Hypoxia Induces Tumor Aggressiveness and the Expansion of CD133-Positive Cells in a Hypoxia-Inducible Factor-1α-Dependent Manner in Pancreatic Cancer Cells

Hashimoto, Okito / Shimizu, Kazuya / Semba, Shuho / Chiba, Sachie / Ku Yonson / Yokozaki, Hiroshi / Hori, Yuichi

Pathobiology, 78(4):181-192

2011-01-19

Copyright (c) 2011 S. Karger AG, Basel

10.1159/000325538

http://www.lib.kobe-u.ac.jp/handle_kernel/90001571

PDF issue: 2018-10-18
Hypoxia Induces Tumor Aggressiveness and the Expansion of CD133-Positive Cells in a Hypoxia-Inducible Factor-1α Dependent Manner in Pancreatic Cancer Cells

Okito Hashimoto\textsuperscript{a} Kazuya Shimizu\textsuperscript{b} Shuho Semba\textsuperscript{a} Sachie Chiba\textsuperscript{c} Yonson Ku\textsuperscript{c} Hiroshi Yokozaki\textsuperscript{a} Yuichi Hori\textsuperscript{c}

\textsuperscript{a}Department of Pathology, Division of Pathology and \textsuperscript{c}Department of Surgery, Division of Hepato-Biliary-Pancreatic Surgery, Kobe University Graduate School of Medicine, \textsuperscript{b}Department of Internal Medicine, Kobe Medical Center, Kobe, Japan

**Short title:** Hypoxia induces CD133\textsuperscript{+} cells in pancreatic cancer

Corresponding author. Yuichi Hori, MD, PhD
Department of Surgery, Division of Hepato-Biliary-Pancreatic Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017 (Japan)
Tel. +81 78 382 6302, Fax +81 78 382 6307. E-Mail: horiy@med.kobe-u.ac.jp

**Key Words:** CD133 \cdot Hypoxia \cdot Pancreatic cancer \cdot Cancer stem cell \cdot Hypoxia inducible factor-1α \cdot HIF-1α \cdot CXCR4
Abstract

Background: Intratumoral hypoxia is known to be increased aggressiveness and distant metastasis. However, the interplay underlying these actions is still unknown. Objective: We explored that cancer cells might acquire stem-like phenotype under hypoxia, consequently leading to an aggressive phenotype, including invasiveness and metastasis.

Methods: Under normoxia (20% O₂) or hypoxia (1% O₂) condition, the expression of CD133 (cancer stem cell marker), CXC chemokine receptor 4 (CXCR4), hypoxia inducible factor-1α (HIF-1α) were examined by RT-PCR and immunostaining using human pancreatic cancer cell lines. We also examined if hypoxia facilitates the invasiveness of CD133⁺ cancer cells. Furthermore, we transfected dominant active HIF-1α (HIF-1αΔODD) by the retroviral gene transfer and examined the effects both \textit{in vitro} and \textit{in vivo}. Results: Compared with normoxia condition, hypoxia elevated the expression of CD133, CXCR4 and HIF-1α. Moreover, hypoxia facilitated the invasiveness of CD133⁺ pancreatic cancer cells. The behavior of HIF-1αΔODD transfected cells under normoxia was compatible with those of the parent cells under hypoxia. Furthermore, xenograft model by HIF-1αΔODD cells showed aggressiveness,
including metastasis and highly tumorigenic ability. **Conclusion:** Hypoxia induces tumor aggressiveness associated with the expansion of CD133⁺ pancreatic cancer cells in a predominantly HIF-1α dependent manner.
Introduction

The presence of intratumoral hypoxia is a negative prognostic indicator for many patients as it has been associated with an increased degree of local failure following radiotherapy, chemotherapy and increased distant metastasis. Hypoxia can drive the metastatic phenotype secondary to both genetic instability and the clonal selection of aggressive tumor cell phenotypes [1]. However, the direct link between hypoxia, genetic instability and an aggressive phenotype remains to be elucidated [2-4].

A subpopulation of cancer stem cells (CSCs) in solid tumors, also referred to as tumor-initiating cells, has been isolated in many human tumors using the cell surface marker, CD133. CD133 is a marker of most tumor stem cells, especially human pancreatic cancer stem cells [5,6] as well as mouse pancreatic tissue-specific progenitor cells [7]. CSCs are defined by their capacity for self-renewal, their potential to develop into any cells in the tumor, and their proliferative capacity to drive the continued expansion of the tumor population [8]. Another important phenomenon, namely, whether or not the hypoxic regions of tumors harbor CSCs as niches, appears to be a common feature of stem cells and it certainly provides some of the critical stem cell
Several lines of evidence suggest that hypoxia promotes the persistence of stem cells and CSCs. Indeed, hypoxic conditions altered the gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype [12], while also promoting a dedifferentiated phenotype in ductal breast carcinoma \textit{in situ} [13]. A highly tumorigenic fraction of cells from a neuroblastoma cell line are preferentially found in hypoxic regions of the tumor [14]. More recent studies have also reported that hypoxia could up-regulate the stem cell surface marker CD133 in human brain tumors, such as medulloblastoma and glioblastoma [15-17].

The present study hypothesizes that cancer cells might acquire a stem-like phenotype under hypoxia, consequently leading to an aggressive phenotype. A subpopulation of human pancreatic cancer cells was observed to increase the expression of CD133 under hypoxia and thus show increased invasiveness. Furthermore, these responses to hypoxia occurred predominantly in a hypoxia inducible factor-1\(\alpha\) (HIF-1\(\alpha\))-dependent manner.
Materials and Methods

All mouse experimental protocols were conducted according to the Guidelines for Animal Experimentation at Kobe University.

Cell Culture

Human pancreatic cancer cell lines, PANC-1 and MIAPaCa-2 (American Type Culture Collection (ATCC), Rockville, MD, USA), were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). PANC-1 cells are derived from human pancreatic ductal adenocarcinoma and show adherent epithelial clusters. Doubling time is about 52hrs. MIAPaCa-2 cells are from human pancreas carcinoma and show adherent single cells with loosely attached clusters. Doubling time is about 40hrs. For hypoxic condition, the cells were moved to a hypoxic chamber (ASTEC, Japan) containing 1% O₂, 5% CO₂ and 94% N₂ for the indicated period to induce hypoxia. The dissolved oxygen concentration was determined by an oxygen meter (Multi 3410 SET4, WTW, Germany).
**Plasmids and Retroviral-Mediated Gene Transfer**

The pHA-HIF-1α and pHA-HIF-1αΔODD plasmids were gifts from Dr. L. E. Huang (University of Utah) [18]. The gene transfer experiments were carried out by using a retroviral vector. Retroviral vectors encoding SV40 T early region (including small t and largeT antigens), cDNA of HIF-1α and HIF-1αΔODD were subcloned into CX4bleo. Both the infection with retroviral vectors and selection were performed as described previously [19]. The infection efficiencies were > 80%, based on the findings obtained when using GFP-expressing retroviral vectors.

**In Vitro Invasion Assays**

A 24-well BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA, USA) was used to study effects of hypoxia on the invasive ability of tumor cells (5 × 10^4 cells/assays). A suspension of tumor cells in 500 µl serum-free DMEM was added to the upper chambers, whereas the lower chambers were each filled with 750 µl chemoattractant medium (DMEM plus 10% FBS). Thereafter, the cells were incubated for 24 h or 48 h. The cells that did not demonstrate invasion were then removed from
the inserts with a cotton swab. Thereafter, the cells that had invaded into the lower surface of Matrigel-coated membrane were then fixed with 4% paraformaldehyde (PFA), stained with hematoxylin, and counted under a light microscope. In order to detect and count CD133\(^+\) and/or CXCR4\(^+\) cells, we stained the cells that had invaded the Matrigel-coated membrane with CD133 and CXCR4 antibodies. The membranes were then removed with a scalpel and mounted on a glass slide with DAPI. We performed CD133\(^+\) and/or CXCR4\(^+\) cell counting by either light microscopy (AX80; Olympus, Japan) or confocal immunofluorescence microscopy (LSM 510; Carl Zeiss, Jena, Germany). In some experiments, the cells were incubated with AMD3100 (0, 1, 10, 100 µg/ml; Sigma-Aldrich, St Louis, MO, USA), a small molecular weight CXCR4 antagonist [17].

**Quantitative Real-Time Polymerase Chain Reaction (q-PCR) Assay**

Total RNA was extracted using the RNeasy Mini kit (QIAGEN, Valencia, CA, USA). The primer sequences were CD133:

5’-TCCACAGAAATTTACCTACATTGG-3’ (forward), 5’-
CAGCAGAGAGCAGATGACCA-3’(reverse) (77 bp), CXCR4: 5’-
CCAGTAGCCACCGCATCT-3’(forward), 5’- ATAGTCCCTGAGCCCAT
TT-3’(reverse) (99 bp), VEGF: 5’- CGCAAGAAATCCCGGTATAA-3’(forward), 5’-
TCTCCGCTCTGAGCAAGG-3’(reverse) (111 bp), and ß-actin: 5’-
TTAAGGAGAGCTGTGCTACG-3’(forward), 5’-
GTTGAAGGTAGTTTCGTGGAT-3’(reverse) (215 bp). Q-PCR was performed with a
Quantitect SYBR Green RT-PCR kit (QIAGEN, Valencia, CA, USA) in an ABI Prism
7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A
reaction mixture was first incubated at 50°C for 30 minutes for reverse transcription.
PCR was then initiated at 95°C for 15 minutes to activate modified Taq polymerase
followed by a 45-cycle amplification (94°C for 15 seconds, 55°C for 30 seconds, and
72°C for 30 seconds). At the end of the thermal cycles a dissociation protocol was
performed, starting at 60°C and then measuring fluorescence with 0.5°C increments, to
ensure that a single product was detected for each primer pair. The results are presented
as the parameter threshold cycle (C_T) values. ΔC_T was the difference in the C_T values
derived from the specific gene being assayed and ß-actin, whereas ΔΔC_T represented
the difference between the paired samples, as calculated by the formula $\Delta\Delta C_T = \Delta C_T$ of a sample-$\Delta C_T$ of a reference. The amount of target gene, normalized to a β-actin and relative to a reference, was expressed as $2^{-\Delta\Delta CT}$.

**siRNA Transfection**

PANC-1 or MIAPaCa-2 cells (1 x $10^5$ cells) were plated 24h before transfection in DMEM supplemented with 10% FBS. Transfection was performed in serum-free Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) with 150 pmol Stealth RNAi mixture for HIF-1α (Invitrogen, Carlsbad, CA, USA; HSS104774, HSS104775 and HSS179231) and 5 µl Lipofectamine (Invitrogen, Carlsbad, CA, USA) for 24h. Then, the transfection medium was replaced with DMEM supplemented with 10% FBS, and exposed to 1% hypoxia for 48h. Controls were transfected with the scrambled siRNA. We used BLOCK-iT Fluorescent Oligo (Invitrogen, Carlsbad, CA, USA) to monitor the transfection efficiency of Stealth RNAi.

**Implantation into SCID Mice**
Regarding the tumorigenic ability, HIF-1αΔODD transfected cells and mock cells were resuspended in 250 μl DMEM, and then made up to 500 μl with Matrigel. 1 x 10^3 and 1 x 10^5 cells were injected subcutaneously into the right and left flanks of scid mice using a 23-gauge needle. Animals underwent autopsy at 28 days after cell implantation and tumor growth was accessed. In addition, a suspension of 1 x 10^6 cells in a volume of 100 μl DMEM were engrafted orthotopically into the pancreatic tail of scid mice. Two weeks after the engraftment, the mice were sacrificed. The tissue specimens were fixed in formaldehyde and examined histologically.

**Immunohistochemistry**

The cells grown on Lab-Tek chamber slides (Nalge Nunc, International, Rochester, NY, USA) in hypoxia (1% O₂) or normoxia (20% O₂) were fixed in 4% PFA for 5 min. The grafts were fixed in 4% PFA and embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA). They were then sectioned to generate 10-μm-thick tissue sections. Primary antibodies were used at the following dilutions: mouse anti- CD133/1, 1:100 (Miltenyi Biotec, Bergisch Gladbach, Germany), rabbit
anti-CXCR4, 1:100 (abcam, Cambridge, UK), rabbit anti-HIF-1α, 1:100 (Santa Cruz, CA, USA), rabbit anti-CA9, 1:500 (Novus, Littleton, CO, USA), anti-HA-Tag antibody, 1:500 (Cell Signaling Technology, Danvers, MA, USA). Cellular hypoxia was detected by hypoxyprobe-1 Omni kit (Natural Pharmacia International, Inc., Burlingen, MA, USA). Hypoxyprobe (pimonidazole) was administered in vitro at a concentration of 200 µM for 2 h. Thereafter, the cells were stained using a rabbit anti-pimonidazole, 1:500 (Natural Pharmacia International, Inc., Burlingen, MA, USA). Tissue sections and fixed cells were incubated with the secondary antibodies labeled with Alexa-conjugated goat anti-rabbit, anti-mouse IgG 1:200 (Molecular Probes, Eugene, OR, USA). All images were generated by confocal immunofluorescence microscopy (LSM 510; Carl Zeiss, Jena, Germany).

**Western Blotting**

The cells were washed with ice-cold PBS and scraped off and then put into RIPA lysis buffer (50 mM Tris-HCl; pH 7.5, 125 mM NaCl, 0.1% Triton X-100, 5 mM EDTA) including protease inhibitors. The proteins were separated by SDS-PAGE and
transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Non-specific binding was blocked by incubation for 1 h at room with 5% non-fat dry milk in TBST. The membranes were then probed in primary antibodies overnight at 4°C; anti-HIF-1α (1:1000), anti-CXCR4 (1:500), anti-β-actin (1:5000; Sigma-Aldrich, St Louis, MO, USA) and anti-CD133 (K-18) (1:200; Santa Cruz, CA, USA). After probing with appropriate secondary antibodies, the signals were then detected by a chemiluminescence substrate (GE Healthcare, Uppsala, Sweden) and the LAS 3000 mini system (FUJIFILM, Japan).

**Statistical Analyses**

All values were expressed as the means ± standard error (SE). Statistical significance was determined by two-tailed unpaired Student’s *t*-test, and differences were considered to be statistically significant at *p* < 0.05.

**Results**

*Hypoxia Enhances the Expression of CD133 in Pancreatic Cancer Cell Lines*
The dissolved oxygen concentration in the medium was $5.96 \pm 0.03$ mg/l at 20% $O_2$ (normoxia) and $0.05 \pm 0.03$ mg/l at 1% $O_2$ (hypoxia), respectively. By FACS analysis, CD133$^+$ cells under 20% $O_2$ in PANC-1 and MIAPaCa-2 cells were $2.4 \pm 0.3$% and $1.1 \pm 0.2$%, respectively. The cells under 1% $O_2$ for 48 h significantly increased the expression levels of CD133 in comparison to those under 20% $O_2$, when analyzed by q-PCR, Western blotting and immunohistochemical staining (fig. 1a, b). More importantly, the up-regulation of the CD133 expression levels was reversible when the cells were re-oxygenated under 20% $O_2$ for 48 h. Immunofluorescence staining by hypoxyprobe (pimonidazole) confirmed these data (fig. 1c). These results indicate that hypoxia promotes the CD133 expression in human pancreatic cancer cell lines, which is consistent with a previous report on human glioma cell lines [12].

*Hypoxia Facilitates the Invasiveness of CD133$^+$ Pancreatic Cancer Cells*

As shown in fig. 2a and b, the invasive activity of the pancreatic cancer cells significantly increased under hypoxia, compared to normoxia. We next stained the invading cells with a CD133 antibody and examined the percentage of CD133$^+$ or
CD133− cells. While 92.0 ± 5.0 % of the invasive MIAPaCa-2 cells were CD133+ under hypoxia, 62.0 ± 5.0 % of invasive PANC-1 cells were CD133+ (fig. 2c, d). These results suggest that CD133+ invading cancer cells are enriched in pancreatic cancer cell lines under conditions of hypoxia, especially in MIAPaCa-2 cells, compared to normoxia.

*Invading Cancer Cells Express CXCR4 under Hypoxia in Vitro*

Recent reports have suggested CXCR4 to be a key regulator of tumor invasiveness leading to local progression and tumor metastasis [20,21]. CXCR4 gene and gene product expression increased under hypoxia in a time-dependent manner, in comparison to normoxia (fig. 3a, b). In addition, these phenotypes were also reversible when the cells were re-oxygenated under 20% O2 for 48 h, which was consistent with CD133 gene expression (fig. S1). On the other hand, other invasion related genes, including *E-cadherin, N-cadherin, snail, twist, matrix metalloproteinase-2 (MMP-2), and c-Met* did not significantly change the expression level (data not shown) [22]. We next stained and quantified the invading cells with CD133 and CXCR4 antibodies. Almost all of the invading cells under hypoxia were CXCR4 positive in both cell lines.
In PANC-1 cells, the CD133⁺CXCR4⁺ and CD133⁻CXCR4⁺ subpopulations comprised 62.0 ± 5.0 % and 38.0 ± 3.0 % of the cells, respectively. On the other hand, in MIAPaCa-2 cells, the CD133⁺CXCR4⁺ and CD133⁻CXCR4⁺ subpopulations were 92.0 ± 5.0 % and 8.0 ± 2.0 % of the cells, respectively (fig. 3c). We also examined whether CD133⁺CXCR4⁺ cancer cells exist in invasive lesions of human pancreatic cancers. We observed infiltrating CD133⁺CXCR4⁺ pancreatic cancer cells in the surrounding stroma (n = 5) (fig. S4a).

Acquired Tumor Aggressiveness Including Invasiveness and Remote Metastasis under Hypoxia are HIF-1α Dependent

It is evident that the critical molecular mediator of hypoxia, HIF-1α regulates multiple steps of tumorigenesis including tumor formation, progression, and response to therapy. The present study first confirmed the up-regulation of HIF-1α under hypoxic conditions (fig. S2). Moreover, we performed knockdown experiments with HIF-1α siRNA and observed CD133 gene expression significantly reduced. In addition, CXCR4 and vascular endothelial growth factor (VEGF) expression, which is the direct target of
HIF-1α, also reduced (fig. S3). In addition, CD133⁺ cells co-expressing hypoxic markers, such as HIF-1α and CA9 were enriched in the invasive lesions of human pancreatic cancer (n = 10) (fig. S4b).

HIF-1α is rapidly degraded under normoxic condition, with a half-life of a few minutes. The oxygen-dependent degradation domain deficient HIF-1α (HIF-1αΔODD), which is stable and functional even under normoxia was overexpressed to elucidate the involvement of HIF-1α in the CD133 gene expression. As expected, the expression of HIF-1α protein was detected only in HIF-1αΔODD transfected cells under 20% O₂ normoxia (fig. 4a). A q-PCR analysis showed that not only CD133, but also VEGF expression increased in HIF-1αΔODD transfected cells (fig. 4a). Furthermore, the induction of CD133 expression was confirmed by immunohistochemical staining (fig. 4b). Of note, the cells expressing dominant active HIF-1α co-expressed CD133 on the cell surface (fig. 4c). Consistent with the HIF-1α overexpression, the number of invading CD133⁺ cells increased even under normoxia (fig. 5d, e). The data on HIF-1αΔODD transfected cells under normoxia was compatible with those of the parent cells under hypoxia (fig. 1b, 2b). Another target of HIF-1α, CXCR4 genes and gene
products also increased in HIF-1αΔODD cells (fig. 5a). We next tested whether the CXCR4 antagonist, AMD3100, could reduce the number of invading CD133⁺ HIF-1αΔODD transfected cells. The dose dependency of AMD3100 on cell growth was initially determined, and cell growth was unaffected at a concentration of 1.0 µg/ml (fig. 5b). We also investigated the effect of AMD3100 on CD133 gene expression by quantitative RT-PCR. A concentration of 1.0 µg/ml of AMD3100 had no effect on CD133 gene expression (fig. 5c). Notably, the treatment of with 1.0 µg/ml of AMD3100, drastically reduced the number of invading CD133⁺ cell (fig. 5d, e). HIF-1αΔODD or mock expressing cells were engrafted into the pancreas of immunodeficient mice. Interestingly, four out of 10 mice engrafted with HIF-1αΔODD expressing cells showed metastatic lesions in the liver (n = 4/10), while none of all mice engrafted with mock cells showed any liver metastasis (n = 0/10) after two weeks of engraftment (fig. 4d, S5). These metastatic cells in the liver as well as the primary orthotopic pancreas tumor expressed CD133 under HIF-1α activation (fig. 4d). Furthermore, tumorigenic ability of HIF-1αΔODD expressing cells is shown in figure 4e. 10^3 and 10^5 HIF-1αΔODD expressing cells showed highly tumorigenic ability than mock cells. H. & E. staining of
the tumor indicated epithelial cancer cells (fig. 4e) and phenotypically indistinguishable from tumors derived from mock cells. These results indicate that the tumor aggressiveness, including invasiveness and remote metastasis under hypoxia, were predominantly HIF-1α dependent.
Discussion

Pancreatic cancer is currently the fourth leading cause of cancer-related mortality and characterized by intratumoral hypovascularity. In addition, human pancreatic tumor oxygenation measured intraoperatively with an electrode shows high levels of hypoxia in pancreatic cancers [23]. Cancer cells might acquire stemness or express “stem cell-like” properties under hypoxia, consequently leading to an aggressive phenotype in solid tumors. The current study initially confirmed that hypoxia induced an increased number of invading CD133+ cancer cells, hypoxic cells themselves expressed CD133 \textit{in vitro}, and that CD133+ cancer cells were preferentially found in the invasive legions of human pancreatic cancer specimen. CD133 is a marker protein typically used to identify and isolate human pancreatic cancer stem cells [5], as well as mouse pancreatic tissue-specific stem cells [7]. Furthermore, DNA microarray data of CD133\textsuperscript{high}PDGFR' stem/progenitor cells from the fetal mouse pancreas showed the up-regulation of the “stemness genes”, including \textit{Bmi1, Notch1-3, β-catenin, Frizzled, APC, Ptc1, Smo}, indicating that CD133 is a putative stem cell marker [7]. Although the concept of CSCs or tissue specific stem cells in solid organs has been
extended from hematopoietic stem cells, only rarely stem cells have been identified as clonogenic precursors that give rise to both self-renewing and differentiated progeny. Future studies must transplant individual CD133+ cancer cells and confirm the tumor progression to definitively prove that CD133+ cancer cells under hypoxia indeed have stem cell properties. In the present study, we cannot conclude that CD133+ cancer cells under hypoxia are CSCs. However, our preliminary data indicate that cancer cells under hypoxia preferably segregate asymmetrically, while cancer cells under normoxia do not, suggesting that cancer cells might acquire a stem-like phenotype under hypoxia (data not shown).

More importantly, the present study demonstrated that these phenotypes under hypoxia are reversible when the cells are re-oxygenated under 20% O2 normoxia conditions. These results are consistent with a recent report using human glioma cell lines [24]. What remains unclear is whether CSCs are the direct progeny of mutated stem cells or more mature cells that reacquire stem cell properties during tumor formation. Recently, Zhu et al. reported that CD133 marks stem cells in the adult mouse small intestine that are susceptible to transformation into tumors retaining a fraction of
mutant CD133 tumor cells [25]. In view of the *in vitro* results that the appearance of CD133+ cell population is reflected by the O_2_ concentration and these phenotypes are reversible without a significant impact on cell number, it is possible that mature CD133- cancer cells can reacquire stem cell properties under hypoxia.

We next questioned how hypoxia is involved in the stem cell microenvironment. The existence of a stem cell niche, consisting of specialized cells that participate in stem cell regulation, has been verified for mammalian adult stem cells in the intestinal, neural epidermal, and hematopoietic systems [10]. In light of these findings, it has been proposed that there are also a CSC niche, however, their location and models for the relationship between cancer cells and their niches remains unresolved [26]. The present study demonstrated for the first time that CD133+ cells themselves are hypoxic cells *in vitro*, as characterized by pimonidazole labeling. Furthermore, the number of hypoxic CD133+ cells, which are double positive with HIF-1α or CA9, has been observed to increase in the invasive lesion of human pancreatic cancer (fig. S4), in comparison to the bulk tumors, thus indicating that hypoxic CD133+ cells might be an indicator of malignant potential in human pancreatic
cancer cells. In general, it is thought that soluble factors from tumor-associated fibroblasts or stromal cells in the hypoxic regions of tumor may promote self-renewal of tumor cells \textit{in vivo}. Furthermore, Das \textit{et al.} reported that migrating neuroblastoma cells under hypoxic conditions home to hypoxic regions in the tumor when injected into tumor-bearing nude mice [14]. Although this study did not rule out the possibility that tumor-associated fibroblasts or stromal cells in the hypoxic regions function as a CSCs niche, the \textit{in vitro} data suggests the possibility that CSCs may be niche independent and the hypoxic cancer cells themselves can acquire the ability to cell-autonomously provide the necessary factors for expansion and self-renewal that are normally restricted by the niche.

The appearance of CD133$^+$CXCR4$^+$ cells in the present study also confirmed that an aggressive phenotype similar to the invasiveness of CD133$^+$ cells was reacquired under hypoxia, since Hermann \textit{et al.} reported the CD133$^+$CXCR4$^+$ cell population to be a highly metastatic human pancreatic cancer cell line which is highly invasive when orthotopically injected into nude mice [5]. Another report indicated the CXCR4 expression to increase liver and lung metastasis in a mouse model of pancreatic cancer.
In the present study, the CD133+CXCR4+ subpopulation was predominant in the invaded MIA PaCa-2 cells, but not in PANC-1 cells, thus suggesting that the CD133+CXCR4+ subpopulation was invasive. On the other hand, almost all of the invading cells under hypoxia were CXCR4+ in both cell lines, indicating that CXCR4, but not CD133, might define the invasive phenotype in pancreatic cancer.

HIF-1α is the best characterized among the transcriptional regulators under hypoxia [28, 29]. HIF-1α regulates the transcription of many genes involved in the maintenance of stem cell pools, cellular de-differentiation, genetic instability, metabolic reprogramming, invasion and metastasis [30, 31]. HIF-1α is also up-regulated in various types of human stem cells including embryonic stem cells, hematopoietic stem/progenitor cells and mesenchymal stem cells [32]. HIF-1α gene expression is up-regulated in CD133highPDGFRα mouse pancreatic stem/progenitor cells [7]. HIF-1αΔODD was used to determine whether or not this behavior under hypoxic conditions is also present in a HIF-1α dependent. The CD133 gene expression and invasive CD133+ cell number indicated that the HIF-1αΔODD transfected cells behavior under normoxia was compatible with those under hypoxia. Although the direct
interaction between HIF-1α and CD133 is still unknown, the current data and a recent report by Soeda et al. strongly support the interplay between these molecules [33]. In the current study, HIF-1α knockdown partially abrogated the elevated CD133 expression under hypoxia conditions (fig. S3b), thus suggesting that additional mechanisms regulate CD133 expression. Griguer et al. reported that CD133 expression in human glioma cells is regulated by hypoxia and mitochondrial dysfunction [24]. One possibility is to assume that CD133 expression in pancreatic cancer is also regulated by not only hypoxia, but also by mitochondrial dysfunction. Another possibility is the involvement of HIF-2α. HIF-2α maintains the undifferentiated state of cells and regulates tumorigenic capacity through the up-regulation of Oct4, an important stem cell factor [34,35]. We detected the baseline HIF-2α expression by RT-PCR, but no changes were observed when pancreatic cancer cells were cultured under hypoxic conditions in the current study (data not shown). Of interest, the CD133+HIF-1α+ cell population in the primary xenograft following HIF-1αΔODD gene transfer increased more than those in the non-treated xenograft, and consequently gave rise to liver metastasis. Taken together, these findings suggest that the acquired aggressiveness
occur predominantly in a HIF-1α-dependent manner. CXCR4 is a direct HIF target [36].

As shown in figure 5, CXCR4 antagonist had no effect on CD133 gene expression in HIF-1αΔODD cells, indicating that CXCR4 and CD133 were independently induced by HIF-1α.

We herein demonstrated that human pancreatic cancer cells up-regulated the stem cell marker, CD133 under hypoxia, while also showing tumor invasiveness. Furthermore, these phenotypes, including invasiveness and metastasis, occur in a predominantly HIF-1α-dependent manner.

Acknowledgements

We thank Dr. E Huang (Dept. of Neurosurgery, University of Utah) for the gift of HIF-1α and HIF-1αΔODD plasmids. We are grateful to Dr. Tsuyoshi Akagi (KAN Research Institute, Inc. Kobe, Japan) for retroviral gene transfer. This research was supported by grants-in aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to Hori Y(C) (21591773), Ku Y(C)(20591611). Ku Y was also supported by grants for Global Center of Excellence
Program for Education and Research on Signal Transduction Medicine in the Coming Generation “Bringing up clinician-scientists in the alliance between basic and clinical medicine”.

References


16 McCord AM, Jamal M, Shankavarum UT, Lang FF, Camphausen K, Tofilon


Huang LE, Gu J, Schau M, Bunn HF: Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 1998;95:7987-7992.


30 Semenza GL: Regulation of cancer cell metabolism by hypoxia-inducible


**Figure legends**

**Fig. 1.** Effects of hypoxia and re-oxygenation on CD133 expression. Human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2 were cultured under 20% O\textsubscript{2} (normoxia) or 1% O\textsubscript{2} (hypoxia) for 48 h. Re-oxygenation conditions indicates the cells were cultured under 1% O\textsubscript{2} (hypoxia) for 48 h, then re-oxygenized under 20% O\textsubscript{2} (normoxia) for an additional 48 h. **a** A quantitative analysis of stem cell marker, the CD133 gene expression by q-PCR and CD133 gene product by Western blotting under 1% O\textsubscript{2} (Means ± SE, n = 5, *p < 0.01 in comparison to cells under 20% O\textsubscript{2}; and **p < 0.01 in comparison to cells under 1% O\textsubscript{2}). **b** The up-regulation of the CD133 gene product under hypoxia by immunostaining. Original magnification was 100x. **c** PANC-1 cells were cultured in the presence of hypoxyprobe, pimonidazole. Pimonidazole accumulated in CD133\textsuperscript{+} cancer cells under 1% O\textsubscript{2}. Original magnification was 200x.

**Fig. 2.** Hypoxia facilitates the invasiveness of CD133\textsuperscript{+} pancreatic cancer cells. The invasiveness of PANC-1 cells and MIA PaCa-2 cells were assessed by a BioCoat Matrigel invasion chamber assay. **a** Invading cells were stained with hematoxylin, and **b** counted under a light microscope (Means ± SE, n = 5, *p < 0.01 in comparison to cells
under 20% $O_2$). c CD133 expression on invading cells were detected by immunostaining, and d counted under a light microscopy (Means ± SE, n = 5, * $p < 0.01$ in comparison to cells under 20% $O_2$). Original magnification was 100x.

**Fig. 3.** Invading CD133$^+$ pancreatic cancer cells co-express CXCR4 under hypoxia. a CXCR4 gene expression in PANC-1 and MIAPaCa-2 were up-regulated under hypoxia in a time-dependent manner (Means ± SE, n = 5, * $p < 0.05$ in comparison to cells under 20% $O_2$). b CXCR4 gene products by immunoblotting were increased under 1% $O_2$ as well. β-actin was shown as an internal control. c Quantification of the invading cell subpopulation. Invading cells were stained with CD133 and CXCR4 antibodies, and each subpopulation was counted under microscopy (Means ± SE, n = 5).

**Fig. 4.** Both acquired stem-like phenotype and tumor aggressiveness including invasiveness and metastasis of pancreatic cancer under hypoxia occurs in a HIF-1α-dependent manner. a MIA PaCa-2 cells were transfected with HIF-1α or oxygen-dependent degradation domain deficient HIF-1α (HIF-1αΔODD) plasmids, then
cultured under normoxia for 48 h. HIF-1α protein level was detected only in HIF-1αΔODD transfected cells under normoxia. β-actin was shown as an internal control. CD133 and HIF-1α target, VEGF gene expressions were increased in HIF-1αΔODD transfected cells under normoxia (Means ± SE, n = 5, * p < 0.05 in comparison to HIF-1α transfected cells). b Overexpression of HIF-1αΔODD significantly induced the number of CD133+ cells. Original magnification was 200x. c Overexpression of HIF-1αΔODD induced a significant increase of CD133 expression. Original magnification was 630x. d Metastatic lesions in the liver (arrows) after orthotopic xenograftment. Xenografted primary pancreatic tumor and liver metastasis were immunostained for CD133 (red), HA-Tag (green). All nuclei were counterstained with DAPI (blue). Original magnification was 200x. e Dominant active HIF-1αΔODD transfected cells showed highly tumorigenic ability. A representative tumorigenic ability of HIF-1αΔODD expressing cells. 10^3 and 10^5 HIF-1αΔODD expressing cells on the right side of the flank showed highly tumorigenic ability than mock cells on the left side of the flank. H. & E. staining of the tumor generated from HIF-1αΔODD expressing cells indicated epithelial pancreatic cancer cells. Original magnification was
Figure 5. Effect of CXCR4 expression on pancreatic cancer under hypoxia is in a HIF-1α-dependent manner. a The CXCR4 genes and gene products were increased in the HIF-1αΔODD transfected cells (Means ± SE, n = 5, * p < 0.05 in comparison to the HIF-1α transfected cells). b The dose dependency of the CXCR4 antagonist, AMD3100, on the HIF-1αΔODD transfected cell growth after 48h culture (Means ± SE, n = 5, * p < 0.05 in comparison to untreated control). c Effect of AMD3100 on CD133 gene expression (Means ± SE, n = 5). A quantitative analysis was performed by q-PCR. d The total number of invaded cells and invaded CD133^+ cells increased in the HIF-1αΔODD transfected MIA PaCa-2 cells. e Treatment with 1.0 µg / ml AMD3100, drastically inhibited the degree of invasiveness of CD133^+ cells (Means ± SE, n = 5, * p < 0.01 in comparison to that observed in HIF-1α transfected cells; and ** p < 0.01 in comparison to HIF-1αΔODD transfected cells).
Figure a: Relative CD133 expression levels in PANC-1 and MIAPaCa-2 cells under different oxygen conditions.

Figure b: CD133 immunostaining images showing the distribution of CD133 expression under various oxygen conditions.

Figure c: Immunofluorescence images showing CD133 and pimonidazole staining in cells exposed to different oxygen concentrations.