ANTICANCER RESEARCH *38*: 1391-1399 (2018)
doi:10.21873/anticanres.12362

Suppressive Effect of Delta-Tocotrienol on Hypoxia Adaptation of Prostate Cancer Stem-like Cells

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Abstract. *Background/Aim: A hallmark of the progression of prostate cancer to advanced disease is the acquisition of androgen-independent growth. This malignant phenotype is characterized by resistance to conventional treatments and predisposes to formation of hypoxic regions containing stem-like cancer cells. Unfortunately, an effective therapy to target prostate cancer stem cells under hypoxia has not yet been established. In this report, we studied whether δ-tocotrienol (T3), a vitamin E family member that has exhibited the most potent anti-cancer activity, could suppress the survival of prostate cancer stem-like cells. Materials and Methods: PC3 stem-like cells were isolated from PC3 parental cells using a three-dimensional culture system. The stemness of the isolated PC3 stem-like cells was confirmed by evaluation of resistance to an anticancer agent (docetaxel) and tumor formation capacity in a xenograft model. The effects of δ-T3 on PC3 stem-like cells under a hypoxia condition were examined by WST-8 (cell viability), real-time reverse transcription-polymerase chain reaction (PCR) and western blotting. Results: δ-T3 demonstrated a cytotoxic effect on prostate cancer stem-like cells in a dose dependent manner and a reduction in the protein levels of hypoxia-inducible factor (HIF)-1α and HIF-2α. Additionally, a specific inhibitor toward HIF-1α induced cytotoxicity on PC3 cells, but selective inhibition of HIF-2α had no effect. Conclusion: Overall, these results suggest that δ-T3 could inhibit the survival of prostate cancer stem-like cells under hypoxia, primarily through the inactivation of HIF-1α signaling.*

Prostate cancer remains the most common solid tumor and the second major cause of cancer death among men in developed countries (1.1 million new cases in 2012 resulting in 307,000 deaths) (1). Prostate cancer cells are androgen-responsive requiring androgens for growth and survival; hence the initial treatment of this cancer involves hormonal therapy (2). Androgen deprivation therapy is the first-line treatment for advanced prostate cancer. Although this therapy is initially effective, the response is short-lived, and eventually prostate cancer cells become androgen-independent (2). The consequent progression to a more aggressive stage of the disease, referred to as castration-resistant prostate cancer, is characterized by metastasis and severe chemoresistance (3). At this stage, castration-resistant prostate cancer remains incurable, punctuating the need for effective prevention and treatment strategies.

Cancer tissues are comprised of phenotypically heterogeneous cell populations, including a minority of cells displaying stemness/tumor initiating properties, high self-renewal and tumorigenicity, defined as cancer stem cells (4). Although initially described in leukemia (5), these cells have subsequently been identified in a number of solid tumors, including prostate cancer (6-8). There is evidence to indicate that cancer stem cells may be the primary mediators of tumor initiation, progression, recurrence, metastasis and resistance to treatment (4, 9). Prostate cancer stem cells in particular are able to survive chemotherapy or radiotherapy. They are also suggested to be responsible for the development of castration-resistant disease and the subsequent poor prognosis for patients with advanced prostate cancer (10). It has been previously shown that cancer stem cells prefer to reside in specific hypoxic regions within the tumor microenvironment (11). Furthermore, it has been demonstrated that hypoxic microenvironment is required to

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Key Words: Cancer stem cell, prostate cancer, tocotrienol, hypoxia adaptation, hypoxia-inducible factor.

produce cancer stem cells in solid tumors (12). Therefore, adaptation of prostate cancer stem-like cells to hypoxia is regarded as a potential therapeutic target.

Tocotrienol (T3), a member of the vitamin E family, has antioxidant properties and also, exerts anticancer effect irrespective of its antioxidant activity (13). T3s have four different isomers (α, β, γ and δ-T3) that differ from each other only in the location and number of methyl groups on their chromanol rings (14). *In vitro* studies have indicated that the various forms of T3s exerts differential anticancer effects, and to date, δ-T3 has demonstrated the most potent anti-proliferative activities against various types of cancer, including prostate cancer, both *in vitro* and *in vivo* (13,15). It has been recently reported that γ-T3 activity is targeted to prostate cancer stem-like cells (16), thus it is easily speculated that δ-T3 might act as an effective anticancer agent targeting stem-like cells. We have also previously reported that a redox-silent analogue of α-T3, 6-*O*-carboxypropyl-α-tocotrienol effectively inhibits hypoxia adaptation of several cancer cell types, including prostate cancer cells (17). In this context, this study aimed to investigate the potential effect of δ-T3 on hypoxia adaptation of prostate cancer stem-like cells.

Materials and Methods

Chemicals. All reagents were purchased from Nakarai Tesque (Kyoto, Japan), unless otherwise indicated. Anti-α-tubulin antibody was obtained from Cell Signaling Technology (Danvers, MA, USA), while anti-hypoxia-inducible factor (HIF)-1α and HIF-2α antibodies from Novus Biologicals (Littleton, CO, USA) and Acris (Herford, Germany), respectively. Also, anti-cluster of differentiation (CD)44 and CD133 antibodies were purchased from Abcam (Cambridge, UK). As secondary antibodies, anti-mouse IgG-peroxidase and rabbit IgG-peroxidase antibodies were obtained from MBL (Nagoya, Japan). BAY 87-2243 (a HIF-1α inhibitor) was purchased from Sellckchem (San Francisco, CA, USA). δ-T3 from Annato oil was a generous gift from Dr Terao (Cyclochem Co., Kobe, Japan). PCR primers were purchased from Sigma Genosys (Hokkaido, Japan).

Isolation and characterization of PC3 stem-like cell. PC3 cells (American Type Culture Collection, Manassas, VA, USA) were used as a typical human androgen-independent prostate cancer cell line. The cells were routinely grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. To isolate PC3 stem-like cells, we utilized the sphere forming capacity of the cells in a three dimensional (3D) culture system (18). To examine sphere formation capacity of PC3 cells, cells were trypsinized, washed, and resuspended in stem cell medium, consisted of DMEM/F12 (Wako CO., Osaka, Japan) medium, 4 μg/ml insulin (Sigma), B27 supplement (1×) (Sigma), 20 ng/ml epidermal growth factor (EGF) (Sigma), and 20 ng/ml basic fibroblast growth factor (FGF) (Thermo Fisher Scientific, Kanagawa, Japan). Cells were suspended in stem cell medium at a density of 1,000 cells/ml, and each cell suspension was transferred into each well of a 24-well low attachment culture plate (Thermo Fisher Scientific, Kanagawa, Japan). Cells were checked for 14 days, and subsequently, those showing the greatest capacity to form spheroids were resuspended in stem cell medium and transferred in 96-well low attachment culture plates, at a density of 1 cell/well. Wells containing single cells were monitored. After 14 days of culture, large-sized colonies were selected and cultured in DMEM/F12 medium containing 10% FBS, using a two dimensional (2D) culture system. Subsequently, mRNA levels of cancer stem cell markers, CD24, CD44, CD133 and sex determining region Y-box2 (SOX2), were examined. The colony showing the highest expression level of each marker was then utilized as a PC3 stem-like cell population. In order to confirm some cancer stem cell phenotypes, the selected PC3 stem-like cells were examined for hypoxia adaptation, chemoresistance, and tumor formation capability in a xenograft model. Cell up to four passages were used, since the spheroid formation capacity of PC3 stem-like cells in a 2D culture using 10% FBS DMEM/F12 medium was retained for four passages. Cells were incubated at 37˚C in 5% CO_2.

Exposure to hypoxia. A hypoxic condition (less than 1% O_2, 5% CO_2) was created by AnaeroPack system (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan), according to the manufacturer's instructions (19). After cells were pre-incubated for 6 h under hypoxia condition to induce hypoxia adaptation, the cells were exposed to hypoxia for 12-48 h.

Cell growth assay. PC3 stem-like cells were seeded in a 96-well plate (3×10^4 cells/well), cultured for 24 h under normoxic conditions, pre-incubated for 6 h and subsequently treated with each agent for 24-48 h under hypoxic conditions. Afterwards, to determine cell growth, WST-8 assay (Wako Co., Osaka, Japan) was carried out. In brief, after each treatment, 10 μl of WST-8 solution were applied to each well containing 100 μl of cell suspension, and the cells were further incubated for 30 min at 37˚C in 5% CO_2. Color development was monitored at 450 nm using a multi-well plate reader.

Isolation of RNA and real-time polymerase chain reaction (PCR). Total RNA was isolated from PC3 stem-like cells using a Tissue Total RNA Extraction Mini Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). Total RNA (100 ng for each sample) was used for cDNA synthesis using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). cDNA template was analyzed by real-time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan Ltd, Tokyo, Japan) and THUNDER-BIRD™ SYBR qPCR Mix (Toyobo, Osaka, Japan), according to the following program: 10 s at 95˚C, followed by 40 cycles of 15 s at 95˚C and 1 min at 60˚C. Primer sets are shown in Table I. Gene expression data was normalized to the expression of the reference gene ribosomal protein L32 (RPL32).

Western blotting. After each treatment, cell lysates were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) including phosphatase inhibitor cocktails 1 and 2 and a protease inhibitor cocktail (containing AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A) (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 15,000 × *g* for 30 min at 4˚C, supernatants (10 μg total protein content) were separated by 5% or 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Atto, Tokyo, Japan). After overnight incubation with Blocking One (Nakalai Tesque, Kyoto, Japan),

Table I. *List of primer sequences.*

Gene	Primer	Sequence (5'-3')
RPL32	Forward primer	AACCCTGTTGTCAATGCCTC
	Reverse primer	CATCTCCTTCTCGGCATCA
CD24	Forward primer	GCTCCTACCCACGCAGATTTA
	Reverse primer	TTGGTGGTGGCATTAGTTGG
CD44	Forward primer	AATGGCCCAGATGGAGAAAG
	Reverse primer	GGGAGGTGTTGGGATGTGAGG
CD133	Forward primer	GTCCTGGGGCTGCTGCTGTTTAT
	Reverse primer	TCTGTCGCTGGTGCATTTCT
SOX2	Forward primer	AACCCCAAGATGCACAACTC
	Reverse primer	CGGGGCCGGTATTTATAATC

RPL32, Ribosomal protein L32; CD, cluster of differentiation; SOX2, sex determining region Y-box2.

membrane was incubated with the indicated primary antibody for 1 h at room temperature, followed by a secondary antibody incubation for 1 h at room temperature. Detection of immunoreactive bands was accomplished using Western Bright Sirius (Advansta, Menlo Park, CA, USA), CanGet Signal (Toyobo, Tokyo, Japan) and cooled CCD camera-linked Cool Saver system (Atto, Tokyo, Japan). A two-dimensional densitometric evaluation was performed, using Atto Image Analyzer System (Atto, Tokyo, Japan). Molecular size was estimated using Protein Dual Color Standards, and protein concentrations were determined by the DC Protein Assay kit (both obtained from Bio-Rad, Hercules, CA, USA).

Xenograft tumor formation. BALB/cAJcl-*nu/nu* nude 7-week-old male mice (CLEA Japan Inc, Tokyo, Japan) were used in this study. All mice were housed under controlled temperature (22±1˚C), relative humidity (55±5%) and a 12 h diurnal system. They were supplied with a commercial pellet (CE-2, CLEA Japan Inc) and permitted sterilized water *ad libitum*. After acclimatization for one week, mice were subcutaneously implanted with 1×10^5 or 10^6 PC3 stem-like cells and PC3 parental cells resuspended in PBS/Matrigel mixture (1:1 volume), into the flanks. Mice were inspected for tumor appearance, by observation and palpation, and tumor growth was measured every three days using a caliper. On day 21 after implantation, mice were sacrificed, tumor weights were measured to confirm tumor growth, and the presence of tumor nodule was checked by necropsy. All animal work was performed according to experimental protocols approved by the Chiba University Animal Research Ethics Board.

Statistical analysis. Differences among groups were analyzed by one-way ANOVA followed by the Tukey-Kramer test, and differences between two groups were analyzed by one-way ANOVA followed by Student's *t*-test. All statistical analyses were performed using Ekuseru-Toukei software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences with *p*-values of 0.05 or less were considered statistically significant. All experiments were conducted with a minimum of three samples from three independent experiments and the data are expressed as the mean±SD. The number of samples for each experiment is shown in the respective figure legend.

Results

Isolation of PC3 stem-like cells from PC3 cells based on spheroid formation capability. There are several established methods to isolate cancer stem-like cells from parental cancer cell populations (20). Herein, isolation of PC3 stem-like cells was based on their ability to form spheroids in a 3D culture system using stem cell medium without FBS (18, 21). As shown in Figure 1A, the selected cell clone had high spheroid formation capability. In order to check the identity of the isolated cells, mRNA levels of a panel of established cancer stem cell markers were compared between the selected PC3 cell population and parental PC3 cells (Figure 1B). mRNA levels of *CD24*, *CD133*, and *SOX2* were increased (56%, 53%, and 96%, respectively, $p<0.05$) in the selected stem-like cell population. CD44 had also elevated expression (42%), though not statistically significant. Since literature evidence indicates that a population of prostate cancer cells positive for CD44/CD133 surface antigens is a candidate for prostate cancer stem-like cells (8), western blot analysis of CD44 and CD133 protein levels was performed. Though not statistically significant, both proteins had increased levels in the isolated cell population (CD44, 40%; CD133, 82%) compared to the parental cells, supporting their stem-like quality (Figure 1C).

Cancer stem-like cell properties in PC3 stem-like cells. Anticancer drug resistance of PC3 stem-like cells was confirmed by comparing the cytotoxicity of docetaxel, a widely used anticancer drug (22), on PC3 stem-like cells and PC3 parental cells. As shown in Figure 2A, docetaxel did not exhibit cytotoxicity against PC3 stem-like cells, at concentrations of 0-1,000 nM; however, at the two highest doses, 100 and 1,000 nM, it was cytotoxic against PC3 parental cells (100 nM, 20% decrease; 1,000 nM, 38% decrease *vs*. control, $p<0.05$). As shown in Figure 2B, further investigation of the cells in a xenograft model, showed statistically significant higher tumor formation capacity of PC3 stem-like cells than PC3 parental cells (10^5 transplanted cells: parental cells 48.7±32.6 mg *vs*. stem-like cells 117.2±28.6 mg, $p<0.05$; 106 transplanted cells: parental cells 128.8±51.1 mg *vs*. stem-like cells 433.9±235.5 mg, $p<0.05$).

Hypoxia adaptation in PC3 stem-like cells. In order to investigate hypoxia adaptation, viability of PC3 stem-like cells cultured under hypoxic condition was compared to the viability of PC3 parental cells under the same conditions. As shown in Figure 3A, hypoxia induced an 86% increase ($p<0.05$) on the latter compared to the former. Linked with this observation, the expression of HIFs, critical transcription factors for cell survival under hypoxia (23), were significantly higher in PC3 stem-like cells than in PC3 parental cells. However, the increase of HIF-1α expression

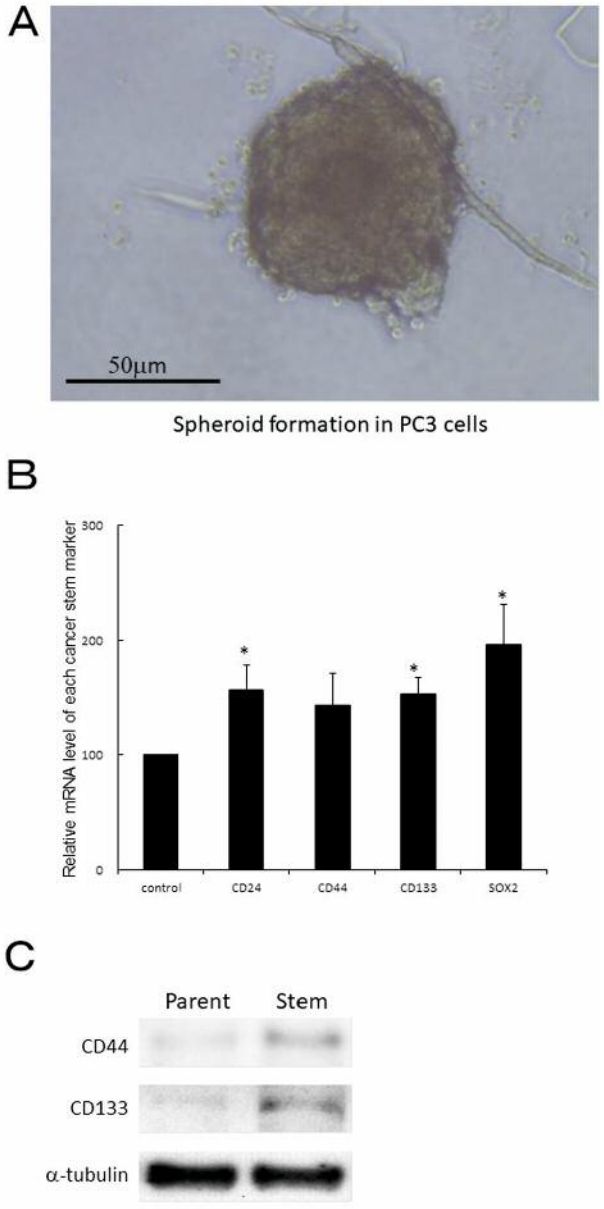

Figure 1. *Isolation of PC3 stem-like cells from PC3 parental cells in a three-dimensional (3D) culture. Spheroid formation of PC3 stem-like cells in 3D culture (A). Scale bar represents 50 μm. mRNA levels of four cancer stem cell markers in PC3 stem-like cells and PC3 parental cells (B). Data (percentage relative to PC3 parental cells, 100%) are presented as the mean±SD (n=3). *p<0.05, compared to PC3 parental cells (control). CD44 and CD133 protein levels as representative prostate cancer stem cell markers were determined (C). Each band is representative one of two independent experiments. Parent, PC3 parental cells; Stem, PC3 stem-like cells.*

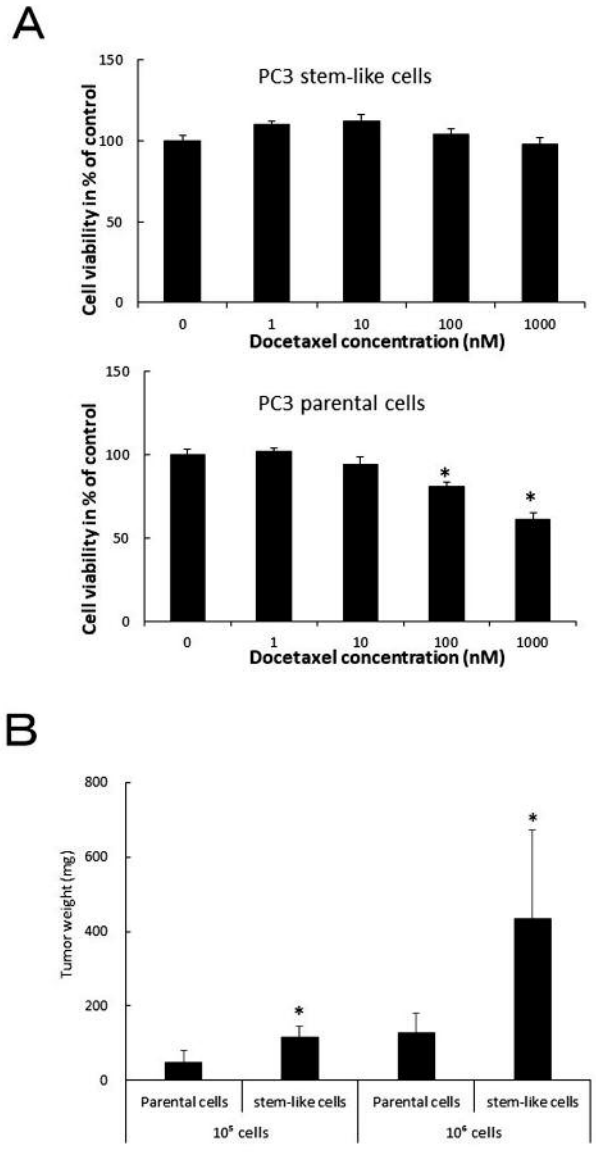

Figure 2. *Cancer stem-like properties in PC3 stem-like cells isolated from PC3 parental cells. Cells were pre-incubated for 6 h under hypoxic condition and subsequently treated with docetaxel under hypoxia, for 48 h. The effect of docetaxel on PC3 parental and PC3 stem-like cell viability was determined (A). Results are presented as percentage relative to non-treated group (designated as 100%). Values represent the mean±SD (n=5). *p<0.05, compared to non-treated group. PC3 stem-like cells and PC3 parental cells (1×10^5 cells or 1×10^6 cells) were transplanted subcutaneously to backs of nude mice. Three weeks later, tumor weights were measured for the evaluation of tumor formation capability of PC3 stem-like cells in a xenograft model (B). Data are presented as the mean±SD (n=4). *p<0.05, compared to PC3 parental cells.*

observed in the stem-like cells compared to parental cells (67%, $p<0.05$) was higher than that of HIF-2α (30%, $p<0.05$) (Figure 3B).

Effect of δ-T3 on hypoxia adaptation in PC3 stem-like cells. As previously reported, hypoxic niches were shown to be enriched of cancer stem cells (24). Similarly, in the present

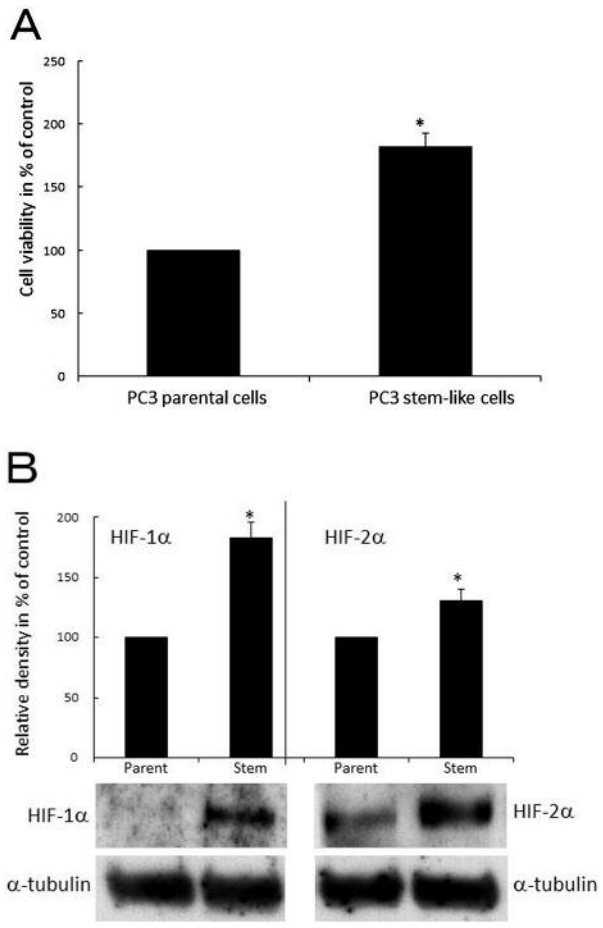

Figure 3. *Hypoxia adaptation of PC3 stem-like cells. PC3 stem-like cells and PC3 parental cells were pre-incubated for 6 h under hypoxic condition and subsequently cultured under hypoxia for 48 h. After that, cell viability was determined (A). Data are expressed as the mean±SD (n=5). *p<0.05, compared to PC3 parental cells. PC3 stem-like cells and PC3 parental cells were cultured for 12 h under the hypoxic condition, and subsequently HIF-1α and HIF-2α protein levels were determined (B). The relative intensity of bands was evaluated as percentage in relation to PC3 parental cells (100%). Values are presented as the mean±SD (n=3). *p<0.05, compared to PC3 parental cells.*

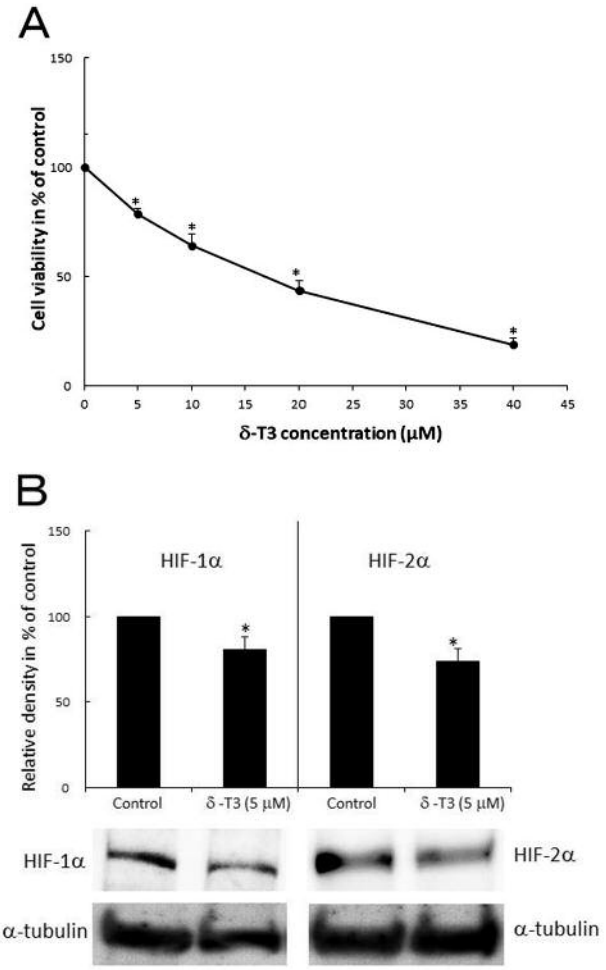

Figure 4. *Effect of δ-T3 on hypoxia adaptation in PC3 stem-like cells. PC3 stem-like cells were pre-incubated for 6 h under hypoxic condition and subsequently treated with δ-T3 at indicated doses for 24 h under hypoxia. Cell viability was determined. Results (percentage relative to non-treated group, 100%) are presented the mean±SD (n=5). *p<0.05, compared to non-treated group (A). In the same context, PC3 stem-like cells were pre-incubated under hypoxia condition for 6 h and subsequently treated with δ-T3 (5 μM) for 12 h. HIF-1α and HIF-2α protein levels were determined. The relative intensity of bands was evaluated as percentage in relation to the control (100%). Values are presented as the mean±SD (n=3). *p<0.05, compared to non-treated group (B).*

study it was observed a higher hypoxia adaptation capability of PC3 stem-like cells, compared to the parental cells. Thus, it was further investigated whether δ-T3 could act as an effective anti-prostate cancer agent *via* the inhibition of hypoxia adaptation in prostate cancer stem-like cells. As shown in Figure 4A, δ-T3 showed effective cytotoxicity against PC3 stem-like cells under hypoxic condition, in a dose-dependent manner, and the highest dose (40 μM) treatment reduced cell viability by 31% compared to non-treated cells ($p<0.05$). In agreement with this finding, δ-T3 treatment significantly suppressed protein level of both HIF-1α and HIF-2α in PC3 stem-like cells ($p<0.05$), resulting in approximately 25% decrease in the expression of both HIF proteins compared to non-treated cells (Figure 4B).

Different contributions of HIF-1α and HIF-2α to hypoxia adaptation in PC3 stem-like cells. Finally, the contribution of HIF subtypes to the adaptation of PC3 stem-like cells to hypoxia was investigated. YC-1, a dual inhibitor of HIF-1α and HIF-2α, and BAY87-2243, a specific HIF-1α inhibitor, were shown to reduce PC3 stem-like cell viability under hypoxic conditions, in a dose-dependent manner. At the highest

treatment dose (YC-1 40 μM and BAY 87-2243 10 μM), cell viability was significantly decreased (39% and 76%, respectively, $p<0.05$) compared to non-treated group (Figures 5A and 5B). On the other hand, the selective inhibition of HIF-2α had slight influence on cell viability in the stem cells within the treatment doses (Figure 5C).

Discussion

The main aim of the present study was to examine whether δ-T3 could effectively suppress PC3 stem-like cells. In order to achieve this aim, a brief method to isolate PC3 stem-like cells from PC3 parental cells was established, based on their capacity to form spheroids in 3D culture. Furthermore, it was confirmed that compared to PC3 parental cells, PC3 stem-like cells exhibited greater adaptation to hypoxia, one of the typical properties of cancer stem-like cells. Finally, δ-T3 was shown to inhibit hypoxia adaptation of PC3 stem-like cells, primarily *via* the suppression of HIF-1α.

Cancer stem cells comprise a small subpopulation of cancer cells capable of self-renewal and unlimited replication, thus initiating tumor formation (4, 20). They have been well-characterized in multiple malignancies including prostate tumors (5-8). Cancer stem cells also contribute to tumor resistance to conventional treatments and may survive initial therapies, leading to recurrence, progression, and metastasis in prostate and several other cancer types, after therapy (10, 11, 25). Therefore, identification of cancer stem cells and understanding their role in cancer growth could reveal novel targeting strategies for prostate cancer therapy. There have been reported several effective methods for isolating several types of cancer stem-like cells (21). Spheroid formation in defined serum-free medium has been used to identify suspected prostate cancer stem cells, and to measure the stem cell or early progenitor cell activity (8,18). Based on these, we successfully used this approach to isolate PC3 stem-like cells in this study. The present study showed that the isolated PC3 stem-like cells, when compared with parental cells, expressed cancer stem cell markers such as CD24, CD44, CD133 and SOX2. These markers have been shown to be expressed in typical prostate cancer stem cells (8,18,25). Furthermore, PC3 stem-like cells exhibited resistance to the conventional drug docetaxel and showed higher tumorigenicity in the xenograft model, compared to PC3 parental cells, consistent with cancer stem cell-like features (16).

Solid tumors are heterogeneous and possess poorly vascularized hypoxic regions. The surrounding hypoxic microenvironment plays an important role in determining the properties of cancer stem cells, since they can easily acquire phenotypes necessary for adaptation to hypoxia (11, 25). Particularly, in prostate cancer, hypoxia has been associated with treatment failure and drug resistance (26). Furthermore,

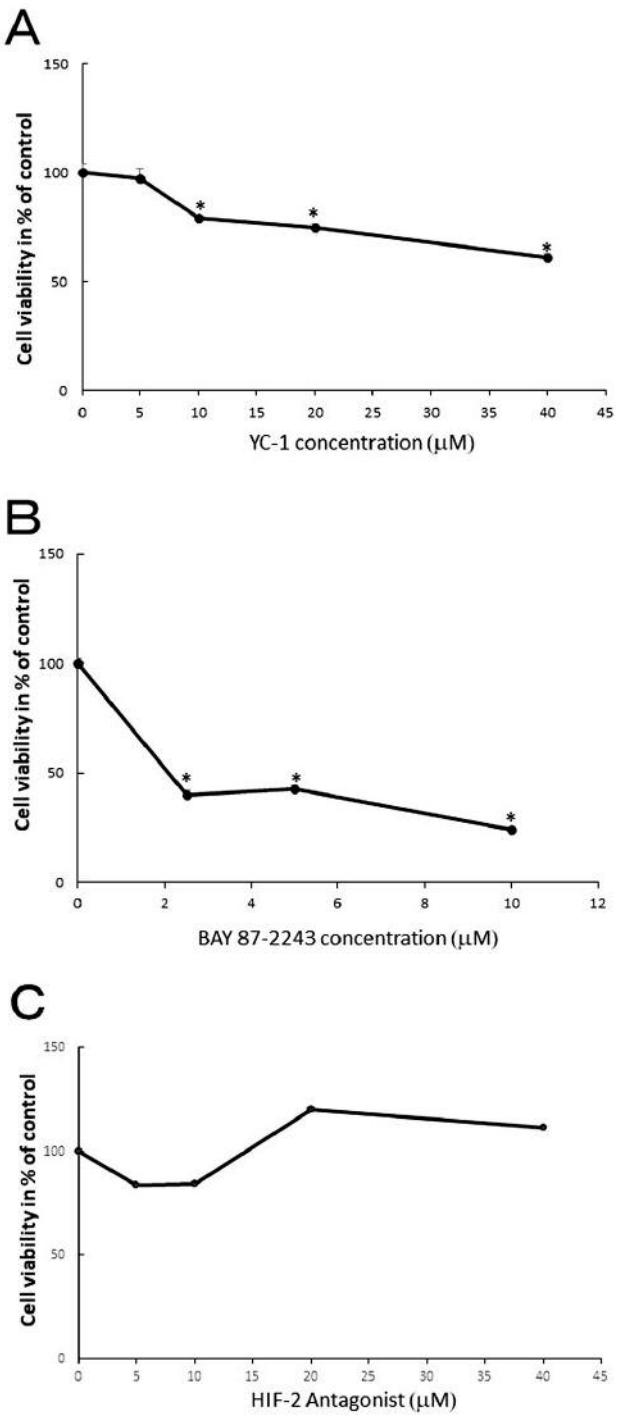

Figure 5. *Different contributions of HIF-1α and HIF-2α to hypoxia adaptation in PC3 stem-like cells. Effect of YC-1 on cell viability in the cells under hypoxic condition (A). Effect of BAY87-2243 on cell viability in the cells under the hypoxic condition (B). Effect of HIF-2α antagonist on cell viability in the cells under the hypoxic condition (C). The PC3 stem-like cells were pre-incubated under hypoxia for 6 h and subsequently treated with each HIF inhibitor at indicated doses under the hypoxic condition, for 24 h. Cell viability was determined. Results (percentage relative to non-treated group as 100%) are presented as the mean±SD (n=5) *p<0.05, compared to non-treated group.*

hypoxia can select for androgen independent prostate cancer cells, which have a survival advantage and are more likely to invade and metastasize (27). A similar hypoxic response was revealed in the present study; PC3 stem-like cells showed a higher survival activity than PC3 parental cells under hypoxic condition, indicating greater adaptation to hypoxia.

Hypoxic stress is a characteristic of many disease states, and HIFs (hypoxia-inducible factor), primarily the two major isoforms of the α-subunit, HIF-1α and HIF-2α direct critical adaptations that allow cell, to survive under these conditions (23, 28). The increased expression and transcriptional activity of HIF-1α can contribute to a more aggressive malignant behavior, characterized by metastasis and chemo-resistance, in androgen-independent prostate cancers (29). Moreover, hypoxia has been proposed as a critical component of cancer stem cell niches, and induction of HIFs has been shown not only to enhance cell proliferation and survival, but also able to promote cancer stem cell maintenance (12, 28). The present data demonstrated that the induction of HIF-1α and HIF-2α in PC3 stem-like cells under hypoxic conditions was higher than that in PC3 parental cells. This study also shows that the induction of HIF-2α was lower than that of HIF-1α. These observations indicate that induction of HIFs, especially HIF-1α is required for PC3 stem-like cell adaptation to hypoxia. Although HIF-1α and HIF-2α are structurally similar, they have different target genes and mechanisms of regulation in glioblastoma, renal cell carcinoma, and pancreatic cancer cells (30). HIF-1α drives the acute response to hypoxia, whilst HIF-2α has a major role in adaptation of lung epithelial cells to chronic hypoxic conditions (31).

Ample evidence has demonstrated that HIF-1α and HIF-2α are both critical for survival and maintaining the stemness properties of cancer stem cells (28, 30). Increased HIF-1α expression has been observed prior to cancer stem cell proliferation, and is essential for the survival of pancreatic cancer stem and glioma stem cells under hypoxic conditions (32, 33). Likewise, HIF-2α has been shown to regulate octamer-binding transcription factor 4 (Oct-4), essential for maintaining stem cell pluripotency, *via* binding to its promoter, and this event is necessary for cancer stem cell survival (34). HIF-1α has been implicated as an oncogenic factor, stimulating prostate cancer progression by inducing the expression of stem cell markers (35). Based on previous as well as on the present evidence, it can be hypothesized that HIF-1α is necessary for PC3 stem-like cells to control their survival in hypoxia, while HIF-2α may be more specific for stemness traits. However, further study is necessary to clarify the exact role of HIF-2α in the adaptation of PC3 stem-like cells to hypoxia.

Cancer stem cells are thought to be responsible for tumor initiation, progression, and metastasis; therefore, the successful targeting and elimination of cancer stem cells forms the foundation of an effective treatment for various types of solid tumors (36). In particular, impaired potential of the self-renewal capacity (*in vitro* spheroid formation), one of the characteristics of cancer stem cells clearly indicates the effectiveness in targeting prostate cancer stem cells (37). It has been reported that γ-T3, a subtype of T3s, suppressed prostate cancer stem cells, as evidenced by the down-regulation of the cancer stem cell markers, reduction of sphere formation ability and tumorigenicity of the cancer stem cells (16). Also, we have reported that δ-T3 has a potent anti-prostate cancer activity (13) and a redox-silent analogue of T3 effectively inhibits hypoxia adaptation in prostate cancer cells (17). In this study, we emphasized the role of differential HIF expression in PC3 stem-like cells. δ-T3 treatment was able to effectively reduce hypoxia adaptation of PC3 stem-like cells, since it suppressed the HIF-1α and HIF-2α protein levels in PC3 stem-like cells under hypoxic condition. It has been recently reported that a redox-silent analogue of T3 reduced the expression level of HIF-1α in prostate cancer cells under hypoxia, partly due to the inactivation of the non-receptor tyrosine kinase family (17). Hence, δ-T3 might interfere with PC3 stem-like cells adaptation to hypoxia *via* the inhibition of HIF-1α levels through inactivation of the non-receptor tyrosine kinase family. However, the exact mechanisms leading to this effect remain to be elucidated.

In conclusion, δ-T3 acts as an effective inhibitor of hypoxic adaptation of PC3 stem-like cells *in vitro*. Moreover, the inhibitory effect of δ-T3 is predominantly dependent on the suppression of HIF-1α induction in PC3 stem-like cells under hypoxic condition. Overall, it seems that δ-T3 might act as an effective anti-prostate cancer agent by inhibiting hypoxia adaptation of prostate cancer stem-like cells.

Acknowledgements

This study was supported by JSPS KAKENHI, Grand-in-Aid for JSPS Fellows (15K12329), and supported by the Inoue Enryou Memorial Foundation of Toyo University.

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Received October 16, 2017
Revised December 22, 2017
Accepted January 11, 2018