

Distinct Populations of Cancer Stem Cells Determine Tumor Growth and Metastatic Activity in Human Pancreatic Cancer

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SUMMARY

Pancreatic adenocarcinoma is currently the fourth leading cause for cancer-related mortality. Stem cells have been implicated in pancreatic tumor growth, but the specific role of these cancer stem cells in tumor biology, including metastasis, is still uncertain. We found that human pancreatic cancer tissue contains cancer stem cells defined by CD133 expression that are exclusively tumorigenic and highly resistant to standard chemotherapy. In the invasive front of pancreatic tumors, a distinct subpopulation of CD133⁺ CXCR4⁺ cancer stem cells was identified that determines the metastatic phenotype of the individual tumor. Depletion of the cancer stem cell pool for these migrating cancer stem cells virtually abrogated the metastatic phenotype of pancreatic tumors without affecting their tumorigenic potential. In conclusion, we demonstrate that a subpopulation of migrating CD133⁺ CXCR4⁺ cancer stem cells is essential for tumor metastasis. Strategies aimed at modulating the SDF-1/CXCR4 axis may have important clinical applications to inhibit metastasis of cancer stem cells.

INTRODUCTION

Pancreatic adenocarcinoma is currently the fourth leading cause for cancer-related mortality. The mortality rate approaches 100% due to the propensity for early metastatic spread and because the disease is highly resistant to radiation and chemotherapy. Despite increasing knowledge in tumor biology, the treatment efficacy in pancreatic cancers has not improved significantly over the past decade. Although the first-line agent gemcitabine has produced clinical response such as reduced pain and weight gain (Matano et al., 2000), the prognosis remains dismal with a 5 year survival rate of 1%–4% and a median survival period of 4–6 months (Ahlgren, 1996; Jemal et al., 2004; Rosenberg, 1997; Rothenberg et al., 1996; Warshaw and

Fernandez-del Castillo, 1992). More recently, however, increasing evidence suggests that stem cells may play a decisive role not only in the generation of complex multicellular organisms but also in the development and progression of tumors (Clarke et al., 2006; Jordan et al., 2006). Cells bearing stem cell properties may represent an integral part of the development and perpetuation of various human cancers (Al-Hajj et al., 2003; Kim et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004). The current consensus definition describes a cancer stem cell (CSC) as a cell within a tumor that is able to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor (Clarke et al., 2006). The implementation of this concept explains the use of alternative terms in literature, such as “tumor-initiating cell” and “tumorigenic cell” to describe putative CSC (Al-Hajj et al., 2003; Kim et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004).

The stem cell hypothesis has recently been explored in pancreatic cancer (Li et al., 2007). However, CSC may not only be associated with tumor initiation and growth but may also play a crucial role in tumor metastasis. Indeed, if CSC represent the only cell population with tumor-initiating potential, one would hypothesize that solely CSC are capable of generating tumor metastases. In the present study, we provide multiple lines of evidence for the crucial role of CSC in pancreatic tumor growth and, even more importantly, define a subpopulation of migrating CSC in tumor metastasis.

RESULTS

Identification and Characterization of Human Pancreatic CSC

In a first step, we aimed to identify pancreatic CSC in tissue samples derived from patients with pancreatic cancer (patient characteristics are depicted in Table S1 in the Supplemental Data available with this article online). CSC were identified by means of the surface marker CD133, which is expressed by normal and malignant stem cells of the neural, hematopoietic, epithelial, and endothelial lineages (Fargeas et al., 2003; Yin et al., 1997). Flow cytometry demonstrated presence of a rare CSC population (Figure 1A, left and middle panels).

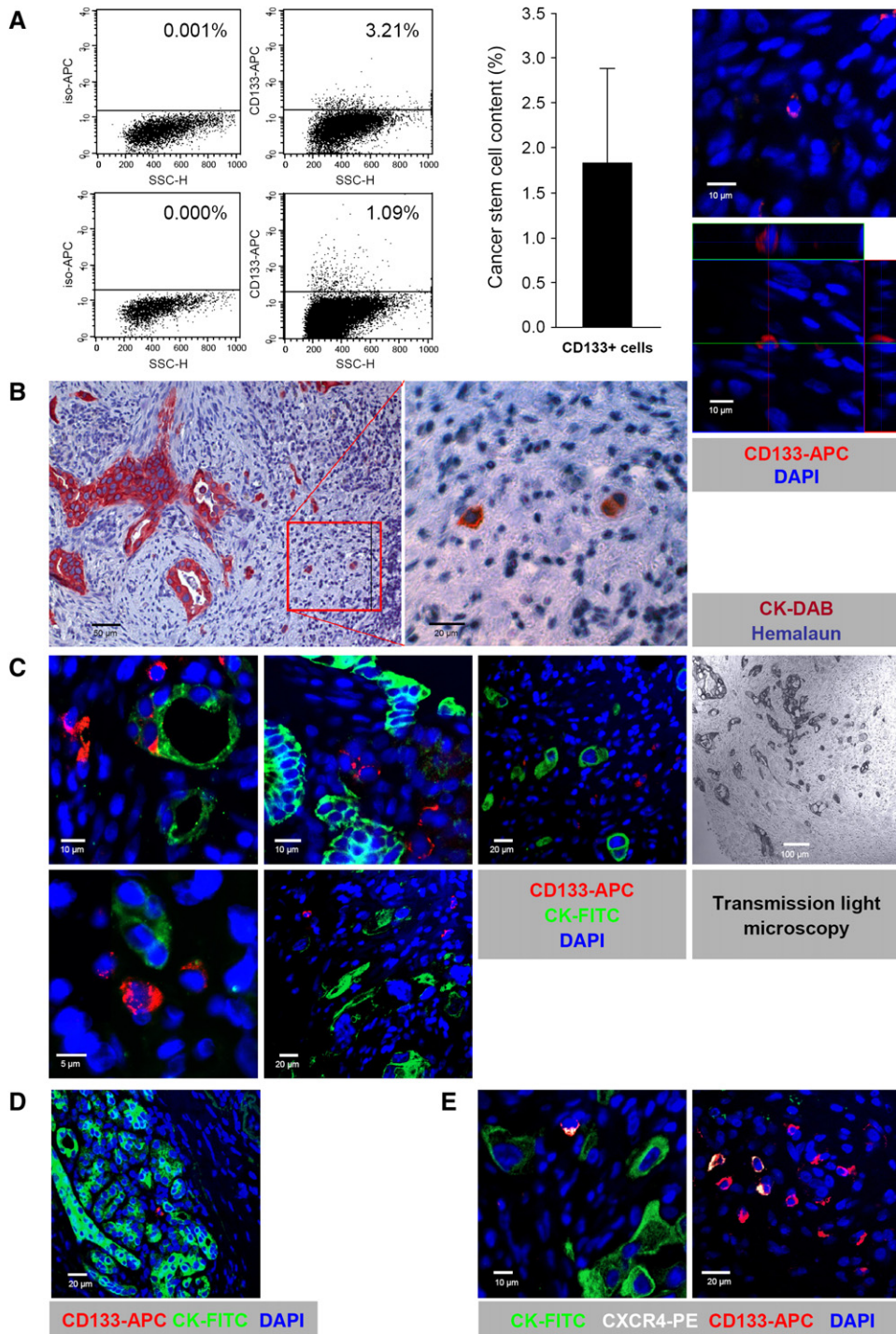


Figure 1. Detection of CD133⁺ Cells in Patient-Derived Pancreatic Cancer Samples

(A) To assess the number of CD133⁺ cells, digested tissue samples were either stained for CD133 and analyzed by flow cytometry or paraffin sections were stained for CD133 (red) and counterstained with DAPI (blue). Representative examples from primary tumors of two individual patients are provided in the left (flow cytometry) and right (histology) panels. Three-dimensional reconstruction of a tissue section is provided in the right lower panel. Quantification of the flow cytometry data is shown in the middle panel (n = five analyzed patients).

(B) Immunohistological staining for the epithelial marker cytokeratin (dark red) and counterstaining with hemalaun illustrates the invasive front in a patient with pancreatic carcinoma. Single cells positive for cytokeratin are shown in high magnification in the right panel.

(C) An overview of the analyzed section was obtained by transmission light microscopy. Detailed analysis of CSC located in this representative invasive front: double staining for cytokeratin (green) and CD133 (red) revealed that a large number CD133⁺ cells are located in the border zone of the tumor and are rarely present in structures with epithelial differentiation but rather reside as single cells in the infiltrated stromal tissue.

Consistently, histological analysis revealed that CD133⁺ CSC were anatomically localized in the bulk tumor (Figure 1A, right panel). To more thoroughly investigate CD133⁺ cells in the invasive border zone of pancreatic cancer samples, we analyzed the invasive front in serial pancreatic cancer sections by staining for cytokeratin. As illustrated in Figure 1B, the invasive front is characterized by disseminated cytokeratin-positive tumor cells. All seven samples from patients with pancreatic cancer reproducibly demonstrated the presence of rare CD133⁺ cells in these areas with histological evidence for cell dissemination (Figure 1C). CD133⁺ cells were often found in close proximity to more differentiated epithelial cells but were essentially negative for the epithelial marker cytokeratin (Moll, 1998). In contrast, CD133 expression in healthy pancreatic tissues was a very rare event (barely detectable upon extensive analysis of histological sections with a mean of 0.27 ± 0.52 cells per high-power field [= 0.021 mm^2]) as compared to tumor tissue (3.60 ± 2.58 cells per high-power field) (Figure 1D). The increased number of CD133⁺ cells in cancer tissue most likely results from their oncogenic transformation. Disseminated tumor cells play a critical role in tumor metastasis, and migrating CSC may represent a specific subset of CSC. Because stromal cell-derived factor 1 (SDF-1) is an important mediator in cell migration, we investigated the expression of its specific receptor CXCR4 in CSC. Indeed, costaining for CXCR4 indicated that CD133⁺ cells in the invasive front strongly express CXCR4 (Figure 1E), whereas CXCR4 expression in CD133⁺ cells of the bulk tumor was rarely found. These results already suggest a potential role of CXCR4⁺ CSC in tumor dissemination.

In a further step, we evaluated the tumorigenic potential of isolated pancreatic CD133⁺ CSC. Specifically, we investigated the ability of tumor-derived CD133⁺ and CD133⁻ cells to orthotopically engraft and give rise to pancreatic tumors in athymic mice. CD133⁺ CSC were isolated by magnetic bead sorting, which resulted in a considerable enrichment of CD133⁺ cells (regular purity > 90%) and, even more importantly, a highly effective negative selection (purity > 99.8%) of CD133⁻ cells (Figure 2A). Potential contamination of the CD133⁺ cell population by hematopoietic and endothelial progenitors was excluded by flow cytometry. CD133⁺ cells were only rarely positive for the panhematopoietic marker CD45 (<0.5%), and fewer than 2% were putative CD31⁺ endothelial progenitors (Figure 2B, left panel). CD133⁺ cells did not express the epithelial differentiation marker cytokeratin (Figure 2B, right panel). In a mouse model of pancreatic cancer, as many as 10^6 patient-derived CD133⁻ pancreatic cancer cells did not induce tumor formation. In contrast, 10^6 unseparated cells or as few as 5×10^2 CD133⁺ cells resuspended in Matrigel generated visible tumors after 3 weeks (Figure 2C; Figure S1A). These data indicate that cells capable

of initiating pancreatic cancer are highly enriched with a CD133⁺ cell population. Interestingly, despite the higher number of CD133⁺ cells present in 10^6 unseparated cells (mean 1.8×10^4 cells), tumor formation following injection of purified CD133⁺ cells was faster and more efficient than tumor formation obtained with the total pancreatic cancer cell population (Figure 2C). We observed a close correlation between the percentage of CD133⁺ cells in the primary tumor as assessed by flow cytometry and the tumorigenicity of the unfractionated cells ($r = 0.92$; $p < 0.05$) (Figure S1B). Additionally, immunohistochemistry and subsequent microscopic analysis revealed that tumor xenografts derived from CD133⁺ cells consistently reproduced the primary tumor at the histological level (Figure 2D). Thus, the tumorigenic population in pancreatic cancer is restricted to CD133⁺ cells, which can reproduce the original tumor in permissive recipients.

To investigate whether CD133⁺ pancreatic cancer cells display long-term tumorigenic potential, we evaluated their ability to generate tumors after serial transplantations. For this purpose, 5×10^2 CD133⁺ or 10^6 CD133⁻ cells from primary tumor xenografts were transplanted into secondary mice. Indeed, injected CD133⁺ tumor cells engrafted and generated tumors that grew rapidly and required the mouse to be sacrificed within 3 weeks (Figure 2C). Additionally, CD133⁺ tumor cells obtained from similar CD133⁺-derived secondary xenografts were subsequently transplanted into third-generation mice. The implanted CSC were negative for cytokeratin, whereas the developing tumors also contain cytokeratin positive differentiated tumor cells as their progeny (Figure 2D). Moreover, tumor morphology was indistinguishable from tumors generated by unfractionated cells. During the *in vivo* passaging, CD133⁺ cells did not lose their tumorigenic potential but instead increased their aggressiveness, as indicated by faster tumor growth (Figure 2E). This was not related to a higher content of CD133⁺ CSC but may be related to *in vivo* selection of a highly tumorigenic subpopulation of CD133⁺ cells (Figure S1C). Thus, the CD133⁺ cell population resident in the pancreatic tumor mass is able to generate serial xenografts showing a virtually unlimited growth potential.

In Vitro Expansion of Human Pancreatic CSC

To be able to study the cell biology of CSC more extensively, we investigated whether human pancreatic cancer cell lines also contain CSC recapitulating the features of primary cells isolated from patients with pancreatic cancer. Flow cytometric analysis revealed that the highly aggressive human pancreatic cancer cell line L3.6pl also contains a reproducible number of CD133⁺ CSC (Figure 3A). Orthotopic implantation of MACS-isolated cells revealed that as few as 10^3 CD133⁺ L3.6pl cells were capable of inducing tumor formation (Figure 3B), whereas

(D) Normal pancreatic tissue also contains very few CD133⁺ cells. Double staining for CD133 (red) and cytokeratin (green) showed a stem cell only in every third microscopic field.

(E) Triple staining for CD133 (red), CXCR4 (white), and cytokeratin (green) identifies a distinct subpopulation of CSC in the invasive front. Error bars represent standard deviations.

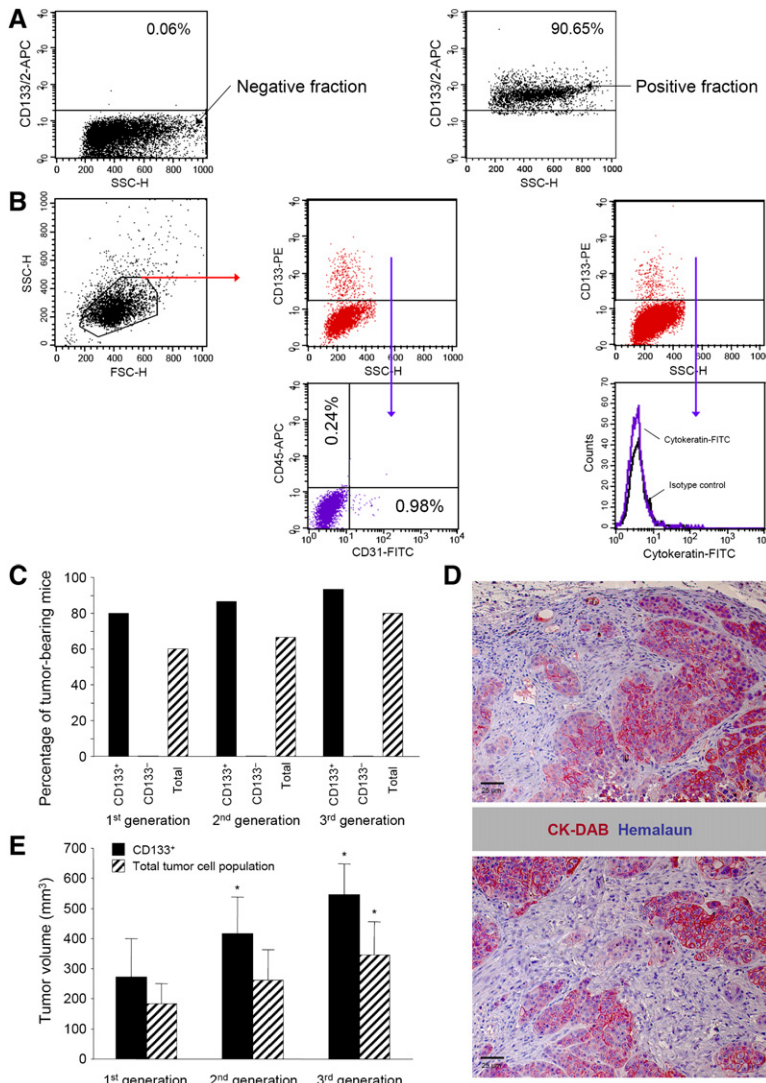


Figure 2. Tumorigenic Potential of CD133⁺ Patient-Derived Pancreatic Cancer Cells

(A) CD133⁺ cells were isolated from freshly resected tumors by magnetic bead selection using the CD133/1 antibody. Samples were then stained with the CD133/2 antibody and analyzed by flow cytometry indicating excellent depletion for CD133⁺ CSC in the negative fraction (left panel) as well as high enrichment for CD133⁺ CSC in the positive fraction (right panel).

(B) Flow cytometry for the pan-endothelial marker CD31 and the pan-leukocyte marker CD45 was performed to detect potential contamination of isolated cells with endothelial or hematopoietic progenitors (left panel). Staining for cytokeratin revealed no expression in CSC (right panel). Experiments were performed in duplicates.

(C) After MACS isolation of first-generation human pancreatic cancer cells, we orthotopically implanted CD133⁺, CD133⁻, or the unfractionated cells into the pancreas of NMRI nu/nu mice. Only CD133⁺ and the entire tumor cell population were capable of inducing tumor formation. Isolation of second- and third-generation tumor cells from transplanted mice revealed similar results (n = seven analyzed patients; experiments were performed in triplicates).

(D) Histological analysis of first-generation tumors (upper panel) and third-generation tumors (lower panel) by staining for cytokeratin and counterstaining for hemalaun revealed that although implanted CSC were negative for cytokeratin, the developing tumors also contain cytokeratin-positive differentiated tumor cells as their progeny.

(E) Tumor size increased using later generations of tumor cells regardless of whether isolated CSC or the entire tumor cell population was injected. Experiments were performed at least in duplicates. Asterisks indicate p < 0.05 versus first generation. Error bars represent standard deviations.

as many as 10⁶ CD133⁻ L3.6pl cells failed to do so. Retransplantation of isolated tumor cells also resulted in reproducible tumor formation in secondary recipients. These data indicate that the L3.6pl cell line is a suitable model to study the biology of pancreatic CSC.

Because normal and neoplastic stem cells from neural and epithelial organs can be expanded as sphere-like cellular aggregates (Dontu et al., 2003; Singh et al., 2004) in serum-free medium containing EGF and FGF-2, we cultivated the L3.6pl pancreatic cancer cells using culture conditions (ultra low adhesion plates) that favor the proliferation of undifferentiated cells. Within 2 to 3 weeks of culture, we obtained pancreatic spheres formed by aggregates of growing undifferentiated CD133⁺ cells (Figure S3Cc). CD133⁻ pancreatic cancer cells invariably died in such serum-free conditions but could grow for several weeks in serum-containing medium. The standard culture of unselected cells allowed the persistence of the

CD133⁺ population at low levels (mean 0.8 ± 0.3%). When CD133⁺ cells were grown as pancreatic spheres, these cells remained CD133⁺ and expressed negligible amounts of cytokeratin. In contrast, the vast majority of cells obtained under standard culture conditions did express cytokeratin (data not shown). Single cells obtained from these dissociated spheres could be clonally expanded. To determine the differentiation potential of these CD133⁺ cells, tumor spheres were cultivated without EGF and FGF-2 in the presence of 10% serum. After several days of culture, cells differentiated into large adherent cells (Figure S3Cc). Upon differentiation, pancreatic cancer cells expressed cytokeratin and acquired a morphology closely resembling the major pancreatic cancer cell population. Therefore, the ability to grow exponentially and the lack of cytokeratin expression as well as the tumorigenic potential suggest that CD133⁺ pancreatic spheres are aggregates of primitive cancer cells.

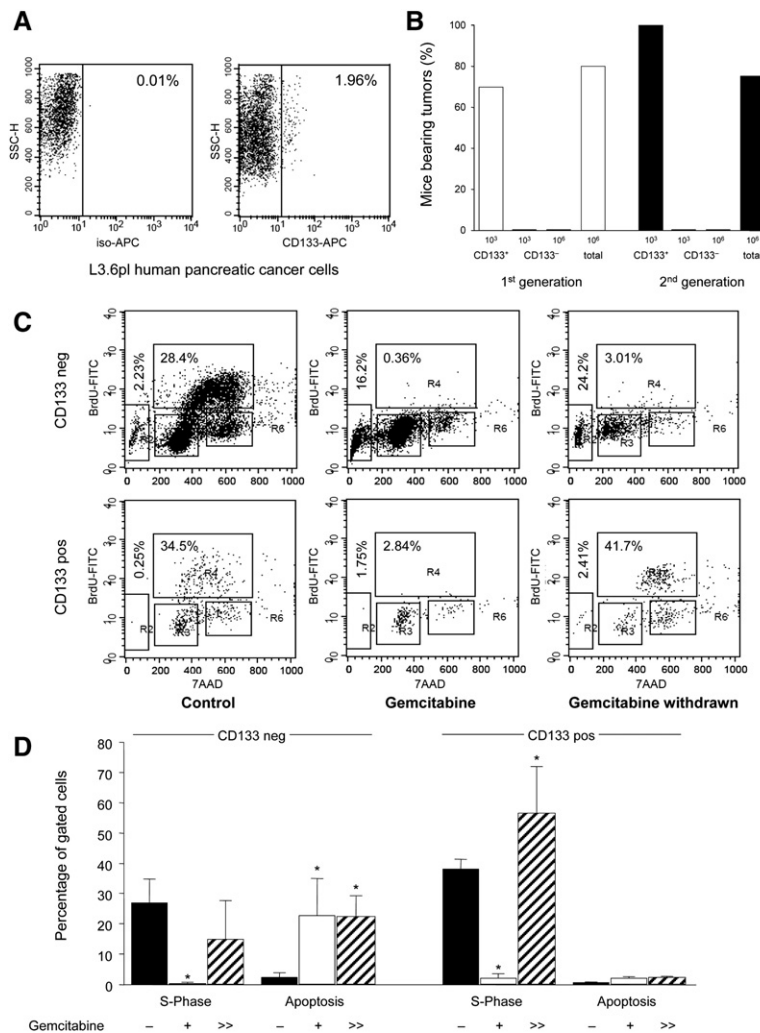


Figure 3. Human Pancreatic CSC Derived from Pancreatic Cancer Cell Lines

(A) The established human pancreatic cancer cell line L3.6pl also contained a distinct population of CD133⁺ cells under standard culture conditions accounting for 1% to 2% (right panel) as determined by flow cytometry. Isotype control ruled out unspecific binding of epitopes (left panel). Experiments were performed in triplicates.

(B) After MACS isolation of first-generation L3.6pl cells, we orthotopically implanted CD133⁺ cells and CD133⁻ cells into the pancreas of mice, respectively. Only CD133⁺ cells or the total tumor cell population were capable of inducing tumor formation (n ≥ 6). Isolation of second-generation L3.6pl cells showed similar results concerning CD133⁻ cells (0%), but caused tumor formation in all the mice receiving CD133⁺ cells (n ≥ 4).

(C) Flow cytometry was used for cell-cycle analysis of CD133⁺ and CD133⁻ L3.6pl cells before and after exposure to gemcitabine. Cells were stained for 7-AAD as marker for apoptosis and for BrdU as marker for DNA synthesis. Cells in S phase are depicted in gate R4, and apoptotic cells are depicted in gate R2. Baseline data are provided in the left panel. Cells were then treated with gemcitabine for 24 hr (middle panel) followed by 24 hr of recovery after withdrawal of gemcitabine (right panel).

(D) Quantification of the cell-cycle analysis. > > indicates withdrawal of gemcitabine for 24 hr. Results are based on three independent experiments. Asterisks indicate p < 0.01 versus control. Error bars represent standard deviations.

Resistance of Human Pancreatic CSC to Standard Chemotherapy

Pancreatic cancers are fundamentally resilient to treatment because the malignant cells survive chemotherapy. Because CSC may play a crucial role in treatment resistance, we performed cell-cycle analyses by flow cytometry. Both CD133⁺ and CD133⁻ cells were exposed to the standard chemotherapeutic agent gemcitabine. We found that CD133⁺ cells showed dramatic drug resistance to gemcitabine compared to autologous CD133⁻ cells (Figures 3C and 3D). Prolonged treatment with gemcitabine favored the survival of CSC so that after 5 days about 50% of the cultivated cells were CSC (Figure 4A). Consistently, primary tumor cells from patients with pancreatic cancer also exhibit a strong resistance to gemcitabine (Figure S2). To demonstrate the in vivo relevance of these findings, we analyzed tumor samples from mice bearing pancreatic cancer after injection of 10⁶ L3.6pl cells and receiving either vehicle or gemcitabine treatment (biweekly with 125 mg/kg gemcitabine by i.p. administration for 21 days). Although the tumors were significantly smaller compared to vehicle-treated mice (100.5 ± 36.4 versus

183.8 ± 66.9 mm³; p < 0.05), a profound enrichment of CD133⁺ CSC had occurred (Figure 4B).

Role of Human Pancreatic CSC in Tumor Metastasis

On the basis of the provided histological evidence for the existence of CXCR4⁺ CSC in the invasive front of human tumor specimens (Figure 1E), we hypothesized that a specific subset of CXCR4⁺ CD133⁺ CSC plays a crucial role in tumor metastasis. For this purpose we used a model involving two related human pancreatic cancer cell lines with opposing metastatic activity. The L3.6pl cells are derived from their parental FG cells by multiple in vivo cycles to select for highly metastatic cells (Brunns et al., 1999). While quantitative differences in the CSC content between these two related cell lines were detectable only under certain culture conditions (data not shown), the qualitative differences in the CSC characteristics were highly consistent and indeed remarkable. While L3.6pl-derived CD133⁺ CSC displayed a strong migratory activity, FG-derived CD133⁺ CSC showed significantly lower invasive activity. Intriguingly, only the L3.6pl-derived CD133⁺ CSC

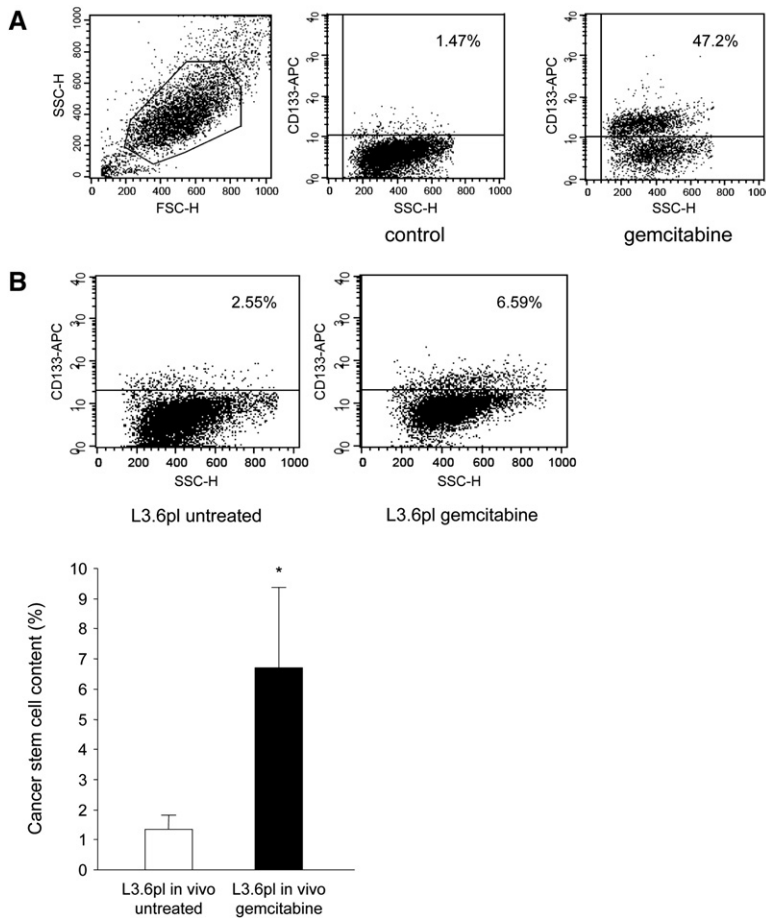


Figure 4. Standard Chemotherapy Enriches for CSC

(A) Prolonged treatment of L3.6pl cell culture with gemcitabine favored survival of CD133⁺ CSC, with the result of up to 50% of the entire cell count being CD133⁺ ($n \geq 4$).

(B) In vivo L3.6pl-derived tumor xenograft experiments showed that gemcitabine resulted in a significant enrichment for CD133⁺ cells. Gemcitabine (biweekly 125 mg/kg i.p.) or vehicle treatment was initiated 9 days after orthotopic tumor cell implantation. CSC content was determined after 3 weeks of treatment by flow cytometry. Representative images (upper panel) and the quantification (lower panel) are shown. Asterisks indicate $p < 0.01$ versus control; $n \geq$ three animals per group. Error bars represent standard deviations.

predominantly expressed the CXCR4 receptor, and their strong migratory activity was primarily mediated by CXCR4 (Figure 5A). Similar findings were observed for several other pancreatic cancer cell lines as well as primary specimens from patients with pancreatic cancer or colon cancer (Figure S3). MiaPaCa cells, a cell line with a very invasive growth pattern, also contained a distinct subpopulation of migrating CSC, and the migratory activity of these cells was clearly dependent on CXCR4 (Figure 5B). Indeed, SDF-1 as the specific ligand for the CXCR4 receptor was the most potent inducer of migration for CD133⁺ CSC, whereas c-Met, which is activated by the hepatocyte growth factor (HGF), was not detectable on pancreatic cancer cells (Figure 6A) and indeed did not show a significant induction of cell migration as compared to control (data not shown). These observations indicate that migration of CD133⁺ cells is primarily mediated through the SDF-1/CXCR4 system.

On the basis of these data, we hypothesized that the phenotypic differences between metastatic L3.6pl cells and their parental FG cells are primarily due to the presence of highly invasive CD133⁺ CXCR4⁺ CSC in the L3.6pl cancer cell population. To test this hypothesis, we isolated from L3.6pl cells both CD133⁺ CXCR4⁺ cells and CD133⁺ CXCR4⁻ cells to high purity using high-speed FACS (Figure 6B) and orthotopically injected into athymic

mice either 5×10^2 tumor-initiating CD133⁺ CXCR4⁻ cells together with 5×10^2 migrating CD133⁺ CXCR4⁺ cells (CXCR4⁺) or only 10^3 tumor-initiating CD133⁺ CXCR4⁻ cells (CXCR4⁻). Both groups showed similar tumor development (262 ± 64 versus 256 ± 73 mm²; $p = 0.75$). Flow cytometric analysis of portal vein blood samples following development of tumors identified only in the CXCR4⁺ group a reproducible population of circulating CD133⁺ CXCR4⁺ CSC (0.04 ± 0.03 of gated cells) (Figure 6C), whereas in the CXCR4⁻ group, no CD133⁺ CSC, either CXCR4⁺ or CXCR4⁻, could be detected in the circulating blood. These data support the hypothesis that CXCR4⁺ CD133⁺ CSC represent a distinct invasive CSC population and cannot be derived from CD133⁺ CXCR4⁻ CSC. Tumors were removed by partial resection of the pancreas and complete splenectomy to allow isolated growth of metastases. Another 2 weeks later, thorough macroscopic and microscopic analysis of the livers revealed that only mice in the CXCR4⁺ group developed liver metastases, whereas mice receiving CD133⁺ CXCR4⁻ cells did not show any trace of liver metastasis (Figure 6D). To provide further evidence that inhibition of CXCR4⁺ CSC abrogates tumor metastasis, we performed a study using continuous pharmacological inhibition of the CXCR4 receptor by AMD3100 and, thus, permanent inhibition of tumor (stem) cell migration. Indeed, we found that

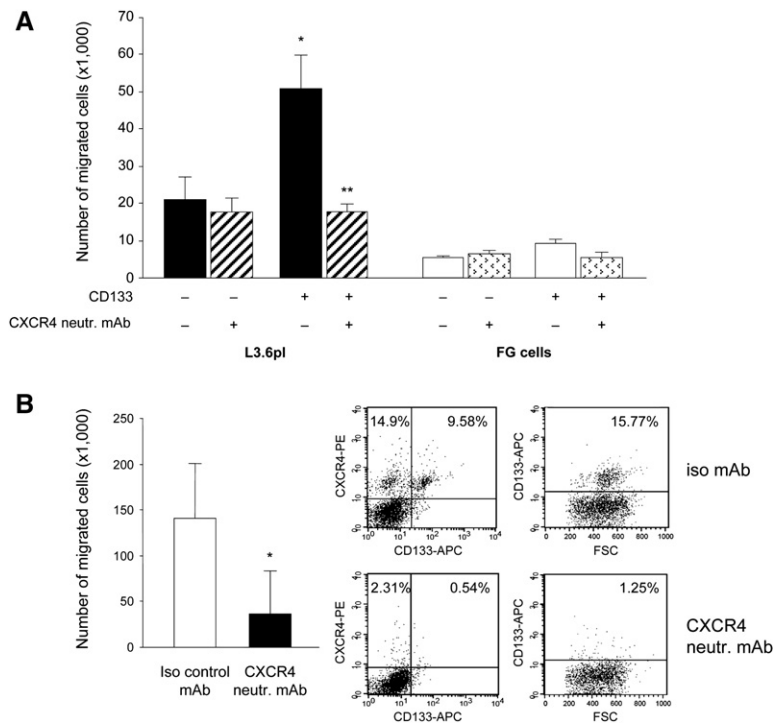


Figure 5. Identification of Migrating Pancreatic CSC

(A) The migratory capacity of L3.6pl and FG cells was assessed in a modified Boyden chamber assay. Prior to the experiment, cells were separated into CD133⁺ and CD133⁻ cells by MACS isolation. The involvement of the CXCR4 receptor was determined by simultaneous administration of the CXCR4 neutralizing antibody. Single asterisk indicates $p < 0.01$ versus CD133⁻ cells. Double asterisk indicates $p < 0.01$ versus no CXCR4 neutralizing antibody.

(B) The migratory activity of MiaPaCa cells in the presence or absence of CXCR4 neutralizing antibodies is shown in the left panel. In the lower Boyden chamber, a dramatic increase in the number of CXCR4⁺ migrating CSC was observed (right upper panel) that was abrogated in the presence of CXCR4 neutralizing antibodies (right lower panel). Asterisk indicates $p < 0.05$ versus iso control mAb.

Error bars represent standard deviations.

concomitant AMD3100 treatment significantly reduced tumor metastasis (Figure S4A). To demonstrate the clinical relevance of our experimental findings, we performed additional studies in patients with pancreatic cancer. Intriguingly, we found that tumor cells with a high number of CXCR4⁺ CSC showed a markedly increased migratory activity and that these patients suffered from a more advanced metastatic disease (Figures S4B and S4C).

DISCUSSION

Here we demonstrate that tumorigenic pancreatic cells are included in a rare population of undifferentiated cells that express CD133 (Fargeas et al., 2003; Yin et al., 1997). Freshly isolated patient-derived CD133⁺ pancreatic CSC were highly tumorigenic, and as few as 500 cells were capable of inducing orthotopic tumor formation in athymic mice. In contrast, as many as 10^6 CD133⁻ tumor cells did not result in any tumor formation. Most importantly, we show here that CSC do not represent a homogeneous population of tumor-initiating cells. Instead, we define a subpopulation of migrating CSC that are characterized by expression of the CXCR4 receptor and are critically involved in tumor metastasis. Indeed, elimination of this subpopulation of CSC virtually abrogated the metastatic activity of pancreatic cancer cells.

Our data are well in line with the CSC hypothesis that suggests that tumors are generated and maintained by a small subset of undifferentiated cells with the ability to self-renew and differentiate into the bulk tumor population (Clarke et al., 2006; Dalerba et al., 2007; Dick, 2005; Kim et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004). As in other cancers such as leu-

kemia, breast, brain, and colon cancer, stem and early progenitor cells also seem to be the target of oncogenic transformation in pancreatic cancer. It is certainly possible that CSC arise by mutations from normal stem cells. On the other hand, several lines of evidence suggest that CSC could also arise from mutated progenitor cells (Passegue et al., 2003; Prindull, 2005). However, to become a CSC, a progenitor cell must acquire mutations that cause it to regain the property of self-renewal. Indeed, many studies have demonstrated that the expression of stem-cell-like properties in tumor cells does not necessarily suggest that these cells originated from stem cells. In experimental settings, the expression of cooperating oncogenes in lineage-restricted progenitor cells can yield tumors with the cytopathological characteristics of highly malignant tumors (Barnett et al., 1998). These studies suggest that CSC are not necessarily derived from bona fide tissue-specific stem cells but instead may arise from a committed progenitor cell that reacquired stem-cell-like properties during oncogenic transformation. At the moment, the state of knowledge of tissue stem cells and progenitor cells residing in the pancreas still needs to be markedly advanced to permit side-by-side analysis of these cell populations with pancreatic tumor cells before this issue can be solved.

CD133 is expressed by normal tissue-resident as well as hematopoietic stem cells (Peichev et al., 2000; Uchida et al., 2000; Yin et al., 1997). As demonstrated for hematopoietic and endothelial progenitors, CD133 is also expressed on early progenitors but usually is no longer detectable upon differentiation (Peichev et al., 2000). In tumor cells, CD133 has been used for the identification of a subpopulation of highly tumorigenic cells as

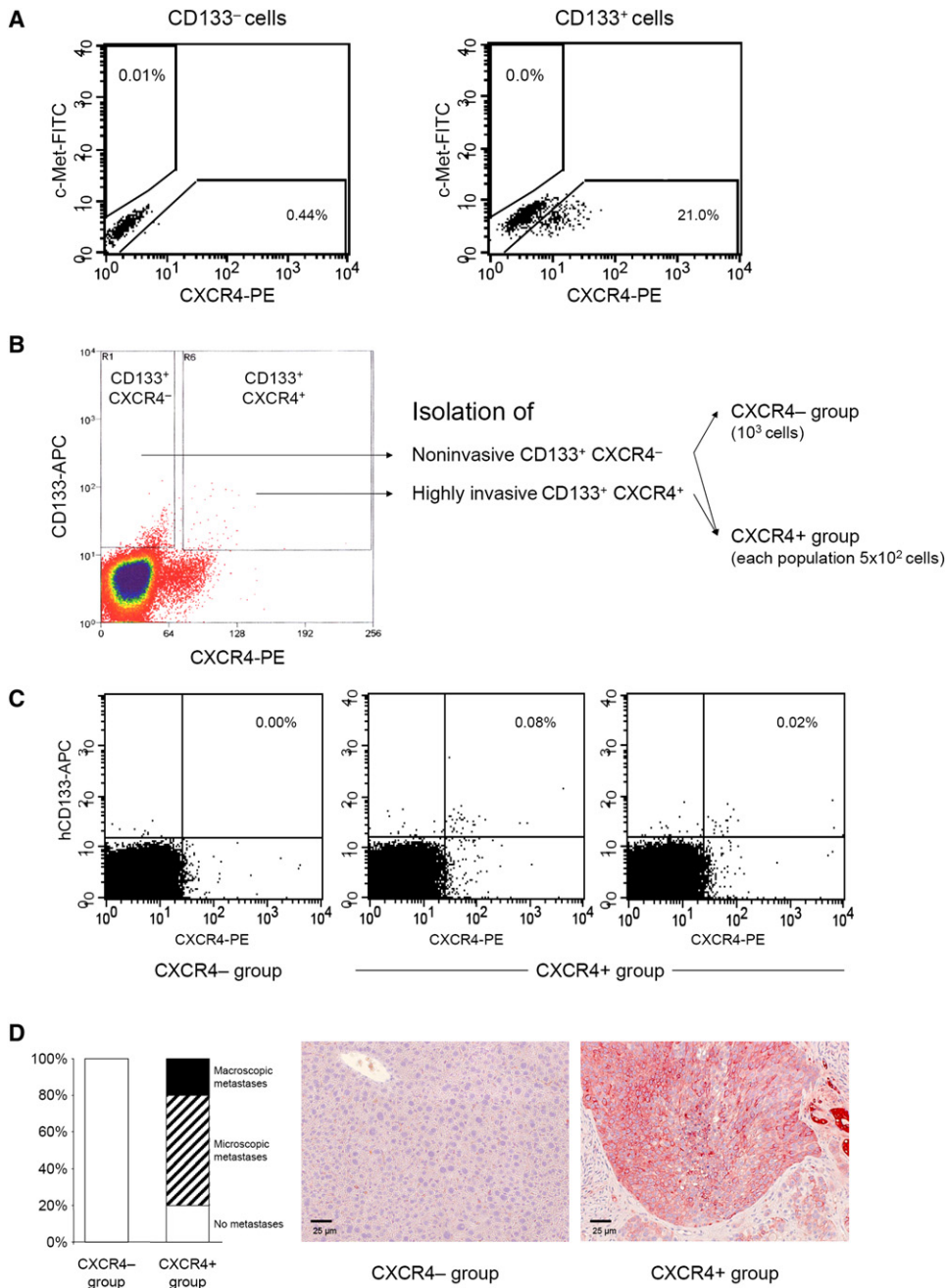


Figure 6. Role of Migrating Pancreatic CSC in Tumor Metastasis

(A) Expression of the hepatocyte growth factor receptor c-Met and the SDF-1 receptor CXCR4 on CD133⁻ (left panel) and CD133⁺ L3.6pl cells (right panel). (B) High-speed FACS was used to deplete CD133⁺ L3.6pl CSC for the distinct population of CD133⁺ CXCR4⁺ migrating CSC (gate R6) followed by orthotopic implantation of either the complete CSC pool or CSC depleted for CD133⁺ CXCR4⁺ into the pancreas of NMRI nu/nu mice. (C) Portal vein blood was investigated for the presence of circulating migrating L3.6pl CSC characterized by double staining for CD133 and CXCR4. (D) Histological analysis of the liver for metastatic lesions was performed by staining for cytokeratin (red) and counterstaining with hemalaun. The metastatic status of the mice in the two groups is provided separately for macroscopic (black) and microscopic (hatched) evidence for metastasis (left panel) (n = five per group). Representative images are from a mouse receiving CD133⁺ L3.6pl CSC depleted for CD133⁺ CXCR4⁺ migrating CSC and a mouse also receiving CD133⁺ CXCR4⁺ migrating CSC (right panel).

demonstrated for neural cancers and, more recently, also for cancers of the colon (O'Brien et al., 2007; Singh et al., 2004). However, it is not expressed on tumor cells upon their differentiation. Thus, the present data from pancre-

atic cancer are well in line with these earlier findings as we identified a subpopulation of CD133⁺ cells that bear self-renewal capacity as they can be clonally expanded, are exclusively tumorigenic, and are able to differentiate

into CD133⁻ tumor cells. A very recent study in pancreatic cancer has utilized a marker combination of CD44⁺ CD24⁺ ESA⁺ for the identification of CSC (Li et al., 2007). By flow cytometry analysis, we showed that these two populations overlap but are not identical.

CSC are likely to undergo symmetric and asymmetric divisions in vivo, which results in the expansion of the tumorigenic cell population while producing a progeny of more differentiated cells that constitute the prevalent population of the tumor cell mass (Dalerba et al., 2007). It is becoming increasingly evident that cancer treatments that fail to eliminate CSC may allow regrowth of the tumor. Indeed, in cases in which bulk disease is eradicated and chemotherapy is given, only to be followed by a relapse, a plausible explanation is that the CSC have not been destroyed completely. In the present experiments, we found substantial resistance of pancreatic CSC to standard chemotherapy. Even very high concentrations of gemcitabine in vitro (up to 100 µg/ml) were not capable of inducing significant apoptosis and/or cell death in CSC, whereas virtually all other tumor cells were eradicated within a few days of gemcitabine treatment. Consistently, in vivo administration of gemcitabine in a mouse model of orthotopic pancreatic cancer resulted in a marked relative enrichment for pancreatic CSC as primarily nontumorigenic CD133⁻ cells were eliminated. Therefore, the further molecular characterization of the tumorigenic CD133⁺ pancreatic cancer cells identified in the present study will be crucial for the development of new therapeutic strategies to cope with this challenge in the future. Most importantly, the demonstrated possibility of expanding pancreatic CSC in vitro has considerable therapeutic implications for the evaluation of drug efficacy. This ability to maintain and even expand tumorigenic pancreatic cancer cells in vitro should be exploited for further preclinical studies.

However, the cancer stem cell concept will have to consider the existence of two forms of CSC in tumor progression, a stationary as well as an invasive CSC. The stationary CSC are still embedded in the epithelial tissue and are probably already active in benign precursor lesions (He et al., 2007). However, these stationary CSC cannot disseminate. In contrast, invasive CSC are located predominantly at the tumor-host interface. These invasive CSC, which combine the two perhaps most decisive traits, stemness and invasiveness, hold important clues for the further understanding of malignant progression. These cells have also been termed migrating CSC, and, indeed, we here provide evidence for their existence in pancreatic cancer and their important role in metastasis. Invasion and metastasis are not random processes but are highly organ specific and pathophysiologically organized involving multiple steps and numerous interactions between cancer cells and the host. The SDF-1/CXCR4 axis was originally detected in leukocytes being responsible for trafficking and homing of hematopoietic progenitors (Loetscher et al., 2000). Homozygous CXCR4-deficient mice are lethal, displaying diverse deformities (Zou et al., 1998). Recent reports suggest that CXCR4 may well be a key regulator of tumor invasiveness leading to local progression

and tumor metastasis (Burger and Kipps, 2006). Hypoxia-inducible factor (HIF-1) overexpression or functional loss of the HIF-suppressing pVHL-protein (von Hippel-Lindau) result in CXCR4 expression in renal cell carcinoma, further supporting a potential role of increased CXCR4 expression in tumor spread (Staller et al., 2003; Zagzag et al., 2005). From a clinical perspective, enhanced CXCR4 expression is associated with poor outcome in renal cancer (Zagzag et al., 2005). SDF-1, the specific ligand of CXCR4, is strongly expressed in lung, liver, bone marrow, and lymph nodes, sites that are commonly affected by pancreatic cancer metastases. In analogy to the directed homing of leukocytes, these gradients of SDF-1 may attract tumor cells and regulate proliferation and invasion at specific metastatic sites (Bachelder et al., 2002; Muller et al., 2001; Schneider et al., 2002), which has been shown for pancreatic cancer in a mouse model of experimental metastasis (Saur et al., 2005). This SDF-1/CXCR4 system also appears to regulate metastasis of other cancer types, including cancers of colon, lung, and breast (Kucia et al., 2005; Muller et al., 2001; Zeelenberg et al., 2003). Consistently, we demonstrate that the migration of invasive CSC is primarily mediated through activation of the CXCR4 receptor. It is most intriguing that in an in vivo model of orthotopic pancreatic cancer, implantation of a CSC population, which was depleted for these migrating CSC (CD133⁺ CXCR4⁻), virtually abrogated the metastatic activity of the developing tumors. These data demonstrate for the first time that in the utilized in vivo system a specific CD133⁺ CXCR4⁺ CSC subpopulation is the only population responsible for tumor metastasis. Together with data emerging from other tumor entities, this creates a considerable interest in the specific targeting and eradication of CSC (Clarke et al., 2006; Dalerba et al., 2007).

EXPERIMENTAL PROCEDURES

Human Pancreatic Cancer Cells and Culture Conditions

We used a highly metastatic cell line (L3.6pl) isolated after successive cycles of selections in nude mice (Bruns et al., 1999). The original COLO 357 human pancreatic cancer cell line established from a celiac lymph node metastasis of well-differentiated, mucin-containing pancreatic cancer cells was injected into the spleen of nude mice to isolate liver metastases designated as the L3.3 line. We then orthotopically injected the L3.3 cells into the pancreas of athymic mice and isolated liver metastases, which we designated as L3.4pl (pancreas-liver). After two additional orthotopic injection-selection cycles, we isolated the cells designated as L3.6pl, which produced spontaneous liver metastasis at a significantly higher incidence than the original COLO 357 cells (here referred to as FG cells). The highly metastatic L3.6pl human pancreatic cancer cells as well as the parental FG cells were maintained in DMEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 12% fetal calf serum (Biochrom, Berlin, Germany), glutamax (Invitrogen), sodium pyruvate, nonessential amino acids, and penicillin-streptomycin mixture, and a 2-fold vitamin solution (all from PAN, Aidenbach, Germany). Adherent monolayer cultures were maintained on plastic dishes and incubated at 37°C and 5% CO₂. Cultures were maintained for less than 6 weeks after recovery from frozen stocks.

To propagate the CSC fraction of the tumor cells, culture conditions favoring proliferation of undifferentiated cells were selected. To avoid adhesion and subsequent differentiation of CSC, we cultivated the

cells as hanging drops at a density of 10^3 cells/25 μ l, as previously described, for at least 14 days and until formation of spheres could be observed (Denham et al., 2006). Cells were grown in a NS-A basal serum-free medium (Euroclone, Irvine, UK), containing 2 mM L-glutamine, 0.6% glucose, 9.6 μ g/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin sodium salt (Sigma, St Louis, MO), and supplemented with 20 ng/ml EGF (PeproTech EC, London, UK) and 10 ng/ml FGF-2 (PeproTech EC). Cultures of differentiated tumor cells were obtained from tumor spheres after removal of growth factors and addition of 5% FCS and cultured for 7 days.

Prospective Isolation of Putative CSC

CSC were isolated by magnetic bead sorting using the MidiMACS system (Miltenyi Biotech). For this purpose, tumor cell suspensions from solid tumors were prepared by mechanical dissociation and incubated with collagenase type IV (Sigma) for 1 hr under continuous shaking at 37°C. In other experiments, cultured tumor cells were trypsinized, washed, and resuspended in PBS (Invitrogen). Cells were then incubated with a monoclonal CD133 antibody labeled with MicroBeads (Miltenyi Biotech) for 30 min at 4°C, and CD133⁺ cells were enriched using a MidiMACS magnet and MS columns (Miltenyi Biotech). All MACS procedures were performed according to the manufacturer's instructions. The purity of isolated cells was determined by standard flow cytometry analysis using an APC-labeled antibody against human CD133/2 (clone 293C3). The purity of isolated CD133⁺ CSC regularly exceeded 90%. For fluorescence activated cell sorting (FACS), cells were stained with APC-labeled CD133 antibodies and sorted by using a modular MoFlo device and Summit software (Cell Sorting Facility of the Institute of Molecular Immunology at Helmholtz Center for Environment and Health, Munich, Germany).

Antibodies

To characterize pancreatic CSC, the following antibodies were used: anti-CD133/1-APC (clone AC133) for cell isolation and characterization and anti-CD133/2-APC (clone 293C3; both from Miltenyi Biotech, Bergisch Gladbach, Germany) for determination of the purity of the isolated cells; anti-c-Met-FITC, anti-CD24-FITC, anti-CD31-PE, or anti-CD44-PE (all from ebiosciences San Diego, CA); anti-CD45-PE, anti-cytokeratin [5, 6, 8, 17, 19]-FITC (both from Dako, Hamburg, Germany); anti-CXCR4-PE (R&D systems, Minneapolis, MN); anti-ESA-APC (biomeda, Foster City, CA); or appropriate isotype-matched control antibodies. Samples were analyzed by flow cytometry, using a FACSCalibur (Becton Dickinson, Heidelberg, Germany), and data were analyzed with CELLQuest Pro software (Becton Dickinson).

Transmigration Assay

A total of 5×10^5 isolated tumor cells were resuspended in 250 μ l of DMEM containing 1% FCS and placed in the upper chamber of a modified Boyden chamber filled with Matrigel (BioCoat invasion assay, 8 μ m pore size, Becton Dickinson Labware, Two Oak Park, Bedford, MA). The upper chamber was placed in a 24-well culture dish containing 500 μ l of the medium described above supplemented with 100 ng/ml SDF-1 (PeproTech EC). After 24 hr of incubation at 37°C, transmigrated cells were pelleted and counted using a Neubauer chamber.

Cell-Cycle Analysis

L3.6 pl cells were cultivated under normal conditions as described above. Gemcitabine was added at a concentration of 100 ng/ml for 24 hr, and cells were then pulsed with BrdU (BD) for 2 hr. In some experiments, gemcitabine was withdrawn after 24 hr, and normal culture medium was added for another 24 hr. Cell-cycle analysis was performed with the BrdU flow cytometry kit (BD) according to the manufacturer's instructions.

Animals and Orthotopic Implantation of Tumor Cells

Athymic 8- to 10-week-old mice (NMRI-nu/nu) were purchased from Charles River (Sulzfeld, Germany). The mice were housed and main-

tained in laminar flow cabinets under specific pathogen-free conditions in accordance with current regulations and standards of the Government of Upper Bavaria, Germany. The mice were used in accordance with institutional guidelines when they were 8–12 weeks of age. To generate tumors, suspensions consisting of single cells with >90% viability were orthotopically injected into the pancreas as described previously (Bruns et al., 1999). The size and weight of the primary pancreatic tumors, the incidence of regional (celiac and para-aortal) lymph node metastasis, and the number of liver metastases were recorded. Histopathology confirmed the nature of the disease. In some experiments, 7 days after implantation of tumor cells into the pancreas, mice were randomized to treatment with twice-a-week intraperitoneal injections of gemcitabine for 21 days at 125 mg/kg or vehicle.

Histological Analysis

For histological evaluation of the tumors, one part of the tumor tissue was fixed in formalin and embedded in paraffin. Another part of the tumor was embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) and stored at -80°C . Liver metastases were counted following staining of liver sections for cytokeratin 5, 6, 8, 17, 19 (Dako) and hemalaun (Sigma). For the detection of CSC, sections were double stained with APC-labeled CD133 monoclonal antibodies (Miltenyi) and antibodies against cytokeratin or CXCR4. Nuclei were identified by DAPI (Vector Labs, Burlingame, CA). CSC frequency was defined as the number of CD133⁺ cells per high-power field. All images were generated on a Zeiss LSM 510 meta confocal laser scanning microscope.

Statistical Analysis

Results for continuous variables are expressed as means \pm standard deviation. Treatment groups were compared with the independent samples' t test. In case of nonnormal distribution, the Mann-Whitney U test was used. Pairwise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. P values < 0.05 were considered statistically significant. All analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL).

Supplemental Data

Supplemental Data include one table and four figures and can be found with this article online at <http://www.cellstemcell.com/cgi/content/full/1/3/313/DC1/>.

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