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ORIGINAL ARTICLE

Phloretin Inhibits the Human Prostate Cancer Cells Through the Generation of Reactive Oxygen Species

Ukjin Kim[1] · C-Yoon Kim[2] · Ji Min Lee[1] · Hanseul Oh[1,3] · Bokyeong Ryu[1] · Jin Kim[1] · Jae-Hak Park[1]

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Abstract

Phloretin is a flavonoid with known anticancer activities. However, we do not fully understand how phloretin mitigates prostate cancer on the molecular level. In the present study, we examined changes in proliferation, colony formation, and migration after phloretin treatment in human prostate cancer cells PC3 and DU145. We measured reactive oxygen species (ROS) and gene expression. Phloretin increased ROS and suppressed cell proliferation, migration, and colony formation in both cell lines. Additionally, phloretin treatment increased oxidative stress, as demonstrated through lower antioxidant enzymes (catalase, SOD2, Gpx1, Gpx3). In addition, their regulator CISD2 decreased in expression. We also found that increased ROS significantly downregulated multiple components of the Wnt/β-catenin signaling pathway (β-catenin, TCF4, FoxA2, c-Myc) and Twist1. Thus, anticancer activity of phloretin against human prostate cancer cells occurs through generating ROS to influence Wnt/β-catenin signaling. The results of this study suggest that phloretin has a therapeutic effect on prostate cancer in vitro, inhibiting the proliferation and migration of cancer cell lines PC3 and DU145. The mechanism of phloretin appears to be increasing ROS production. We thus recommend phloretin as a promising anticancer therapeutic agent.

Keywords Phloretin · Prostate cancer · ROS · Redox homeostasis · Wnt/β-catenin signaling

Introduction

According to annual reports (1975–2011) from the United States government, prostate cancer occurs in men at significantly higher rates than other cancers [1]. Globally, prostate cancer is the second most common tumor and the sixth leading cause of death among men [2]. The strong association between this cancer and age is due to the large role of signaling pathways involving reactive oxygen species (ROS) in cancer development and progression [3]. In general, ROS influences cancer development through promoting cell proliferation, invasion, and metastasis while inhibiting apoptosis. However, ROS also has anti-cancer effects, including inducing cell cycle arrest, apoptosis, and necrosis [4].

Oxidative stress also regulates β-catenin action [5]. This effector protein interacts with TCF/LEF-1 to activate Wnt target genes during Wnt/β-catenin signaling, a pathway that is associated with both normal prostate development and prostate cancer progression [6]. During this signaling process, β-catenin degradation is inhibited, leading to intracellular or nuclear accumulation [7]. Specifically, prostate cancer progression is linked to nuclear β-catenin accumulation [8]. Research using HEK293 (human embryonic kidney) cells revealed that H_2O_2-induced oxidative stress suppressed Wnt/β-catenin signaling, whereas the activation of this pathway inhibited H_2O_2-induced apoptosis [9].

Phloretin (CAS number, 60–82-2) is a flavonoid found in Rosaceae (e.g., apples and pears) with known anti-inflammatory and immunosuppressive effects in lymphoid- and myeloid-derived cell lines [10]. Moreover, phloretin exhibits anticancer activity via inducing apoptosis in human leukemia cells, bladder cancer, and colorectal cancer [11, 12], as well as inhibiting growth, invasion, and migration in human

✉ Jae-Hak Park
pjhak@snu.ac.kr

[1] Department of Laboratory Animal Medicine, Research Institute for Veterinary Science, BK21 PLUS Program for Creative Veterinary Science Research, College of Veterinary Medicine, Seoul National University, Seoul 08826, Republic of Korea

[2] Department of Stem Cell Biology, School of Medicine, Konkuk University, Seoul 05029, Republic of Korea

[3] National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungcheongbuk, Republic of Korea

liver cells [13]. However, we do not know what role, if any, ROS generation plays in phloretin antitumor function. In this study, we demonstrated how phloretin affects prostate cancer cells and described associated molecular mechanisms. Our work is the first to provide empirical evidence that phloretin inhibits prostate cancer through generating ROS and down-regulating Wnt/β-catenin signaling.

Materials and Methods

Reagents

Human prostate cancer cell lines PC-3 and DU145, and African green monkey kidney-derived Vero cell were acquired from the American Type Culture Collection (Manassas, VA, USA). PC-3 and DU145 cells were cultured in RPMI 1640 medium (Welgene, Gyeongsan, South Korea) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (PS) (Gibco) at 37 °C in 95% air/5% CO_2. Vero cell was cultured in DMEM medium (Welgene) supplemented with 10% FBS and 1% PS at 37 °C in 95% air/5% CO_2. Phloretin (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) (Duksan Pure Chemical Co., Ansan, South Korea) to obtain concentration of 100 mM. The final DMSO concentration in the solution was 0.1%. DMSO at the same final concentration of 0.1% was used as control.

Cell Proliferation Assay

The proliferation capacity of cells was analyzed using thiazolyl blue tetrazolium bromide (MTT) (Sigma) assay based on the ability of live cells to convert tetrazolium salt into purple formazan. Briefly, PC-3, DU145, and Vero cells were seeded into 96-well cell culture plates (SPL Life Science, Pocheon, South Korea) at a density of 1.6×10^4 per well in 200 μl media. After 24 h of culturing, the medium was replaced to FBS free media for 24 h. Cells were treated with phloretin at concentration of 20, 50, 100 μM or vehicle (DMSO) control and cultured for 24 h. The medium was then changed with 100 μl of MTT (diluted to 1 mg/ml in FBS-free medium from a stock solution of 5 mg/ml) and incubated at 37 °C for 3 h. The supernatant was eliminated and 100 μl of DMSO was supplemented to each well to dissolve the formazan crystals. Plates were agitated at room temperature for 5 min. The absorbance was read at 570 nm on an Epoch BioTek microplate reader (BioTek, Winooski, VT, USA). All treatments were performed in triplicates.

Clonogenic Assay

To determine the long-term effects, Clonogenic assay using logarithmically-growing PC-3 and DU145 cells was performed. In brief, approximately 1000 cells obtained from sub-confluent cell culture flask (SPL) were seeded per 60 mm cell culture dishes (SPL) in 5 ml of medium. 24 h after seeding the cells, phloretin at concentrations of 20, 50, 100 μM or vehicle (DMSO) was added to the medium. The cells were allowed to form colonies for 7 days and were rinsed with fresh medium every 3 days. When the colonies were discrete and well defined, the dishes were washed with phosphate buffered saline (PBS) (Gibco) solution, fixed with methanol (Merck, Darmstadt, Germany), stained with hematoxylin (Yeong-Dong Diagnostics, Yongin, South Korea). The colonies per dish were counted using inverted microscope (Olympus IX70). Colonies with 50 cells or more were counted. Plating efficiency (PE) is the ratio of the number of colonies to the number of cells seeded. The number of colonies that arise after treatment of cells, expressed in terms of PE, is called the surviving fraction (SF).

Cell Migration Assay

Cell motility was analyzed using an in vitro wound healing assay. PC-3 and DU145 cells were seeded into 6-well cell culture plate (SPL) and grown to 90% or above confluence. Monolayers of prostate cells were then wounded using a pipette tip. Cell repair was monitored using an inverted microscope (Olympus IX70, Tokyo, Japan) following 24 h exposure to phloretin at concentrations of 20, 50, 100 μM or vehicle (DMSO). All treatments were performed in triplicates. The wounded areas were measured using Image J software (https://imagej.nih.gov/ij/).

ROS Measurement

The generation of intracellular ROS was determined using 2′,7′-dichlorofluorescin diacetate (DCFH-DA (Sigma) which is converted to fluorescent 2′,7′-dichlorofluorescin (DCF) in the presence of peroxides. After exposure to different concentrations of phloretin for 24 h, PC-3 and DU145 cells were treated with 10 μM DCFH-DA for 1 h at 37 °C and washed with PBS. The cells were detached with trypsin-EDTA (Gibco), and intracellular ROS was detected using a fluorescence spectrometer Victor 3 (Perkin Elmer, Waltham, MA, USA) at 485 nm exposure and 535 nm emission.

Real-Time Reverse Transcription-Polymerases Chain Reaction (PCR)

Total RNA was extracted using a Hybrid-R RNA extraction kit (GeneAll Biotechnology, Seoul, South Korea). cDNA was

synthesized by M-MLV cDNA Synthesis kit (Enzynomics, Daejeon, South Korea) according to the supplier's intructions. Quantitative real-time PCR was performed using TOPreal™ qPCR 2X PreMIX (Enzynomics) on a CFX Connect Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). Primers used were 5'-GGAAGCCATCAAAC GTGACT-3′ (sense) and 5'-CTGATTTGGACAAG CAGCAA-3′ (antisense) for human SOD2 (NCBI gene ID: 6648); 5'-ACAGCAAACCGCACGCTATG-3′ (sense) and 5'-CAGTGGTCAGGACATCAGCTTTC-3′ (antisense) for human Catalase (NCBI gene ID: 847); 5'-CGCTTCCA GACCATTGACATC-3′ (sense) and 5'-CGAGGTGG TATTTTCTGTAAGATCA-3′ (antisense) for human Gpx1 (NCBI gene ID: 2876); 5'-ACATGCCTACAGGTATGCGT-3′ (sense) and 5'-GAGCAGAACAATTGGACCTA-3′ (antisense) for human Gpx3 (NCBI gene ID: 2878); 5'-TTGGCTACCTTGCAGTTCGT-3′ (sense) and 5'-ATGTGAACCATCGCAGGCA-3′ (antisense) for human CISD2 (NCBI gene ID: 493856); 5'-CTCGGACAAGCTGA GCAAGA-3′ (sense) and 5'-GCTCTGGAGGACCT GGTAGA-3′ (antisense) for human Twist1 (NCBI gene ID: 7291); 5'-ATGACTCGAGCTCAGAGGGT-3′ (sense) and 5'-ATTGCACGTGTGGCAAGTTC-3′ (antisense) for human β-catenin (NCBI gene ID: 399274); 5'-CCTGGCACCGTAGG ACAAAT-3′ (sense) and 5'-TGGGACCATATGGGGAGGG-3′ (antisense) for human TCF4 (NCBI gene ID: 6934); 5'-GTGAAGATGGAAGGGCACGA-3′ (sense) and 5'-CATGTTGCTCACGGAGGAGT-3′ (antisense) for human FoxA2 (NCBI gene ID: 3170); 5'-CATGTACGTTGCTA TCCAGGC-3′ (sense) and 5'-CTCCTTAATGTCAC GCACGAT-3′ (antisense) for human β-actin (NCBI gene ID: 60). Ratio of target gene fold-change was normalized to human β-actin expression using comparative CT ($2^{-\Delta\Delta Ct}$) method.

Statistical Analysis

All data are presented as mean ± standard error. Statistical significance ($P < 0.05$) was further analyzed with Student's t test using computer program GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Results

Phloretin Inhibits Cell Proliferation and Colony Formation in the PC3 and DU145 Cell Lines

We first analyzed the effect of phloretin on cell proliferation and colony formation. In the PC3 and DU145 prostate cancer cell lines, cell growth was inhibited by phloretin at various concentrations for 24 h (Fig. 1). As a negative control for prostate cancer, phloretin was treated in Vero cells derived from African green monkey and as shown in Fig. 1, it did not affect the proliferation of Vero cells. Phloretin also inhibited colony formation in two prostate cancer cell lines (Fig. 2a) and we counted the number of surviving colonies (Fig. 2b).

Phloretin Inhibits Migration of PC3 and DU145 Cell Lines

We evaluated the migration of cells by in vitro wound healing assay by treating phloretin with PC3 and DU145 cells. After 24 h of incubation at various concentrations of phloretin, the migration of prostate cancer cells to the denuded area was inhibited in a dose-dependent manner (Fig. 3a). The area where the wound was recovered was quantitatively analyzed (Fig. 3b).

Phloreitn Induces ROS Accumulation in PC3 and DU145 Cell Lines through Inhibiting Antioxidant Enzyme Gene Expression

According to reports, ROS accumulation is known to partially affect the proliferation of cells. We treated PC3 and DU145 cells with various concentrations of phloretin and measured the amount of ROS present in the cells using DCFH-DA, a

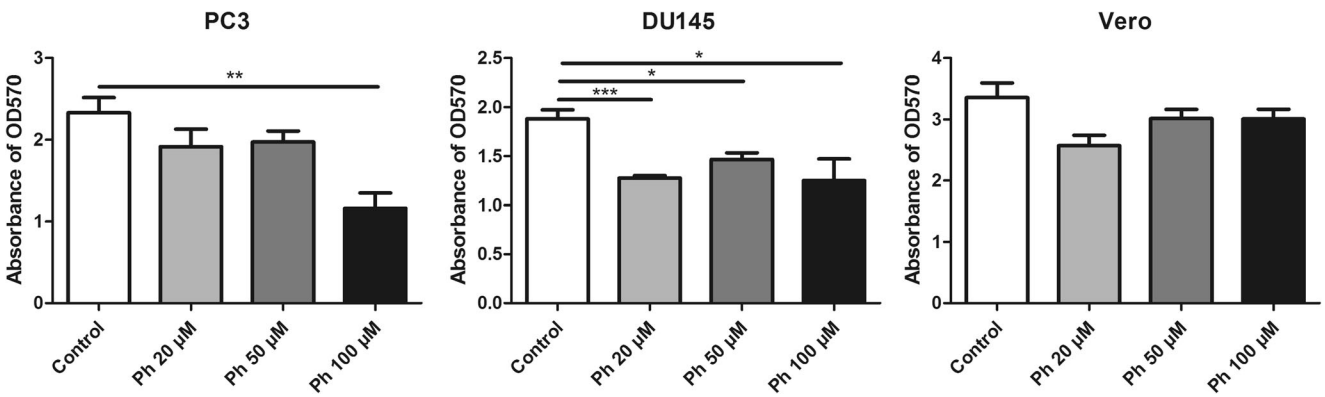

Fig. 1 The effect of phloretin on cell proliferation. The inhibition of proliferation of PC3 and DU145 by phloretin was confirmed by MTT assay. But, phloretin has no effect of normal cell line Vero cells derived from normal African green monkeys. Results are presented as means ± SEM

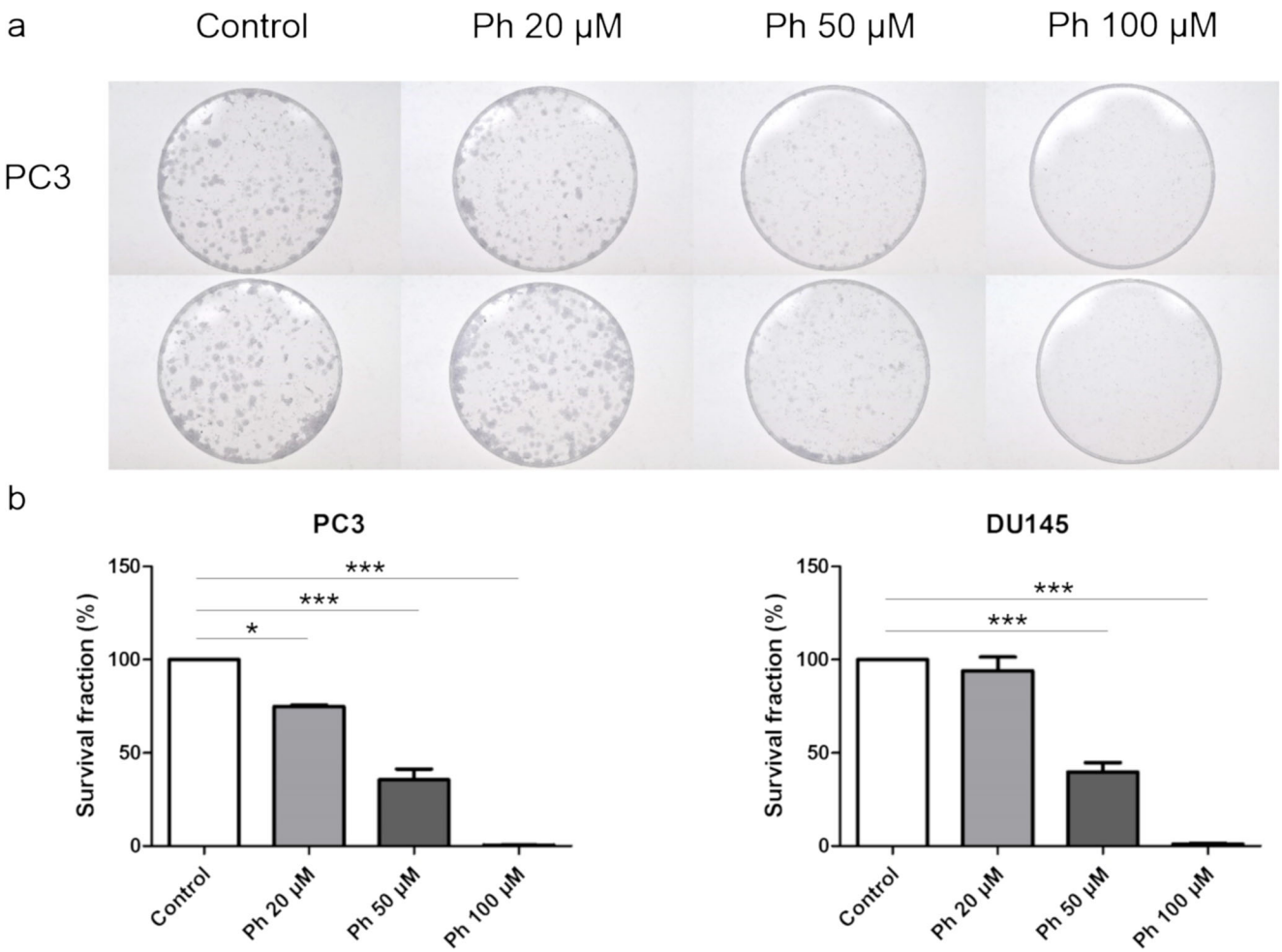

Fig. 2 The effect of phloretin on colony formation. To determine long term effect of phloretin, PC3 and DU145 cells were allowed to form colonies for 7 days. Colony formation of prostate cancer cell lines was inhibited in a concentration-dependent manner. Results are presented as means ± SEM

fluorescent dye, after 24 h. As expected, ROS levels increased after treatment with phloretin in PC3 and DU145 (Fig. 4). In addition, gene expression of SOD2, Catalase, Gpx1, Gpx3 and CISD2 genes related to ROS production was analyzed. Phloretin treatment resulted in decreased expression of SOD2, Catalase, Gpx1 and CISD2 in PC3 and DU145 (Fig. 5). However, Gpx3 expression increased at a medium concentration of 50 μM and decreased at a high concentration of 100 μM. Presumably, the production of ROS by phloretin seems to be related to the expression of antioxidant enzyme genes.

Phloretin Downregulates the mRNA Levels of Wnt/β-Catenin Signaling in PC3 and DU145 Cell Lines

When the Wnt/β-catenin pathway is activated, β-catenin enters the nucleus and increases the expression of the target gene in the down-stream. We therefore analyzed the expression of β-catenin, TCF4, FoxA2, and c-Myc when phlroetin was treated with PC3 and DU145 cells (Fig. 6). The expression of β-catenin, TCF4, and FoxA2 decreased in PC3 and DU145 after treatment with phloretin. However, c-Myc decreased in PC3 but increased in DU145. Wnt/β-catenin signaling has been reported to be associated with the epithelial-mesenchymal transition (EMT) of prostate cancer [14]. The expression of Twist associated with EMT was analyzed and the expression was decreased in both PC3 and DU145.

Discussion

First, we confirmed that phloretin inhibited the proliferation of prostate cancer cell lines PC3 and DU145. To verify whether this effect is specific to cancer cells, we then followed procedures in previous reports [15, 16], to repeat phloretin treatment in Vero cells, a normal line derived from African green monkeys. Phloretin did not affect Vero cell proliferation, indicating specificity to prostate cancer cells. Our findings corroborate

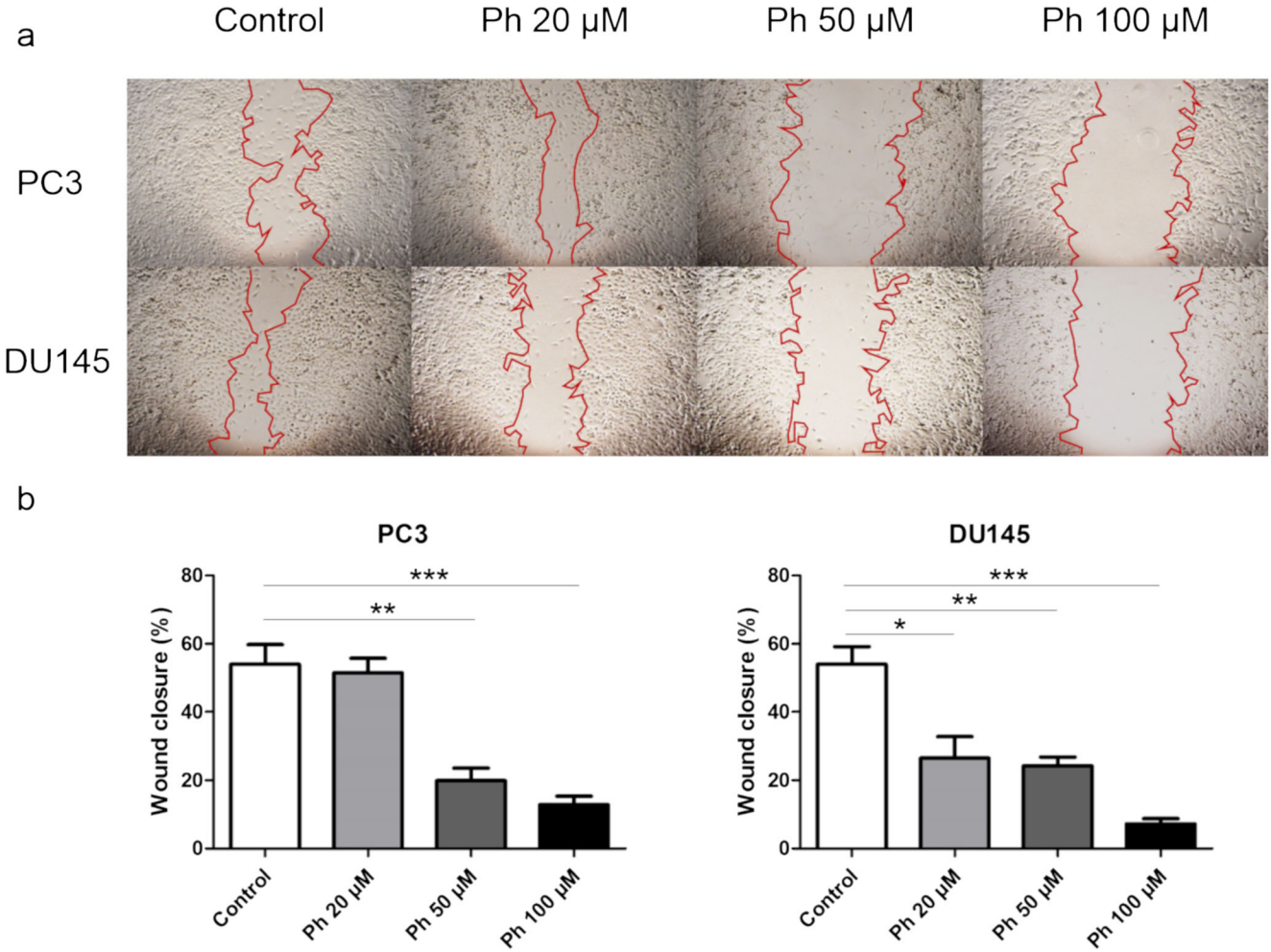

Fig. 3 The effect of phloretin on migration of PC3 and DU145. Phloretin suppressed the migration of PC3 and DU145 human prostate cancer cells. Cells that migrated to the wounded region were photographed (magnification, ×40). Results are presented as means ± SEM

earlier reports of phloretin inhibiting human glioblastoma cell proliferation [17].

Furthermore, we confirmed that the anticancer effect of phloretin is related to ROS production, as previously described [17]. Overall, medical trials have not found a significant relation between prostate cancer incidence and the intake of antioxidant supplements such as Vitamin C [18]. However, this lack of a connection may be because ROS anticancer effects depend on tumor stage [19]. For instance, antioxidants significantly reduced prostate cancer in patients with normal prostate-specific antigen (PSA), a biomarker of prostate cancer risk, whereas antioxidants significantly *increased* prostate cancer in patients with above-baseline PSA levels [18]. Previously, we used a Gpx3-knockout TRAMP mouse model

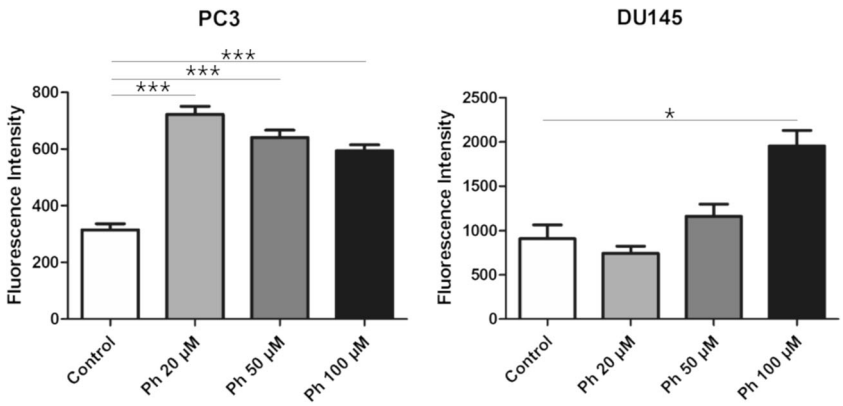

Fig. 4 Phloretin induced generation of ROS in the PC3 and DU145 cell lines. Fluorescence spectrometer was used to determine ROS generation after staining with DCFH-DA. Results are presented as means ± SEM

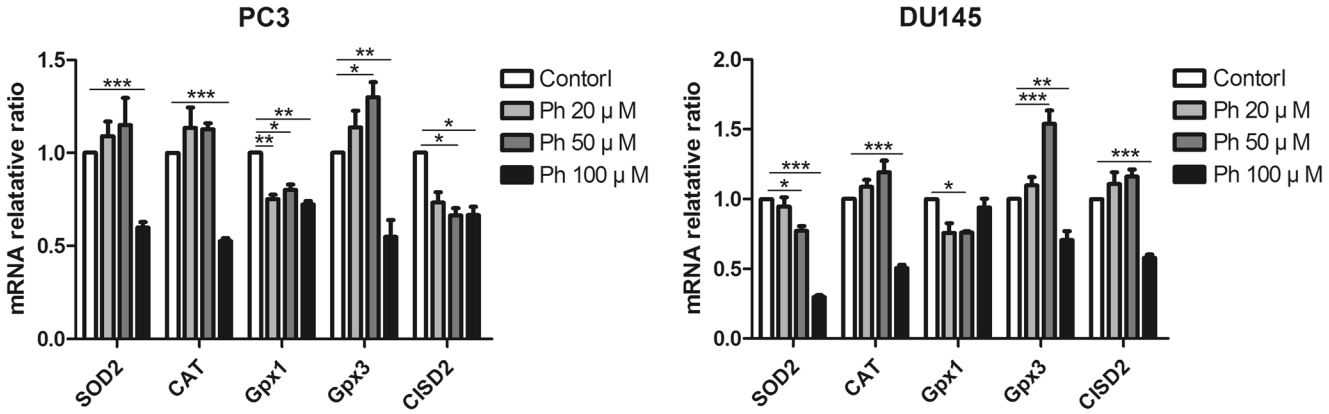

Fig. 5 The effect of phloretin on mRNA expression levels of SOD2, CAT, Gpx1, Gpx3 and CISD2 in PC3 and DU145 cells. Phloretin downregulated the mRNA expression levels of anti-oxidant enzymes in PC3 and DU145 cells. The mRNA expression levels of CISD2 was downregulated in PC3 and DU145 cell. Results are presented as means ± SEM

(highly susceptible to tumors) to show that a decrease in Gpx3 expression is associated with prostate cancer development [20]. The downregulation of Gpx3 dampened ROS removal processes, thus elevating oxidative stress in TRAMP mice, and such an outcome during the pre-neoplastic stage likely contributed to cancer development. Together, these data suggest that antioxidants and ROS signaling may exert different effects depending on the stage of prostate cancer.

The redox-sensitive protein CISD2 belongs to the CDGH iron sulfur domain-containing family [21] and is found in mitochondria [22] and ER [23], both major ROS sources. In lung cancer cells, CISD2 depletion corresponds to an increase in ROS production [24]. Likewise in prostate cancer cells, CISD2 probably acts to maintain redox homeostasis through removing ROS. This elevated antioxidant ability in cancer cells increase drug resistance and ensure their survival [19]. We found that phloretin treatment led to ROS production in prostate cancer cells, suggesting that ROS concentrations may have exceeded the functional capacity of antioxidant enzymes. Corroborating this hypothesis, CISD2 expression was downregulated, in conjunction with the expression of antioxidant enzymes SOD2, catalase, Gpx1, and Gpx3. However, treatment with 20 μM and 50 μM phloretin increased Gpx3 expression, possibly to compensate for lower SOD2, catalase, and Gpx1 levels. Indeed, previous research on Nkx3.1 knockout mice also found evidence of this compensatory mechanism; elevated Qscn6 expression caused oxidative stress, but while Gpx2 and Prdx6 decreased, Gpx3 expression increased [25].

Our analysis of Wnt/β-catenin signaling confirmed its importance in prostate cancer [8]. In both cancer cell lines (DU145 and PC3), phloretin decreased the expression of β-catenin and two targets, TCF4 and FoxA2. However, c-Myc expression decreased in PC3 and increased in DU145. This β-

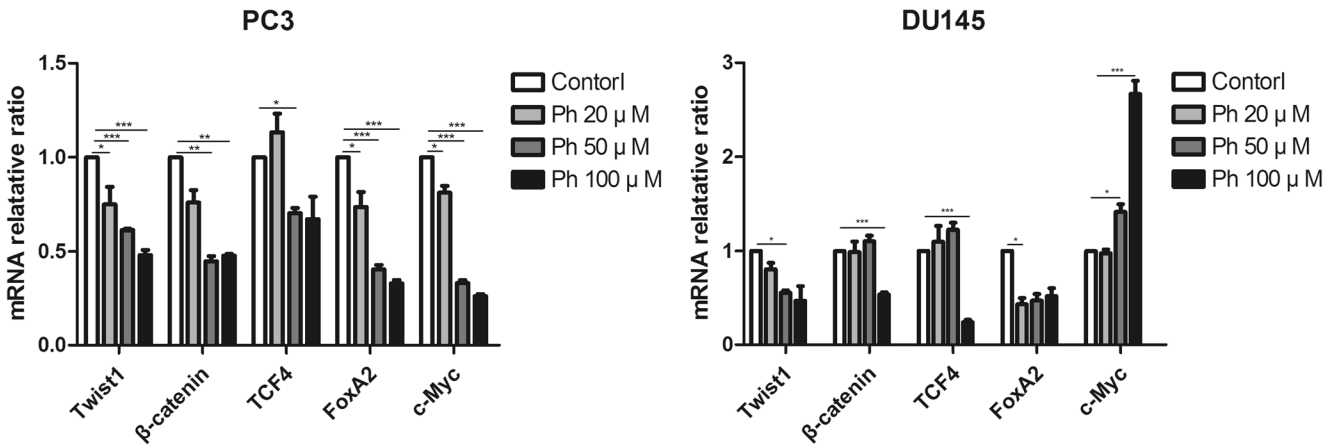

Fig. 6 The effect of phloretin on mRNA expression levels of Twist1, β-catenin, TCF4, FoxA2, and c-Myc in PC3 and DU145 cells. The mRNA expression levels of Twist1, β-catenin, TCF4 and FoxA2 in PC3 and DU145 cells were downregulated. However, c-Myc decreased in PC3 but increased in DU145. Results are presented as means ± SEM

catenin target is associated with apoptosis [26]. We observed that c-Myc inhibition suppressed apoptosis in DU145 (though no change was detected in PC3). Furthermore, when DU145 cells were treated with the anticancer agent Paclitaxel, c-Myc expression increased in conjunction with apoptosis [27]. Overexpression of c-Myc caused an increase in colcemid-induced apoptosis was increased [28]. Taken together, these results suggest that c-Myc overexpression is associated with apoptosis in DU145, but not in PC3. Furthermore, phloretin reduced the expression of Twist1. Twist1 is associated with the epithelial mesenchymal transition and invasion of prostate cancer [29]. Further studies are needed to determine whether Phloretin inhibits prostate cancer invasion and metastasis.

Collectively, the results of this study suggest that phloretin has a therapeutic effect on prostate cancer in vitro, inhibiting the proliferation and migration of cancer cell lines PC3 and DU145. The mechanism of phloretin appears to be increasing ROS production. In addition, phloretin reduced the expression of SOD2, catalase, Gpx1, and Gpx3, along with their regulator CISD2. Phloretin also inhibited Wnt/β-catenin signaling in PC3 and DU145, while reducing Twist1 expression. We thus recommend phloretin as a promising anticancer therapeutic agent.

Author Contributions Jae-Hak Park conceived and designed the experiments and is GUARANTOR for the article. Ukjin Kim, C-Yoon Kim, Ji Min Lee, Hanseul Oh, Bokyeong Ryu and Jin Kim performed experiments. Ukjin Kim analyzed the data and wrote the manuscript.

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Compliance with Ethical Standards

Conflict of Interest None of the authors declare competing financial interests.

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