Original article

Phenethyl isothiocyanate suppresses cancer stem cell properties in vitro and in a xenograft model

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Background: Cancer stem cells (CSCs) are a subset of cells within the bulk of a tumor that have the ability to self-renew and differentiate, and are thus associated with cancer invasion, metastasis, and recurrence. Phenethyl isothiocyanate (PEITC) is a natural compound found in cruciferous vegetables such as broccoli and is used as a cancer chemopreventive agent; however, its effects on CSCs are little known.

Purpose: To evaluate the effect of PEITC on CSCs in this study by examining CSC properties.

Methods: NCCIT human embryonic carcinoma cells were treated with PEITC, and the expression of pluripotency factors Oct4, Sox-2, and Nanog were evaluated by luciferase assay and western blot. Effect of PEITC on self-renewal capacity and clonogenicity were assessed with the sphere formation, soft agar assay, and clonogenic assay in an epithelial cell adhesion molecule (EpCAM)-expressing CSC model derived from HCT116 colon cancer cells using a cell sorting system. The effect of PEITC was also investigated in a mouse xenograft model obtained by injecting nude mice with EpCAM-expressing cells.

Results: We found that PEITC treatment suppressed expression of the all three pluripotency factors in the NCCIT cells, in which pluripotency factors are highly expressed. Moreover, PEITC suppressed the self-renewal capacity and clonogenicity in the EpCAM-expressing CSC model. EpCAM was used as a specific CSC marker in this study. Importantly, PEITC markedly suppressed both tumor growth and expression of three pluripotency factors in a mouse xenograft model.

Conclusion: These results demonstrate that PEITC might be able to slow down or prevent cancer recurrence by suppressing CSC stemness.

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Introduction

Epidemiological studies have shown that consuming fruits and vegetables can help reduce cancer risk. Glucosinolates are rich sources of sulfur-containing compounds, are found in cruciferous vegetables such as broccoli, Brussels sprouts, and watercress and have inhibitory effects on the initiation, promotion, and progression stage of cancer (Murillo and Mehta, 2001; Navarro et al., 2011).

Cruciferous vegetables contain a variety of glucosinolates, each of which forms a different isothiocyanate when hydrolyzed (Fenwick et al., 1983). Although derived from the same botanical family, the glucosinolate content of different cruciferous vegetables varies substantially depending on the variety, cultivation, climate, and agronomic factors associated with plant growth (Fenwick et al., 1983; Navarro et al., 2011). Phenethyl isothiocyanate (PEITC), a naturally occurring isothiocyanate generated from glucosinaturin (phenethyl-glucosinolate) via hydrolysis by the enzyme myrosinase, is present in watercress at higher concentrations than in other cruciferous vegetables (Fenwick et al., 1983; Navarro et al., 2011). PEITC has been shown to induce cell cycle arrest, apoptosis, and expression of phase II detoxifying enzymes, as well as to block
inflammation (Cheung and Kong, 2010). However, the inhibitory effects of PEITC on cancer stem cells (CSCs) are little known. Ju and colleagues have indirectly demonstrated that PEITC has a suppressive activity on drug-resistant colorectal cancer cells, which have common features with CSCs (Ju et al., 2016).

Current treatment options for cancer patients include surgery and radio-, chemo-, immune, and hormone therapies. However, therapeutic resistance and tumor recurrence are major obstacles for long-term treatment success (Urruticoechea et al., 2010). Although the preceding underlying mechanisms are unknown, accumulating evidence suggests that CSCs are responsible for these phenomena. CSCs are a sub-population of tumor cells that exhibit embryonic stem cell (ESC) characteristics such as self-renewal and differentiation (Reya et al., 2001) and contribute to tumor initiation as well as invasion, metastasis, and recurrence (Gangemi et al., 2009; Reya et al., 2001). Therefore, agents that target CSCs might have the potential to suppress cancer recurrence as well as to cure cancer.

Pluripotency-associated transcription factors such as octamer-binding transcription factor (Oct4), sex determining region Y-box (Sox), and Nanog play critical roles in self-renewal and maintenance of stemness in ESCs and CSCs (Ben-Porath et al., 2008; Liu et al., 2013). These factors are expressed in malignant or poorly differentiated tumors and are closely associated with tumor development, including self-renewal capacity and oncogenic potential (Ben-Porath et al., 2008; Reya et al., 2001). Epithelial cell adhesion molecule (EpCAM) is a transmembrane protein that is mainly expressed in normal epithelial cells and can interfere with cadherin-mediated cell-to-cell contact (Munz et al., 2009). Numerous reports have shown that EpCAM is an oncogenic signal transducer that is highly expressed in various types of cancer and is associated with enhanced proliferation, migration, metastasis, and invasion by promoting epithelial mesenchymal transition (EMT) and expression of cancer-related target genes (Munz et al., 2009). Moreover, it is frequently overexpressed in CSCs isolated from colonic, breast, pancreatic, and prostate carcinomas (Gires et al., 2009) and plays an important role in regulating cancer-initiating abilities in colonic CSCs (Lin et al., 2012; Munz et al., 2009). Silencing of EpCAM by RNA interference resulted in decreased proliferation, migratory, and invasive capacities (Gires et al., 2009; Munz et al., 2009). Therefore, therapeutic strategies targeting the suppression of CSC pluripotency factors and EpCAM may be effective for cancer chemoprevention and treatment.

We evaluated the effect of PEITC on CSCs in the present study by examining CSC properties such as expression of the pluripotency factors Oct4, Sox-2, and Nanog; clonogenicity; and self-renewal ability in CSC models established from sorted EpCAM+ HCT116 colorectal cancer cells and NCCIT human embryonic carcinoma cells treated with PEITC. We also assessed whether PEITC inhibits tumor growth in a xenograft mouse model established by inoculation of EpCAM+ HCT116 cells.

Materials and methods

Cell culture and treatment

The 293T human embryonic kidney cell line and NCCIT and HCT116 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). 293T cells were cultured in MEM containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 × nonessential amino acids, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). NCCIT cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2.5 g/l d-glucose, and antibiotics. HCT116 cells were cultured in MEM supplemented with 10% FBS and antibiotics. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. PEITC (99% purity; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO for in vitro experiments. The DMSO concentration did not exceed 0.1% and was equal across all treatment groups, including the control group.

Transient transfection and luciferase (luc) assay

The transcriptional activities of Oct4 and Nanog after 24 h of treatment with PEITC were measured with the luciferase (luc) reporter assay. Oct4 transcriptional activity was measured as previously described (Yun et al., 2015b). Briefly, 293T cells were transfected with both 0.5 μg pcDNA3-mOct4 and 2 μg pGL3-Oct4(10×)TATAluc reporter vector, and 1 μg pRL-CMV; NCCIT cells were transfected with 2 μg pGL3-Oct4(10×)TATAluc reporter vector and 1 μg pRL-CMV. To measure Nanog transcriptional activity, 293T cells were transfected with both 0.7 μg pBABE-HA-Nanog and 0.7 μg pGL3-Nanog(5×)TATAluc reporter vector, and 0.1 μg pRL-CMV; NCCIT cells were transfected with 1 μg pGL3-Nanog(5×)TATAluc reporter vector and 0.2 μg pRL-CMV. Oct4 and Nanog constructs were provided by Dr. Jungho Kim (Sogang University, Seoul, Korea).

Cell sorting and sphere formation

HCT116 cells were harvested, sorted, and stained as previously described with slight modifications (Yun et al., 2015a). Briefly, cells were incubated with a phycoerythrin (PE)-conjugated antibody against human EpCAM (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4 °C on a rotator. Stained cells were sorted using an S3e cell sorter (Bio-Rad, Hercules, CA, USA).

For the sphere formation assay, sorted EpCAM+ cells were cultured on ultra-low attachment 100-mm dishes (Corning Inc., Corning, NY, USA) in stem cell medium (SCM) containing serum-free DMEM/F12 (Corning Inc.) supplemented with 20 ng/ml epidermal growth factor (EGF), 20 ng/ml of bFGF, 0.5 × B27 (all from Invitrogen, Carlsbad, CA, USA), and antibiotics. After 6 days of enrichment, cells were collected by low-speed centrifugation and dissociated with 0.25% trypsin. Single cells were used for subsequent experiments.

Clonogenic and soft agar assays

Single cells isolated from spheres were seeded at a density of 3 × 103 cells/well in a 6-well plate and cultured for 6–8 days. To measure the inhibitory effects of PEITC, the cells were treated with 10 or 20 μM PEITC in fresh culture medium, which was refreshed every 3 days. After 6–8 days, cell colonies were stained with Coomassie Brilliant Blue R-250 as previously described (Kang et al., 2009).

For the soft agar assay, single cells from spheres (3 × 103 cells) were resuspended in sphere-forming medium containing 0.6% soft agar (Sigma-Aldrich) and PEITC (10 or 20 μM) and layered on 1.2% base soft agar containing SCM in 6-well plates. After 6–8 days, the cells were stained with crystal violet solution (0.05% Crystal Violet, 1% formaldehyde, and 1% MeOH in PBS) for 20 min and then measured using an i-MAX Gel Image Analysis System (Core Bio System, Seoul, Korea) and Alpha View software (Alpha Innotech, San Leandro, CA, USA).

Mouse tumor xenografts and treatment

Male nude mice (BALB/c nu/nu; 5 weeks old) were purchased from Orient Bio (Seongnam, Korea) and maintained in high-efficiency particulate air resistance filter-top cages with free access to food and water on a 12:12-h light/dark cycle. The mice were
acclimated for at least 1 week before they were used in experiments. Animal handling and treatment protocols were approved by the Institutional Animal Care and Use Committee of Korea Institute of Science and Technology (2015–026). For the xenograft model, mice were injected subcutaneously into the flank with 3 × 10^6 EpCAM^+ cells in 100 μL PBS, and tumor volumes and body weight were recorded once a week. Tumor volumes were measured using calipers and determined using the formula (0.5 × length × width^2). When tumor size reached 50 mm^3, the mice were randomized into two groups (8 mice/group): the treatment group received PEITC (20 mg/kg) in solvent composed of 10% cremophor (Sigma-Alrich), 10% EtOH, and 80% PBS by intraperitoneal injection five times weekly (Monday to Friday), and the control group received an equal volume of solvent. On day 26, the mice were sacrificed 48 h after the last PEITC administration and the tumors were excised. Part of each tumor was immediately frozen in liquid nitrogen and stored at −70 °C for western blot analysis, whereas the remaining portion was fixed in 4% formaldehyde solution. Fixed tumors were embedded in paraffin and sectioned at a thickness of 4–5 μm, and processed for hematoxylin and eosin (H&E) staining and immunohistochemistry.

*H&E staining*

Paraffin-embedded tumor tissue sections mounted on slides were deparaffinized and stained with H&E as previously described (Kim et al., 2015). Histological analysis was carried out using an Axio Zoom.V16 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

*Immunocytochemistry and immunohistochemistry*

Immunocytochemistry was performed as previously described (Kim et al., 2015; Yun et al., 2010) with slight modifications. Spheres were plated on coverslips coated with Matrigel matrix (BD Biosciences, San Jose, CA, USA) for 24 h, then incubated with primary antibodies against Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Nanog (Cell Signaling Technology, Danvers, MA, USA), EpCAM (Cell Signaling Technology), or Sox-2 (Thermo Fisher Scientific, Waltham, MA, USA). Immunoreactivity was detected with Alexa Fluor 594-conjugated anti-rabbit and Alexa Fluor 488-conjugated anti-mouse secondary antibodies (Thermo Fisher Scientific). Samples were imaged using a Nikon TE2000-U fluorescence microscope (Nikon, Kanagawa, Japan).

For immunohistochemistry, slides were incubated with primary antibodies against Oct4, Sox-2, and Nanog overnight at 4 °C, then washed with PBS containing 0.05% Triton X-100 followed by incubation with secondary antibodies using the Vectastain Elite ABC kit (Vector Laboratories). Samples were developed by treatment with diaminobenzidine, counterstained with hematoxylin, and visualized with the Axio Zoom.V16 fluorescence microscope.

*Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay*

Apoptosis was detected using an in situ TUNEL detection kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s protocol. TUNEL-positive cells in the tumor tissue were visualized using a TCS SPS confocal microscope system (Leica, Wetzlar, Germany) and quantified by calculating the percentage of TUNEL-positive cells from three different images for each condition.

*Western blot analysis*

Single cells dissociated from spheres were seeded on ultra-low attachment 100-mm plates at a density of 6 × 10^5 cells/plate and treated with 10 or 20 μM PEITC for 6 days. Fresh medium containing the same concentration of PEITC was added every 3 days. The cells were lysed in RIPA buffer containing protease inhibitor (Sigma-Aldrich) and sonicated. Frozen tumor samples were homogenized in RIPA buffer containing protease inhibitor using a modular homogenizer (CAT Scientific, Paso Robles, CA, USA). All samples were used for western blotting, which was carried out as previously described (Yun et al., 2010; Yun et al., 2015b) using antibodies against the following proteins: Oct4 and β-actin (Santa Cruz Biotechnology); ATP-binding cassette subfamily G member (ABC)2 (Abcam, Cambridge, MA, USA); Sox-2, Nanog, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and α-tubulin (Cell Signaling Technology). Anti-rabbit and -mouse secondary antibodies were from Santa Cruz Biotechnology. Protein bands were detected with the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific) and visualized using a ChemiDoc XRS+ imaging system with Image Lab software (Bio-Rad) or a Las-4000 image reader with Multi Gauge 3.1 software (Fujifilm, Tokyo, Japan).

**Statistical analysis**

Data are expressed as the mean ± SD. Differences among mean values were evaluated by the paired or unpaired t test with Mann-Whitney test or by one-way analysis of variance with Dunnett’s multiple comparisons test using GraphPad Prism 6 software (GraphPad Inc., La Jolla, CA, USA). P < 0.05 was considered statistically significant.

**Results**

**CSC markers are downregulated by PEITC treatment**

We previously established a transient co-transfection screening system for Oct4 transcriptional activation based on luciferase (luc) activity in 293T cells (Yun et al., 2015b). Of various phytochemicals tested in our preliminary study, PEITC was chosen for further experimentation due to its potent inhibitory effect on Oct4 transcription (data not shown).

First, we evaluated the effect of PEITC on the cell viability of NCCIT cells, using an MTT cell viability assay. PEITC-treated cells exhibited a significant decrease in cell viability ([C]₀ = concentration required to inhibit cell growth by 50%; 15.4 μM for 24 h, 10.2 μM for 48 h) at the indicated concentrations (Supplementary Fig. 1). We next evaluated whether PEITC reduces the transcription levels of pluripotency factors, including Oct4 and Nanog. We found that PEITC treatment reduced Oct4 transcription in a dose-dependent manner in 293T cells transfected with Oct4 expression constructs (Fig. 1A). Moreover, Nanog, another pluripotency factor, was downregulated in a dose-dependent manner in 293T cells transfected with Nanog expression constructs and treated with PEITC (Fig. 1B). To examine whether PEITC acts on pluripotent cells, we next determined the effect of PEITC on endogenous Oct4 and Nanog expression in NCCIT cells, in which pluripotency factors are highly expressed. After transfection with either Oct4 or Nanog luc reporter vector, PEITC treatment also significantly decreased Oct4 and Nanog endogenous transcription levels (Fig. 1A and B).

To investigate whether PEITC regulates protein expression of pluripotency factors, we conducted western blot analysis of NCCIT whole cell lysates after treatment with PEITC for 24 h. The protein expression levels of Oct4, Sox-2, and Nanog were decreased by PEITC treatment (Fig. 1C). These results suggested that PEITC suppresses the three pluripotency factors.
**PEITC suppresses CSC properties in vitro**

Fluorescence- or magnetic-activated cell sorting based on cell surface markers is a useful tool for CSC isolation (Tirino et al., 2012). In this study, we used a cell sorting system to sort CSC model cells using a PE-conjugated antibody against the cell surface marker EpCAM, which is overexpressed in various tumors and CSCs (Lin et al., 2012; Munz et al., 2009). Additionally, Lin and colleagues further identified and characterized the CSC properties of EpCAM-enriched HCT116 cells (Lin et al., 2012). As shown in Fig. 2A, 40–60% of EpCAM* cells were sorted from total expression in HCT116 cells, with a purity of at least 95%. We confirmed these EpCAM* cells to have CSC properties by sphere culture, western blotting, and immunocytochemistry (Fig. 2B–D). A CSC model was established from sorted EpCAM* sphere-forming cells cultured on ultra-low attachment dishes. Three-dimensional sphere culture systems allow examination of self-renewal capacity (Weiswald et al., 2015). After 6 days of enrichment in SCM, sphere cells were harvested and dissociated (Fig. 2B). Evaluation of the expression of the three pluripotency factors in our sphere model revealed the expression in the 1st and 2nd generation sphere cells (Fig. 2C). Moreover, we observed EpCAM fluorescence at the surface of sphere cells, and Oct4, Sox-2, and Nanog fluorescence were detected internally in sphere cells (Fig. 2D). These data demonstrated that EpCAM* cells have common features with CSCs.

We further investigated the effects of PEITC on CSC properties including self-renewal capacity, clonogenicity, upregulation of pluripotency factors, and therapeutic resistance using EpCAM* cells. As shown in Fig. 3A, treatment with PEITC dose-dependently reduced the self-renewal capacity of sphere cells as evidenced by sphere growth inhibition. We also observed the inhibition of sphere growth upon PEITC treatment on soft agar (Fig. 3B), which stimulates self-renewal capacity and clonogenic potential. Similarly, clonogenic potential was reduced dose-dependently in treated cells (Fig. 3C). Moreover, 20 μM PEITC inhibited the expression of the three pluripotency factors as well as that of the ABCG2 transporter, a marker of multidrug resistance (Fig. 3D). Taken together, the results in vitro demonstrated that PEITC suppresses CSC properties in EpCAM* cells.

**PEITC suppresses CSC properties in a mouse xenograft model**

To determine whether PEITC suppresses CSC properties in vivo, athymic nude mice were treated with vehicle or PEITC (20 mg/kg) by intraperitoneal injection after EpCAM* cell inoculation. The treatment induced no statistically significant changes in body weight induced (Fig. 4A), and no signs of acute or delayed toxicity were detected. However, tumor growth was markedly suppressed by PEITC as compared to vehicle treatment over 4 weeks (Fig. 4B). Moreover, a histological examination of H&E-stained tumor sections showed high cellular densities in the vehicle-treated group, with uniformly distributed nuclei. In contrast, in the PEITC-treated group, the cell density was lower and cells were more scattered, while TUNEL assay revealed an increase in apoptosis (Fig. 4C). Taken together, these findings showed a decrease in tumor growth and changes in cell numbers by PEITC, which might be due to the induction of apoptosis.

Furthermore, western blotting and immunohistochemistry of tumor sections showed that Oct4, Sox-2, and Nanog levels were markedly downregulated in the PEITC treatment group (Fig. 4D and E). Overall, these data demonstrated that PEITC suppressed CSC properties in vivo.

**Discussion**

Tumors consist of phenotypically and functionally different cell types that may have distinct origins (Visvader, 2011). Multiple
studies have shown that cancer cell subsets—including CSCs—within the bulk of the tumor have self-renewal capacity and continuously differentiate. CSCs play a key role in tumorigenesis but also tumor invasion, metastasis, therapeutic resistance, and recurrence by increasing the expression of pluripotency factors as well as self-renewal, differentiation potential, and clonogenicity (Liu et al., 2013; Reya et al., 2001). Therefore, therapeutics that target CSCs hold great promise for effective cancer chemoprevention and treatment.

A multitude of studies have revealed that phytochemicals found in certain food substances can exert considerable biological activities in various diseases including cancer and on CSCs (Kawasaki et al., 2013).
Fig. 4. PEITC inhibits EpCAM+ cell tumor growth in a xenograft model. EpCAM+ cells (3 × 106 cells/100 μl PBS) were subcutaneously inoculated into the flanks of male immunodeficient mice. When tumors reached 50 mm³, the mice were randomly divided into two groups that were injected daily with vehicle (control solvent) or 20 mg/kg PE. (A) Changes in body weight and (B) tumor volume after 26 days of treatment (bar = 10 mm). Tumor volumes represent mean ± SD (n = 8). (C) Immunohistochemical analysis. Tumor sections were stained with H&E (bar = 50 μm) and analyzed by the TUNEL assay (green) with DAPI co-staining (bar = 50 μm) and (D) Oct4, Sox-2, and Nanog immunolabeling (bar = 50 μm) with hematoxylin counterstaining. TUNEL-positive cells were counted in three different images and data are expressed as the mean ± SD. (E) Western blot analysis. Protein levels were measured from tumor samples from different mice. Band intensity was normalized to β-actin; bars indicate the mean ± SD (n = 8). *P < 0.05, **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
et al., 2008). Especially, isothiocyanates, which are small molecules that are abundantly present in cruciferous vegetables, display gene modulatory and signaling activities that inhibit various cancer cells (Cheung and Kong, 2010). For example, the isothiocyanates sulforaphane and benzyl isothiocyanate inhibit CSC growth (Kim et al., 2013; Rausch et al., 2010). In addition, PEITC has been reported to induce apoptosis in various cancer cell types and xenograft models (Cheung and Kong, 2010). It has been reported that PEITC attenuates the growth of sphere-forming cervical CSCs isolated from HeLa cells via death receptor signaling (Wang et al., 2014) and that it significantly reduces the side population of colorectal cancer cells in vitro and tumor growth in vivo (Ju et al., 2016). Thus, the biological benefits of phytochemicals, particularly, isothiocyanates, could be exploited to achieve CSC elimination. In present study, we demonstrated that PEITC suppresses CSC properties in vitro and in vivo.

Various approaches to eradicating CSCs have been developed, including biomarker-based therapies (ABCG2, aldehyde dehydrogenase (ALDH), CD44, CD133), targeting of signal pathways (Wnt/β-catenin, Notch, Hedgehog), overcoming the mechanisms of resistance (targeting drug efflux mechanisms), and differentiation therapy (Hu and Fu, 2012). Moreover, knockdown of pluripotency factors has been used to arrest cell growth and apoptosis in adherent cancer cells, ESCs, and CSCs (Chen et al., 2012; Liu et al., 2013; Yun et al., 2015b). Our previous study showed that inhibiting Oct4 treatment with curcumin activated the caspase cascade and PARP cleavage-dependent apoptosis as well as GSK-3β, thereby blocking Wnt/β-catenin signaling and caspase activation in NCCIT cells (Yun et al., 2015b). Here, we focused on Oct4, Nanog, Sox-2, ABCG2, and EpCAM as CSC markers to investigate the effects of PEITC on CSCs. Our data showed that PEITC decreased the CSC markers in vitro and in vivo with induction of apoptosis in tumor sections (Fig. 4C, right panel), which is possibly related to suppressing the expression of pluripotency factors; however, further in-depth studies are needed.

EpCAM can undergo intramembrane proteolysis by PS-2, resulting in the release of its intracellular domain (i.e., EpICD). EpICD is translocated to the nucleus in a complex with FHL2 and β-catenin that may crossstalk with the Wnt/β-catenin signaling pathway. In the nucleus, the EpICD complex interacts with Lefty to regulate the transcription of target genes such as c-Myc, Oct4, Sox-2, and Nanog (Munz et al., 2009). Our results showed that PEITC inhibited sphere formation and significantly reduced the expression of the three pluripotency factors; however, β-catenin and EpCAM levels were slightly decreased in the sphere and tumor sections (Figs. 3 and 4; data not shown). A previous study showed the nuclear β-catenin level to be unchanged in HT-29 colon cancer cells following PEITC treatment (Cheung et al., 2008), suggesting that PEITC does not directly affect Wnt/β-catenin signaling. Therefore, PEITC might inhibit the interaction between EpICD and β-catenin, consequently suppressing the transcription of target genes, including the three pluripotency factors.

Our study revealed that ABCG2 as well as Oct4, Nanog, and Sox-2 were downregulated by PEITC treatment (Fig. 3D). Previous studies have reported that CSCs exhibit drug resistance via the expression of ABCG2, which is closely correlated with the expression of pluripotency factors. In mouse ESCs, inhibition of ABCG2 resulted in the downregulation of Nanog, whereas PI3K/Akt inhibition suppressed the side-population phenotype in flow cytometry analysis and caused the downregulation of ABCG2, Oct4, and Sox-2 expression (Susanto et al., 2008). Sox-2 silencing also suppressed Oct4, Nanog, and ABCG2 levels in thyroid carcinoma cells (Carina et al., 2013). Meanwhile, Oct4 was found to mediate chemoresistance via an Oct4-PI3K/Akt-ABCG2 pathway in drug-resistant hepatocellular carcinoma cells (Wang et al., 2010). All of these findings support that PI3K signaling could be important in the regulation of CSC properties, including drug resistance, and of the complex relationships between ABCG2 and pluripotency factors, which modulate stemness regulatory networks that suppress CSC properties. Moreover, EpCAM+ prostate cancer cells possess stem-cell-like properties that regulate proliferation, invasion, metastasis, and chemo-/radio-sensitivity via the activation of the PI3K/Akt/mTOR signaling pathway (Ni et al., 2013). Several evidences have indicated that this pathway plays a key role in CSC biology (Martelli et al., 2011). Inhibition of the PI3K pathway led to a relative decrease in CD133+/CD44+ stem-like populations in prostate cancer cell lines (Dubrovskova et al., 2009). PEITC was also shown to inhibit PI3K, thereby blocking Akt phosphorylation (Kim et al., 2006). Thus, the effect of PEITC on CSC markers could be mediated by the inhibition of PI3K/Akt signaling. Further study is required to unravel the role of PI3K/Akt signaling in the effect of PEITC in EpCAM+ HCT116 cells.

Conclusion

In summary, our results demonstrate that PEITC suppresses CSC properties in vitro and in a mouse model in vivo, including expression and activation of Oct4, Sox-2, and Nanog as well as self-renewal, clonogenicity, and tumor growth. Our findings provide evidence that PEITC has therapeutic effects on CSCs. Based on these and previous findings (Ju et al., 2016; Wang et al., 2014), PEITC might be useful for targeting CSCs to overcome therapeutic resistance and slow down or prevent recurrence.

Conflict of interest statement

The authors declare no competing financial interest.

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Supplementary materials

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