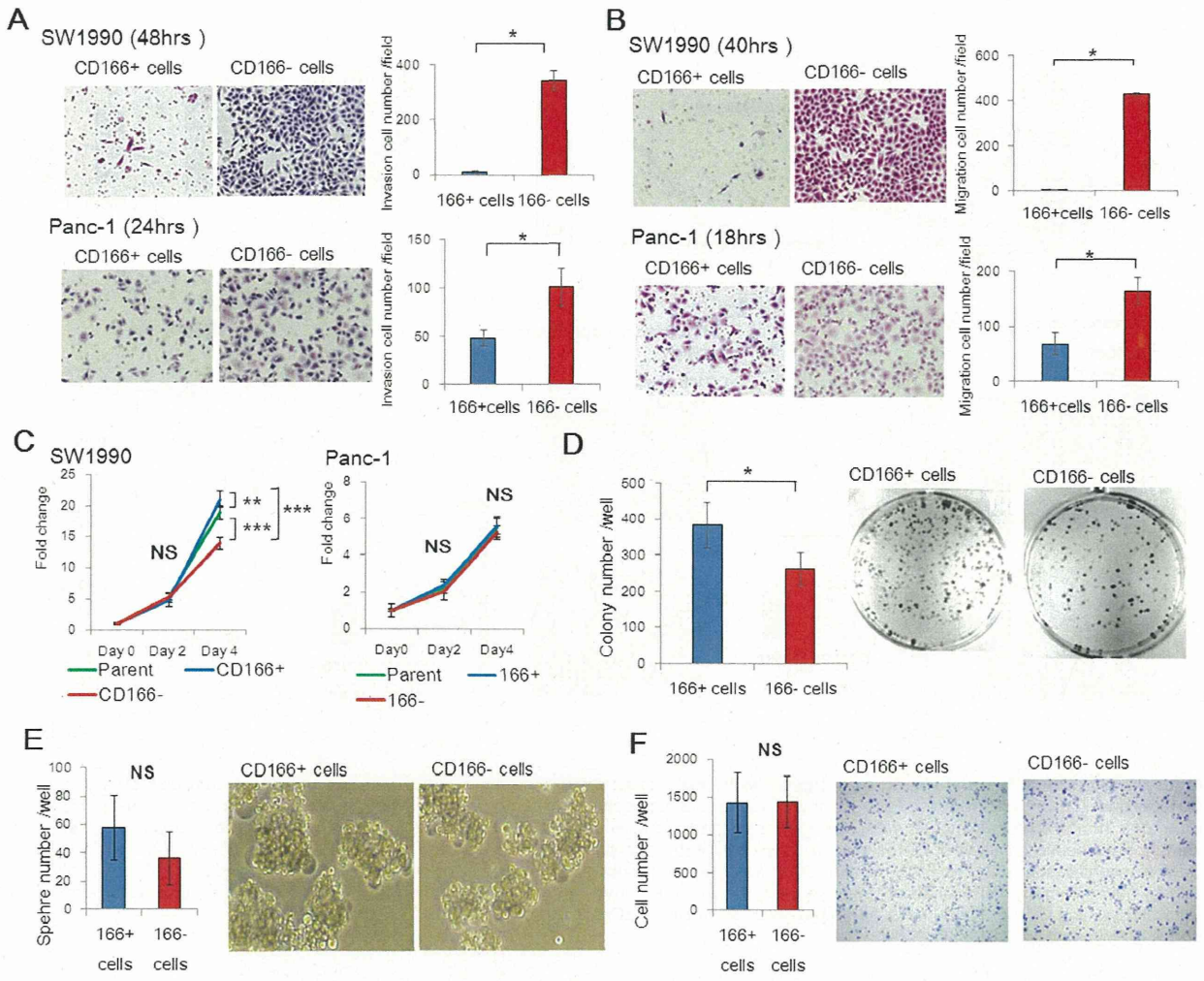

Figure 3. CD166- cells show high invasive and migratory activities. (A) Invasion assays of CD166+ and CD166- cells derived from SW1990 and Panc-1 cell lines were performed by culturing the cells on Matrigel-coated transwell inserts. After the indicated times, the cells on the lower membrane of the inserts were stained with H&E (representative images are shown) and quantified. (B) Migration assays of CD166+ and CD166- cells from SW1990 and Panc-1 cell lines were performed by culturing the cells on inserts. After the indicated times, the cells on the lower membrane of the inserts were stained with H&E (representative images are shown) and quantified. Original magnification: 200×. (C) Proliferation of CD166+ and CD166- cells derived from SW1990 and Panc-1 cell lines was measured at the indicated times post-initial seeding. (D) Quantification and representative images of the colony formation capacities of CD166+ and CD166- Panc-1 cells. (E) Quantification and representative images of the sphere formation capacities of CD166+ and CD166- Panc-1 cells. (F) Quantification and representative images of the adhesive capacities of CD166+ and CD166- Panc-1 cells. Original magnification: 40×. Data represent the mean ± SD; *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$; NS, not significant.
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To explore whether CD166 plays a role in metastasis, we used two previously established metastatic pancreatic cancer cell lines that were generated from liver metastases in nude mouse xenograft models [15]. These cell lines, metastatic Panc-1 and metastatic SUIT-2, showed a stronger liver metastatic potential compared with that of their parental cell lines. In the metastatic Panc-1 cell line, the CD166 expression rate was significantly increased compared with that of the parental cells (99.2% vs. 46.9%, Figure 6B). In the SUIT-2 cell line, most cells expressed CD166 in both parental SUIT-2 cells (99.3%) and metastatic SUIT-2 cells (99.4%). qRT-PCR analysis showed that the levels of CD166 mRNA in both metastatic Panc-1 and metastatic SUIT-2 cells were significantly greater than those in their parental cell lines ($p<0.0001$ and $p=0.0015$ for Panc-1 and SUIT-2, respectively, Figure 6C).

Discussion

In the present study, immunohistochemistry showed that $CD166^{high}$ was often found in pancreatic cancer tissues compared to normal pancreatic tissues. Our flow cytometric analyses of resected pancreatic cancer tissues revealed that the percentage of CD166+ cells ranged from 33.8 to 70.2% among EpCAM+ cells, suggesting that CD166 expression was frequent in pancreatic cancers. However, the role of CD166 expression has not been clarified in pancreatic cancer. Previously, the roles of CD166 have been reported in several other types of cancer. However, the findings are controversial, because the cells expressing CD166

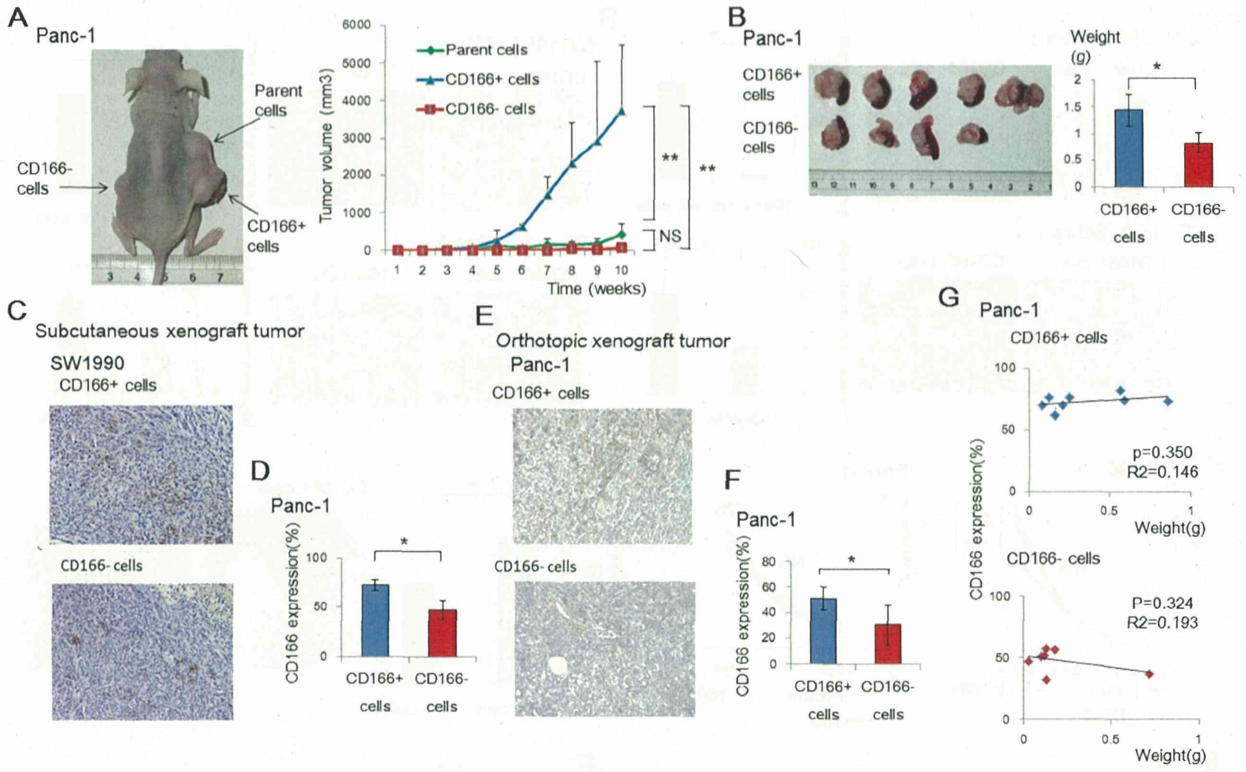

Figure 4. CD166+ cells show strong tumorigenicity in mouse xenograft models. (A) Mice were subcutaneously transplanted with parental, CD166+, and CD166- cells from the Panc-1 cell line (representative image), and tumor volumes were regularly measured for 7 weeks. (B) Mouse orthotopic tumor xenograft models were also generated from CD166+ and CD166- Panc-1 cells. Tumors were excised and wet weighed. (C, E) Immunohistochemical analysis of CD166 in subcutaneous (C) and orthotopic (E) tumors derived from CD166+ and CD166- SW1990 and Panc-1 cells. Original magnification: 100×. (D, F) CD166 expression was analyzed in subcutaneous (D) and orthotopic (F) tumors derived from CD166+ and CD166- cells by flow cytometry. (G) Analysis of the relationship between tumor weight and the positivity rate of CD166 cells in orthotopic tumors derived from CD166+ and CD166- Panc-1 cells. All graphs show the mean ± SD; *, $p<0.05$, **, $p<0.01$.
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show strong tumorigenicities and inhibition of CD166 enhances invasive and migratory activities [6,7,9,10].

Several studies have reported that CD166+ cancer cells in colon and prostate cancers might represent CSCs, because these cells exhibit strong *in vivo* tumorigenicity [6,7]. In the present study, we found that the CD166+ pancreatic cancer cells had stronger tumorigenicity than that of their CD166- counterparts *in vivo*. We also found no differences in the expression rates of other

Table 3. Tumorigenic potential of CD166+/CD166- cells derived from the Panc-1 cell line.

		No. of mice with tumor formation (Tumor volume>100 mm3)		
	No. of injected cells	Euthanized at 6 weeks	8 weeks	10 weeks
Parent	4×10 4	0/5	0/5	0/5
	2×10 5	0/5	2/5	4/5
	1×10 6	0/5	3/5	4/5
CD166+ cells	4×10 4	0/5	0/5	0/5
	2×10 5	0/5	0/5	0/5
	1×10 6	4/5	4/5	4/5
CD166- cells	4×10 4	0/5	0/5	0/5
	2×10 5	0/5	0/5	0/5
	1×10 6	0/5	0/5	1/5

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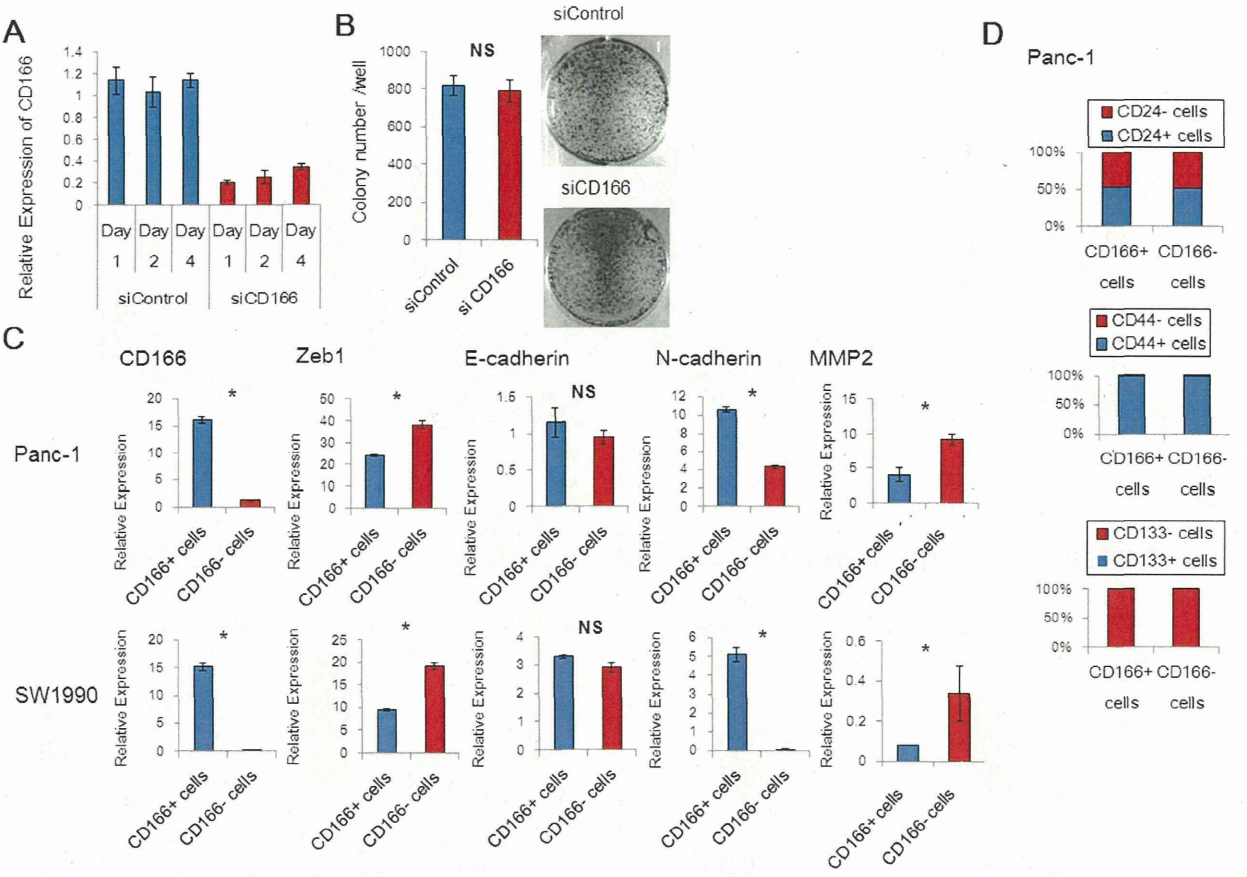

Figure 5. CD166- Cells Over-express the EMT Activator Zeb1. (A) qRT-PCR analysis of the mRNA levels of CD166 in SUIT-2 cells after RNA interference was performed at the indicated days post-transfection. Control (siControl) or CD166 silenced cells (siCD166) were analyzed by (B) colony formation assays at the indicated days post-transfection. (C) qRT-PCR analysis of EMT markers E-cadherin, N-cadherin, Zeb-1, and MMP2 in CD166+ and CD166- Panc-1 and SW1990 cells. (D) The relationships between expression of CD166 and CSC markers CD24, CD44, and CD133 in Panc-1 cells were analyzed by flow cytometry. Data represent the mean ± SD; *, $p<0.05$; NS, not significant.
doi:10.1371/journal.pone.0107247.g005

pancreatic CSC candidate markers, CD24, CD44, and CD133 [23,24], between CD166+ and CD166- cells. These results suggest that CD166 might be an independent marker of pancreatic CSCs. The present study revealed that CD166+ cells showed greater proliferation and colony formation abilities than those of CD166- cells *in vitro*. Although a greater sphere formation ability of CD166+ cancer cells has been reported in colon cancer, prostate cancer, and head and neck squamous cell carcinoma [6,7,30], our study showed that there was no significant difference in the sphere formation capacities of CD166+ and CD166- cells.

On the other hand, we showed that the CD166- population of pancreatic cancer cells had stronger invasive and migratory activities compared with those of the CD166+ population *in vitro*. The relationship between invasive/migratory abilities and CD166 expression has been previously reported in other types of cancer including endothelial-like yolk sac cells [31], epithelial ovarian carcinoma cells [9], and glioblastoma cells [10]. We also investigated the expression levels of EMT-associated genes in relation to the status of CD166 expression. We found that an EMT activator, Zeb1, was over-expressed in CD166- cells compared with that in CD166+ cells. However, there were no differences in morphology or expression of the epithelial marker E-cadherin. The role of CD166 might be related to pancreatic CSCs or EMT, but there are some controversial points.

To identify other key molecules involved in CD166 expression, our study showed that the level of MMP2 mRNA was greater in CD166- cells than that in CD166+ cells. Additionally, microarray analysis identified several genes that were differentially expressed in CD166+ and CD166- cells, including four genes, TSPAN8, BST2, BMP7, and ColA1, which are related to invasive and migratory activities or tumorigenicity [25–29]. Swart et al. and Hong et al. reported an association of CD166 with adhesiveness [5,12]. Adhesiveness might cause the functional differences between CD166+ and CD166- cells; however, Hong et al. reported that knockdown of CD166 by RNA interference reduces cell adhesion but does not affect growth or invasion of pancreatic cancer cells [12]. Our study revealed that CD166+ cells showed strong proliferative activities, but there was no significant difference in the adhesive capabilities of CD166+ and CD166- cells. Further investigation is needed to clarify the functional difference between CD166+ and CD166- pancreatic cancer cells.

We examined the relationship between CD166 expression and metastatic potential in two previously established metastatic pancreatic cancer cell lines [15]. In these cell lines, the levels of

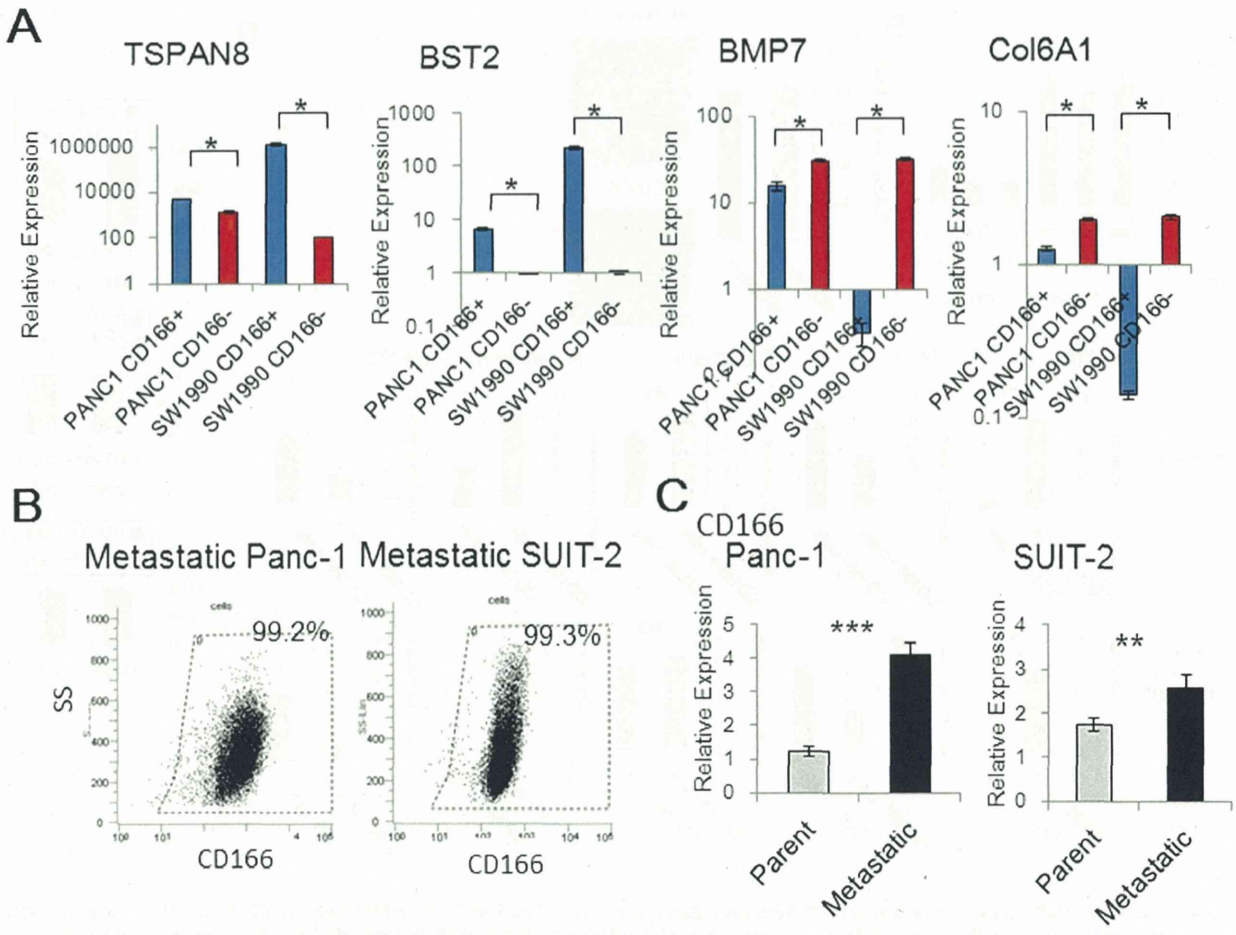

Figure 6. Over-expression of TSPAN8 and BST2 in CD166+ cells, and BMP7 and Col6A1 in CD166- cells. (A) qRT-PCR was used to analyze the mRNA levels of TSPAN8, BST2, BMP7, and Col6A1, which were found to be up-regulated by more than 2-fold in CD166+ and CD166- cells in comparisons of microarray data. (B) The positivity rate of CD166 in metastatic Panc-1 and metastatic SUIT-2 cell lines was measured by flow cytometry. (C) qRT-PCR analysis was also used to examine CD166 mRNA levels in the metastatic cell lines. Data represent the mean ± SD; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.
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CD166 mRNA expression were greater than those in their parental cell lines. Therefore, CD166 expression might be associated with the metastatic behavior of pancreatic cancer cells.

In conclusion, we have revealed that CD166+ pancreatic cancer cells are highly tumorigenic, whereas CD166- pancreatic cancer cells exhibit stronger invasive and migratory activities. Although further investigations are needed to uncover the mechanisms underlying these functional differences, this study demonstrates that CD166 expression is related to different functions in pancreatic cancer cells.

Supporting Information

Figure S1 Analysis of the relationships between CD166 positivity rates and malignant potential indicators (invasion, migration, and proliferation) in pancreatic cancer cell lines.
(TIF)

Figure S2 Mice were subcutaneously transplanted with parental, CD166+, and CD166− cells from the SW1990 cell line (representative image) and tumor volumes were regularly measured for 7 weeks. Data represent the mean ± SD; NS, not significant.
(TIF)

Figure S3 Effects of CD166 silencing by RNA interference on pancreatic cancer cell behavior. Control (siControl) or CD166 silenced cells (siCD166) were analyzed by (A) invasion assays and (B) migration assays at the indicated days post-transfection. Original magnification: 200×. (C) Proliferation assay. Data represent the mean ± SD; NS, not significant.
(TIF)

Figure S4 Effect of CD166 knockdown in SUIT-2 cells on the expression levels of TSPAN8, BST2, BMP7, and Col6A1. SUIT-2 cells were transfected with CD166-targeting (siCD166) or control siRNA (siControl), and the expression levels of the four genes were assessed by qRT-PCR. Data represent the mean ± SD; ***, $p<0.001$; NS, not significant.
(TIF)

Table S1 Relationships between CD166 expression and clinicopathological factors.
(DOCX)

Table S2 CD166 positivity rates and malignant potential indicators (invasion, migration, and proliferation) in each pancreatic cancer cell line as reported previously.
(DOCX)

Table S3 Tumorigenic potential of CD166+/− cells derived from the SW1990 cell line.
(DOCX)

Table S4 Differentially expressed genes by >2-fold in CD166+ cells. ($p<0.05$).
(DOCX)

Table S5 Differentially expressed genes by >2-fold in CD166- cells. ($p<0.05$).
(DOCX)

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Author Contributions

Conceived and designed the experiments: KF KO. Performed the experiments: KF CU. Analyzed the data: MS KH KS YO. Contributed reagents/materials/analysis tools: KS TO ST YO. Contributed to the writing of the manuscript: KF KO MS KM YO MT.

References

1. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M (2011) Pancreatic cancer. Lancet 378: 607–620.
2. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, et al. (2010) Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: A randomized controlled trial. JAMA 304: 1073–1081.
3. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414: 105–111.
4. Ohneda O, Ohneda K, Arai F, Lee J, Miyamoto T, et al. (2001) ALCAM (CD166): Its role in hematopoietic and endothelial development. Blood 98: 2134–2142.
5. Swart GW (2002) Activated leukocyte cell adhesion molecule (CD166/ALCAM): Developmental and mechanistic aspects of cell clustering and cell migration. Eur J Cell Biol 81: 313–321.
6. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, et al. (2007) Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 104: 10158–10163.
7. Rajasekhar VK, Studer L, Gerald W, Socci ND, Scher HI (2011) Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF-kappaB signalling. Nat Commun 2: 162.
8. Weidle UH, Eggle D, Klostermann S, Swart GW (2010) ALCAM/CD166: Cancer-related issues. Cancer Genomics Proteomics 7: 231–243.
9. Rosso O, Piazza T, Bongarzone I, Rossello A, Mezzanzanica D, et al. (2007) The ALCAM shedding by the metalloprotease ADAM17/TACE is involved in motility of ovarian carcinoma cells. Mol Cancer Res 5: 1246–1253.
10. Kijima N, Hosen N, Kagawa N, Hashimoto N, Nakano A, et al. (2012) CD166/activated leukocyte cell adhesion molecule is expressed on glioblastoma progenitor cells and involved in the regulation of tumor cell invasion. Neuro Oncol 14: 1254–1264.
11. Kahlert C, Weber H, Mogler C, Bergmann F, Schirmacher P, et al. (2009) Increased expression of ALCAM/CD166 in pancreatic cancer is an independent prognostic marker for poor survival and early tumour relapse. Br J Cancer 101: 457–464.
12. Hong X, Michalski CW, Kong B, Zhang W, Raggi MC, et al. (2010) ALCAM is associated with chemoresistance and tumor cell adhesion in pancreatic cancer. J Surg Oncol 101: 564–569.
13. Tachezy M, Zander H, Marx AH, Stahl PR, Gebauer F, et al. (2012) ALCAM (CD166) expression and serum levels in pancreatic cancer. PLoS One 7: e39018.
14. Bachem MG, Schunemann M, Ramadani M, Siech M, Beger H, et al. (2005) Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. Gastroenterology 128: 907–921.
15. Eguchi D, Ohuchida K, Kozono S, Ikenaga N, Shindo K, et al. (2013) MAL2 expression predicts distant metastasis and short survival in pancreatic cancer. Surgery 154: 573–582.
16. Ohuchida K, Mizumoto K, Murakami M, Qian LW, Sato N, et al. (2004) Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. Cancer Res 64: 3215–3222.
17. Ikenaga N, Ohuchida K, Mizumoto K, Cui L, Kayashima T, et al. (2010) CD10+ pancreatic stellate cells enhance the progression of pancreatic cancer. Gastroenterology 139: 1041–51, 1051.e1–8.
18. Zhang L, Mizumoto K, Sato N, Ogawa T, Kusumoto M, et al. (1999) Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin. Cancer Lett 142: 129–137.
19. Kozono S, Ohuchida K, Eguchi D, Ikenaga N, Fujiwara K, et al. (2013) Pirfenidone inhibits pancreatic cancer desmoplasia by regulating stellate cells. Cancer Res 73: 2345–2356.
20. Sato N, Maehara N, Mizumoto K, Nagai E, Yasoshima T, et al. (2001) Telomerase activity of cultured human pancreatic carcinoma cell lines correlates with their potential for migration and invasion. Cancer 91: 496–504.
21. Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139: 871–890.
22. Liu J, Ben QW, Yao WY, Zhang JJ, Chen DF, et al. (2012) BMP2 induces PANC-1 cell invasion by MMP-2 overexpression through ROS and ERK. Front Biosci (Landmark Ed) 17: 2541–2549.
23. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, et al. (2007) Identification of pancreatic cancer stem cells. Cancer Res 67: 1030–1037.
24. Hori Y (2013) Prominin-1 (CD133) reveals new faces of pancreatic progenitor cells and cancer stem cells: Current knowledge and therapeutic perspectives. Adv Exp Med Biol 777: 185–196.
25. Wang H, Rana S, Giese N, Buchler MW, Zoller M (2013) Tspan8, CD44v6 and alpha6beta4 are biomarkers of migrating pancreatic cancer-initiating cells. Int J Cancer 133: 416–426.
26. Gu G, Zhao D, Yin Z, Liu P (2012) BST-2 binding with cellular MT1-MMP blocks cell growth and migration via decreasing MMP2 activity. J Cell Biochem 113: 1013–1021.
27. Notting I, Buijs J, Mintardjo R, van der Horst G, Vukicevic S, et al. (2007) Bone morphogenetic protein 7 inhibits tumor growth of human uveal melanoma in vivo. Invest Ophthalmol Vis Sci 48: 4882–4889.
28. Sakai H, Furihata M, Matsuda C, Takahashi M, Miyazaki H, et al. (2012) Augmented autocrine bone morphogenic protein (BMP) 7 signaling increases the metastatic potential of mouse breast cancer cells. Clin Exp Metastasis 29: 327–338.
29. Chiu KH, Chang YH, Wu YS, Lee SH, Liao PC (2011) Quantitative secretome analysis reveals that COL6A1 is a metastasis-associated protein using stacking gel-aided purification combined with iTRAQ labeling. J Proteome Res 10: 1110–1125.
30. Yan M, Yang X, Wang L, Clark D, Zuo H, et al. (2013) Plasma membrane proteomics of tumor spheres identify CD166 as a novel marker for cancer stem-like cells in head and neck squamous cell carcinoma. Mol Cell Proteomics 12: 3271–3284.
31. Ikeda K, Quertermous T (2004) Molecular isolation and characterization of a soluble isoform of activated leukocyte cell adhesion molecule that modulates endothelial cell function. J Biol Chem 279: 55315–55323.