CD24 was detected using an antibody and ALHD bright cells were detected using an ALDHEFLOUR assay. CD24+ALHD bright or double positive cells were greater in CFPAC-1-CD44 high cells (4.3%) compared to CFPAC1-CD44 low cells (1.2%), (data not shown),

The CD44 high population in AsPC-1 cells showed a clear mesenchymal phenotype and lack of E-cadherin; however, the CD44 low population remained rather intermediate with continued higher expression of CD44s than CD44v and intermediate levels of E-cadherin and vimentin (Fig. 2C). The lack of sphere forming activity by serial passages in the AsPC-1 high cells (data not shown) suggests that expression of CD44s may play a role but is not in itself sufficient for tumor sphere formation and stemness.

CD44 high/mesenchymal cells are more tumorigenic and are less responsive to gemcitabine in vivo.
To compare tumor progression \textit{in vivo}, we orthotopically implanted either CD44 high or CD44 low populations of CFPAC-1 cells that express firefly luciferase into the pancreas of athymic nude mice. Bioluminescent imaging was performed weekly to monitor tumor progression. Six weeks after tumor cell implantation, mice were euthanized and tumor growth and metastasis were evaluated by tissue examination, tumor weight and histopathology. The quantification data of the bioluminescent imaging for the 12 mice in each of the CD44 high and CD44 low groups is shown in Fig. 4A (right panel) and the representative bioluminescent images up to six weeks for a representative mouse in the CD44 high and CD44 low groups is shown in Fig. 4A (left panel). Tumor growth and metastatic spread were more rapid in mice implanted with CD44 high expressing cells. By week 6, mice implanted with CD44 high cells developed larger tumors and showed greater local invasion (Fig. 4A and 4B) and liver metastasis (Fig. 4C.). Bioluminescent imaging of resected livers showed that 9 of 12 mice had metastatic foci in CD44 high group while only 2 of 12 mice from the CD44 low group showed positive bioluminescent imaging (Fig 4C.). Histologically, the metastatic liver foci were within the lymphatic tissue surrounding the liver or showed bile duct involvement with surrounding hepatic invasion (Fig. 4D). Macroscopic observation also showed evidence of peritoneal metastases and tumor attachment to the intestines and by histology of several mesenteric lymph nodes in some mice.

We next compared the response to gemcitabine for orthotopically implanted CD44 high and CD44 low cells \textit{in vivo}. Mice were treated with vehicle (saline) alone or gemcitabine once a week beginning two weeks after implantation. Tumors in the placebo group grew and progressed more rapidly (Fig. 4E and 4F). These control
groups of mice were euthanized at six weeks after implantation of tumor cells due to tumor burden and signs of physical stress in the group implanted with CD44 high cells. The gemcitabine treated group of mice continued to receive weekly treatment. In the first six weeks both CD44 high and low groups of mice responded to gemcitabine and tumor growth was inhibited or regressed (Fig. 4E). However, in mice treated weekly with gemcitabine, the CD44 high group mice displayed signs of tumor growth by week twelve and tumors continued to grow exponentially until the end of the study at week twenty-two at which time these mice formed ascites. Interestingly, tumors remained small to non-detectable in the gemcitabine treated CD44 low group of mice through twenty-two weeks (Fig. 4E and 4F).

To determine whether CD44 expression had an effect on tumor cell proliferation, immunohistochemistry staining for Ki67 was performed. The results showed that tumor cell proliferation was significantly lower in CD44 low tumors when treated with gemcitabine; whereas, tumors that grew from the CD44 high group following gemcitabine treatment had proliferation rate similar to the saline treated group (Fig. 5A). To further investigate the role of CD44 in tumor recurrence after gemcitabine treatment, tumor tissues were analyzed by immunohistochemistry. CD44 low tumors showed less intensity of staining in saline treated controls compared to CD44 high tumors (Fig. 5B). However in tumors that grew following gemcitabine treatment, almost all cells showed intense CD44 staining (Fig. 5B). Western blot analysis of tissue from tumors derived from CD44 high cells showed a switch from expressing predominantly CD44s to expressing high levels of CD44 variant isoforms and these tumors also expressed the epithelial marker, E-cadherin (Fig. 5C). The expression of epithelial variant form of
CD44 v8-10 and ESRP1 in CD44 high tumors was confirmed by RT-PCR analysis (Fig. 5D). This suggests that these rapidly growing tumors undergo a CD44 isoform switch towards expressing high levels of CD44 variant isoforms and a phenotypic change to a more epithelial-like tumor cell. An epithelial phenotype may be required for tumor cells rapid growth. Thus, the greater level of CD44 expression is associated with an increase in tumor growth; however, the specific roles of the CD44 isoforms in engraftment and growth need to be further determined.

Desmoplasia commonly develops in pancreatic cancer and is thought to contribute to chemoresistance. It is possible that CD44 may play a role in this process. To investigate this possibility tumors derived from CD44 high and CD44 low cells were stained with Sirius Red to detect collagen. No differences of the collagen staining were found between saline treated CD44 high and CD44 low tumors, however, gemcitabine treated tumors displayed large area of fibrosis-like pathology and collagen staining (Fig. 5E). This suggests that CD44 expression level does not play a role in the development of desmoplasia although gemcitabine treatment induces fibrosis perhaps as a result of cell death in tumor areas.

Knockdown CD44 inhibits tumorigenecity and increases sensitivity to gemcitabine

To further look at the role of CD44 in mediating tumorigenic properties, CD44 was knocked down in CD44 high cells with shRNA (Fig. 6F). These studies show that knocking down CD44 significantly reduced the colony forming property in CFPAC-1 CD44 high cells (Fig. 6A) and enhanced their response to gemcitabine similar to the
level of CD44 low cells (Fig. 6B). To test our hypothesis that CD44 high cells may contain a sub population of cells that possess stem-like properties and are originally resistant to gemcitabine, cells were exposed to high dose of gemcitabine (50 ng/ml) for 2 weeks. We found that colonies only grew appreciably from the CD44 high cells and that knocking down CD44 from CD44 high cells abolished the colony forming activity in the presence of high dose of gemcitabine (Fig. 6C). The invasive and sphere forming properties of these cells were tested by *in vitro* Matrigel invasion and sphere forming assays. Knocking down CD44 significantly inhibited the invasiveness in both CFPAC-1 and AsPC-1 CD44 high cells (Fig. 6D) and prevented the sphere forming activity of CFPAC-1 (Fig. 6E). However, knocking down CD44 itself was not sufficient to completely reverse the EMT phenotype of these cells, as the mesenchymal marker, vimentin expression was only slightly reduced and the epithelial marker, E-cadherin was not re-expressed in the CD44 knock down clones (Fig. 6F).

**DISCUSSION**

In this study, we show that the phenotypic and tumorigenic properties of PDAC cells are related to the level of expression and type of CD44 isoform. An initial observation from analyses of a panel of PDAC cell lines was that cells showing an EMT phenotype express higher levels of CD44 with a predominance of the standard isoform. To further look at heterogeneity of CD44 expression we sorted out CD44 high and low expressing cells from two separate PDAC cell lines, CFPAC-1 and AsPC-1. CD44 high cells express predominantly a CD44s isoform, display an EMT phenotype and are more invasive *in vitro*. In comparison, CD44 low cells express relative low level of CD44s and
low levels of larger size CD44 variants, are more epithelial like and are less invasive.

Our finding that PDAC cells with an EMT phenotype express mainly CD44s isoform is in agreement with a study by Brown et al. (20) showing that induction of EMT in HMLE cells was associated with a switch from expressing mainly CD44v isoforms to expressing predominantly CD44s. Our study is also in agreement with that of Mani et al. (1) showing that EMT was associated with higher level in expression of CD44. Interestingly, the study by Mani et al (1) shows that induction of EMT generates cells that have properties of stem cells. Here we also show using the CFPAC-1 model that CD44 high but not CD44 low expressing cells were able to grow in serial passages as tumor spheres. However, only a low percentage of cells (4.3%) from the CD44 high group show co-expression of other stem cell markers including CD24 and ALDH. To this point, knockdown of CD44 in CD44 high cells was sufficient to prevent growth of these cells as tumor spheres. However, CD44 high cells from ASPC-1 cells were not able to form tumor spheres, These results suggests that CD44 may play a role but is not sufficient by itself for a stem cell phenotype.

In addition to expressing high levels of CD44s, PDAC cells with an EMT phenotype express small molecular size exon variant isoforms of CD44v3 or CD44v6. This phenomenon appears unique for cells with a mesenchymal phenotype since cells with an epithelial phenotype express CD44v isoforms that contain predominantly multiple variant exons. A number of studies suggest that expression of CD44 variants including CD44v3 and v6 are associated with metastatic lesions (16, 17, 19, 32). The functional differences of CD44 molecules that possess single or multiple variant exons is not clear and needs further investigation. The high level of expression in CD44 high
cells suggests that CD44s isoform plays an important role in tumor cell plasticity contributing to increased invasiveness. However, this study does not rule out the possibility that minor variant isoforms contribute significantly to these properties.

The functional significance of the CD44 in CFPAC-1 cells was further assessed using an orthotopic mouse model. Tumors developed from CD44s/EMT cells grew and metastasized more rapidly than that CD44 low expressing cells. However, once tumors grew in vivo, the tumor cells underwent a phenotypic change. Western blot analysis of tumor specimens from the CD44 high group express higher level of CD44 variant isoforms (v8-10) than the standard form and showed an increase in expression of the epithelial marker, E-cadherin, which is expressed at a low level or not present in the original CD44 high cells. This phenomenon suggests that cells from CD44 high-induced tumors undergo MET and that a CD44 isoform switch may be required for this to occur. Since these tumors appear to be resistant to gemcitabine this suggests an uncoupling of CD44s expression and gemcitabine resistance. However these tumors continue to express high levels of CD44 but as variant isoforms. Thus the level of CD44 may be important in maintaining resistance. Desmoplasia an important component of PDAC (37, 38) did not appear to differ appreciably in tumors grown from CD44 high or CD44 low cells but was dramatically increased by gemcitabine treatment.

EMT and MET are dynamic processes in tumor progression, CD44 may play a role in regulating these processes. EMT is involved in invasion, tumor metastasis and stem cell properties; whereas, conversion back to an epithelial phenotype may favor
tumor growth (39). Tumor cells once engrafted, may need CD44v to convert EMT-like cells to epithelial-like cells (MET); otherwise, CD44s may be required to facilitate cells to undergo EMT, which increases local invasion and for metastasizing to the distal regions. Thus, the total level of CD44 as well as the isoform may be important in tumor progression. EMT appears in this study to be closely associated with loss of ESRP1 and a decrease in expression of CD44 multi-exon variants. ESRP1 has been reported to play a role in the isoform switch of CD44 (6, 20, 21, 40). Expressing ESRP1 in PDAC cells was shown to decrease proliferation, invasion and metastases (41). We showed here that ESRP1 expression was associated with cells that are epithelial-like and express lower levels of total CD44, of which most express CD44v molecules that possess multiple variant exons. ESRP1 expression was decreased or lost in the mesenchymal-like cells that mainly express CD44s. We also demonstrated that the tumors derived from CD44 high cells underwent CD44 isoform switch and gained the ESRP1 expression when grown as orthotopic implants. This observation is consistent with the premise that the molecular basis of CD44 isoform switch is regulated by ESRP1. Therefore, regulation of ESRP1 expression likely controls, at least in part, the type of CD44 isoform expressed and down regulation of ESRP1 likely facilitates expression of mainly the CD44s form.

CD44 is highly expressed in variety of tumors, tumor initiating cells and recurrent tumors, suggesting that it could be a therapeutic target for cancer. Continued progression and recurrence of cancers following chemotherapy is thought to be due to inherent or acquired resistance to the therapeutic agent. In this study, we treated the
CD44 high and CD44 low tumor bearing mice with gemcitabine. Although both CD44 high and low tumors responded to gemcitabine at the beginning, CD44 high tumors gradually develop resistance after 12 weeks of treatment. CD44 low tumors continued to be sensitive to gemcitabine through 22 weeks suggesting that CD44 expression level is related to chemoresistance and disease progression. We have also demonstrated that knocking down CD44 in CD44 high cells attenuated colony forming activity and invasiveness and increased the sensitivity to gemcitabine. A subpopulation of CD44 high cells may possess stem cell properties that are inherently resistant to gemcitabine. Preclinical studies show promising results by targeting CD44 using CD44 antibodies (24, 27). These studies demonstrated that relapsing tumors are sensitive to anti-CD44 treatment; therefore, targeting CD44 could have a therapeutic benefit. Our study suggests the possibility that targeting the CD44 high expressing tumor cells may increase and prolong response to therapy and perhaps prevent the outgrowth of a gemcitabine resistant population. In our studies CD44 high cells formed tumors that gradually developed resistance to gemcitabine. It is of interest that the gemcitabine resistant tumors continued to express high total levels of CD44 composed of CD44s but also showed a dramatic increase in the levels of CD44v that possess multiple variant CD44 exons.

In summary, PDAC cell lines show heterogeneity in regards to CD44 isoform and level of expression. _In vitro_, CD44 high expressing cells show predominantly a CD44s isoform, display an EMT phenotype, and _in vivo_ are highly invasive and form tumors and metastases more rapidly. Mice implanted with CD44 high cells show initial
response to gemcitabine but gradually show rapid expansion and loss of response to
gemcitabine. Mice implanted with CD44 low cells showed a continued response to
gemcitabine over a 22-week period. This suggests that CD44 low expressing phenotype
will have a prolonged response to chemotherapy. Molecular analyses show that CD44
high tumors continue to express high levels of CD44 but with a switch to expressing
increased levels of CD44v isoforms. Knockdown of CD44 in CD44 high cells causes
these cells to be less invasive and more sensitive to gemcitabine. This study suggests
that targeting CD44 in PDAC that show a CD44 high expression level may benefit
therapy and improve overall response.

FIGURE LEGENDS

Figure 1. Association of CD44 isoforms with PDAC cell phenotypes.
A, Western blot analysis of a panel of PDAC cell lines for the expression of CD44 and
EMT markers, ZEB1, E-cadherin and vimentin. β-actin was used for the protein loading
control. B, The diagrams of PCR primer design for CD44s and CD44v mRNA
expression. C, RT-PCR analysis of CD44 isoforms and ESRP1 expressed in a panel of
PDAC cell lines. CD44v isoforms labeled as CD44v (seq) were confirmed by DNA
sequencing. D, E and F, Quantitative RT-PCR with Syber green supermix for the
expression of CD44s, CD44v3 and CD44v6 containing isoforms. Data represent fold
change (mean ± SD) calculated with 2^ΔΔCt method of triplicate experiment.

Figure 2. Isolation of CD44 high and low cells
A, Cells were labeled with FITC conjugated monoclonal anti-CD44 antibody and CD44 high and CD44 low cells were separated as gated by Fluorescence-Activated Cell Sorting. B, Light microscope photos represent the morphology of CD44 high and CD44 low cells after cell sorting. C, Expression of CD44s, CD44v and EMT marker proteins from cell lines after cell sorting was analyzed by Western blotting. p, parental cells; Hi, CD44 high cells; Lo, CD44 low cells.

Figure 3. In vitro tumorigenic and invasive properties of CD44 high and CD44 low cells

A, Clonogenic assays were performed as described previously (34). Briefly, cells were seeded at 50, 250 and 500 cells per 6 cm² dishes and cultured for 2 weeks. The cells were stained with crystal violet and colonies ≥ 1mm were counted. The data were presented as mean ± SD of experiments performed in triplicates. B, 3 x 10⁴/well cells were seeded in 24 well Matrigel invasion chambers for 24 h. Invaded cells were counted under microscope and the data represent mean ± SD from triplicate experiments. Upper panel, represent photos of invasion assays, lower panel, quantification data of invasion assays. C, Tumor sphere forming assay of CFPAC-1 cells. 2000 cells were plated in a low-attach 24 well plate and cultured in the medium described in materials and methods for 7-10 days and spheres ≥ 30 μm were counted. P1~P4 are the passage numbers, every passage lasted for 7-10 days. D, Confocal microscopy images of immunofluorescence staining of CD44 and vimentin in tumor spheres of CD44 high population cells.
Figure 4. **CD44 mediate tumor growth, invasion and chemoresistance in vivo.**

2.5x 10^5 CF/CD44-Hi and CF/CD44-Lo cells were implanted into the tail of pancreas of nude mice. Primary tumor and metastases were examined grossly and pathologically at week 6 after implantation. A, Represent bioluminescent images of tumors from CD44 high and CD44 low cells monitored weekly by bioluminescent imaging ((left). Tumor growth curve plotted from bioluminescent data of CF/CD44-Hi and CF/CD44-Lo groups. The data represent means (n=12) of each group, Bars, SE. *, p<0.05; **, p< 0.01, CF/CD44-Lo vs. CF/CD44-Hi group. B, Tumors collected at the end of experiment from CF/CD44-Hi and CF/CD44-Lo groups (left) and a quantification data of tumor weight of 12 mice each group (right). C, Bioluminescent images of liver metastases taken at the end of experiment from CF/CD44-Hi and CF/CD44-Lo mice. D, Representative photos of H&E stained liver sections. The arrows indicate the metastatic foci. E, Tumor growth curve plotted from bioluminescent data of CF/CD44-Hi and CF/CD44-Lo groups treated with saline for 6 weeks or gemcitabine for 20 weeks (n=6 each group). **, p<0.01; ***, p< 0.001, compared to CF/CD44-Hi group. F, A quantification data of tumor weight (mean ±SE, n=6 each group).

Figure 5. A, Representative pictures of immunostaining of tumor sections with monoclonal antibody to Ki67 (left) and Quantification data of Ki67 stained tumor sections analyzed by ImmunoRatio online software (right). The data was presented as the mean +/- the SD. B, Representative pictures of immunohistochemistry of tumor sections with CD44 monoclonal antibody. C, Tumor tissue lysates from saline treated
group and collected 6 weeks after implantation. 50 μg of the tissue lysates were analyzed by Western blot with indicated antibodies. β-actin was used for the protein loading control. Last two lanes labeled, as “Cell” are lysates from CF/CD44-high and CD44-low cells used for the implantation. D, RT-PCR analysis of tumor tissues treated with saline using the primer 1 as indicated in figure 1b and CD44 isoforms were separated in 1.2 % agarose gel. CD44v isoforms labeled as CD44 v8-10 (seq) were confirmed by DNA sequencing. E, Representative photos of Sirius Red staining of tumor sections (Left panel) and a quantification data (right panel) analyzed using Image J program (NIH).

**Figure 6.** A and B, 500 cells were plated per 6 cm² dishes. The next day cells were treated with one dose of 0, 2.0 and 5.0 ng/ml gemcitabine and cultured for 2 weeks. The cells were stained with crystal violet and colonies ≥ 1mm were counted. A, The data were presented as mean ± SD of experiments performed in triplicates. B, The data were calculated as % of colony number relative to control (gemcitabine 0 dose). C, 5000 cells were plated per 6 cm² dishes. The next day cells were treated with gemcitabine (50 ng/ml) and gemcitabine was replenished every week for 2 weeks. Colonies ≥ 1mm were counted. The data represent the mean +/- SD for triplicate experiments. D, 3 x 10⁴/well cells were seeded in 24 well Matrigel invasion chambers for 24 h. Invaded cells were counted under microscope and the data represent mean ± SD from triplicate experiments. *, p<0.05; **, p<0.01; and ***, p< 0.001 compared to CD44-Hi cells. E, Tumor sphere forming assay of CF/CD44-Hi, Lo and shCD44 cells. 2000 cells were plated in a low-attach 24 well plate and cultured in the medium described in materials
and methods and passage every 7-10 days and 2000 cell were re-plated. The 
passages-4 spheres (≥ 30 μm) were counted. F, CD44 levels and EMT marker proteins 
in CD44 knocking down CF/CD44-Hi and As/CD44-Hi cells analyzed by Western 
blotting. cl.1 and cl.2 represents shCD44 clone 1 and clone 2 respectively.

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Fig. 4

A

Week 1  Week 2  Week 3  Week 4  Week 5  Week 6

p/sec/cm²/sr

B

No.  1  2  3  4  5  6  7  8  9  10  11  12

CD44-Hi

CD44-Lo

C

No.  1  2  3  4  5  6  7  8  9  10  11  12

CD44-Hi

CD44-Lo

Luciferase image of positive liver:
CD44-Hi, 9 of 12; CD44-Lo, 2 of 12, P=0.0123, Fisher’s Exact Test.

D

Examples of Live metastasis of CD44-Hi tumor (H&E)

E

F

p<0.01

p<0.001
CD44 expression level and isoform contributes to pancreatic cancer cell plasticity, invasiveness and response to therapy

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