Susceptibility of pancreatic cancer stem cells to reprogramming

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Takahashi and Yamanaka(1) first discovered that adult mice fibroblasts could be reprogrammed into a pluripotent state using only four key transgenes (4F: Oct3/4, Sox2, Klf4 and cMyc) and termed these cells “induced pluripotent stem (iPS) cells.” Thereafter, numerous studies have shown that various types of human differentiated cells from normal tissues can also be induced into iPS cells; thus, iPS cell technology has now become the most promising innovation for regenerative medicine.(2,3) In malignant cells, Carette et al.(4) first applied 4F reprogramming to chronic myeloid leukemia cells and showed that ectopic expression of 4F-enabled differentiation into three germ layer cell types. Our previous studies have demonstrated that reprogramming of gastrointestinal cancer cells can decrease their chemoresistant and tumorigenic features in both in vitro and in vivo.(5,6) These results suggest that reprogramming of gastrointestinal cancer cells modifies their malignant phenotypes.

Many types of malignant tumors, including pancreatic cancer, have poor clinical outcomes despite advances in chemotherapeutic agents.(7,8) Many studies have indicated that this is partly because of the existence of a specific subpopulation of cells known as cancer stem cells (CSC), which are characterized by their quiescence, self-renewal capacity, heterogeneity, and multipotency rather than pluripotency, similar to the properties of normal somatic stem cells. In addition, CSC are now well known to show relative resistance to most conventional chemotherapeutic agents.(9) Hence, to create a durable clinical response, it is very important to develop effective treatments that target and eliminate CSC. Although our previous studies have demonstrated that reprogramming of serial gastrointestinal cancer cells can decrease their chemoresistance and tumorigenicity, no previous reports have shown whether CSC, which should be targeted, are sensitive to reprogramming.

To isolate CSC from a heterogeneous population prospectively, many cell surface markers are commonly used in various types of malignant cells. Li et al.(10) report that in pancreatic cancer, c-Met, a member of the receptor tyrosine kinase family, is currently the most specific marker of pancreatic CSC in vitro and in vivo. Here we studied the susceptibility of pancreatic CSC and non-CSC to reprogramming using the CSC marker c-Met and demonstrated that pancreatic CSC are favorable targets for reprogramming.

Material and Methods

RNA isolation, RT-PCR and quantitative RT-PCR analysis. Total RNA was prepared using TRIzol reagent (Life Technologies,
Gaithersburg, MD, USA), and RT was performed using the SuperScript III RT First-Strand Synthesis System according to the manufacturer’s instructions (Life Technologies). To confirm PCR amplification, 35 cycles of PCR were performed using a PCR kit (Takara, Kyoto, Japan) on the GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 10 s, 60°C for 10 s and 72°C for 60 s. An 8-μL aliquot of each reaction mixture was size-fractionated on a 2% agarose gel and visualized using ethidium bromide staining. To confirm mRNA quality, PCR amplification was performed for GAPDH using specific primers. For quantitative assessment, gene expression was evaluated by quantitative RT-PCR (qRT-PCR) using the LightCycler CYBR Green Master kit (Roche Diagnostics, Mannheim, Germany) for cDNA amplification of specific target genes. The expression of mRNA copies was normalized against GAPDH mRNA expression. All primer sequences are listed in Table S1.

Immunocytochemistry. The immunocytochemical examination was performed using antibodies to detect pluripotent markers (anti-Oct3/4, anti-Sox2, anti-Nanog or anti-Tra-1-60) in accordance with the manufacturer’s instructions (Cell Signaling Technology, Beverly, MA, USA).

Other methods. Additional methods are described in Data S1.

Results

PANC-1 cells induced with reprogramming factors presented induced pluripotent stem-like phenotype. We previously reported that reprogramming using retroviral or lentiviral vectors, which are DNA viruses, altered their malignant phenotypes. However, potential risks of exogenous genomic insertion remain. Because Sendai viral vectors, which are RNA viruses, have the advantage of avoiding undesirable genomic alteration,(11) we applied Sendai viral vectors for reprogramming and estimated the optimum conditions needed.

PANC-1 cells were transfected with Sendai virus carrying blue fluorescent protein (BFP) within a certain range of multiplicity of infection (MOI: 0, 3, 30, 100) on day 0. Fluorescence microscopy confirmed that >80% of PANC-1 cells efficiently expressed BFP at an MOI of 30 by day 3 (Fig. S1). In contrast, <30% of PANC-1 cells expressed BFP at an MOI of 3. Thus, we used an MOI dose of 30 in accordance with our protocol (Fig. 1a). We infected PANC-1 cells using a series of Sendai viruses carrying four defined transgenes, OCT3/4, SOX2, KLF4 and cMYC, on day 0. RT-PCR detected expression of these four transgenes on day 3 (Fig. 1b). According to our reprogramming protocol, PANC-1 cells were cultured on a mouse embryo fibroblast feeder with human ES medium supplemented with 0.5 μM PD0325901, 3 μM CHIR99021 and 0.5 μM A83-01 from day 7. By day 21, a number of iPSC colonies with a clear, flat, well-defined appearance and high density of granulated cells had gradually formed (Fig. 1c).

Because many types of pluripotent stem cells, including iPSC cells, are well known to exhibit alkaline phosphatase (ALP) activity, an ALP staining assay is often used as a screening for undifferentiated status. We subjected the iPSC colonies to the ALP staining assay that showed strongly positive ALP activity (Fig. 1d). The ALP-positive colonies were also tested for expression of other undifferentiated cell markers, Oct3/4, Sox2, Nanog and Tra-1-60, by immunofluorescence staining (Fig. 1e). Efficient expression of not only exogenous Oct3/4 and Sox2 but also endogenous Nanog and Tra-1-60 in the iPSC cells was observed.

To assess the differentiation potential of the iPSC colonies, the colonies were picked up and exposed to 5 days of floating culture conditions followed by 7 days of adherent culture conditions. We termed the cells arising from the subsequent culture “post-iPSC cells” and compared them with their parental cells using qRT-PCR. The relative expression levels of three serial germ layer markers (TUBB3 [ectoderm], T [mesoderm] and GATA4 [endoderm]) were significantly higher in the post-iPSC cells than in their parental cells (Fig. 1f). Thus, induced PANC-1 cells were positive for ALP activity and other undifferentiated markers, and simultaneously exhibited potential for differentiation into the other two germ layer derivatives, which indicated that they had become pluripotent stem cells.

c-Met is a good marker of cancer stem cells in pancreatic cancer. Recent studies have reported that c-Met, or the combination of c-Met and CD44, is one of the most specific markers

![Image](https://www.wileyonlinelibrary.com/journal/cas)
CSC in pancreatic cancer. Therefore, we aimed to separate pancreatic CSC using these two cell surface markers by flow cytometry (Fig. 2a). The population expressing c-Met accounted for 1.2% of all cells. In contrast, almost all PANC-1 cells expressed CD44; thus, this marker was not suitable for isolating CSC. Consequently, for further analysis, we focused on c-Met as a pancreatic CSC marker.

To determine the CSC characteristics of cells expressing high levels of c-Met (c-Met [high] population) and of those exhibiting low c-Met expression (c-Met [low] population), we performed

Fig. 2. c-Met (high) population among PANC-1 cells represents high CSC-like phenotypes. (a) Typical FACS plot showing c-Met (high) and CD44 (+) frequencies in PANC-1 cells. (b) Representative images of spheres. Bar = 50 μm. Total number of spheres. N = 6 per group. Data are represented as the mean ± SD and were analyzed by two-tailed unpaired t-tests (**P < 0.01, c-Met [high] vs c-Met [low]). (c) Cell sensitivities to gemcitabine. n = 3. Data are represented as the mean ± SD and were analyzed by two-way measures analysis of variance (**P < 0.01, c-Met [high] vs c-Met [low]). (d) qRT-PCR of endogenous reprogramming transcription factors. n = 3. Data are represented as the mean ± SD and were analyzed by two-tailed unpaired t-tests (**P < 0.01, c-Met [high] vs c-Met [low]). (e) Quantitative RT-PCR of endogenous reprogramming transcription factors. n = 3. Data are represented as the mean ± SD and were analyzed by two-tailed unpaired t-tests (**P < 0.01, c-Met [high] vs c-Met [low]). (f) 1 × 10^6 of c-Met (high) and c-Met (low) cells were transplanted in NOD-SCID mice, and the xenograft tumor volume was monitored up to week 5. n = 10. Data are represented as the mean ± SD and were analyzed by two-way repeated measures analysis of variance (**P < 0.01, c-Met [high] vs c-Met [low]). (g) The weights of tumors were measured on week 5 after resection. n = 10. Data are represented as the mean ± SD and were analyzed using the Mann–Whitney U-test (**P < 0.01, c-Met [high] vs c-Met [low]). CSC, cancer stem cell.
sphere formation assays that reflected the cells’ self-renewal capacity in vitro. The sphere formation assays demonstrated that the c-Met (high) population formed twice as many spheres as primary spheres \( (P < 0.01) \) and three times as many spheres as secondary spheres as did the c-Met (low) population \( (P < 0.01) \); Fig. 2b). We also tested cell viability on exposure to a chemotherapeutic agent, gemcitabine, using the MTT assays. The MTT assays showed that although gemcitabine inhibited proliferation of both cell populations in a dose-dependent manner, the c-Met (high) cells exhibited greater resistance to gemcitabine than did the c-Met (low) cells \( (P < 0.05) \); Fig. 2c).

Cancer stem cells are also known to endogenously express stemness markers, such as OCT3/4, SOX2 and NANOG, in many cancers.\(^{14,15}\) We quantified the expression levels of endogenous OCT3/4, SOX2, KLF4, cMYC and NANOG in the two populations. The results demonstrated that the expression levels of these key genes were significantly higher in c-Met (high) cells than in c-Met (low) cells (Fig. 2d).

Depending on its ligand, hepatocyte growth factor, c-Met not only is a CSC marker but also functions as a tyrosine kinase. Li et al.\(^{14}\) previously reported that c-Met signaling induced expression of reprogramming transcription factors associated with stemness, including OCT3/4, SOX2 and NANOG. We tested the efficacy of c-Met signaling inhibition on expression of reprogramming factors in the c-Met (high) population. SU11274, the c-Met inhibitor, was shown to be efficacious in the c-Met (low) population \( (P < 0.01) \); Fig. 2b). We also tested cell viability on exposure to a chemotherapeutic agent, gemcitabine, using the MTT assays. The MTT assays showed that although gemcitabine inhibited proliferation of both cell populations in a dose-dependent manner, the c-Met (high) cells exhibited greater resistance to gemcitabine than did the c-Met (low) cells \( (P < 0.05) \); Fig. 2c).

To address this question, we separated the two cell populations according to c-Met expression using the autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and evaluated their reprogramming efficiency. In the present study, because we demonstrated that the c-Met inhibitor, SU11274, inhibited expression of endogenous reprogramming factors, we simultaneously evaluated the efficacy of c-Met signaling inhibition on the reprogramming efficiency in both cell populations \( (P < 0.01) \). Transfer efficiency and cell viability have been assumed to be critical for successful reprogramming. To test directly whether the immature status or the transfer efficiency of a given cell type affects reprogramming, PANC-1 cells were sorted into c-Met (high) and c-Met (low) populations followed by being transfected with \( \text{SeV-BFP} \) at MOI 30, respectively. Fluorescence imaging and flow cytometry analysis on day 3 revealed that >95% of PANC-1 cells were positive for BFP in two populations, and no significant difference in transfer efficiency was observed (Fig. S3b,e). Furthermore, to evaluate the cell viability of these two populations under floating culture conditions, the PANC-1 cells of these two populations cultured under 6-day floating conditions were reseeded at a density of 1000 cells/well onto six-well attachment plates supplemented with DMEM containing 10% FBS. After culturing for 3 days, the colonies that appeared were stained with crystal violet, and the number of stained colonies was counted. Crystal violet staining showed that there was no significant difference in the numbers of stained colonies between these two populations (Fig. S3d).

Differentiation and reprogramming of cells are accompanied by drastic epigenetic modifications. Trimethylation of histone 3 lysine 4 (H3K4 me3) is frequently observed in the promoter regions of pluripotent stem cells and is known to be a hallmark of transcriptional activation in general. We measured H3K4 me3 occupancy in the promoter region of the stemness gene, SOX2, among c-Met (low) cells, c-Met (high) cells and iPSC cells using ChIP analysis. The ChIP analysis showed that H3K4 me3 was almost absent in the promoter regions of SOX2 of c-Met (low) cells and was enriched up to 0.2% in c-Met (high) cells \( (P < 0.01) \); Fig. S3e). In addition, not surprisingly, H3K4 me3 occupancy in the promoter regions of SOX2 of iPSC cells was dramatically increased relative to that in the other two populations \( (P < 0.01) \); Fig. S3e). This result was compatible with the results of the expression levels of SOX2 gene in each cell type \( (P < 0.01) \); Fig. S3f).

Together, these data indicate that the c-Met signaling pathway affects CSC properties and has an important role in the susceptibility to reprogramming in PANC-1 cells.
Discussion

In this study, we demonstrated that PANC-1 cells, which represent a pancreatic cancer cell line, could be induced to form iPSc-like colonies expressing embryonic stem cell markers homogeneously and to possess the potential to differentiate into three germ layer cells by 4F transduction. In addition, we showed that the c-Met (high) population, which was enriched in pancreatic CSC, was more susceptible to reprogramming than was the c-Met (low) counterparts. Although several studies, including ours, have demonstrated that several types of malignant tumor cells are capable of being induced into a pluripotent state by 4F reprogramming, it was assumed that c-Met signaling affects reprogramming efficiency, not directly but through stemness genes, such as OCT3/4 and SOX2. Interestingly, 3.5% of c-Met (low) cells were also positive for ALP staining. Of course, c-Met is not the master marker that can be used to distinguish pancreatic CSC from non-CSC completely, which may imply that reprogramming could detect unknown CSC in the c-Met (low) population.

We also studied reprogramming efficiency in other pancreatic cancer cell lines, MIA PaCa-2, PSN-1 and AsPC-1. However, we found that these cell lines were resistant to reprogramming. These results may be partly explained by innate genetic mutations related to the epithelial-to-mesenchymal transition pathway, such as OCT3/4 and SOX2. Interestingly, 3.5% of c-Met (low) cells were positive for ALP staining. Of course, c-Met is not the master marker that can be used to distinguish pancreatic CSC from non-CSC completely, which may imply that reprogramming could detect unknown CSC in the c-Met (low) population.

Some studies on the deep relationships between core reprogramming factors and CSC phenotype or carcinogenesis have been reported. Ohshima et al. demonstrated that induction of OCT3/4, SOX2 and KLF4 to colon cancer cells and cultivation with serum-containing medium, not human ES medium, induced CSC-like phenotype cells. Ohnishi et al. also report that insufficient reprogramming to normal cells by 4F is linked to carcinogenesis with epigenetic alterations. However, if reprogramming is achieved, the malignant phenotypes of the reprogrammed cells may decrease. Our previous study demonstrated that reprogrammed PANC-1 cells decreased their proliferation in vitro and tumorigenicity in vivo, indicating the rationale for further studies to achieve the high therapeutic efficiency.

Pancreatic cancer cells induced by 4F have shown decreased malignancy in vitro and in vivo. Together with our previous data, the results of the present study indicate that reprogramming technology may be useful for extensive epigenetic modification of the malignant features of pancreatic cancer cells with the aim of targeting CSC.

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Disclosure Statement

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Supporting Information

Additional supporting information may be found in the online version of this article:
Fig. S1. Representative fluorescence images of PANC-1 cells expressing blue fluorescent protein.
Fig. S2. CSC properties in the c-Met (high) population. CSC, cancer stem cell.
Fig. S3. Reprogramming efficiency of the c-Met (high) and c-Met (low) populations.
Table S1. A list of primer sequences.
Data S1. Supporting Methods.