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Gamma-tocotrienol as an effective agent in targeting prostate cancer stem cell-like population

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Emerging evidence supports that prostate cancer originates from a rare subpopulation of cells, namely prostate cancer stem cells (CSCs). Conventional therapies for prostate cancer are believed to mainly target the majority of differentiated tumor cells but spare CSCs, which may account for the subsequent disease relapse after treatment. Therefore, successful elimination of CSCs may be an effective strategy to achieve complete remission from this disease. Gamma-tocotrienols (γ-T3) is one of the vitamin-E constituents, which have been shown to have anticancer effects against a wide range of human cancers. Recently, we have reported that γ-T3 treatment not only inhibits prostate cancer cell invasion but also sensitizes the cells to docetaxel-induced apoptosis, suggesting that γ-T3 may be an effective therapeutic agent against advanced stage prostate cancer. Here, we demonstrate for the first time that γ-T3 can downregulate the expression of prostate CSC markers (CD133/CD44) in androgen-independent prostate cancer cell lines (PC-3 and DU145), as evident from Western blotting analysis. Meanwhile, the spheroid formation ability of the prostate cancer cells was significantly hampered by γ-T3 treatment. In addition, pretreatment of PC-3 cells with γ-T3 was found to suppress tumor initiation ability of the cells. More importantly, although CD133-enriched PC-3 cells were highly resistant to docetaxel treatment, these cells were as sensitive to γ-T3 treatment as the CD133-depleted population. Our data suggest that γ-T3 may be an effective agent in targeting prostate CSCs, which may account for its anticancer and chemosensitizing effects reported in previous studies.

Prostate cancer (PCa) is responsible for the largest number of deaths among all other cancers, except for lung cancer. Because of the slow growing nature of the tumor, many prostate cancer patients have already developed metastatic disease on diagnosis and will inevitably enter the hormone refractory stage after hormone ablation therapy. There is currently no curative treatment against hormone refractory prostate cancer (HRPC). The most effective treatment regime for patients with HRPC, docetaxel-based chemotherapy, can only improve the median survival time by 3 months.[1,2] Therefore, effective treatment strategies against metastatic HRPC are urgently needed.

The reason why current therapies fail in treating metastatic HRPC is not completely understood; however, increasing evidence has suggested that current therapies are not only successful in targeting differentiated tumor cells but also sparing the putative cancer stem/progenitor cells.[3] Similar to normal stem cells, cancer stem cells (CSCs) are thought to be quiescent compared with mature cancer cells.[4–6] This property makes CSCs resistant to chemotherapeutic drugs, which mainly target actively replicating cells. Moreover, the expression of multiple drug resistant gene (*MDR1*)[7] and ABC transporter[8] also help protect CSCs from cytotoxic drugs. In addition, two of the recent studies demonstrated that prostate CSCs are androgen independent[9,10] and may not respond to hormone ablation as mature tumor cells do. Owning to their ability to self renew and differentiate, CSCs are capable of regenerating the heterogeneous tumor population (with both androgen-dependent and androgen-independent cells) after hormone ablation, which accounts for tumor relapse. Therefore, elimination of the bulk of frequently replicating tumor cells as well as the rare subset of slow, dividing stem-like cells

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that are responsible for tumor regeneration may represent a better therapeutic strategy in the treatment of prostate cancer.

Vitamin E is a well-known antioxidant composed of two main components—tocopherols (T) and tocotrienols (T3). Increasing evidence suggests that T3s possess substantial anticancer effects in many types of cancers, including breast, liver, lung, gastric, colon, skin and prostate cancer.[11–17] Recently, we have reported that among the four isoforms (α, β, γ and δ) of T3, the gamma isoform (γ-T3) possesses the most potent anticancer effect against prostate cancer cells.[18] By comparing the effect of the four T3 isomers on nonmalignant prostate epithelial cells and prostate cancer cell lines, we found that γ-T3 is more effective in inhibiting both the growth and survival of prostate cancer cells.[18] Meanwhile, γ-T3 treatment also inhibited prostate cancer cell invasion by suppressing the epithelial to mesenchymal transition.[18] More importantly, γ-T3 promoted docetaxel-induced apoptosis in prostate cancer cells,[18] as well as docetaxel and dacarbazine-induced apoptosis in melanoma cells,[17] suggesting that it may be used as a potential chemosensitizing agent. Although T3 has been shown to regulate a number of signaling pathways, such as NK-kappaB[19] and PI3K,[20] the exact mechanisms underlying its anticancer effect are still largely unknown.

Because CSCs are believed to contribute to chemoresistance, we reasoned that the chemosensitizing effect of γ-T3 may be mediated through targeting of prostate CSCs. In this study, we show that γ-T3 treatment significantly downregulates protein expression of prostate CSC markers CD44 and CD133 in prostate cancer cells. Meanwhile, γ-T3 treatment not only suppresses the spheroid formation ability of prostate cancer cells but also interferes with their ability to form tumors *in vivo*. Interestingly, although CSC-enriched spheroids are highly resistant to docetaxel, treatment of γ-T3 at IC_{50} induced a drastic dissociation of matured spheroids. These results were further confirmed using CD133-enriched and CD133-depleted populations of PC-3 cells. Our overall findings support that γ-T3 may possess anti-CSC effects, which may contribute to its anticancer and chemosensitizing effect against prostate cancer.

Material and Methods

Gamma-Tocotrienol

The γ-T3 was provided by Davos Life Science Pvt., Ltd, Singapore, and was dissolved in absolute ethanol (100 mM) and stored at −20°C. Using the corresponding T3 isomers as reference standards, the purity of γ-T3 was verified to be ≥97% by high-performance liquid chromatograph1y.

Cell lines and culture conditions

Prostate cancer cell lines PC-3, DU145 and bladder cancer cell line MGH-U1 (ATCC, Rockville, MD) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 1% (wt/vol) penicillin-streptomycin (Invitrogen) and 5% fetal bovine serum (Invitrogen). All cell types were kept at 37°C in a 5% CO_2 environment.

Generation of PC-3 cells stably expressing luciferase

A luciferase-expressing PC-3 cell line, PC-3 luc, was generated using the Viralpower Lentiviral gene expression system (Invitrogen) according to the manufacturer's instructions. Briefly, supernatant containing the lentivirus was mixed with polybrene (8 μg/mL) and used to infect PC-3 cells. After infection, positive transfectants were selected as a pool by treatment with blasticidine (10 μg/mL) for 6 days.

Cell viability assay

Cell viability on γ-T3 treatment was measured by a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO) assay as described previously.[21] Briefly, cells were seeded on 96-well plates and treated with different concentrations of γ-T3 for the indicated time. At the end of the treatment, MTT was added to each well and incubated for 4 hr at room temperature (RT). DMSO was then added to each well to dissolve the formazan crystals. The plate was incubated for a further 5 min at RT, and the optical density (OD) was measured at a wavelength of 570 nm on a Labsystem multiskan microplate reader (Merck Eurolab, Dietikon, Schweiz). All individual wells were set in triplicate. The percentage of cell viability was presented as the OD ratio between the treated and untreated cells at the indicated concentrations.

Western blotting

Detailed experimental procedures have been described previously.[21] Briefly, whole cell lysates were prepared by resuspending cell pellets in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid and 0.1% SDS). Protein concentration was determined using D_C Protein Assay kit (Bio-Rad, Hercules, CA). Protein extract was loaded onto an SDS-polyacrylamide gel, separated by electrophoresis and then transferred to a PVDF membrane (Amersham, Piscataway, NJ). The membrane was then incubated with primary antibodies against CD133 (Miltenyi Biotec, Auburn, CA), Bcl-2, PARP, cleaved caspase 3, 7 and 9 (Cell Signaling Technology, Beverly, MA), Id-1, CD44, β-catenin and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at RT. After washing with TBS-T, the membrane was incubated with either anti-mouse or rabbit IgG secondary antibodies, and the signals were visualized using the ECL plus western blotting system (Amersham, Piscataway, NJ).

Spheroid formation assay

The spheroid formation assay was modified from a previously reported protocol.[22] Briefly, cells were trypsinized, washed with 1 × PBS and resuspended in DMEM F12 medium. Two hundred cells were added to each well of a 24-well plate precoated with polyHEMA (Sigma). Cells were

grown in DMEM/F12 (Invitrogen) supplemented with 4 μg/mL insulin (Sigma), B27 (Invitrogen), 20 ng/mL EGF (Sigma) and 20 ng/mL basic FGF (Invitrogen). Fresh medium with the above supplements was added every day. The γ-T3 was added at the indicated time points, and the number of spheroids was counted at the Day 14 of the assay or at the end of the treatment. Each experiment was repeated in triplicate, and each data point represents the mean and SD. Statistical difference was determined by Student's *t* test and was considered as significance if $p < 0.05$.

Subcutaneous and orthotopic implantation of PC-3 cells

For subcutaneous implantation of PC-3 cells, nude mice were divided into three groups. Mice in Groups 1 and 2 were injected subcutaneously with 1×10^6 PC3-Luc cells pretreated either with ethanol or with 5 μg/mL of γ-T3 for 48 hr. In Group 3, nude mice were orally fed γ-T3 (100 mg/kg/d) for 3 weeks. Then, 1×10^6 PC3-Luc cells were injected subcutaneously into the nude mice, and the mice were fed with γ-T3 (100 mg/kg/d) for another 4 weeks. Tumors volume was measured at the end of study. To detect the bioluminescent signal of the cells, mice were anesthetized and injected with 80 mg/kg of D-luciferin solution by intraperitoneal (Xenogen Corporation, Cranbury, NJ). The signal was detected by a Xenogen IVIS 100 series imaging system (units of photons per second per unit area).

The orthotopic model was established with procedures described previously,[24] with some modifications. Briefly, 8-week-old CB-17 SCID mice were anesthetized and placed under a dissecting microscope (Olympus, Tokyo, Japan). An incision at the midline of the abdomen was made, exposing the dorsal prostate at the base of the bladder. Equal amounts of viable PC3-luc cells (2.5×10^4) with or without prior γ-T3 treatment for 24 hr were injected into the dorsal prostates of the mice. The organs were replaced, and the abdomen was closed. Tumor development was monitored by measuring the bioluminescent signal every 2 weeks for 6 weeks after tumor implantation. Statistical difference was determined by two-tailed *t* test and was considered as significance if $p < 0.05$. All surgical and animal handling procedures were carried out according to the guidelines of the Committee on the Use of Live Animals in Teaching and Research (CULATR), the University of Hong Kong.

Isolation of CD133-enriched and CD133-depleted population

CD133-enriched and CD133-depleted populations were isolated from the PC3 cell line using the magnetic cell separation kit (Miltenyi Biotec GmbH, Germany). PC3 cells (1×10^8) were suspended in 300 μL of PBS buffer, pH 7.2, containing 0.5% BSA and 2 mM EDTA. One hundred microliters of FcR Blocking Reagent and CD133 microbeads were added to the cells. The cell mixture was mixed well and incubated for 30 min at 4°C. After incubation, cells were washed with 10 mL of buffer and centrifuged at 300*g* for 10 min. The cell pellet was resuspended in 500 μL of PBS buffer. A column coated with CD133 microbeads was rinsed with 500 μL of PBS buffer before cell loading. The cell suspension was loaded onto the column, and the flow-through containing the CD133-depleted population was collected. The column was washed with three column volumes of PBS buffer. The column was removed from the separator and placed on a collection tube. The CD133-enriched population was eluted by adding 1 mL of PBS buffer and firmly pushing the plunger. To increase the purity of the CD133-positive population, the eluent was run through a second column.

Results

Effect of γ-T3 on CSC marker expression

To test if γ-T3 affects CSC properties, we first investigated the effect of γ-T3 on the expression of prostate CSC markers in PC-3 cells, which has been reported to contain CSCs.[25] PC-3 cells were first treated with increasing doses of γ-T3 (0, 2.5 and 5 μg/mL) for 24, 48 and 72 hr. After treatment, expression of the two established prostate CSC markers, CD44 and CD133, were examined by Western blotting. As shown in Figure 1*a*, protein expression of CD44 was significantly downregulated after γ-T3 treatment in a time- and dose-dependent manner. Similarly, γ-T3 treatment was also found to suppress the expression of CD133 (Fig. 1*a*), suggesting that γ-T3 may have an anti-CSC effect.

To understand if the decrease in CSC marker expression is due to induction of cell death by γ-T3, cell viability assays and Western blotting of common apoptotic markers were performed. Interestingly, both methods failed to detect a drastic reduction of cell viability by γ-T3 (Fig. 1*b*,*c*) at the selected dosages, although apoptosis can be detected at a higher dosage (Supporting Information Fig. 1), suggesting that the decrease in CSC marker expression may not due to induction of cell death by γ-T3. However, we were able to detect the downregulation of a number of proteins that have previously been shown to play key roles in the maintenance of normal and cancer stem cells. As shown in Figure 1*c*, expression of beta-catenin, which has been shown to maintain the CSC phenotype of squamous cell carcinoma,[26] was significantly suppressed by γ-T3 treatment in a dose- and time-dependent manner. Similarly, Id-1 and bcl-2, which are essential for maintaining the stemness of hematopoietic stem cells,[27,28] were also downregulated by γ-T3 treatment. These results, together with the decrease in prostate CSC marker expression, prompted us to speculate that γ-T3 treatment may be able to specifically suppress CSC properties.

γ-T3 suppresses CSC marker expression in other cancer cell lines

Results from the above experiments demonstrated that γ-T3 treatment inhibits CSC marker expression in the androgen-independent prostate cancer cell line PC-3. However, it is possible that the suppressive effect is only specific to PC-3 cells rather than a general effect. Therefore, we decided to

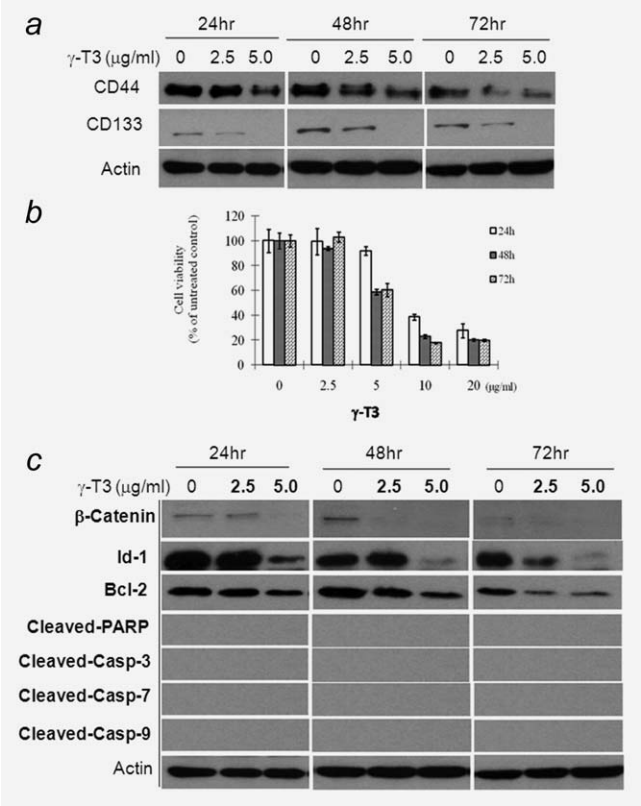


Figure 1. γ-T3 downregulates prostate CSC markers in PC-3 cells. (*a*) Western blotting of prostate CSC markers CD44 and CD133 after γ-T3 treatment. Note that γ-T3 significantly downregulates both stem cell markers in a dose- and time-dependent manner. (*b*) Viability of PC-3 cells after treatment with 2.5 and 5 μg/mL of γ-T3 for 24, 48 and 72 hr was examined by an MTT assay. Each experiment was repeated at least three times. The results are presented as the mean ± SD. (*c*) Western blotting result of stem cell maintenance proteins and apoptotic markers in γ-T3-treated PC-3 cells. Note that no cleaved forms of PARP or caspase-3, caspase-7 and caspase-9 were detected, indicating no induction of apoptosis by γ-T3 treatment.

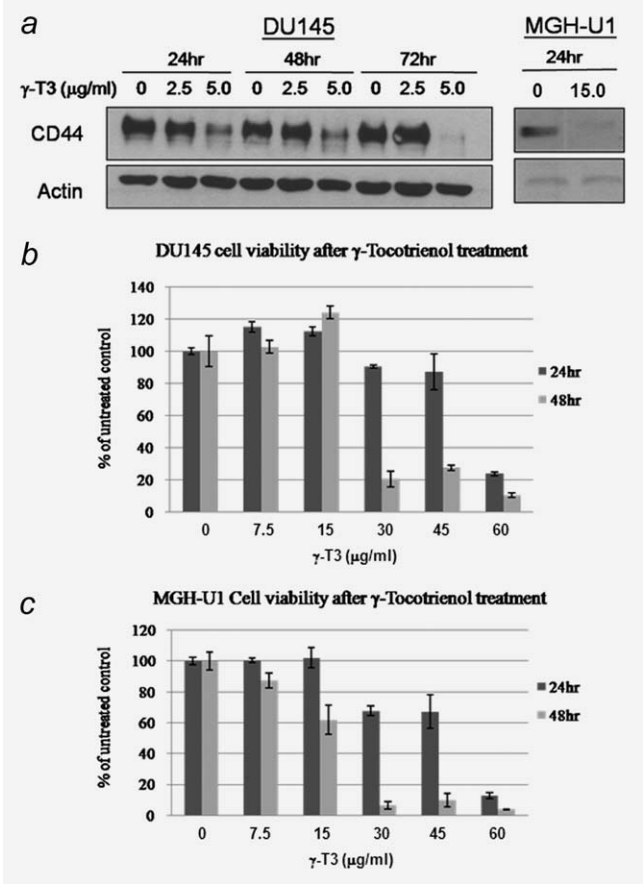


Figure 2. γ-T3 suppresses CSC marker expression in other cancer cell lines. (*a*) Western blotting of CD44 in vehicle and γ-T3 treated DU145 and MGH-U1 cells. CD44 expression of both cell lines was down-regulated after low dose γ-T3 treatment. (N.B. 5 μg/ml of γ-T3 is equivalent to 12.176 μM). MTT assay showing the viability of DU145 (*b*) and MGH-Ul (*c*) cells after treatment with different doses of γ-T3 for 24 and 48 hr.

repeat our experiments using other cancer cell lines. DU145 is another prostate cancer cell line that has been shown to possess CSC properties, and, as shown in Figure 2, at doses that have minimal effects on cell viability, γ-T3 treatment suppresses CD44 expression in a time- and dose-dependent manner. Similar effects were observed in a bladder cancer cell line (MGH-U1) (Fig. 2*a*,*b*), suggesting that the observed effect of γ-T3 on CSCs may not be restricted to prostate cancer.

γ-T3 inhibits prostasphere formation of PC-3 cells under nonadherent culture conditions

The ability to form prostaspheres in nonadherent culture is one of the characteristics of prostate CSCs.[9,29,30] To confirm that γ-T3 treatment can inhibit prostate CSC properties, prostasphere formation of PC-3 cells was studied in the presence or absence of γ-T3. As shown in Figure 3*a*, after culturing the cells for 14 days in nonadherent condition, an average of 21 prostaspheres per well were found in the untreated group. However, no prostaspheres were observed in wells treated with γ-T3 (Fig. 3*a*). Similarly, the spheroid formation ability of DU145 and MGH-U1 was almost completely suppressed by γ-T3 treatment (Fig. 3*b*,*c*). These results suggest that γ-T3 can effectively inhibit spheroid formation in CSCs.

γ-T3 significantly reduces the tumorigenicity of prostate cancer cells *in vivo*

Because CSC is suggested to a play role in cancer initiation, it is possible that γ-T3 treatment may inhibit the tumor formation ability of PC-3 cells. To test this hypothesis, PC-3 cells constitutively expressing the luciferase reporter gene (PC-3-luc) were pretreated with either vehicle or 5 μg/mL of γ-T3 for 48 hr. Subsequently, viable PC-3-luc cells from each group were injected subcutaneously into nude mice. Examination of tumor formation revealed that all mice injected

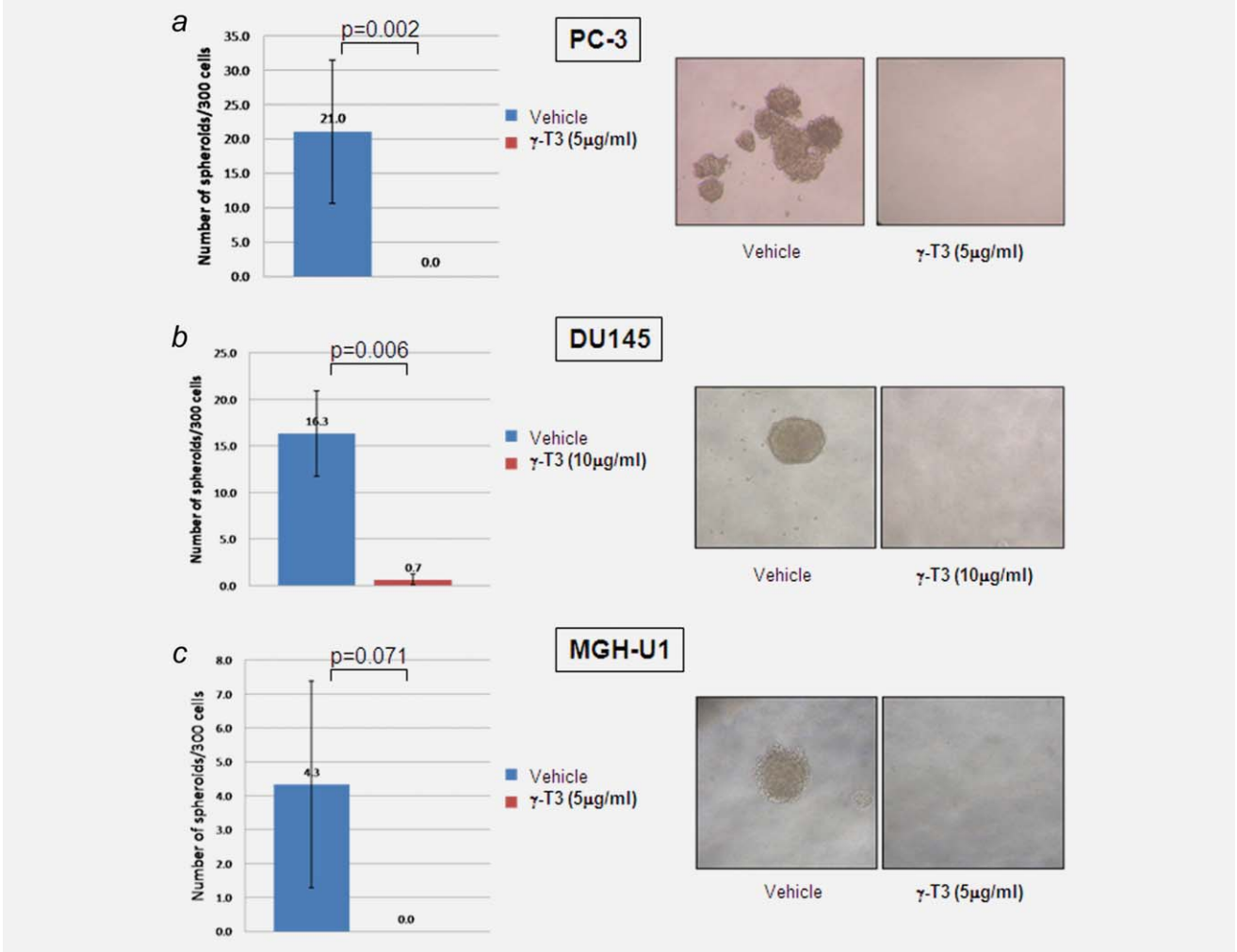
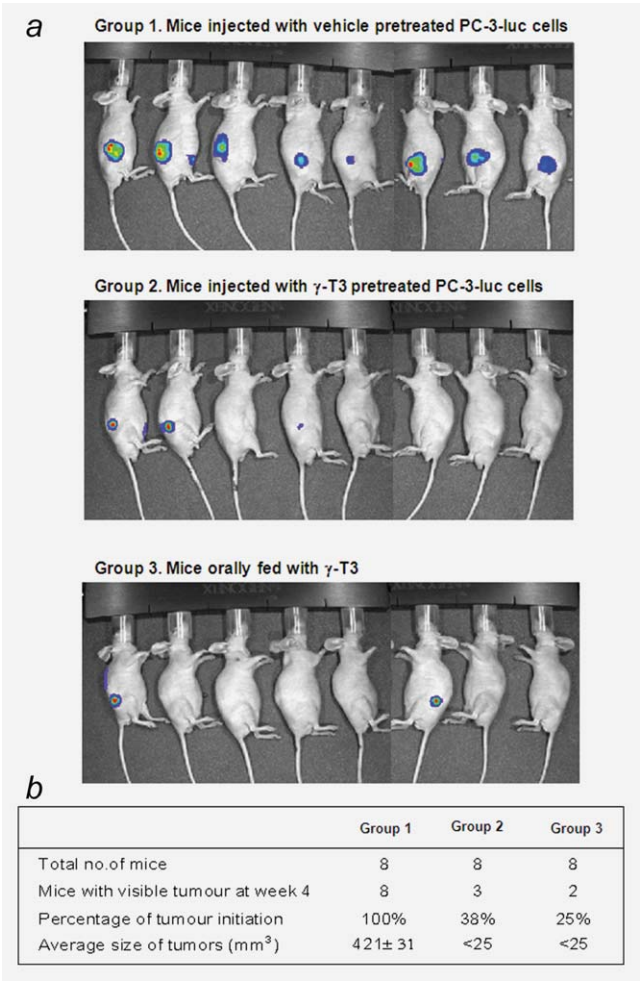


Figure 3. Effect of γ-T3 on CSC properties. (*a*) Spheroid formation assay was performed with cells treated with γ-T3 or vehicle. Two hundred PC-3 cells were seeded onto polyHEMA-coated 12-well plates and treated with either γ-T3 or vehicle for 14 days. The number of prostaspheres formed was counted, and the result was presented as the mean ± SD. Note that γ-T3 treatment efficiently suppresses the spheroid formation ability of PC-3 cells. Image of the prostaspheres was captured under microscope. Note that no prostaspheres can be found in the γ-T3 treated group. Spheroid formation assay was performed with DU145 (*b*) and MGH-U1 (*c*) cells treated with γ-T3 or vehicle. Note that γ-T3 treatment efficiently suppresses the spheroid formation ability of both cell lines. Images of the spheroids were captured under microscope. Note that no spheroids can be found in γ-T3-treated groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with vehicle-pretreated PC-3-luc formed tumors 4 weeks after injection (Fig. 4*a*). However, only three of eight mice injected with γ-T3 pretreated PC-3-luc cells developed tumors (Fig. 4*a*), suggesting that γ-T3 inhibits tumor formation by directly suppressing the tumorigenicity of the PC-3-luc cells. Our findings were further confirmed by orthotopically injecting γ-T3 pretreated PC-3-luc cells into SCID mice, which demonstrated that more than half of the mice (five of seven) implanted with γ-T3 pretreated PC-3-luc failed to develop visible tumors (Fig. 4*c*). The significant decrease in tumor initiation rate indicates that γ-T3 can reduce the tumorigenic potential of highly aggressive PC-3 cells, which is likely due to the decrease of the CSC population after γ-T3 treatment. To see if oral consumption of γ-T3 can achieve the same effect, nude mice were fed γ-T3 (100 mg/kg/d) for 3 weeks before subcutaneous injection of PC-3-luc cells. After injection, the mice were continually fed with γ-T3 for another 3 weeks at the same dosage. Four weeks after implantation, tumor formation was examined by bioluminescence imaging. As shown in Figure 4*a*, only two of eight mice that were fed with γ-T3 had tumors. These data strongly support that γ-T3 oral consumption can also significantly suppress the tumorigenicity of PC-3-luc cells *in vivo*.

γ-T3 effectively eliminates chemoresistant cancer stem-like cells

Recent studies have suggested that the presence of CSCs may contribute to the development of chemoresistance.[23,31] We



	Group 1	Group 2	Group 3
Total no.of mice	8	8	8
Mice with visible tumour at week 4	8	3	2
Percentage of tumour initiation	100%	38%	25%
Average size of tumors (mm^3)	421± 31	<25	<25

Figure 4. γ-T3 inhibits tumorigenicity of PC-3 cells *in vivo*. (*a*) Bioluminescent images of nude mice that were subcutaneously injected with PC3-Luc cells for 4 weeks. In the top panel, nude mice were injected with vehicle treated PC3-Luc cells (Group 1). In the middle panel, nude mice were injected with PC3-Luc cells pretreated with 5 μg/mL of γ-T3 for 48 hr (Group 2). In the bottom panel, nude mice were fed with γ-T3 before injection with PC3-Luc cells (Group 3). (*b*) Summary of tumor incidence at Week 4. Note that γ-T3 pretreatment leads to ≥60% inhibition of tumor incidence ($p = 0.011$). (*c*) Bioluminescent images of SCID mice that were orthotopically injected with PC-3-luc cells for 2 weeks. SCID mice in the upper row were injected with vehicle-treated PC-3-luc cells while mice in the bottom row were injected with γ-T3-treated PC-3-luc cells. Note that γ-T3 pretreatment leads to ≥50% inhibition of tumor incidence ($p = 0.078$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

believe that the previously reported chemosensitization effect of γ-T3[17,18] is a result of elimination of CSCs by γ-T3. To test this hypothesis, we first compared the sensitivity of CD133-enriched and depleted populations of PC-3 cells with docetaxel, a chemotherapeutic drug currently used for the treatment of HRPC. As demonstrated in the cell viability assay, the CD133-enriched population is highly resistant to docetaxel treatment. Cells remain 100% viable when treated at a dosage of 40 ng/mL (Fig. 5*a*). However, a drastic decrease in cell viability was observed when the CD133-depleted population was exposed to the same dosage of docetaxel. These data clearly demonstrate the chemoresistant characteristic of prostate CSCs. More importantly, despite the difference in docetaxel sensitivity, both CD133-enriched and CD133-depleted populations were extremely sensitive to γ-T3 treatment, as evidenced by the >50% inhibition in cell viability in the presence of γ-T3 (Fig. 5*a*). Examination of apoptotic markers produced similar results (Fig. 5*b*), suggesting that γ-T3 is effective in targeting both the docetaxel sensitive and resistant population. To confirm our findings, we tested if γ-T3 can also target the preformed prostasphere, which has been shown to contain an enriched-CSC population.[30,32] Prostaspheres were formed by growing DU145 cells in non-adherent culture for 14 days, at which time each prostasphere had reached a considerable size. As expected, these prostaspheres were highly resistant to docetaxel (Fig. 5*c*). At a dosage of 40 ng/mL, which has been shown to induce apoptosis in DU145 cells,[33] docetaxel failed to induce any observable effect on prostasphere number, suggesting that CSC-enriched cells are highly resistant to docetaxel. However, γ-T3 treatment was found to significantly decrease the spheroid number by more than 70% (Fig. 5*c*). In addition to the decrease in spheroid number, γ-T3 treatment also reduced the size of the spheroids and induced a more diffuse structure (Fig. 5*c*). These findings suggest that the chemosensitizing effect of γ-T3 is likely due to its anti-CSC activity.

Alpha-tocopherol does not interfere with the anti-CSC effect of γ-T3

We have previously demonstrated that alpha-tocopherol (α-T) attenuates the anticancer effect of γ-T3. Although the mechanism for this attenuation is still unclear, it is believed that α-T may compete with γ-T3 for the tocopherol-transport protein, thus inhibiting the intake of γ-T3 by the cancer cells. To test if the anti-CSC effect of γ-T3 was affected by α-T, CD133-enriched or CD133-depleted populations were treated with γ-T3 alone or in combination with α-T. As shown in Figure 6*a*, addition of α-T restored the viability of the CD133-depleted population, an effect similar to that reported previously.[34] Meanwhile, α-T was also found to interfere with γ-T3-induced Id-1 downregulation and apoptosis (Fig. 6*b*). Surprisingly, α-T did not affect the effect of γ-T3 on the CD133-enriched population, as evidenced from the cell viability assay (Fig. 6*a*). Similarly, α-T also failed to protect the CD133-enriched population from γ-T3-induced apoptosis (Fig. 6*b*), suggesting that the CSC-enriched and CD133-depleted populations respond differently to α-T and γ-T3 treatment.

Discussion

T3s are constituents of vitamin E that have long been studied for their antioxidative, anticardiovascular disease, neuroprotective and anticancer effects.[35] The anticancer effect of T3

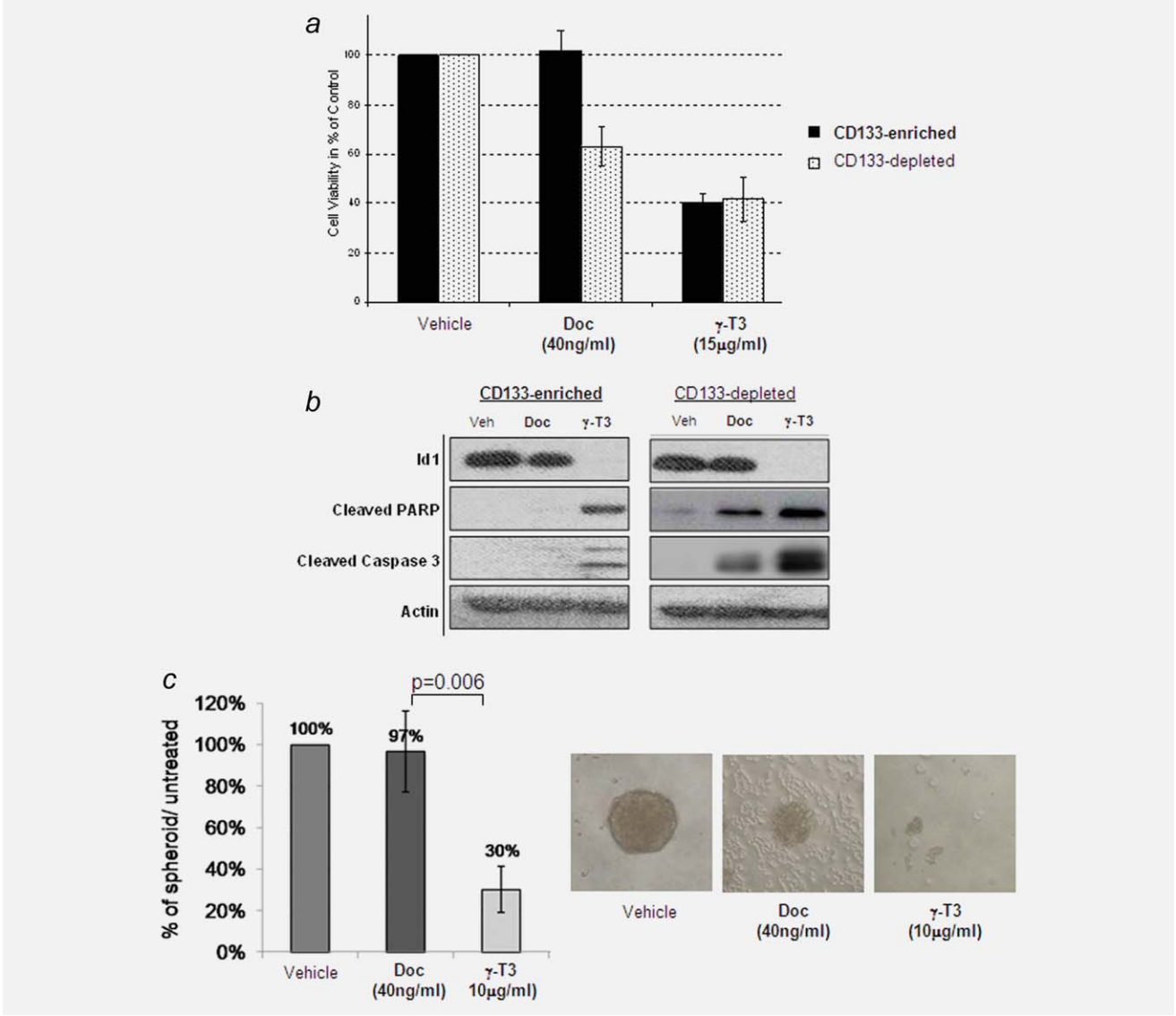


Figure 5. Effect of γ-T3 on CSC-enriched populations. (*a*) The effect of γ-T3 and docetaxel on CD133-enriched and CD133-depleted populations of PC3 cells was determined by an MTT assay. Note that the CD133-enriched population is resistant to docetaxel (40 ng/mL) but is highly sensitive to γ-T3 (15 μg/mL). Each experiment was repeated at least three times. The results are presented as the mean ± SD. (*b*) Analysis of apoptotic markers by Western blotting in CD133-enriched and CD133-depleted cells treated either with γ-T3 or docetaxel. Note that γ-T3, but not docetaxel, induces apoptosis in both CD133-enriched and CD133-depleted cells. Beta-actin was used as a loading control. (*c*) CSC enriched prostaspheres were formed by maintaining DU145 cells in nonadherent culture supplemented with serum replacement medium for 14 days. The prostaspheres were then treated with either vehicle, γ-T3 (10 μg/mL) or docetaxel (Doc, 40 ng/mL) for 48 hr. Spheroids were counted under the microscope before and after treatment. The results were presented as the mean % change in spheroid number to control ± SD. Note that spheroids were highly sensitive to γ-T3 treatment but resistant to docetaxel. The right panel shows images of the prostaspheres after 48 hr of treatment with vehicle, 40 ng/mL of docetaxel or 10 μg/mL of γ-T3. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was found to be mediated through antiproliferation,[16,36,37] induction of apoptosis,[38,39] cell cycle arrest,[13,40] antiangiogenesis[41] and antiinvasion.[18] More recently, γ-T3, one of the four isoforms of T3, was reported to possess chemosensitizing effects,[17,18] which led us to speculate that γ-T3 may be able to target the chemoresistant cancer stem-like cells. Here, we demonstrated for the first time that γ-T3 has anti-CSC effects, as evidenced by the downregulation of CSC markers and the suppression of prostasphere and tumor formation.

Putative CSCs in the prostate were first identified by Collins *et al.*[42] in 2005, where CD44+/alpha2beta1hi/CD133+ were identified as CSC surface markers. These cancer-initiating cells have also been identified in the established androgen-dependent cell line LNCaP[43] and in androgen-

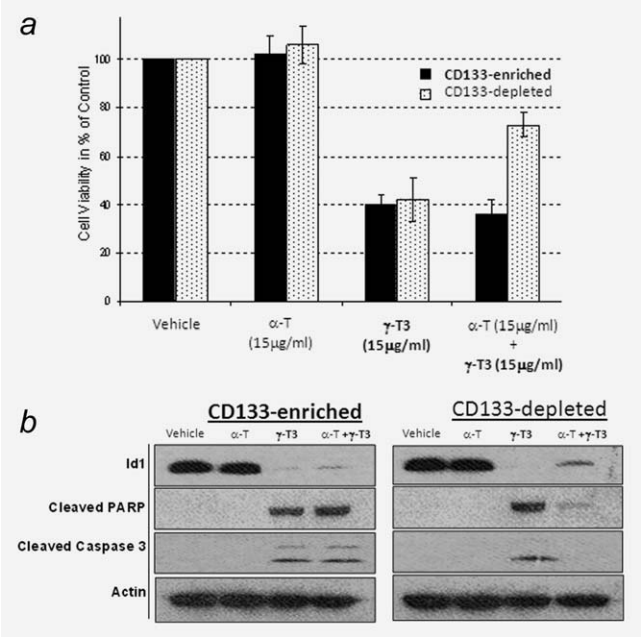


Figure 6. α-T protects CD133-depleted but not CD133-enriched populations of PC-3 cells from γ-T3-induced apoptosis. (*a*) The effect of γ-T3 and α-T on CD133-enriched and CD133-depleted populations was determined by an MTT assay. Note that α-T restores cell viability of the CD133-depleted population, but not the CD133-enriched population. Each experiment was repeated at least three times. The results are presented as the mean ± SD. (*b*) Analysis of apoptotic markers by western blotting in CD133-enriched and CD133-depleted cells treated with γ-T3 alone or in combination with α-T. Note that α-T protects the CD133-depleted but not CD133-enriched population from γ-T3-induced apoptosis. Beta-actin was used as a loading control.

independent prostate cancer cell lines DU145[43,44] and PC-3.[25,45] In this study, we showed that, in PC-3 cells, expression of CSC markers CD44 and CD133 was downregulated by γ-T3 treatment (Fig. 1). We also observed a significant decrease of CD44 in the androgen-independent prostate cancer cell line DU145 and bladder cancer cell line MGH-U1 (Fig. 2). Interestingly, the downregulation of CSC markers by γ-T3 correlated with the suppression of proteins crucial for the maintenance of CSCs. Beta-catenin, for example, has been shown to be important in sustaining the CSC phenotype of squamous carcinoma cells through inhibition of cellular differentiation.[26] Similarly, Id-1 has also been demonstrated to modulate stem cell renewal and differentiation.[27] Therefore, in the presence of γ-T3, the decrease in β-catenin and Id-1 expression may account for the loss of stemness of prostate CSCs.

The ability to form spheres in nonadherent, serum free conditions is a key property of stem cells.[46] Recently, a spheroid formation assay was used to identify and to enrich putative CSCs.[30,32,47,48] In this study, all three malignant cell lines PC-3, DU145 and MGH-U1 were able to form spheroids in nonadherent culture, suggesting the presence of cancer stem-like cells within these cell lines. Because prostaspheres are enriched with CSCs (6.25 and 12.2% of CD133+CD44+ cells in PC-3 and DU145 spheres, respectively),[30] the inhibitory effect of γ-T3 on prostasphere formation supports that γ-T3 may be a potent agent in targeting or eliminating prostate cancer stem-like cells *in vitro* (Fig. 3). Similar effects were also observed in MGH-U1 cells, where γ-T3 treatment resulted in 100% inhibition in spheroid formation (Fig. 3*c*). Although the putative CSCs in bladder are yet to be identified, the suppressive effect of γ-T3 on the stem cell properties of MGH-U1 suggests that the anti-CSC effect of γ-T3 is not restricted to prostate cancer. This is supported by the finding that γ-T3 can also downregulate CD44 expression in bladder cancer cells.

The ability of CSCs to generate serial transplantable tumors *in vivo* suggests that they are likely to be the tumor initiating cells.[49] This hypothesis is supported by the fact that an isolated CSC population is more tumorigenic than a non-CSC counterpart when injected into immunocompromised mice.[25,30,45] In this study, pretreating PC-3 cells with γ-T3 before injection into mice was found to significantly inhibit the tumorigenicity of PC-3 cells (Fig. 4). Despite the fact that all γ-T3 pretreated PC-3 cells can eventually develop detectable tumors (data not shown), the drastic decrease in detectable tumors at early tumor initiation stages, and the delay of tumor formation support our hypothesis that γ-T3 is potent in targeting prostate CSCs. More interestingly, when mice injected with PC-3 cells were fed with γ-T3, most of them failed to develop tumors (Fig. 4*a*), suggesting that γ-T3 intake can significantly inhibit the tumorigenicity of PC-3 cells *in vivo*.

The presence of CSCs is suggested to contribute to chemoresistance. Prostate cancer cells are in general highly resistant to common chemotherapeutic agents. Docetaxel represents the only effective chemodrug that has demonstrated significant improvement in patient survival.[1,2] Based on a previous study, the IC90 dosage of docetaxel for DU145 cells is 1.01 ng/mL.[33] However, in this study, 40 ng/mL of docetaxel was unable to induce any effect on the viability of the CSC-enriched population (Fig. 5). Meanwhile, the same dosage of docetaxel also failed to affect the prostasphere, providing further evidence that CSCs are the origin of docetaxel resistance. Despite their resistance to docetaxel treatment, both CSC-enriched populations and prostaspheres were extremely sensitive to γ-T3 treatment (Fig. 5). Intriguingly, unlike CSC-depleted populations, addition of α-T failed to protect the CSC-enriched population from γ-T3-induced apoptosis (Fig. 6). What accounts for this difference remains unclear. Nonetheless, the results highlight the importance of the anti-CSC effect of γ-T3 in docetaxel chemosensitization.

In summary, we have demonstrated for the first time that γ-T3 treatment not only downregulates prostate CSC marker expression but also effectively inhibits CSC properties. Our results suggest that γ-T3 may be an effective therapeutic agent for the treatment of advanced stage prostate cancer.

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