Antioxidation and Antiapoptosis Characteristics of Heme Oxygenase-1 Enhance Tumorigenesis of Human Prostate Carcinoma Cells

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Abstract

Heme oxygenase-1 (HO-1) has antiinflammatory and antioxidant properties and is deemed as a tissue protector. However, effects of HO-1 in prostate cancer remain in controversy. We evaluated the role of HO-1 in prostate carcinoma *in vitro* and *in vivo*.

Overexpression of HO-1 did not affect prostate cell proliferation in the normal condition but enhanced cell proliferation under serum starvation. HO-1 overexpression enhanced cell invasion of PC-3 cells through epithelial—mesenchymal transition (EMT) induction, which was supported by increased Slug, N-cadherin, and vimentin expressions. In the xenograft animal study, HO-1 overexpression enhanced PC-3 cell tumor growth *in vivo*. HO-1 attenuated reactive oxygen species induced by H_2O_2 or pyocyanin treatment in PC-3 and DU145 cells. HO-1 further reduced PC-3 and DU145 cell apoptosis induced by H_2O_2 or serum starvation. Our results suggested that HO-1 was able to increase prostate carcinoma cell invasion *in vitro* and tumor growth *in vivo*. The EMT induction and antioxidant and antiapoptotic effects of HO-1 in the prostate carcinoma cells may be responsible for these findings.

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Introduction

Heme degradation in human is catalyzed by heme oxygenase (HO) to produce carbon monoxide (CO), biliverdin, and iron [1–3]. HO includes two isozymes, which are HO-1 and HO-2, each has distinct expression pattern in the body. It has been shown that HO-2 represents as a constitutive and noninducible protein with mainly expressing in testis and brain [4]. To the contrary, HO-1 is a highly inducible protein with only very low expression in the cell before

numerous stimulations causing oxidative stress, including heavy metals, cytokines, heme, etc. [5] As a result of the antioxidative stress characteristic, HO-1 is deemed as a tissue protector, especially for kidney, heart, and liver [6]. However, this protective effect, while applying in cancer cells, may lead to increased tumor growth due to the antiapoptosis effect derived from the antioxidative stress of HO-1.

It is reported that the emerging role of HO-1 in tumor biology is multifaceted and tissue-specific [7]. Cytoprotective and antiapoptotic properties of HO-1 in tumor by increasing survival of cancer cells and resistance to anticancer drugs or therapies have been found in several cancer types [8,9]. The possible mechanisms by which HO-1 affects apoptosis of cancer cells may be through the modulation of caspase-3, B-cell lymphoma-2, B-cell lymphoma-extra large, vascular endothelial growth factor, and stromal cell–derived factor-1 [10–13]. Furthermore, HO-1 has also been shown to be the downstream gene of some oncogenes [9,11].

Thus, it is not surprised that HO-1 has been implied to induce tumor progression in renal cancer, glioma, lung cancer, etc. [14]. However, high HO-1 expression has also been shown to link with slower cancer growth in breast cancer and non–small-cell lung cancer [15,16]. For prostate cancer, the role of HO-1 is still controversial. HO-1 has been shown to increase tumor growth and metastasis in prostate cancer [17]; however, Gureron et al. demonstrated the antitumor effect of HO-1 on prostate cancer [18].

In this study, we aimed to further investigate the role of HO-1 in prostatic cancer. HO-1 was overexpressed in PC-3 cells to evaluate the effect of HO-1 on cell proliferation and metastasis *in vitro* and tumor growth *in vivo*. The related mechanisms would also be investigated.

Material and Methods

Cell Culture and Chemicals

The PC-3 and DU145 cells were obtained from the Bioresource Collection and Research Center (Taiwan) and were cultured in the RPMI-1640 medium (Life Technologies, Rockville, MD) with 10% fetal calf serum (FCS; HyClone, UT) as described previously [19]. DAPI (4,6-diamino-2-phenylindole), propidium iodide, H_2O_2, and H2DCFDA (2′,7′-dichlorofluorescin diacetate) were purchased from Sigma–Aldrich Co. (St. Louis, MO). Pyocyanin was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA).

HO-1 Expression Vector and Stable Transfection

The clone of human HO-1 cDNA in pOTB7 vector (MGC:1723) was purchased from Invitrogen (Carlsbad, CA). The human HO-1 expression vector was constructed by cloning a full length of HO-1 cDNA into the pcDNA3.1/zeo expression vector (Invitrogen) with *Eco R1* sites. Proper ligation and orientation was confirmed by extensive restriction mapping and sequencing. Electroporation was conducted using an ECM 830 Square Wave Electroporation System (BTX, San Diego, CA). Transfected PC-3 (PC-HO1-1 and PC-HO1-2) cells were selected by 100 μg/mL of Zeocin (Invitrogen). The DU145 cells were transient transfected with HO-1 expression vector (DU-HO1) for 72 hours without Zeocin selected. For construction of the mock-transfected cells (PC-DNA and DU-DNA), cells were transfected with a controlled pcDNA3.1 expression vector and clonally selected in the same manner as described previously [20].

Cell Proliferation and Viability Assay

The cell proliferations were measured using 3H-thymidine incorporation, CyQUANT cell proliferation assay (Invitrogen), and WST-1 cell proliferation assay (abcam, Cambridge, UK), as described previously [21,22]. Cell viability was analyzed using the MTS assay (Promega Biosciences, Madison, WI).

EdU Staining Proliferation Assay

The EdU fluorescence of cells was detected using Attune NxT acoustic focusing cytometer (Thermo Fisher Scientific Inc., Waltham, MA) as described previously [23]. Briefly, PC-DNA, PC-HO1-1, and PC-HO1-2 cells (5×10^5) were cultured in serum-free medium for 24 hours. After another 48 hours incubated with 10% serum medium, the cells were incubated with EdU (5-ethynyl-2′-deoxyuridine; 10 μM) for further 2 hours. Then, the cells were collected and analyzed using Click-iT EdU Flow Cytometry Assay Kits (Thermo Fisher Scientific Inc.).

ROS Analysis

Cells were cultured in RPMI-1640 medium with 10% FCS for 48 hours, and then the cells were harvested with trypsin and washed twice with PBS. 20 μl of H2DCFDA added to the cell pellet and incubated at 37 °C and 5% CO_2 incubator for 30 min. After adding reactive oxygen species (ROS) inducer (20 μM of pyocyanin or 125 μM of H_2O_2) as indicated for 1 hour, cells were pelleted and then suspended in 500 μl of PBS. The ROS was analyzed using the FACS-Calibur Cytometer (BD Biosciences, Franklin Lakes, NJ). We also analyzed the total ROS induced by H_2O_2 using immunofluorescence reader, Briefly, cells (3×10^3/per well) were cultured in a 96-well plate for 48 hours and then washed twice with PBS. 200 μl of H2DCFDA (20 μM in RPMI 1640 medium with 2% FCS) were added to each well and then incubated for 30 min in incubator with 37 °C and 5% CO_2. The 0, 125, and 50 M of H_2O_2, respectively, in RPMI 1640 medium with 10% FCS were added for 1 hour after cells were washed twice with PBS. The intensity of DCF-DA fluorescence was detected and quantified with the Chameleon Fluoro-Lumino--Photometer (Turku, Finland).

Sub-G1 Cycle Analysis

Cells were treated with 125 μM of H_2O_2 for 16 hours or serum starvation for 5 days to induce cell death. Cell cycle of sub-G1 analysis was performed and quantified using the FACS-Calibur E6147 Cytometer and CellQuest Pro 4.02 software (BD Biosciences) as described previously [21].

Annexin V-FITC Apoptosis Detection

The cell pellets were harvested after treated with H_2O_2 (500 μM) for 12 hours. The detection and quantification of cell apoptosis were performed after treated with Annexin V-FITC (BioVision Inc, Milpitas, CA) using the FACS-Calibur E6147 Cytometer (BD Biosciences) as described previously [22].

Nuclear and Cytoplasmic Extraction

Cells were harvested with trypsin and washed twice with PBS. Nuclear and cytoplasmic fractions were separated using the NE-PER Nuclear and cytoplasmic extraction kit (Thermo, Rockford, NJ) as described previously [24].

Immunoblot Assay

Equal quantities of cell extracts which was measured by BCA protein assay kit were separated onto a 10% SDS-PAGE gel, transferred and analyzed by the Western lightning plus-ECL detection system (Perkin Elmer, Inc., Waltham, MA). Antibodies

against HO (HO-1; Hsp32, Stressgen, Victoria, BC, Canada), PARP, cleaved PARP (BD Biosciences), N-cadherin, Vimentin (Abgent, San Diego, CA), Lamin B1 (Santa Cruz Biotechnology, Santa Cruz, CA), Slug, and β-actin (Millipore, Temecula, CA) were used.

Immunofluorescence

Cells were seeded for 24 hours on sterile glass coverslips. The processes of fixation, permeabilization, and block were performed as described previously [25].

F-actin Staining

Cells were seeded onto glass bottoms of the culture dishes (MatTek, Ashland, MD), then, precoated with fibronectin, and allowed to attach overnight. The F-actin protein expression was revealed by incubation with Texas Red X-Phalloidin and mounted with ProLongR Gold reagent (Invitrogen) as descried previously [26].

Real-time Reverse Transcription—polymerase Chain Reaction

Total RNA from cells was isolated using Trizol reagent. The cDNA was synthesized, and real-time polymerase chain reaction (qPCR) was performed as described previously [27]. The mRNA expressions of genes were assayed using the FAM dye-labeled TaqMan MGB probes for HO-1 (Hs00157965_m1) and β-actin (Hs01060665_g1), purchased from Applied Biosystems (Foster City, CA).

Matrigel Invasion Assay

Cells (1×10^5) migrated to the matrigel-coated transmembrane for 24 hours. The images were captured using a digital camera connected to an inverted microscope (IX71, Olympus, Tokyo, Japan) with PAX-it Digital Image Management & Image Analysis and standardized for light intensity [28].

Xenograft Animal Study

All animal experiments met the Guide for Laboratory Animal Facilities and Care as promulgated by Council of Agriculture Executive Yuan, Taiwan. The protocol was approved by the Chang Gung University Animal Research Committee (Permit Number: CGU15154). All methods were performed in accordance with the "Animal Welfare Law and Policy" (LAW3ANI). The male nude mice (BALB/cAnN-Foxn1, 4 weeks old) were randomized into two groups: PC-DNA ($n = 6$) and PC-HO-1 ($n = 6$). Animal were anesthetized intraperitoneally and equal volumes of cells (4×10^6/100 μl) were injected into the back of mice. Tumor volume was measured using vernier calipers and calculated as $\pi/6 \times$ larger diameter $\times$ (smaller diameter) [2] as described previously [28].

Statistical Analysis

All the results are expressed as the mean ± standard error (SE). Statistical analyses were performed using SigmaStat program for Windows, version 2.03 (SPSS Inc. Chicago, IL). The significance of difference was determined by Student's t-test or one way ANOVA with a P value less than 0.05 (*$P < 0.05$) or 0.01 (**$P < 0.01$).

Results

HO-1 Expression is Predominant in the Cytoplasm of PC-3 Cells With HO-1 Ectopic Overexpression

The expression vector containing human HO-1 cDNA was ectopically stably transfected into PC-3 cells to evaluate the

location of HO-1 protein in cells. Expressions of HO-1 in the selected clones (PC-HO1-1 and PC-HO1-2) were determined by immunoblot (Figure 1A) and RT-PCR (Figure 1B) assays. Further immunoblot assays with subcellular extraction revealed clearly that cytoplasmic and nuclear positive HO-1 was detected PC-3 cells; however, the majority of HO-1 expression in PC-HO1-1 and PC-HO1-2 cells located in the cytoplasm in comparison with PC-DNA cells (mock-transfected PC-3 cells) (Figure 1C). Similar results were found in the immunofluorescence assays in which HO-1 expressed in both nuclear and cytosol area, but predominantly in the cytoplasm in the HO-1-overexpressed PC-3 cells (Figure 1D).

HO-1 Overexpression has No Obvious Effect on Cell Proliferation but Enhances Cell Invasion of PC-3 Cells In Vitro Via Modulation of EMT

The CyQUANT cell proliferation (Figure 2A) and WST-1 cell proliferation (Figure 2B) assays revealed that ectopic HO-1-overexpressed PC3 cells did not affect cell proliferation. Further study using the EdU staining proliferation assays also confirmed that overexpression of HO-1 did not affect cell proliferation assays in vitro (Figure 2C). The matrigel invasion assays indicated that PC-HO1-1 and PC-HO1-2 cells enhanced cell invasion to 1.8- and 2.3-folds, respectively, as compared with PC-DNA cells (Figure 2D). The immunoblot assays showed that ectopic overexpression of HO-1 upregulated N-cadherin, Vimentin, and Slug expressions in PC-3 cells (Figure 2E). Results of F-actin staining further suggested that modulation of epithelial—mesenchymal transition (EMT) altered the organization reminiscent of actin cytoskeletal as HO-1 overexpressed in PC-3 cells (Figure 2F).

HO-1 Enhances Tumorigenesis of PC-3 Cells In Vivo

PC-DNA and PC-HO-1 cells were injected subcutaneously into the back of nude mice to determine the effects of HO-1 on tumorigenesis. After 34 days of growth, the tumor volume of tumors derived from PC-HO1-1 cells was more than 7 times the size of those from the PC-DNA cells (40.90 ± 10.85 mm^3 vs. 285.74 ± 60.90 mm^3) (Figure 3A and B). There was no significant difference in the mean body weight of animals between two groups (Figure 3C). The weight of tumors derived from PC-HO1-1 cells was about 4 times the weight of tumors from the PC-DNA cells' group (Figure 3D). We randomly selected four tissues from each groups, and results of immunoblot assays confirmed that HO-1 was overexpressed in the xenograft tumors derived from PC-HO1-1 cells (Figure 3E).

HO-1 Alleviates ROS Generation in PC-3 Cells

Treatments of 125 μM of H_2O_2 induced 24.8-fold ROS immunofluorescence intensity in PC-DNA cells, while the same dosage of H_2O_2 induced only 15.1-fold and 17.5, respectively, ROS immunofluorescence intensity in PC-HO-1 and PC-HO1-2 cells as compared with the vehicle treatment (Figure 4A). Further flow cytometry assays showed similar results. Ectopic HO-1 overexpression blocked the ROS generation stimulated by 125 μM of H_2O_2 or 20 μM of pyocyanin (a ROS inducer) treatments in PC-HO1-1 (Figure 4B) and PC-HO1-2 (Figure 4C) cells as compared with PC-DNA cells.
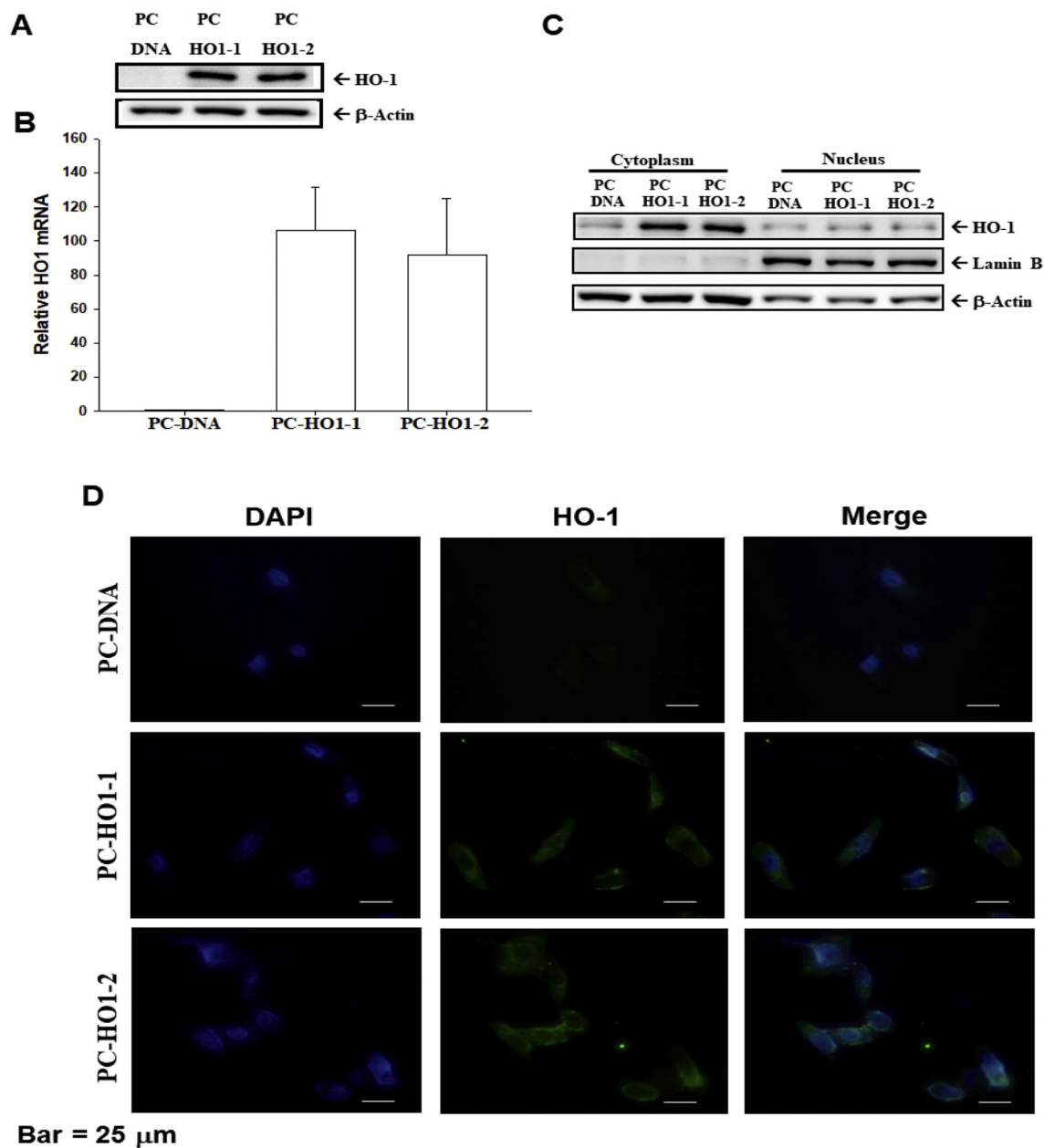
Figure 1. Ectopic HO-1 overexpression is predominant in the cytoplasm areas of prostate carcinoma cells. PC3 cells were transfected stably with HO-1 expression vector (PC-HO1-1 and PC-HO1-2) or pcDNA3.1 (PC-DNA). Expressions of HO-1 were analyzed using (A) immunoblot and (B) RT-qPCR assays. (C) HO-1 exhibition of ectopic overexpression was determined by subcellular extraction and immunoblotting. (D) The location patterns of HO-1 in PC-DNA, PC-HO1-1, and PC-HO1-2 cells were determined by immunofluorescence staining. The blue-stained nuclei of PC-DNA, PC-HO1-1, and PC-HO1-2 cells were stained by DAPI. All images were observed and recorded under the same settings of a fluorescence microscope with magnification of × 400.

HO-1 Prevents Cell Death Induced by Serum Starvation or H_2O_2 Treatment in PC-3 Cells

Although ectopic HO-1 overexpression did not enhance cell proliferation in normal condition as shown in Figure 2, which is contrary to the finding that HO-1 overexpression strengthened tumorigenesis shown in Figure 3, we discovered another possible mechanism for the increased *in vivo* tumorigenesis. Results of CyQUANT cell proliferation assays showed PC-HO1-1 and PC-HO1-2 cells, after 5 days of serum starvation, had higher cell proliferative rate as compared with PC-DNA cells (Figure 5A). Further immunoblot assays indicated that PC-HO1-1 (Figure 5B, top) and PC-HO1-2 (Figure 5B, bottom) cells had less cleaved form

of PARP protein as compared with PC-DNA cells after serum starvation, suggesting that ectopic HO-1 overexpression rendered cells more resistant to apoptosis after serum starvation for 3 or 5 days. This finding was supported by flow cytometry assays showing the sub-G1 cell percentage of PC-DNA cells was much higher than that of PC-HO1-1 and PC-HO1-2 cells after 5 days of serum starvation (Figure 5C, top). Quantitative analysis showed that PC-DNA, PC-HO1-1, and PC-HO1-2 cells induced 11.7%, 5.8%, and 9.5%, respectively, of cell apoptosis after serum starvation (Figure 5C, bottom). Flow cytometry assays revealed that PC-HO1-1 and PC-HO1-2 cells had lower percentage of cells in sub-G1 phase in comparison with PC-DNA cells after 250 μM of H_2O_2 treatment for
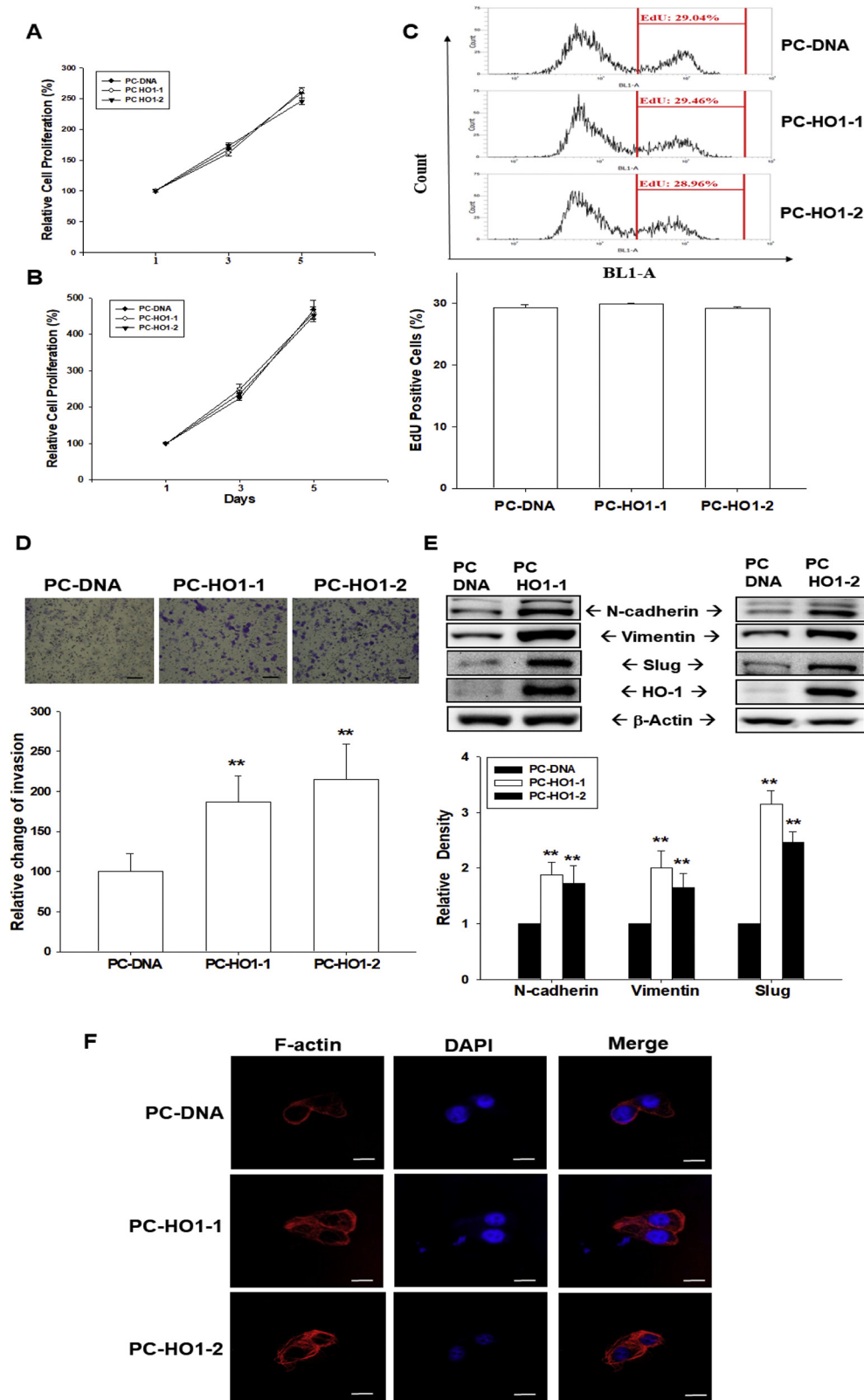
Figure 2. Ectopic overexpression of HO-1 modulates epithelial—mesenchymal transition and F-actin distribution in prostate carcinoma cells. The cell proliferation of PC-DNA (●), PC-HO1-1 (○), and PC-HO1-2 (▼) detected by CyQUANT (A), WST-1 (B), and EdU (C) cell proliferation assay kit. The cell invasion of PC-DNA, PC-HO1-1, and PC-HO1-2 measured using the matrigel invasion assay for 24 hours of incubation. The scale bar is 150 μm. (D). (E) The expressions of N-cadherin, Vimentin, and Slug in PC-DNA, PC-HO1-1, and PC-HO1-2 cells were determined by immunoblot assays. (F) Immunofluorescence staining of F-actin (red) and DAPI (blue) among PC-DNA, PC-HO1-1, and PC-HO1-2 cells. The scale bar is 10 μm. * p< 0.05, **p<0.01.
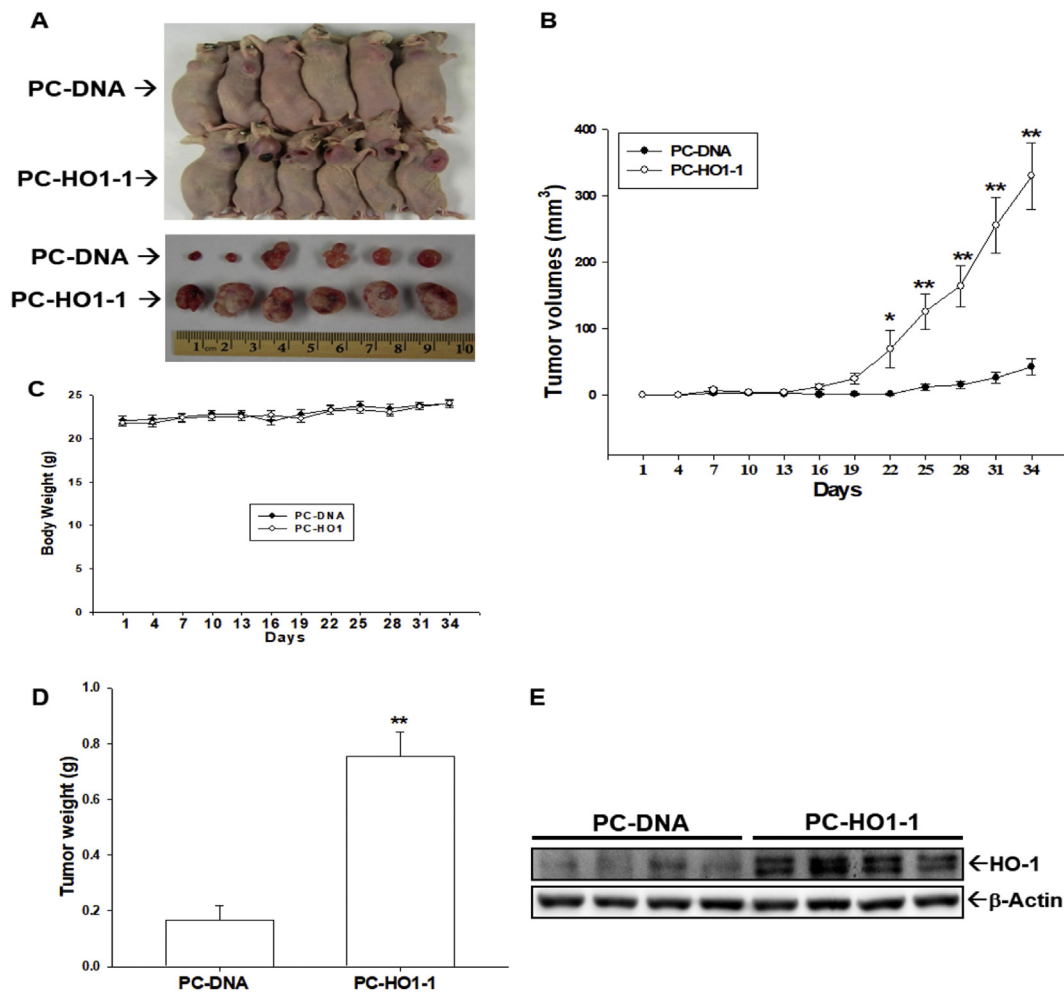
Figure 3. Effects of ectopic HO-1 overexpression on the tumorigenesis of prostate carcinoma cells. (A) Four-week-old male athymic nude (nu/nu) mice were randomized into two groups: PC-DNA ($n = 6$) and PC-HO1 ($n = 6$). PC-DNA cells and PC-HO1 cells (1×10^6) were injected s.c. in the dorsal area, respectively. Tumor growth rates (B) and animal body weight (C) were measured per 3 days starting at 1 week of growth point (day 1) in which the tumors became perceptible under the skin after inoculation. Subsequently, tumor volumes were measured as indicated days. The tumor weight (D) and levels of HO-1 protein expression (E) were determined after animals were sacrificed. * $p < 0.05$, ** $p < 0.01$.

16 hours (Figure 5D). Results of MTS assays also indicated that H_2O_2 treatment could decrease cell viability dose dependently with PC-HO1-1 and PC-HO1-2 cells much more resistant to H_2O_2 treatment (Figure 5E). Results of flow cytometry with double stains for PI and Annexin V-FITC also showed that after 12 hours of 500 μM of H_2O_2, PC-HO1-1 and PC-HO1-2 cells had less apoptotic cells (Figure 5F).
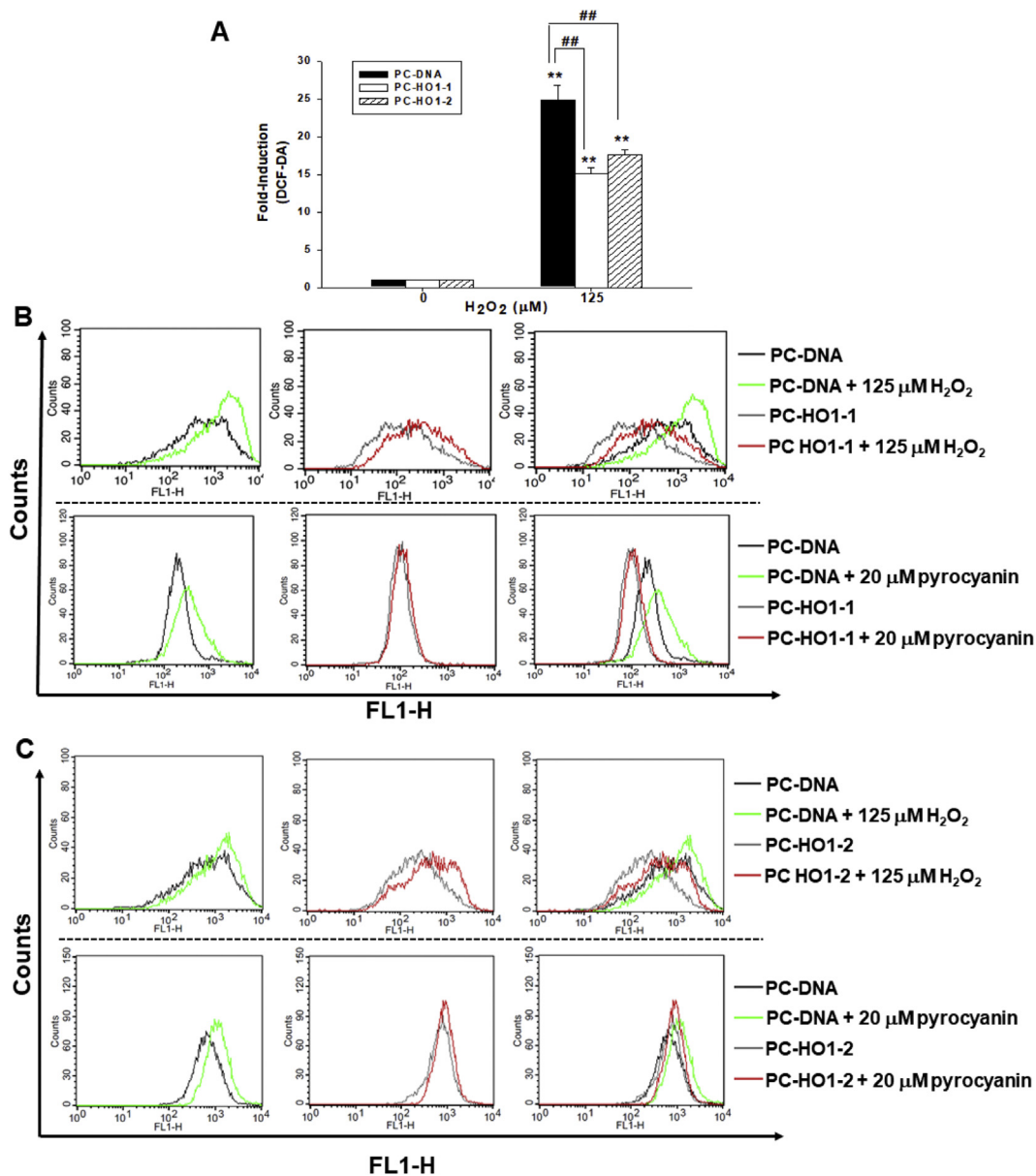
HO-1 Alleviates ROS Generation and Cell Death Induced by Serum Starvation or H_2O_2 Treatment in DU145 Cells

To confirm the antioxidant and antiapoptotic characteristics of HO-1 in prostate carcinoma cells, we repeated the experiments in Figure 5 using another prostate carcinoma cells, DU145. Immunoblot (Figure 6A, top) and RT-qPCR (Figure 6A, bottom) assays were applied to determine expressions of HO-1 after transient overexpression of HO-1 in DU145 (DU-HO1) cells. Results of MTS assays indicated transient overexpression of HO-1 (DU-HO1) could increase cell viability after serum starvation for 3 days as compared with mock-transfected DU145 (DU-DNA) cells (DU-DNA) (Figure 6B). The immunoblot assays also indicated

DU-HO1 cells had less cleaved form of PARP protein after serum starvation for 3 days (Figure 6C). Flow cytometry assays revealed that DU-HO1 cells had lower percentage of cells in sub-G1 phase in comparison with DU-DNA cells after serum starvation for 3 days (Figure 6D). Flow cytometry assays with PI and annexin V-FITC double stains showed transient HO-1 overexpression had less apoptotic cells after 12 hours of 500 μM of H_2O_2 treatment (Figure 6E). Further flow cytometry assays showed ectopic HO-1 overexpression in DU145 cells decreased ROS generation stimulated by 125 μM of H_2O_2 (Figure 6F, top) or 20 μM of pyocyanin (Figure 6F, bottom) treatments.

Discussion

Heme could induce oxidative stress in tissues. Therefore, heme-degradation pathway is deemed as a tissue protection pathway. Accordingly, HO-1 activity, which is responsible for heme degradation and induced by numerous stresses, is vital for cell survival after stress. HO-1 converts prooxidant heme into CO, biliverdin, and iron [1–3]. Iron could further induce ferritin synthesis, which has cytoprotective effect [6]. Biliverdin would be reduced by biliverdin

Figure 4. HO-1 alleviates ROS generation in prostate carcinoma PC-3 cells. (A) Fold change of ROS generation through treatments of 125 μM of H_2O_2 on PC-DNA, PC-HO1-1, and PC-HO1-2 cells detected by DCF-DA dye determining by immunofluorescence reader. The values shown in each bar chart represent the fold induction $\pm$ SE ($n = 6$) of intense fluorescence of cells induced by H_2O_2 relative to that of solvent-treated group (**$P < 0.01$) or relative to that of indicated (#$P < 0.05$; ##$P < 0.01$). Ectopic overexpression of HO-1 blocked ROS generation stimulated by 125 μM of H_2O_2 or 20 μM of pyocyanin determining by flow cytometry in PC-HO1-1 (B) and PC-HO1-2 (C) cells.

reductase to form bilirubin. Both biliverdin and bilirubin are capable of eliminating ROS, thus having antioxidant effect [6]. CO could increase cGMP production and affect numerous signaling pathways. In general, the stress-induced HO-1 activity leads to antiinflammatory, antiapoptotic, and antioxidant effects [6,29].

To study HO-1 effect on PC-3 cells, HO-1 was overexpressed in PC-3 cells and PC-HO1-1 and PC-HO1-2 cells were obtained (Figure 1A and B). As shown in Figure 1C and D, HO-1 expressed predominantly in the cytoplasm of PC-3 cells by immunoblotting and immunofluorescence assays. Our results are in agreement with general concept that HO-1 localizes mainly in the cytoplasma where it is associated with endoplasmic reticulum, caveolin, or mitochondria [2]. However, this result is different from other studies showing

hemin-treated prostate carcinoma cells and the HO-1-overxpressed PC3 cells in xenograft expressed HO-1 mainly located in the nuclei [30,31]. One important aspect of HO-1 effect on tumor biology might be related to its cellular localization, and the subcellular location of HO-1 in carcinoma cells which may account for the divergent effect on the different types of cancer has been discussed [29].

Results of previous studies investigating the functions and expression of HO-1 in prostate carcinoma cells *in vitro* and *in vivo* are still not consistent. For example, a significant elevation of HO-1 expression in malignant prostate cancer epithelial cells has been shown and, thus, HO-1 inhibitor is deemed as a potential therapeutic agent for prostate cancer [32,33]; however, results from other studies
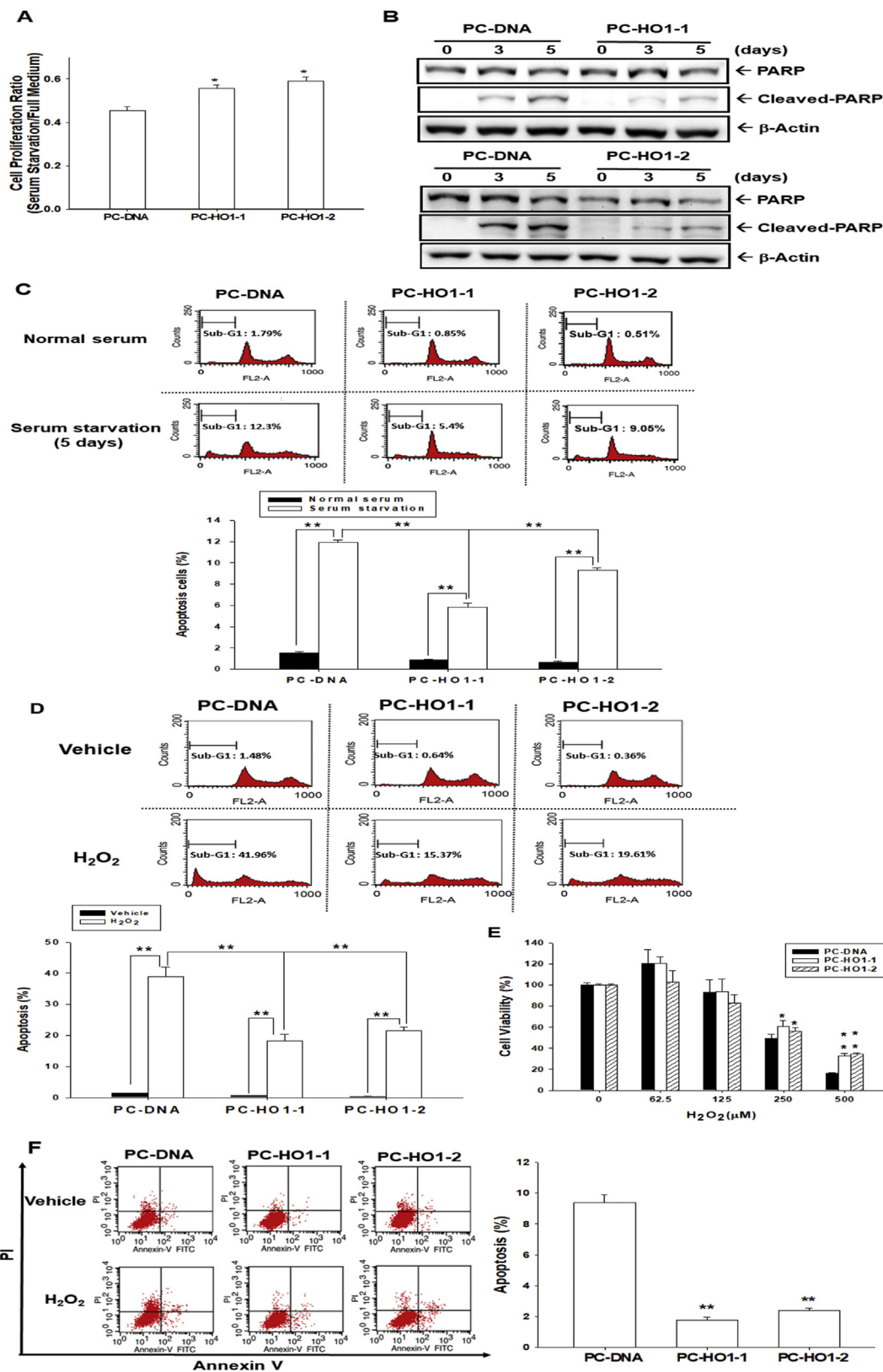
Figure 5. HO-1 attenuates serum starvation and H_2O_2 treatment induced cell apoptosis in PC-3 cells. (A) Cell proliferation ratio of PC-DNA, PC-HO1-1, and PC-HO1-2 cells after 5 days of serum starvation using CyQUANT cell proliferation assay kit. The proliferation ratio of cells incubated in full medium was regarded as 1. (B) PARP protein expressions of PC-DNA, PC-HO1-1, and PC-HO1-2 cells after 3 and 5 days of serum starvation utilizing immunoblot assays. (C) Fluorescence intensity of sub-G1 phase detected PI staining using flow cytometry after 5 days of serum starvation. (D) Fluorescence intensity of sub-G1 phase after 250 μM of H_2O_2 treatment for 16 hours detected by PI dye using flow cytometry. (E) Cell viability with different concentrations of H_2O_2 treated PC-DNA, PC-HO1-2, and PC-HO1-2 cells utilizing MTS assays. (F) Cell apoptosis was determined by Annexin V-FITC in conjunction with PI staining. Fluorescence intensity for Annexin V-FITC is plotted on the x-axis, and PI is plotted on the y-axis. * p< 0.05, **p<0.01.
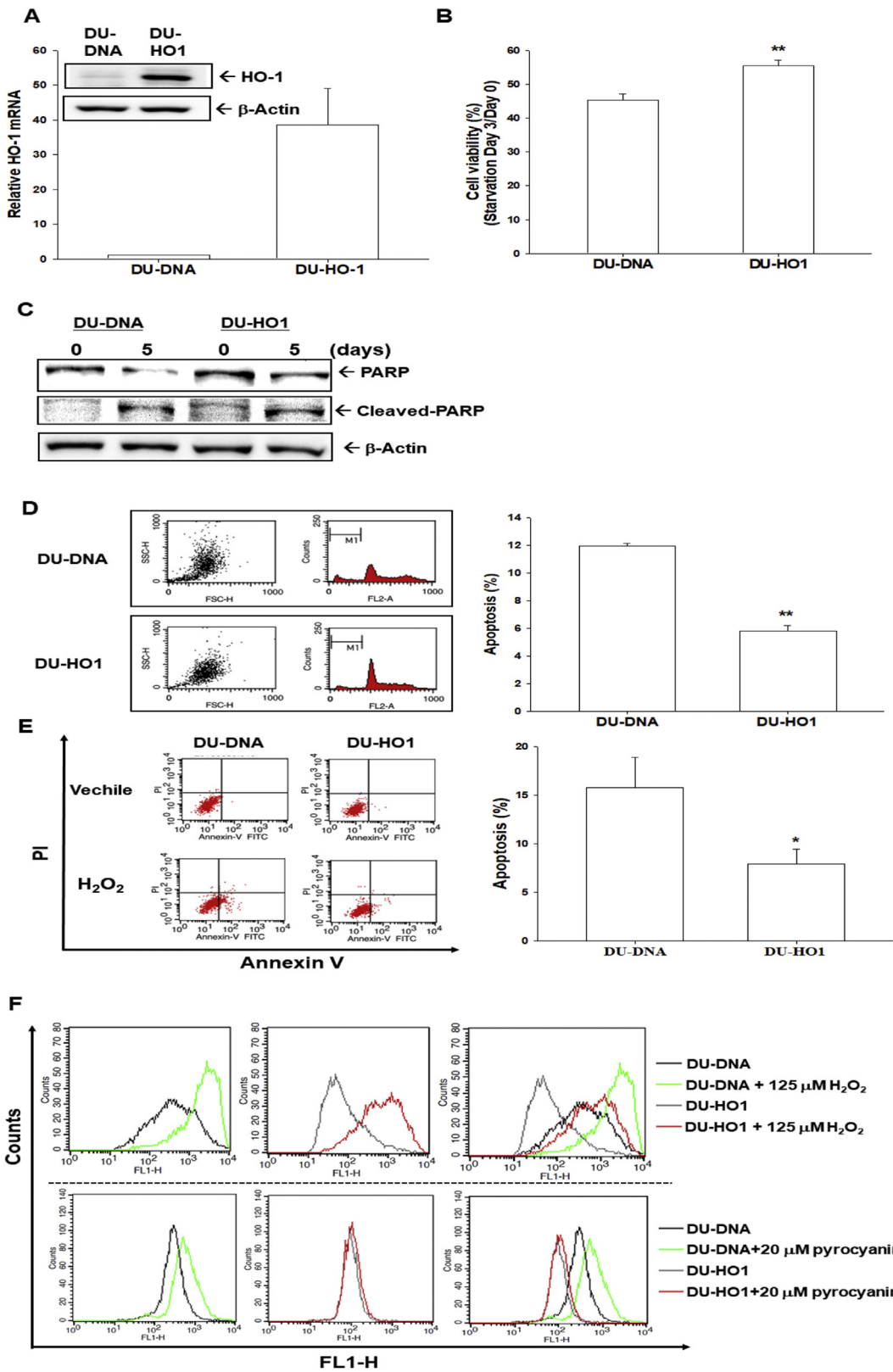
Figure 6. HO-1 attenuates oxidation and apoptosis under serum starvation and H$_2$O$_2$ treatment in prostate carcinoma DU145 cells. DU145 cells were transfected transiently with HO-1 expression vector (DU-HO1) or pcDNA3.1 (DU-DNA) for 72 hours. (A) Expressions of HO-1 were analyzed by immunoblot (top) and RT-qPCR (bottom) assays. (B) Cell viability of DU-DNA and DU-HO1 cells after 3 days of serum starvation utilizing MTS assays. (C). PARP protein expressions of DU-DNA and DU-HO1 cells after 3 and 5 days of serum starvation utilizing immunoblot assays. (D) Percentage of sub-G1 phase after 3 days of serum starvation detected PI staining using flow cytometry. (E) Cell apoptosis of DU-DNA and DU-HO1 cells induced by 500 μM of H$_2$O$_2$ for 12 hours were determined by Annexin V-FITC in conjunction with PI staining. (F) ROS generation of DU-DNA and DU-HO1 cells stimulated by 125 μM of H$_2$O$_2$ (upper) or 20 μM of pyocyanin (bottom) treatments determining by flow cytometry. * p< 0.05, **p<0.01.

indicated HO-1 reduced cell proliferation and metastasis in prostate cancer cells, thus implying stimulation of HO-1 expression may be a therapeutic direction for prostate cancer [18,30,31]. In this study, our result showed that the cell proliferation rate of PC-HO1-1 and PC-HO1-2 cells was not changed as compared with PC-DNA cells *in vitro* (Figure 2A−C). Moreover, PC-HO1 cells had higher invasive potential than PC-DNA cells (Figure 2D).

EMT is the process during which epithelial cells differentiate into mesenchymal cells. EMT is an important process for normal development and wound healing. However, EMT also plays a crucial role in cancer progression. For example, after EMT, cancer cells get more stem-cell like characteristics, which makes cancer cells more resistant to chemotherapy [34−36]. The increased invasiveness of cancer cells after EMT further worsens the clinical outcome [37]. EMT is under delicate control. So far, at least three transcriptional families have been shown to involve in EMT regulations, which include Snail/Slug, ZEB1/2, and Twist families [38]. After EMT, the mesenchymal cell markers, such as vimentin or N-cadherin, would also increase [39]. Figure 2E showed that HO-1 overexpression would increase Slug, N-cadherin, and vimentin expression in PC-3 cells, which demonstrated HO-1 overexpression would induce EMT in PC-3 cells to enhance cell invasiveness shown in Figure 2D. Apart from EMT, HO-1 overexpression also increased F-actin synthesis, thus further increasing cell motility (Figure 2F).

Although the cell proliferation rate did not change after HO-1 overexpression in PC-3 cells *in vitro*, the tumorigenesis ability increased. As shown in Figure 3A, B, and D, the xenograft animal study revealed that tumors from PC-HO1-1 had higher tumor volume as compared with PC-DNA cells. To investigate the reason behind this, we applied H_2O_2 or pyocyanin to treat PC-DNA and PC-HO1-1 cells. Figure 4 showed that H_2O_2 increased ROS in both PC-DNA cells and PC-HO1 cells with the latter having much less amount of ROS, further demonstrating the scavenger role of HO-1 for ROS. The role of ROS in tumorigenesis has been discussed. Because ROS plays a causal role in tumor development but high ROS level has the potential to actually block tumorigenesis [40,41], our results are in agreement with previous studies demonstrating HO-1 can reduce H_2O_2 or pyocyanin-induced oxidative damage [42,43], which may account for the higher tumorigenesis of PC-HO1-1 cells noted *in vivo* shown in Figure 3.

Figure 5A showed that after 5 days of starvation, the cell proliferation rate of PC-HO1-1 and PC-HO1-2 cells was higher than PC-DNA cells. The western blot result further revealed that PC-HO1-1 had lower cleaved PARP than PC-DNA cells (Figure 5B), indicating the lower apoptosis rate of PC-HO1-1 cells. The result was also supported by the flow cytometry data showing in normal medium, the percentage of apoptotic cells was similar in PC-DNA and PC-HO1-1 cells; however, after serum starvation, the apoptotic cell percentage was much higher in PC-DNA cells (Figure 5C). The MTS test showed that after 250−500 μM H_2O_2 treatment, PC-HO1-1 had higher cell proliferative rate than PC-DNA cells (Figure 5D), which is supported by the flow cytometry result showing after H_2O_2 treatment, PC-HO1-1 had lower percentage of apoptotic cells (Figure 5E and F). These results are in agreement with previous studies which indicated HO-1 had antiapoptotic effect [29,44].

The antioxidant and antiapoptotic effects of HO-1 were also evaluated in DU145 cells (Figure 6). Overexpression of HO-1 in DU145 cells decreased cell apoptosis induced by serum starvation or H_2O_2 treatment (Figure 6A−D). The antioxidant effect of HO-1 in DU145 cells was also observed (Figure 6E). Taken together, the result from Figures 4−6 suggested that HO-1 overexpression rendered PC-3 cells more resistant to serum starvation or ROS-induced treatment, which may contribute to the higher tumorigenesis of PC-HO1-1 cells noted *in vivo* (Figure 3).

Conclusion
Our results suggested that although ectopic HO-1 overexpression did not increase cell growth *in vitro*, it did enhance cell proliferation under the stress of serum starvation *in vitro* and tumorigenesis *in vivo*. HO-1 overexpression further enhanced prostate cancer cell invasion through induction of EMT. The antioxidant and antiapoptotic effects of HO-1 shown in our study may attribute to the higher tumorigenesis found in the xenograft animal model.

Authors' Contributions
K.-C.C. and K-H.T. wrote the manuscript and designed this experiment and contributed equally to this work; Y.-H.L., C.-P. H., K-S.C., H.-H.T., T.-H.F., Y.-S.S., and C.-C.C. helped conduct the experiment and analysis data; H.-H.J. was in charge of the whole experiment conduction and paper writing.

Conflicts of Interest
The authors declare that no conflict of interest exists.

Acknowledgment

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.10.008.

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