

## Identification of Pancreatic Cancer Stem Cells

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### Abstract

**Emerging evidence has suggested that the capability of a tumor to grow and propagate is dependent on a small subset of cells within a tumor, termed cancer stem cells. Although data have been provided to support this theory in human blood, brain, and breast cancers, the identity of pancreatic cancer stem cells has not been determined. Using a xenograft model in which primary human pancreatic adenocarcinomas were grown in immunocompromised mice, we identified a highly tumorigenic subpopulation of pancreatic cancer cells expressing the cell surface markers CD44, CD24, and epithelial-specific antigen (ESA). Pancreatic cancer cells with the CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> phenotype (0.2–0.8% of pancreatic cancer cells) had a 100-fold increased tumorigenic potential compared with nontumorigenic cancer cells, with 50% of animals injected with as few as 100 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells forming tumors that were histologically indistinguishable from the human tumors from which they originated. The enhanced ability of CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells to form tumors was confirmed in an orthotopic pancreatic tail injection model. The CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells showed the stem cell properties of self-renewal, the ability to produce differentiated progeny, and increased expression of the developmental signaling molecule sonic hedgehog. Identification of pancreatic cancer stem cells and further elucidation of the signaling pathways that regulate their growth and survival may provide novel therapeutic approaches to treat pancreatic cancer, which is notoriously resistant to standard chemotherapy and radiation.** [Cancer Res 2007;67(3):1030–7]

### Introduction

Pancreatic adenocarcinoma is a highly lethal disease, which is usually diagnosed in an advanced state for which there are little or no effective therapies. It has the worst prognosis of any major malignancy (3% 5-year survival) and is the fourth most common cause of cancer death yearly in the United States, with an annual incidence rate approximating the annual death rate of 31,000 people (1). Despite advances in surgical and medical therapy, little effect has been made on the mortality rate of this disease. One of the major hallmarks of pancreatic cancer is its extensive local tumor invasion and early systemic dissemination. The molecular basis for these characteristics of pancreatic cancer is incompletely understood.

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Attempts to better understand the molecular characteristics of pancreatic cancer have focused on studying gene and protein expression profiles of samples of pancreatic cancer. However, these types of studies have not taken into account the heterogeneity of cancer cells within a particular tumor. Emerging evidence has shown that the capacity of a tumor to grow and propagate is dependent on a small subset of cells. This concept was originally based on the observation that when cancer cells of many different types were assayed for their proliferative potential in various *in vitro* or *in vivo* assays, only a minority of cells showed extensive proliferation (2). This observation caused the idea that malignant tumors are composed of a small subset of distinct cancer stem cells (typically <5% of total tumor cells based on cell surface marker expression), which have great proliferative potential, as well as more differentiated cancer cells, which have very limited proliferative potential.

The existence of cancer stem cells was first proven in the context of acute myelogenous leukemia (3, 4) and subsequently verified in breast (5) and brain tumors (6–8). In 2003, Al-Hajj et al. (5) reported that a phenotypically distinct and relatively rare population of CD44<sup>+</sup>CD24<sup>-</sup>epithelial-specific antigen (ESA)<sup>+</sup> tumor-initiating cells (TIC) was responsible for the propagation of human metastatic breast cancer specimens in immunodeficient nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice. Further evidence in support of a role for stem cells in solid tumors has also come recently from studies of brain tumors (6–8). Singh et al. (6) showed that the neural stem cell antigen CD133 was expressed in brain-derived TICs from pediatric medulloblastomas and astrocytomas. The CD133<sup>+</sup> subpopulations from these tumors could initiate clonally derived neurospheres *in vitro* that showed self-renewal, differentiation, and proliferative characteristics similar to normal brain stem cells (6–8). Furthermore, transplantation of CD133<sup>+</sup>, but not CD133<sup>-</sup>, cells into NOD/SCID mice was sufficient to induce growth of tumors *in vivo* (8). These cells have been termed cancer stem cells because, like normal stem cells, they can both self-renew and produce differentiated progeny. Recently, the identification of cancer stem cells has also been reported in human prostate and ovarian cancers (9, 10).

A practical consequence of this tumor cell heterogeneity is that strategies for inducing cell death must address the unique survival mechanisms of each different cell type within the malignant population. Most traditional cancer treatments have been developed and assayed based on their ability to kill most of the tumor population (i.e., log kill assays). However, these treatments can easily miss the cancer stem cells, which have been shown in several tumor types to be more resistant to standard chemotherapeutic agents (11–13). This model explains why standard chemotherapy may result in tumor shrinkage, but most tumors recur, likely because the cancer stem cell survives and regenerates the tumor. Treatments specifically targeting the cancer stem cell population may be more effective in resulting in solid tumor cure.

To determine if a cancer stem population could be identified in human pancreatic adenocarcinoma, we used a xenograft model in which primary human pancreatic adenocarcinomas were implanted in immunocompromised mice to assess if specific cell surface markers could be used to identify a subpopulation of pancreatic cancer cells with enhanced tumorigenic potential. We identified a CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> subpopulation as putative pancreatic cancer stem cells. The CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells showed the stem cell properties of self-renewal, the ability to produce differentiated progeny, and increased expression of the developmental signaling molecule sonic hedgehog (SHH). Identification of pancreatic cancer stem cells and further elucidation of the signaling pathways that regulate their growth and survival may provide novel therapeutic approaches to treat pancreatic cancer.

## Materials and Methods

**Primary tumor specimen implantation.** Samples of human pancreatic adenocarcinomas were obtained within 30 min following surgical resection according to Institutional Review Board–approved guidelines. Tumors were suspended in sterile RPMI 1640 and mechanically dissociated using scissors and then minced with a sterile scalpel blade over ice to yield 2 × 2-mm pieces. The tumor pieces were washed with serum-free PBS before implantation. Eight-week-old male NOD/SCID mice were anesthetized using an i.p. injection of 100 mg/kg ketamine and 5 mg/kg xylazine. A 5-mm incision was then made in the skin overlying the midabdomen, and three pieces of tumor were implanted s.c. The skin incision was closed with absorbable suture. The mice were monitored weekly for tumor growth for 16 weeks.

**Preparation of single-cell suspensions of tumor cells.** Before digestion with collagenase, xenograft tumors or primary human tumors were cut up into small pieces with scissors and then minced completely using sterile scalpel blades. To obtain single-cell suspensions, the resultant minced tumor pieces were mixed with ultrapure collagenase IV (Worthington Biochemicals, Freehold, NJ) in medium 199 (200 units of collagenase per mL) and allowed to incubate at 37°C for 2.5 to 3 h for enzymatic dissociation. The specimens were further mechanically dissociated every 15 to 20 min by pipetting with a 10-mL pipette. At the end of the incubation, cells were filtered through a 40- $\mu$ m nylon mesh and washed with HBSS/20% fetal bovine serum (FBS) and then washed twice with HBSS.

**Flow cytometry.** Dissociated cells were counted and transferred to a 5-mL tube, washed twice with HBSS containing 2% heat-inactivated FBS, and resuspended in HBSS with 2% FBS at concentration of 10<sup>6</sup> per 100  $\mu$ L. Sandoglobin solution (1 mg/mL) was then added to the sample at a dilution of 1:20 and the sample was incubated on ice for 20 min. The sample was then washed twice with HBSS/2% FBS and resuspended in HBSS/2% FBS. Antibodies were added and incubated for 20 min on ice, and the sample was washed twice with HBSS/2% FBS. When needed, a secondary antibody was added by resuspending the cells in HBSS/2%FBS followed by a 20-min incubation. After another washing, cells were resuspended in HBSS/2% FBS containing 4',6-diamidino-2-phenylindole (DAPI; 1  $\mu$ g/mL final concentration). The antibodies used were anti-CD44 allophycocyanin, anti-CD24 (phycoerythrin), and anti-H2K (PharMingen, Franklin Lakes, NJ) as well as anti-ESA-FITC (Biomedica, Foster City, CA), each at a dilution of 1:40. In all experiments using human xenograft tissue, infiltrating mouse cells were eliminated by discarding H2K (mouse histocompatibility class I) cells during flow cytometry. Dead cells were eliminated by using the viability dye DAPI. Flow cytometry was done using a FACSAria (BD Immunocytometry Systems, Franklin Lakes, NJ). Side scatter and forward scatter profiles were used to eliminate cell doublets. Cells were routinely sorted twice, and the cells were reanalyzed for purity, which typically was >97%.

**Sorted cell implantation into NOD/SCID mice.** Sorted cells were washed with serum-free HBSS after flow cytometry and suspended in serum free-RPMI/Matrigel mixture (1:1 volume) followed by injection s.c. into the right and left midabdominal area using a 23-gauge needle. In separate experiments, mice were anesthetized with an i.p. injection of 100 mg/kg

ketamine and 5 mg/kg xylazine, a median laparotomy was done, and either 1,000 or 5,000 sorted cells (CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> versus CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup>) were resuspended in PBS in a volume of 100  $\mu$ L were injected into the tail of the pancreas using a 30-gauge needle ( $n = 3$  animals per group). Animals underwent autopsy at 28 days after cell implantation and tumor growth was accessed. Tissues were fixed in formaldehyde and examined histologically.

**Immunohistochemistry.** Tissue samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Formalin-fixed, paraffin-embedded sections were cut 4- $\mu$ m thick, mounted on poly-L-lysine-coated slides (Sigma, St. Louis, MO), and dried overnight at 37°C. Sections were then dewaxed in xylene, rehydrated according to standard histopathologic procedures, and stained with H&E. Immunodetection was done using the ChemMate Detection kit (peroxidase/3,3'-diaminobenzidine, rabbit/mouse, DakoCytomation, Carpinteria, CA). Detection of expression levels of S100P and stratifin in sections of a primary tumor and the subsequent tumor derived from CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> sorted cells was done as we described previously (14, 15).

**Cell cycle analysis.** For cell cycle analysis by flow cytometry, cells were fixed with 70% ethanol overnight at 4°C. Cell pellets were then suspended in 300  $\mu$ L PBS containing 10  $\mu$ g/mL propidium iodide (Calbiochem, San Diego, CA) and 100  $\mu$ g/mL RNase to stain nuclear DNA for 30 min at room temperature. DNA content was analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the different phases of the cell cycle was analyzed from DNA histograms using BD CellQuest software (Becton Dickinson). Cell cycle analysis was done on CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> and CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells from three separate pancreatic cancer xenografts.

**Real-time reverse transcription-PCR.** To assess expression levels of SHH, three samples of normal pancreas and three separate samples of pancreatic cancer xenografts were used. Samples of normal human pancreas used as controls were obtained from organ donors provided by the Michigan Transplantation Society and processed similar to samples of pancreatic adenocarcinoma. Single-cell suspensions of the samples were prepared, and ESA<sup>+</sup> normal pancreatic cells, bulk pancreatic cancer cells, and sorted CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> and CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells were used. For real-time reverse transcription-PCR (RT-PCR) analysis, cDNA was first synthesized using equivalent amounts of total RNA (0.5–1  $\mu$ g) with random primers in a 20  $\mu$ L reverse transcriptase reaction mixture (Promega, Madison, WI). Real-time quantitative RT-PCR (Taqman) primers were designed and purchased from Applied Biosystems (Foster City, CA) as Assay-on-Demand Gene Expression Products. Real-time RT-PCRs were done following the supplier's directions. Twenty microliter of PCR mixture contained 10  $\mu$ L of 2 $\times$  Taqman Universal PCR Master Mix, 1  $\mu$ L of 20 $\times$  working stock of expression assay mix, and 50 ng RNA converted DNA. Real-time PCRs were done in a ABI Prism 7900HT Sequencing Detection System (Applied Biosystems). The reaction for each sample was done in triplicate. Fluorescence of the PCR products was detected by same apparatus. The number of cycles that it takes the for amplification plot to reach the threshold limit, the  $C_t$  value, was used for quantification. Ribosomal protein S6 was used for normalization.

**Statistical analysis.** Data are expressed as the mean + SE. Statistically significant differences were determined by Student's  $t$  test and  $\chi^2$  analysis, where appropriate, and defined as  $P < 0.05$ .

## Results

**Establishment of xenografts from human pancreatic tumors.** A total number of 10 human pancreatic adenocarcinoma xenografts were established, 8 from primary tumors and 2 from metastatic lesions (Table 1). Xenografts are critical for these types of studies because of the difficulty in routinely obtaining primary tumors from the pancreas. The validity of using xenografts is supported by previous work showing that pancreatic cancer xenografts retain many of the features of the primary tumor on multiple passaging (16). The initial engraftment rate with implantation of three, 2 × 2-mm-minced pieces of a pancreatic

**Table 1.** Engraftment of human pancreatic cancers into NOD/SCID mice

Tumor no.	Origin	Mice tumor formation	Passage in mice	Diagnosis
1	Primary tumor	Yes	Yes	Adenocarcinoma
2	Primary tumor	Yes	Yes	Adenocarcinoma
3	Metastasis	Yes	Yes	Adenocarcinoma
4	Primary tumor	Yes	Yes	Adenocarcinoma
5	Primary tumor	Yes	Yes	Adenocarcinoma
6	Metastasis	Yes	Yes	Adenocarcinoma
7	Primary tumor	Yes	Yes	Adenocarcinoma
8	Primary tumor	Yes	Yes	Adenocarcinoma
9	Primary tumor	Yes	Yes	Adenocarcinoma
10	Primary tumor	Yes	Yes	Adenocarcinoma

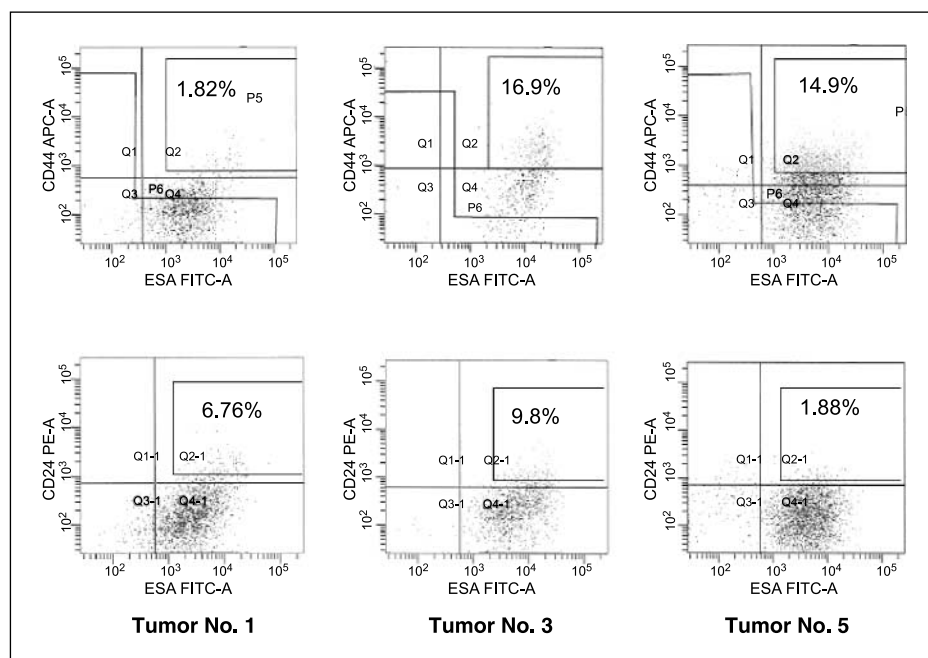
NOTE: Mice were injected with unsorted primary pancreatic adenocarcinomas minced into 2-mm pieces. Cells from all 10 xenografts and one primary tumor were isolated by flow cytometry as described in Fig. 1. All of the tumors were primary pancreatic tumors, except tumors 3 and 6, which were metastatic tumors. All of the tumors were passaged serially in mice.

cancer into a single site in a NOD/SCID mouse was 25% to 30%. Changing this approach to implantation of three minced pieces bilaterally into the midabdomen of four separate NOD/SCID mice resulted in an improvement of the engraftment rate of individual tumors to 100%. We did not observe an improvement in the rate of engraftment with pretreatment of mice with VP16 (etoposide) given via i.p injection (30 mg dose/1 kg mouse) for 5 days before implantation, as has been observed in studies of human breast cancer xenografts (5). After establishment of xenografts, studies were done on passages 1 to 2 of tumors. Results were compared with those obtained from a freshly sorted primary tumor.

**Tumor-initiating capability of sorted pancreatic cancer cells.** To test the hypothesis that there is a small subpopulation of distinct, highly tumorigenic pancreatic cancer cells within a human pancreatic cancer that is responsible for tumor formation, xenografts were digested with ultrapure collagenase IV followed by sorting for the markers CD44, CD24, and ESA, both individually or in combination. Flow cytometric quantification of CD44, CD24, and ESA expression was done on one acutely dissociated tumor and 10 tumor xenografts. Sorted cells were then suspended in a Matrigel mixture (1:1) and s.c. injected into NOD/SCID mice. Tumor growth was monitored weekly for 16 weeks, at which time animals were sacrificed and tumor absence or presence was confirmed by histologic examination. The markers CD44, CD24, and ESA were chosen as a starting point based on prior work on breast cancer stem cells, in which  $ESA^+CD24^-/low CD44^+$  cells generated tumors histologically similar to primary breast tumors when as few as 100 cells were transplanted, whereas tens of thousands of bulk unsorted cancer cells were needed to form tumors in NOD/SCID mice (5). ESA, CD44, and CD24 have been identified as stem cell surface markers, which act as adhesion molecules with multiple signaling functions (17–19).

Depending on the individual tumor, 2% to 9% of sorted human pancreatic cancer cells expressed the cell surface marker CD44, 3% to 28% expressed CD24, and 11% to 70% expressed ESA. When examining expression of multiple surface markers, 1% to 16.9% of sorted cells were  $CD44^+ESA^+$ , 1.8% to 23% were  $CD24^+ESA^+$ , and 0.5% to 2% were  $CD44^+CD24^+$ , whereas only 0.2% to 0.8% of cells were  $CD44^+CD24^+ESA^+$ . Several examples of  $CD44^+/CD24^+/ESA^+$  sorted tumor cells from individual patients are shown in Fig. 1. The percentage of cancer cells expressing these cell surface markers in individual tumors was maintained on passaging. The percentage of cells expressing CD44, CD24, and ESA in the freshly dissociated tumor and xenografts derived from that tumor was similar.

In a dose response of unsorted pancreatic cancer cells (100–10<sup>4</sup>) injected per mouse, no tumor growth was evident at 16 weeks



**Figure 1.** Isolation of tumorigenic pancreatic cancer cells. Flow cytometry was used to isolate subpopulations of human pancreatic cancer cells, which were tested for tumorigenicity in NOD/SCID mice. Cells were stained with antibodies against CD44, CD24, ESA, H2K and DAPI. Dead cells and mouse cells were eliminated from the analyses. Plots are representative examples of patterns of CD44 and ESA staining (top) and CD24 and ESA staining (bottom) of viable human pancreatic cancer cells from three individual patient xenografts, with the frequency of the boxed tumorigenic cancer cell population as a percentage of cancer cells in the specimen shown.

**Table 2.** Tumor formation ability of sorted pancreatic cancer cells using cell surface markers

Cell no.	10 <sup>4</sup>	10 <sup>3</sup>	500	100
Unsorted	4/6	0/6	0/3	0/3
CD44 <sup>+</sup>	8/16	7/16	5/16	4/16
CD44 <sup>-</sup>	2/16	1/16	1/16	0/16
<i>P</i>	0.022*	0.014*	0.07	0.03*
ESA <sup>+</sup>	12/18	13/18	8/18	0/18
ESA <sup>-</sup>	3/18	1/18	1/18	0/18
<i>P</i>	0.002*	0.0001*	0.007*	N/A
CD24 <sup>+</sup>	11/16	10/16	7/16	1/16
CD24 <sup>-</sup>	2/16	1/16	0/16	0/16
<i>P</i>	0.001*	0.001*	0.003*	0.31
CD44 <sup>+</sup> ESA <sup>+</sup>	9/16	10/16	7/16	4/16
CD44 <sup>-</sup> ESA <sup>-</sup>	3/16	2/16	0/16	0/16
<i>P</i>	0.03*	0.004*	0.003*	0.033*
CD24 <sup>+</sup> ESA <sup>+</sup>	6/8	5/8	5/8	2/8
CD24 <sup>-</sup> ESA <sup>-</sup>	2/8	1/8	0/8	0/8
<i>P</i>	0.05*	0.04*	0.007*	0.13
CD44 <sup>+</sup> CD24 <sup>+</sup>	6/8	5/8	4/8*	2/8
CD44 <sup>-</sup> CD24 <sup>-</sup>	1/8	1/8	0/8	0/8
<i>P</i>	0.01*	0.04*	0.02*	0.13
CD44 <sup>+</sup> CD24 <sup>+</sup> ESA <sup>+</sup>	10/12	10/12	7/12	6/12
CD44 <sup>-</sup> CD24 <sup>-</sup> ESA <sup>-</sup>	1/12	0/12	0/12	0/12
<i>P</i>	0.0002*	0.0001*	0.001*	0.004*

NOTE: Cells were isolated by flow cytometry as described in Fig. 1 based on expression of the combinations of the indicated markers and assayed for the ability to form tumors after injection into the subcutaneous of the flank of NOD/SCID mice at 100, 500, 10<sup>3</sup>, and 10<sup>4</sup> cells per injection. Mice were examined for tumor formation by palpation and subsequent autopsy. The analysis was completed 16 wks following injection. Data are expressed as number of tumors formed/number of injections. *P* values are listed comparing tumor formation for each marker at different cell dilutions.

\**P* values <0.05 compared with results with marker-negative cells.

unless at least 10<sup>4</sup> cells were injected, where four of six mice developed tumors (see Table 2). For cancer cells sorted for the markers CD44, CD24, and ESA, expression of individual markers identified cell populations with enhanced tumorigenic potential (Table 2). For example, injection of 100 CD24<sup>+</sup> cells would occasionally form a tumor (1 of 16 animals), whereas no tumors were observed with CD24<sup>-</sup> cells until at least 10<sup>3</sup> cells were injected (1 of 16 animals), whereas 10 of 16 animals developed tumors when injected with 10<sup>3</sup> CD24<sup>+</sup> cells, representing at least a 10-fold increase in tumorigenic potential compared with marker-negative cells (*P* = .001). Similar results were obtained with CD44<sup>+</sup> and ESA<sup>+</sup> cells, with cells expressing CD44<sup>+</sup> showing the highest tumorigenic potential when using a single marker, with 4 of 16 animals developing tumors when injected with as few as 100 cells. Injection of cancer cells expressing dual marker combinations (CD44<sup>+</sup>ESA<sup>+</sup>, CD24<sup>+</sup>ESA<sup>+</sup>, and CD44<sup>+</sup>CD24<sup>+</sup>) resulted in an

enhanced tumorigenic potential compared with single marker sorted cells, with more tumors forming with injection of as few as 100 cells, and no tumors forming in marker-negative cells until at least 10<sup>3</sup> cells were injected (Table 2). The sorted cell population with the highest tumorigenic potential were those cells expressing CD44, CD24, and ESA, where 6 of 12 animals injected with 100 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells formed tumors, and cells negative for expression of these cell surface markers did not develop any tumors until 10<sup>4</sup> CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells were injected, when only 1 of 12 animals developed a tumor (Table 2). Thus, pancreatic cancer cells expressing the cell surface markers CD44, CD24, and ESA had at least a 100-fold increased tumorigenic potential compared with nontumorigenic cells. Findings were similar for all tumors tested, including cells derived from the freshly sorted tumor and the xenografts. We observed that the tumors that developed from the nontumorigenic cells tended to be smaller and to develop more slowly than tumors that developed from tumorigenic cells. This may be accounted for by the reduced proliferative capacity of the nontumorigenic cells or due to the 1% to 3% of tumorigenic cells that invariably contaminate the nontumorigenic cells.

Histologically, the tumors derived from the highly tumorigenic pancreatic cancer cells appeared remarkably similar to histologic sections of the patient's primary tumor. An example of this is shown in Fig. 2A from a representative mouse injected with 500 CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells on the left side of the abdomen and 500 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells on the right side of the abdomen. H&E staining of the tumor generated from the CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells showed epithelial cancer cells and was phenotypically indistinguishable from the patient's primary tumor. Tumors derived from highly tumorigenic pancreatic cancer cells also expressed differentiation markers typically seen in pancreatic adenocarcinoma, as shown in Fig. 2B, where both the primary tumor and the tumor derived from that patient's CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> sorted cells had morphologic characteristics similar to the patient's primary tumor and expressed the differentiation markers S100P and stratifin. These differentiation markers are expressed in the majority of human pancreatic adenocarcinomas (14, 15).

To determine whether differences in tumorigenicity observed between CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> and CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells were due to differences in cell cycle distribution, we analyzed cell cycle distribution by flow cytometry from cells isolated from three different xenografts (Fig. 3A and B). We did not observe any differences in cell cycle distribution between the highly tumorigenic and nontumorigenic populations, showing that neither cell population was enriched for cells at a particular stage of the cell cycle.

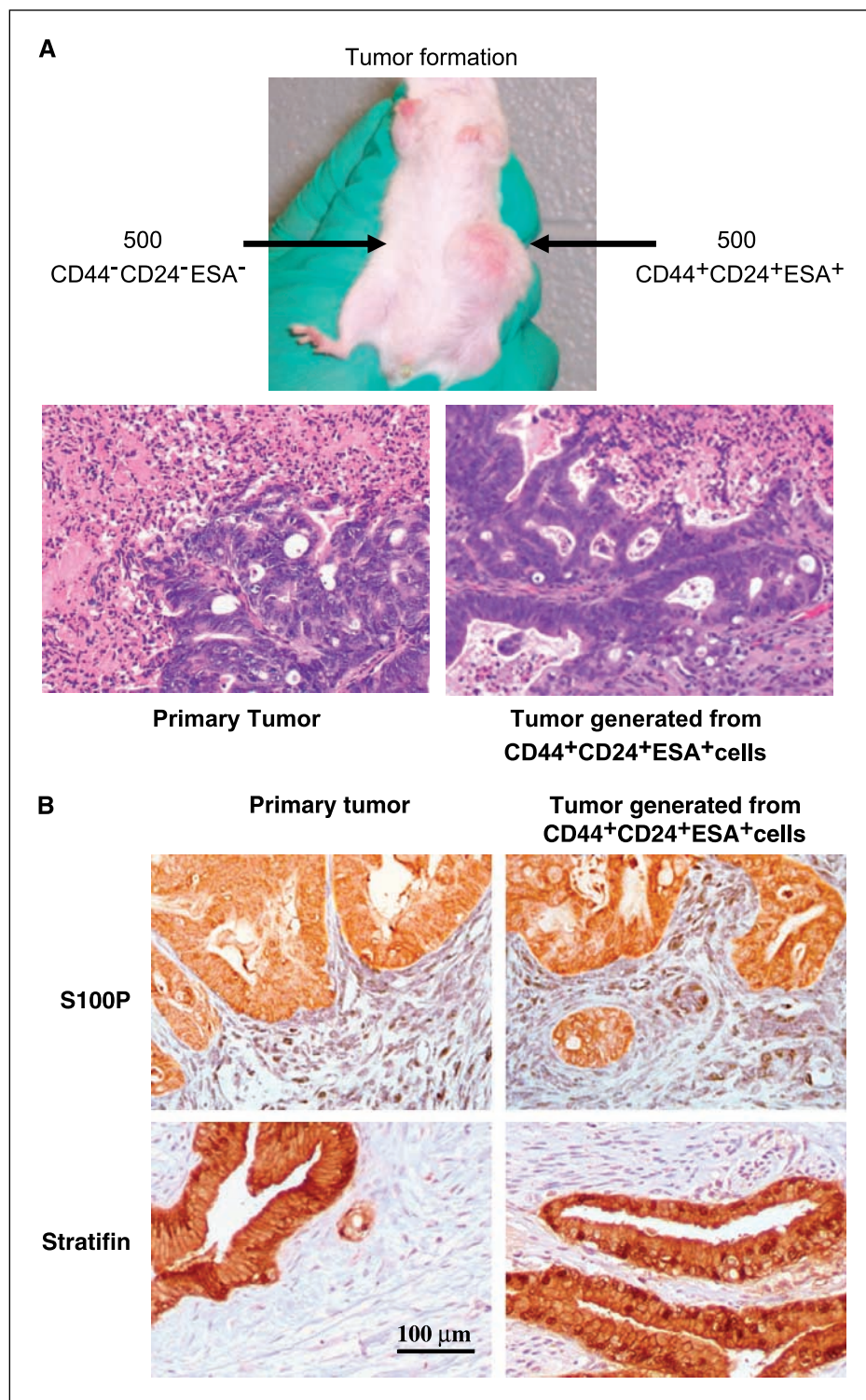
The biological function of stem cells is highly dependent on the local tissue environment or the niche (20). To validate our findings of the tumorigenic potential of the pancreatic cancer cells based on cell surface marker expression, we tested the tumorigenic potential of CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells and CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells when injected directly into the pancreas. Either 1,000 or 5,000 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> or CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> pancreatic cancer cells were injected into the mouse pancreatic tail, and tumor formation was monitored weekly for 4 weeks (*n* = 3 animals per group). At 4 weeks, the animals were sacrificed and tumor formation was assessed. In animals injected with 5,000 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells, macroscopic tumors were evident in two of three mice, whereas none was observed in animals injected with CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells (Fig. 3C and D). Tumor formation was confirmed with histologic analysis (data not shown). These results support the



enhanced tumorigenic potential of CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells in the pancreatic niche.

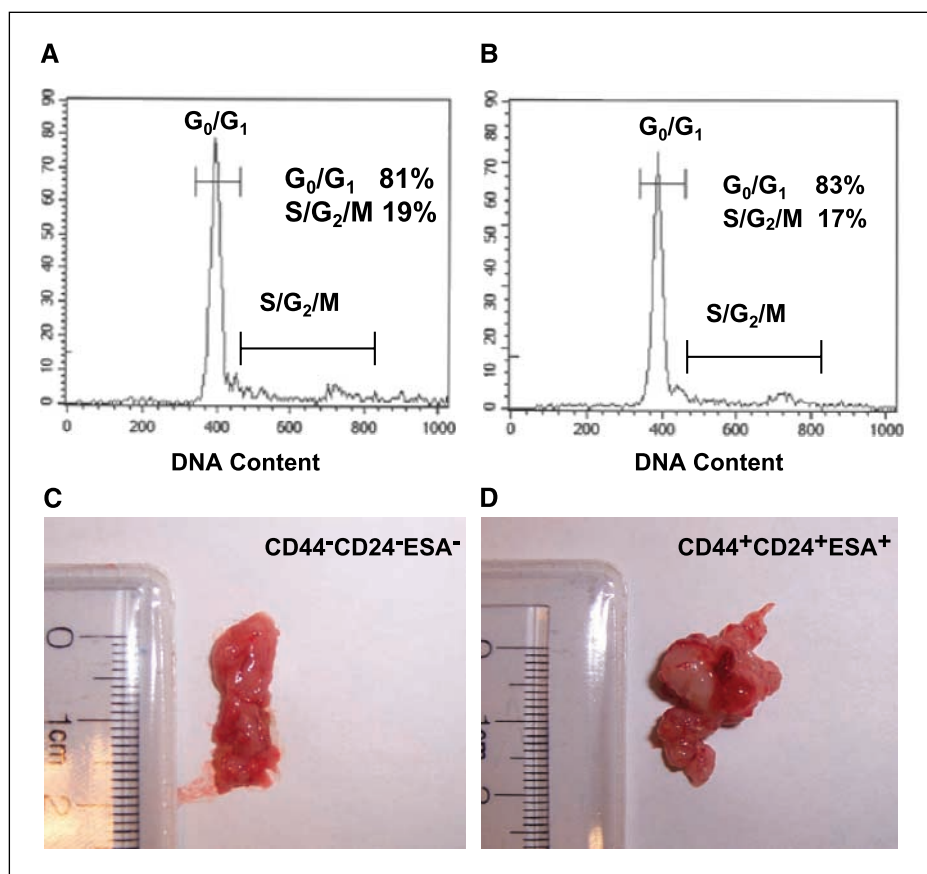
**The tumorigenic cancer cell population generates the phenotypic diversity of the initial tumor.** Normal stem cells are defined by their ability to both self-renew and generate phenotypically diverse progeny. To test if our highly tumorigenic cancer cells also exhibited these properties, CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells (Fig. 4B and E) were injected into mice and the resultant

tumors were analyzed. The pattern of CD44, CD24, and ESA expression evident in the secondary tumors (Fig. 4C and F) was similar to that which was observed in the tumor from which they were derived (Fig. 4A and D). The highly tumorigenic CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells produced additional CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells as well as phenotypically diverse nontumorigenic cells, showing the same phenotypic complexity as the primary tumor from which the tumorigenic cells were derived. The tumors have



**Figure 2.** Tumor formation in NOD/SCID mice injected with highly tumorigenic pancreatic cancer cells. *A*, a representative experiment depicting tumor formation in a mouse at the injection site of 500 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells, with no tumor formation seen at the injection site of 500 CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells. H&E staining of the tumor generated from CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells (*right*) has similar histologic features to the corresponding patient's primary pancreatic tumor (*left*). Magnification,  $\times 200$ . *B*, expression of differentiation markers in tumors derived from highly tumorigenic pancreatic cancer cells. Tissues were examined for the presence of S100P (*top*) and stratifin (*bottom*) in a primary patient tumor (*left*) and a tumor derived from CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells from the same patient (*right*). Antibody localization was done using horseradish peroxidase, with dark brown staining indicating the presence of the specific antigen.

**Figure 3.** Characterization of highly tumorigenic pancreatic cancer cells. *A* and *B*, representative histogram of the DNA content of tumorigenic and nontumorigenic pancreatic cancer cells. The cell cycle status of CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells (*A*) and CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells (*B*) was determined by propidium iodide staining of DNA content. *C* and *D*, CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells initiate tumors on injection into the NOD/SCID mouse pancreatic tail. Mouse injected with 5,000 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells shows tumor formation 28 days following injection (*D*), whereas a tumor did not develop in the mouse injected with the same number of nontumorigenic cells (*C*).



now been passed through four rounds of tumor formation in mice, and similar results have been observed, with no evidence of decrease in the tumorigenicity of the CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells (data not shown). These data suggest that CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells act as cancer stem cells, capable of undergoing both the processes of self-renewal and the creation of differentiated progeny.

**Self-renewal pathways are up-regulated in pancreatic cancer stem cells.** Several developmental signaling molecules have been implicated in the self-renewal process of normal stem cells, including Bmi-1, Notch, hedgehog, PTEN, and Wnt (21–24). Deregulation of these signaling molecules has been associated with tumorigenesis, both in human and rodent models (21, 25–27). In the pancreas, aberrant expression of SHH using a Pdx-1 promoter has been found to produce precursor lesions to pancreatic cancer, termed PanIN lesions, and to develop similar genetic changes to pancreatic adenocarcinoma (28). Furthermore, human pancreatic adenocarcinomas display increased hedgehog pathway activity (29). We next determined if there was increased expression of the developmental signaling molecule SHH in our highly tumorigenic pancreatic cancer cell population. Real-time quantitative RT-PCR was done using three samples of normal pancreas and three separate pancreatic cancer xenografts. For normal pancreas, a single-cell suspension of ESA<sup>+</sup> cells was used so that the epithelial cell population within the pancreas served as a control. Experimental samples included single-cell suspensions of bulk pancreatic cancer cells, CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells, and CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells. We found that SHH expression was up-regulated 4.1-fold in bulk pancreatic cancer cells, 4.0-fold in

CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells, and 46.3-fold in CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells compared with normal pancreatic epithelial cells (Fig. 4G), suggesting that SHH is markedly up-regulated in pancreatic cancer stem cells.

## Discussion

In this report, we have identified a subpopulation of highly tumorigenic cancer cells within human pancreatic adenocarcinomas using a xenograft model in which primary human pancreatic adenocarcinoma cells were implanted in immunocompromised mice. These highly tumorigenic cancer cells were identified by expression of the cell surface markers CD44, CD24, and ESA. These cells displayed several features typically seen in stem cells, including the ability to both self-renew and generate differentiated progeny, the ability to differentiate to recapitulate the phenotype of the tumor from which they were derived, and activation of developmental signaling pathways.

We chose to examine expression of the markers CD44, CD24, and ESA based on studies in breast cancer, in which CD44<sup>+</sup>CD24<sup>-</sup>/low ESA<sup>+</sup> cells were identified as putative cancer stem cells (5). We found that cells that expressed CD44, CD24, and ESA represented the most highly tumorigenic population of pancreatic cancer cells, with injection of as few as 100 triple positive CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells resulting in tumor formation in 6 of 12 of animals, a 100-fold enhanced tumorigenic potential compared with nontumorigenic cells. These markers were found to characterize a highly tumorigenic population that was distinct from those observed in human breast cancer, where in eight of nine patients, the phenotype of the

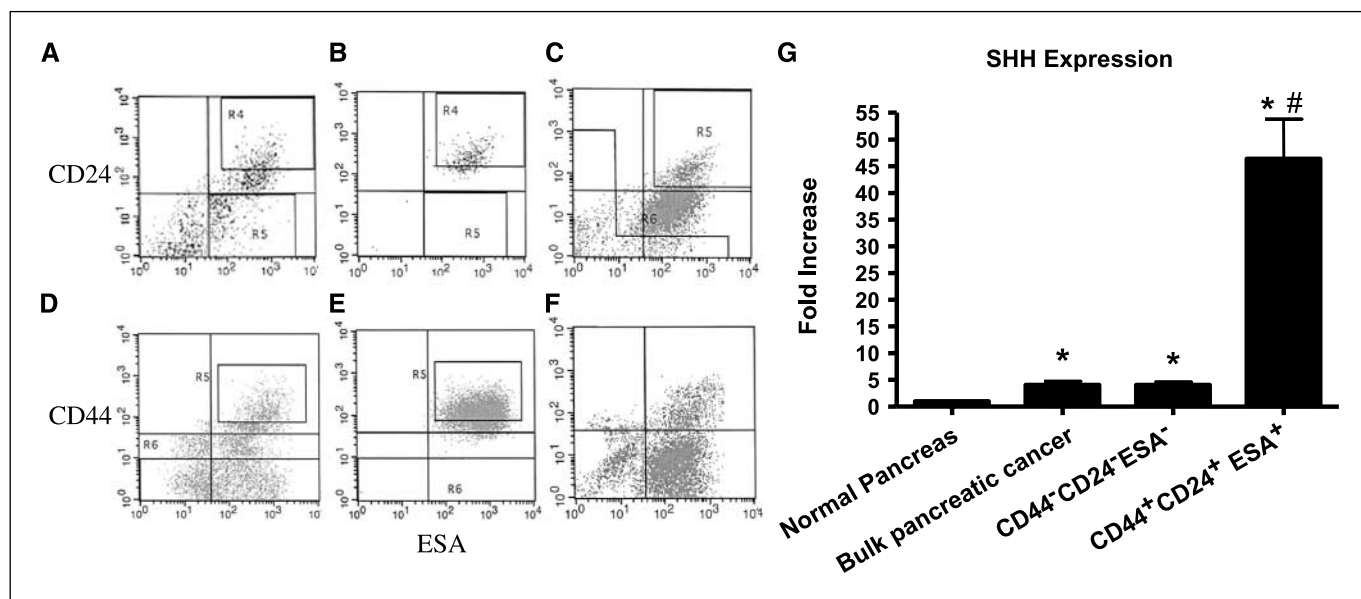
breast cancer stem cell was  $ESA^+CD44^+CD24^-/low$ . Interestingly, in one breast cancer patient studied, the tumorigenic cancer cell population was  $CD44^+CD24^+ESA^+$ . This patient had a particularly virulent subtype of breast cancer, a comedo-type adenocarcinoma of the breast, and in this cancer, >66% of the cells were contained in the tumorigenic fraction (5). Whereas the correlation between ESA and CD24 expression and cancer stem cell function has not been examined in other tumor types,  $CD44^+$  cells have been shown to define a highly tumorigenic cancer cell population in prostate cancer cells with stem cell-like characteristics (9). Other cell surface markers define a highly tumorigenic, stem cell-like population in other human solid tumor types. In human brain tumors and prostate cancer, expression of  $CD133^+$  defined a subpopulation of cancer cells with high tumorigenic potential (6, 30, 31), whereas in melanoma, the cancer stem cell population was enriched in the  $CD20^+$  fraction of cells (32). In human ovarian cancer cells, a side scatter population of cells that bind the Hoechst dye defines a subpopulation of cells with stem cell-like characteristics and enhanced tumorigenicity (10). These studies suggest that several stem cell markers may be shared by cancer stem cells in different tumor types, such as  $CD44$  and  $CD133$ ; however, it is possible that each tumor has its own unique phenotype for markers, as highly tumorigenic breast cancer cells are  $CD24^-$ , whereas their pancreatic counterparts are  $CD24^+$ .

It has been shown previously that cancer stem cells associated with other types of cancers have aberrant activation of developmental signaling pathways, such as hedgehog, the polycomb family, Wnt, and Notch. To determine if our putative pancreatic cancer stem cell population had enhanced expression of developmental genes, we chose to examine expression of SHH, based on previous reports linking hedgehog signaling to pancreatic cancer. Misregulation of hedgehog signaling has also

been shown to play a role in other types of cancer, including basal cell carcinoma, breast cancer, and small cell lung cancer (25, 27). Hedgehog pathway activation occurs in a significant number of primary human pancreatic carcinomas (28, 29) and PanIn lesions, precursor lesions of invasive pancreatic cancer. Additionally, transgenic overexpression of SHH within the pancreas results in PanIn lesions and the accumulation of genetic mutations commonly seen in pancreatic cancer, including *k-ras* mutations and up-regulation of *Her2/neu*, suggesting that Hedgehog signaling is an early mediator of pancreatic cancer tumorigenesis. Inhibition of hedgehog signaling by cyclopamine inhibited pancreatic cancer growth *in vitro* and *in vivo*, suggesting that this signaling pathway has an early and critical role in the genesis of pancreatic cancer (28). We found that SHH was markedly up-regulated in  $CD44^+CD24^+ESA^+$  cells compared with  $CD44^-CD24^-ESA^-$  and bulk pancreatic cancer cells, suggesting that SHH is highly up-regulated in pancreatic cancer stem cells, with persistent, albeit lower, expression in their differentiated progeny.

We cannot state at present whether these pancreatic cancer stem cells arise from a mutated stem cell, or a downstream progenitor or differentiated cell that has regained stem cell-like properties because of genetic alterations. In other cancer types, several studies support the concept that cancer stem cells may arise from self-renewing normal stem cells which are transformed by dysregulation of a self-renewal pathway (3, 21, 26). Determination of the cell of origin in pancreatic cancer will be greatly enhanced by identification of normal pancreatic stem cells, whose isolation has been elusive to date despite extensive efforts.

Although compelling data are provided in this study that a subpopulation of pancreatic cancer cells exists with markedly enhanced tumorigenic potential and stem pancreatic cell properties,



**Figure 4.** Highly tumorigenic pancreatic cancer cells display stem cell-like properties. *A* to *F*, phenotypic diversity in tumors arising from  $CD44^+CD24^+ESA^+$  cells. Plots are representative of the CD44, CD24, and ESA staining patterns of human pancreatic cancer cells. *A* and *D*, staining pattern from a patient tumor that had been passaged once in NOD/SCID mice.  $CD44^+CD24^+ESA^+$  tumorigenic cells from the tumor were then isolated (*B* and *E*) and injected into the flank of NOD/SCID mice. *C* and *F*, the staining pattern of the resultant tumor that arose from the  $CD44^+CD24^+ESA^+$  cells. The tumorigenic cells formed tumors that contained phenotypically diverse cells (*C* and *F*) similar to those seen in the original tumor (*A* and *D*). *G*, mRNA expression of SHH, important in developmental signaling pathways, in normal pancreas, bulk pancreatic cancer cells, nontumorigenic  $CD44^-CD24^-ESA^-$  pancreatic cancer cells and highly tumorigenic  $CD44^+CD24^+ESA^+$  pancreatic cancer cells. Total RNA was isolated and mRNA was quantitated by real-time RT-PCR. Data are expressed as the mean  $\pm$  SE. \*,  $P < 0.05$  versus normal pancreas; #,  $P < 0.05$  comparing  $CD44^-CD24^-ESA^-$  to  $CD44^+CD24^+ESA^+$  cells.

there are potential limitations to our study. The location of the implantation of the sorted cells may have affected our results, as some sites may be more conducive to tumor growth than other sites. The majority of the data derived on the tumorigenic potential of pancreatic cancer cells expressing CD44, CD24, and ESA were derived from s.c. tumor formation. This is not the normal niche of pancreatic cancer cells and may not faithfully recapitulate the environment experienced by the cancer cells in the original tumor. We attempted to address this limitation by verifying our findings with orthotopic implantation of sorted pancreatic cancer cells and monitoring of tumor formation. We observed similar findings of enhanced tumorigenicity with sorted CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup>-positive cells in the orthotopic model as in the s.c. model, suggesting that the findings of enhanced tumorigenicity observed with expression of the markers ESA, CD44, and CD24 was not dependent on the site of implantation. We noted that increased numbers of CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells were needed to generate tumors when injected into the pancreas compared with the subcutaneum. This may be due to one of several potential factors, including impaired cell viability in the setting of pancreatic trauma secondary to the injection, leakage of cells at the site of injection, or alternatively an altered microvascular environment in the pancreas compared with the subcutaneum.

The results from this study have significant implications for the treatment of pancreatic cancer. Studies in other types of tumor suggest that cancer stem cells are resistant to current therapeutic regimens. CD34<sup>+</sup>CD38<sup>-</sup> leukemic cells were significantly less sensitive to daunorubicin or cytarabine than the bulk population of leukemic blast cells (11). Similarly, Matsui et al. (33) have shown that myeloma cancer stem cells are more resistant to therapies standardly used to treat myeloma, including chemotherapy and the proteasome inhibitors. A better understanding of pancreatic cancer stem cells will not only affect our ability to better understand the therapeutics we have in hand, but expression studies of pancreatic cancer stem cells will help us identify novel diagnostic markers and therapeutic targets.

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