Hypoxic Conditions Promote Gemcitabine Sensitivity in a Pancreatic Cancer Stem Cell Line

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Abstract. *Development of an effective therapeutic strategy for refractory pancreatic cancer must consider whether chemosensitivity can be induced in chemoresistant cells. We established a pancreatic cancer stem cell-rich cell line using TIG-1 feeder cells and leukemia inhibitory factor (LIF)-rich SNL76/7 conditioned medium. We generated a cell line, namely YNPC031312-B, following isolation of cells from the malignant ascites of a patient with gemcitabine-resistant pancreatic cancer. A YNPC031312-B-Hypoxia cell line was established by maintaining YNPC031312-B cells under tumor-like hypoxic conditions (1% O_2). Both cell lines exhibited a pancreatic cancer stem cell phenotype: YNPC031312-B cells were $CD24^+CD44^-CD133^+$epithelial cell adhesion molecule $(EpCAM)^+$alkaline $phosphatase^+$Octamer-binding transcription factor (OCT)3/4+and YNPC031312-B-Hypoxia cells were $CD24^+CD44^+CD133^+EpCAM^+$. YNPC031312-B-Hypoxia cells were larger, had superior migratory ability, and higher gemcitabine sensitivity compared to YNPC031312-B cells. The use of LIF or other factors with similar bioactivity under hypoxic conditions may contribute to the phenotypic change to gemcitabine sensitivity. Our results may aid development of new therapeutic strategies targeting refractory pancreatic cancer.*

Pancreatic cancer is one of the most deadly cancer types, with a 5-year survival rate of under 5% (1). Chemoresistant pancreatic cancer is refractory to clinical treatments and has an extremely poor prognosis. Therefore, conversion of chemoresistant pancreatic cancer to a chemosensitive state could improve treatment options for refractory pancreatic cancer.

Pancreatic cancer stem cells are believed to be chemoresistant. Additionally, pancreatic cancer develops in microenvironments with high levels of hypoxia (2). Therefore, different signaling and factors may be activated under these hypoxic conditions when compared with the normoxic conditions commonly used in experimental settings. Recently, a relationship between pancreatic cancer stem cells and hypoxia was identified (3). The influence of hypoxia on pancreatic cancer cell physiology *in vivo* requires further study.

Conditioned medium obtained from SNL76/7 cells contains leukemia inhibitory factor (LIF) (4). LIF is the most pleiotropic member of the interleukin-6 family of cytokines (5), and has been implicated as an important factor for maintaining human induced pluripotent stem cells and embryonic stem cells as naïve cells (6-9). Additionally, embryonic stem-like cells can become established when embryonic fibroblasts are used as a feeder layer for culture of murine stem cells (10). Here, we used TIG-1 feeder cells and LIF-containing medium to maintain cancer tissue stem cells and establish cancer stem cell-rich cell lines. We then used these cell lines to examine phenotypic changes under hypoxic conditions.

Materials and Methods

Cell line establishment. Pancreatic cancer cell-rich cell populations were obtained from the malignant ascites of patients with advanced gemcitabine-resistant pancreatic cancer and collected by gradient centrifugation. These cells were cultured in T-75 flasks in Dulbecco's modified Eagle's medium (Nacalai Tasque, Kyoto, Japan) supplemented with SNL76/7 conditioned medium, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Life Technologies Grand Island, NY, USA). TIG-1 cells

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Key Words: Pancreatic cancer stem cell, gemcitabine resistance, hypoxia, phenotypic change, leukemia inhibitory factor.

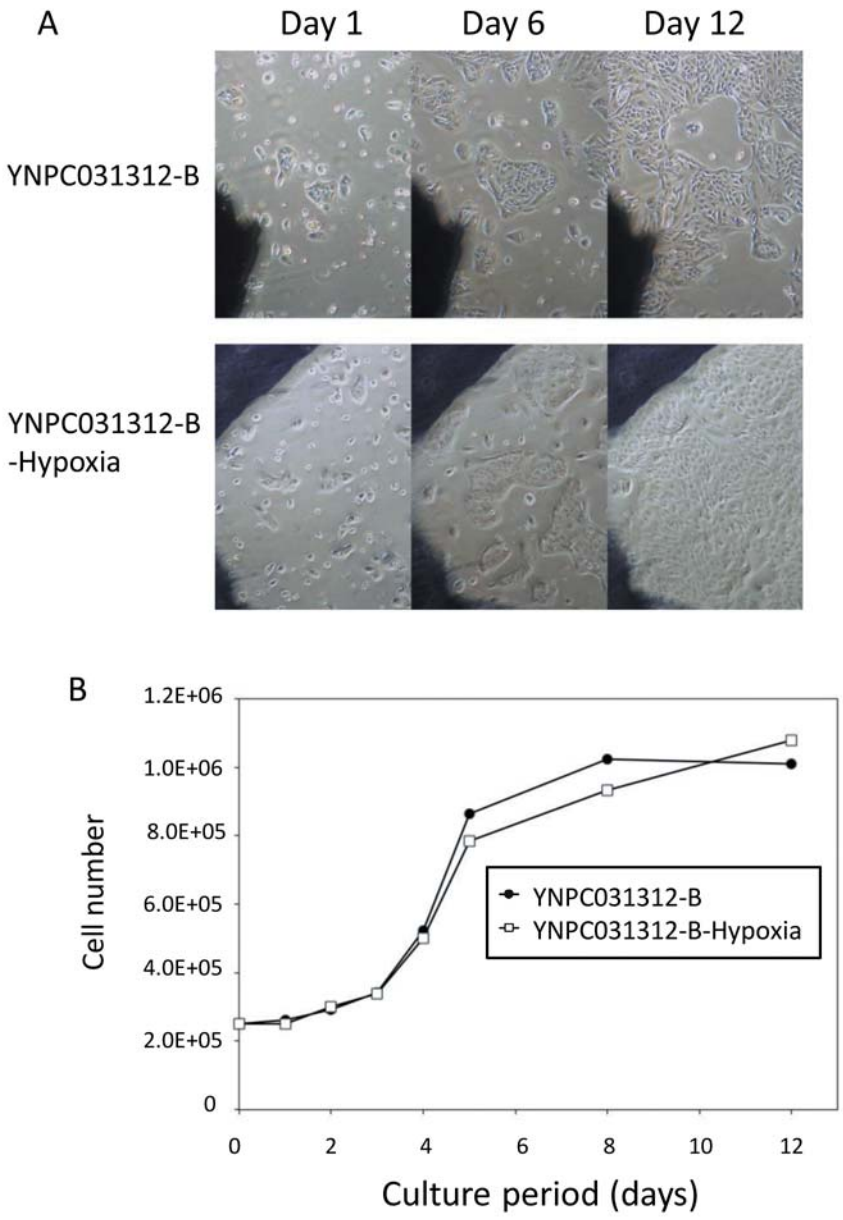

Figure 1. *A: Representative cell morphology in YNPC031312-B cells cultured under usual normoxic conditions and YNPC031312-B-Hypoxia cells cultured under hypoxic conditions (1% O_2) are shown. Original magnification, ×64. B: YNPC031312-B cells (2.5×10^5 cells) were cultured under usual normoxic conditions and YNPC031312-B-Hypoxia cells (2.5×10^5 cells) were cultured under hypoxic conditions (1% O_2) in a 35 mm dish. The cell number at the indicated days during culture was analyzed by cell counting using a light microscope.*

(Health Science Research Resources, Tokyo, Japan) (11) were used as a feeder layer. Independent colonies were harvested, and single clones were obtained using the limiting dilution method. A single clone was passaged under typical normoxic conditions and the YNPC031312-B cell line was established. Several YNPC031312-B cells were adapted to hypoxic conditions and used to establish YNPC031312-B-Hypoxia cells. For the hypoxic condition, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigas incubator (Sanyo, Tokyo, Japan). To assess cell proliferation, cell numbers and cell morphology were investigated by light microscopy. Gemcitabine (Kyowa Hakko Kirin, Tokyo, Japan) was used at 50 μg/ml, 100 μg/ml, and 200 μg/ml in the chemosensitivity test.

Fluorescence immunocytochemistry. YNPC031312-B cells and YNPC031312-B-Hypoxia cells were seeded on chamber slides (Matsunami Glass Co., Osaka, Japan) and cultured under normoxia and hypoxia, respectively. After overnight culture, cells were fixed

Table I. *Differentiation markers and stem cell markers.*

	CD24	CD44	CD133	EpCAM	OCT 3/4	ALP
YNPC031312-B	2+	–	1+	2+	1+	2+
YNPC031312-B-Hypoxia	2+	1+	2+	2+	2+	ND

EpCAM: Epithelial cell adhesion molecule; OCT3/4: octamer-binding transcription factor 3/4; ALP: alkaline phosphatase; ND: Not done.

in 4% paraformaldehyde followed by permeabilization with 0.2% Triton-100, then incubated with primary, followed by secondary antibodies. Primary antibodies used were anti-CD133 (130-098-046; Miltenyi Biotec Inc. CA, USA), anti-CD44 (555479; BD Biosciences, San Jose, CA, USA), anti octamer-binding transcription factor (OCT)3/4 (SC-5279; Santa Cruz Biotechnology, Dallas, TX, USA), anti-human epithelial cell adhesion molecule (EpCAM)/tumor-associated calcium signal transducer (TROP)1 (FAB9601F; R&D system, Minneapolis, MN, USA), anti- CD24 (555427; BD Biosciences), and alkaline photosphatase (ALP) substrate kit III/Vector Blue (VEC SK-5300; Funakoshi Co., Ltd, Tokyo, Japan). Cells were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA). After mounting in Vectorshield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), samples were analyzed by fluorescence microscopy (Axio Imager; Carl Zeiss, Oberkochen, Germany).

Migration assay. Distance moved and velocity of random migration in established YNPC031312-B and YNPC031312-B-Hypoxia cells were confirmed by time-lapse imaging. Images were acquired every 30 s for 15 min using LuminaVision (Mitani Corporation, Tokyo, Japan). Cells were identified in each image and tracked over time using Image-Pro Analyzer software (Nippon Roper KK, Tokyo, Japan). Previously, the usefulness of time-lapse imaging was confirmed by comparing with conventional migration method using transwell insert chamber (12).

Results

Cell morphology and proliferation. YNPC031312-B-Hypoxia cells were larger than YNPC031312-B cells (Figure 1A). On day 12, YNPC031312-B-Hypoxia cell cultures were almost confluent and the total number of cells appeared high compared with YNPC031312-B cell cultures (Figure 1A). However, there was no significant difference in the total numbers of YNPC031312-B and YNPC031312-B-Hypoxia cells (Figure 1B).

Cell surface marker, migration and chemosensitivity. YNPC031312-B cells exhibited a $CD24^+CD44^-CD133^+$ $EpCAM^+ALP^+OCT3/4^+$phenotype, while YNPC031312-B-Hypoxia cells exhibited a $CD24^+CD44^+CD133^+EpCAM^+$ phenotype (Table I). These results demonstrate that YNPC031312-B cells have an undifferentiated phenotype and that both cells express a cancer stem cell phenotype.

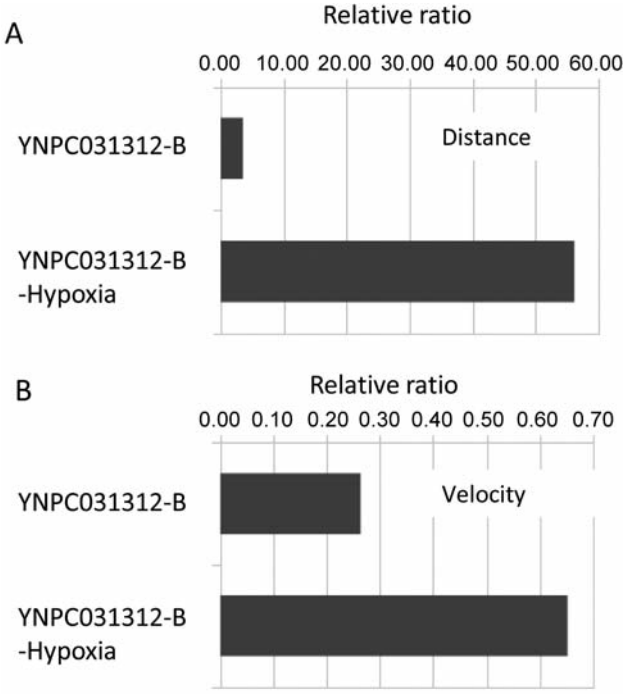

Figure 2. *Distance moved (A) and velocity (B) of random migration in established YNPC031312-B and YNPC031312-B-Hypoxia cells were estimated by time-lapse imaging analysis.*

Distance moved and velocity of random migration were significantly higher in YNPC031312-B-Hypoxia cells compared to YNPC031312-B cells (Figure 2). Chemosensitivity to gemcitabine was significantly enhanced in YNPC031312-B-Hypoxia cells compared with YNPC031312-B cells (Figure 3). These results suggest that phenotypic change occurred in YNPC031312-B-Hypoxia cells.

Discussion

In the present study, we demonstrate two novel findings. One is that a cancer stem cell-rich population can be established using TIG and LIF-rich SNL76/7 conditioned medium. Another is that the cell phenotype was changed

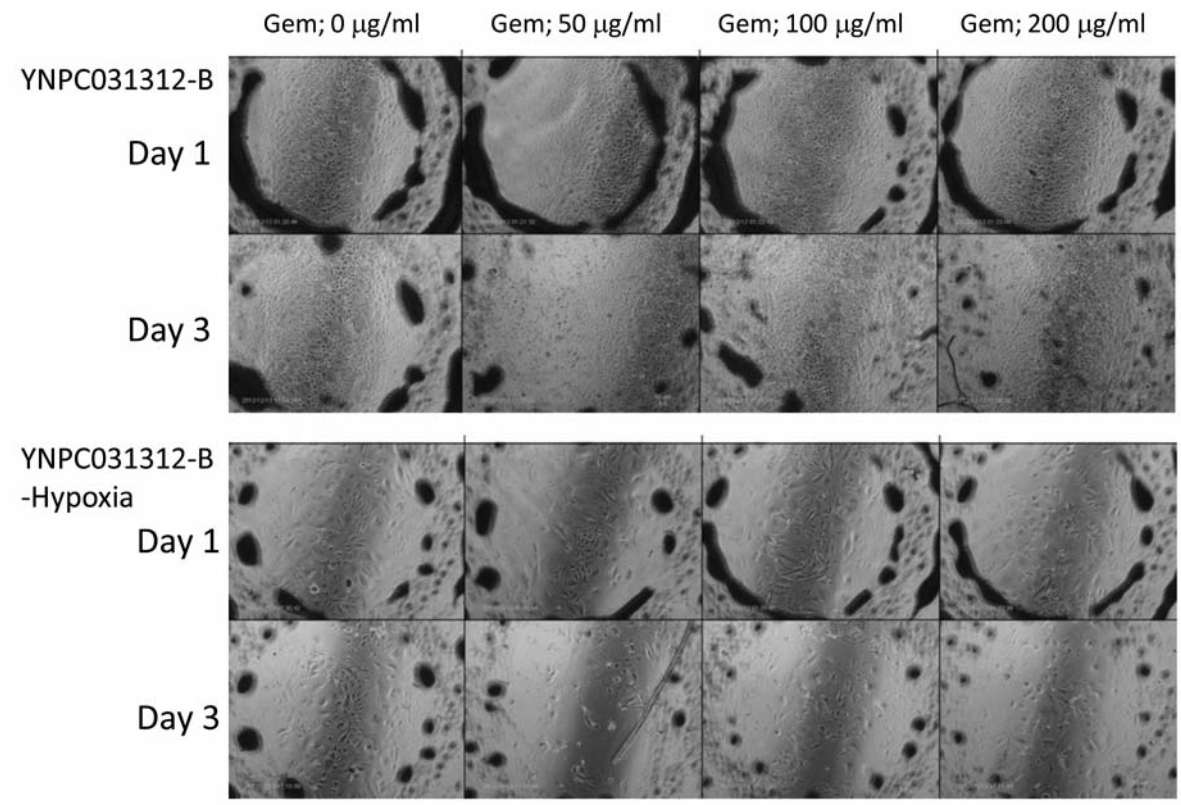

Figure 3. *YNPC031312-B cells under normoxic conditions and YNPC031312-B-Hypoxia cells under hypoxic conditions (1% O_2) were treated with gemcitabine (Gem). Representative images are shown. Original magnification, ×64.*

from gemcitabine resistance to gemcitabine-sensitive by culture under hypoxia.

Cancer cell populations are heterogeneic, and it can be difficult to establish a cancer stem cell-rich cell line directly from such populations. We hypothesized that embryonic fibroblasts and LIF may together maintain a population of undifferentiated cells, including cancer stem cells. Therefore, we used TIG-1 feeder cells and LIF to successfully establish a cancer stem cell-rich cell line. Culture of these cells under hypoxic conditions induced a phenotypic change whereby these gemcitabine-resistant cells developed sensitivity to gemcitabine. Contrastingly, cells established from the same patient in a similar manner without the use of TIG-1 feeder cells and LIF-conditioned medium still exhibited chemoresistance even under hypoxic conditions (data not shown).

Our findings carry important considerations regarding the malignant phenotype. An increased capacity for cellular migration under hypoxia augments the malignant phenotype. Therefore, the enhanced chemosensitivity observed under hypoxic conditions appears inconsistent with the increased migratory capacity. Whether the expression of other genes changed under hypoxic conditions and whether these changes were related to the use of TIG-1 cells and LIF deserves further investigation. Others have suggested that hypoxia-inducible factors (HIFs), transcription factors activated by hypoxic conditions, contribute to drug resistance (13, 14). Further investigation of the interactions among HIFs, LIF, and the use of embryonic fibroblast feeder cells is required to address this.

Our results suggest that LIF or factors with similar bioactivity under hypoxic conditions may contribute to phenotypic changes in chemoresistant pancreatic cancer. The method of cell line establishment we demonstrate here may be technically useful in future studies. Our results may also offer clues to aid development of a new effective therapeutic strategy targeting refractory pancreatic cancer.

Conflicts of Interest

The Authors declare no conflict of interest with regard to this work.

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