Nitric oxide induces cancer stem cell-like phenotypes in human lung cancer cells

Nutrida Yongsanguanchai, Varisa Pongrakhananom, Apiwat Mutirangura, Yon Rojanasakul, and Pithi Chanvorachote

1Pharmaceutical Technology (International) Program, Chulalongkorn University, Bangkok, Thailand; 2Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; 3Cell-Based Drug and Health Products Development Research Unit, Chulalongkorn University, Bangkok, Thailand; 4Center of Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 5School of Pharmacy, West Virginia University, Morgantown, West Virginia

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Yongsanguanchai N, Pongrakhananom V, Mutirangura A, Rojanasakul Y, Chanvorachote P. Nitric oxide induces cancer stem cell-like phenotypes in human lung cancer cells. Am J Physiol Cell Physiol 308: C89–C100, 2015. First published November 19, 2014; doi:10.1152/ajpcell.00187.2014.—Even though tremendous advances have been made in the treatment of cancers during the past decades, the success rate among patients with cancer is still dismal, largely because of problems associated with chemo/radioresistance and relapse. Emerging evidence has indicated that cancer stem cells (CSCs) are behind the resistance and recurrence problems, but our understanding of their regulation is limited. Rapid reversible changes of CSC-like cells within tumors may result from the effects of biological mediators found in the tumor microenvironment. Here we show how nitric oxide (NO), a key cellular regulator whose level is elevated in many tumors, affects CSC-like phenotypes of human non-small cell lung carcinoma H292 and H460 cells. Exposure of NO gradually altered the cell morphology toward mesenchymal stem-like shape. NO exposure promoted CSC-like phenotype, indicated by increased expression of known CSC markers, CD133 and ALDH1A1, in the exposed cells. These effects of NO on stemness were reversible after cessation of the NO treatment for 7 days. Furthermore, such effects were reproducible using another NO donor, S-nitroso-N-acetylpenicillamine. Importantly, inhibition of NO by the known NO scavenger 2-(4-carboxy-phenyl)-4,4,5,5 tetramethylimidazoline-1-oxo-3-oxide strongly inhibited CSC-like aggressive cellular behavior and marker expression. Last, we unveiled the underlying mechanism of NO action through the activation of caveolin-1 (Cav-1), which is upregulated by NO and is responsible for the aggressive behavior of the cells, including anoikis resistance, anchorage-independent cell growth, and increased cell migration and invasion. These findings indicate a novel role of NO in CSC regulation and its importance in aggressive cancer behaviors through Cav-1 upregulation.

Cancer stem cells; nitric oxide; caveolin-1; CD133; ALDH1A1; non-small cell lung carcinoma

According to the American Cancer Society, 2013 annual cancer statistics, lung cancer remains one of the most common malignancies and is the leading cause of cancer-related deaths worldwide (61). Increasing evidence has indicated the role of cancer stem cells (CSCs) in cancer aggressiveness, chemoresistance, and relapse; they are being considered as the underlying causes of the high mortality rate of cancer (29, 47, 50, 55). The concept of a tumor having a heterogeneous cancer cell population with a subpopulation of cells possessing a high tumorigenic potential and stem-like property was first described in 1997 (5). This subpopulation is commonly known as tumor-initiating cells, tumor-propagating cells, or CSCs and has been identified in many types of cancer (16, 23, 40, 46, 56, 58, 63). CSCs have been suggested as the rationale behind chemo/radioresistance and cancer relapse (3, 4, 18, 39, 68) and have been a target of new cancer therapeutic strategies.

Nitric oxide (NO), a free radical gaseous molecule, is renowned for its involvement in various biological, physiological, and pathological processes (52). NO was initially identified as a transcellular messenger molecule synthesized by a family of enzymes called NO synthases (NOS), comprised of inducible NOS, endothelial NOS, and neuronal NOS, through the conversion of l-arginine in the presence of oxygen and NADPH (48, 51). A number of studies pointed out that all three isoforms of NO are involved in the process of cancer development and progression (1, 25, 64, 66). Not only is NOS expression detectable in various cancers, but also the NO level is frequently upregulated in tumor areas (26, 36). Previous studies indicated that NO could render cells resistant to death induced by various stimuli (7, 8, 67). NO also regulates cancer cell migration and invasion (60), and increased NOS expression and activity have been reported in metastatic lung cancer cells (57). Clinical data further support the role of NO in lung cancer and metastasis. A high level of NO was observed in the lung of patients with lung cancer (43, 49), and a positive correlation has been reported between NO, its stable end-products nitrite and nitrate, and its generator NOS with advanced cancer staging and poor survival in patients with lung cancer (2, 15, 24). Animal studies further showed that genetic ablation of NOS suppressed lung tumor formation in mice (35). Together, these studies strongly support the role of NO in tumorigenesis and metastasis although the underlying mechanisms remain obscure.

We hypothesize that NO may mediate its procarcinogenic effects through CSCs because of their importance in cancer aggressiveness described above. We also hypothesize that NO...
may mediate its effects through caveolin-1 (Cav-1) because its expression has been linked to cancer aggressiveness (11, 27, 28, 30, 42, 45, 59) and because NO has been shown to be a key regulator of Cav-1 in human lung cancer cells (7). Cav-1 is a scaffolding protein found in the cellular structure caveolae, which has been shown by our group and others to regulate the aggressiveness of cancer cells by increasing their motility and resistance to anoikis (11, 27, 28, 30, 42, 45, 59). It has also been shown to increase cell survival under nonadherent conditions via Mcl-1 stabilization (12) and upregulation of activated protein kinase B (Akt) (6). In this study, we used molecular and pharmacological approaches to investigate the role of NO in Cav-1 and CSC regulation and their impact on the invasive and anoikis properties of human lung cancer cells. We demonstrated for the first time that NO plays an important role in the CSC-like transformation of lung cancer cells. Cav-1 is upregulated during the transformation and is responsible for the anoikis resistance and invasive properties of the cells although it is not required for the stem-like phenotype.

MATERIALS AND METHODS

Cell culture and NO exposure. Human non-small cell lung cancer cell lines, NCI-H292 and NCI-H460, were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, and 100 μM penicillin and streptomycin. Cell cultures were maintained in a 37°C humidified incubator with 5% CO2. Cells were routinely passaged at preconfluent density using a 0.25% trypsin solution with 0.53 mM EDTA. RPMI 1640 medium, FBS, l-glutamine, penicillin/streptomycin, phosphate-buffered saline (PBS), trypsin, and EDTA were purchased from Gibco (Grand Island, NY). Cells were seeded onto six-well plates at an initial plating density of 2 × 10⁴ cells/well. Cells were allowed to adhere to the surface of the plates for 4 h, after which they were treated with the indicated concentrations of freshly prepared NO donor. The treated cells were subcultured and exposed to fresh NO every 3 days. The cells were subsequently collected at days 7 and 14 posttreatment for further analysis. The NO donor dihydropropenynametranilimide (DPTA) NONOate and NO scavenger 2-(4-carboxy-phenyl)-4,4,5,5 tetramethylimidazoline-1-oxide-3-oxide (PTIO) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas another NO donor S-nitroso-N-acetylpenicillamine (SNAP) was obtained from Invitrogen (Carlsbad, CA).

Cytotoxicity and proliferation assays. For cytotoxicity assay, cells were seeded onto 96-well plates at a density of 1 × 10⁴ cells/well and were allowed to incubate overnight. Cells were then treated with various concentrations of NO donor and analyzed for cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s protocol (Sigma Chemical, St. Louis, MO). The cytotoxicity index was calculated by dividing the absorbance of the treated cells by that of the control cells. For cell proliferation assay, cells were seeded onto 96-well plates at a density of 5 × 10³ cells/well and were left to settle overnight. Cell proliferation was determined by PrestoBlue assay according to the manufacturer’s protocol (Invitrogen).

Cell death assay. Nuclear containing with Hoechst 33342 (Sigma Chemical) and propidium iodide (PI) (Sigma Chemical) was used to determine apoptotic and necrotic cell death. Cells were incubated with 10 μM Hoechst 33342 and 5 μM PI for 30 min at 37°C. They were visualized and imaged under a fluorescence microscope (Olympus IX51 with DP70).

Migration assay. Wound-healing assay was used to determine cell migration. Briefly, confluent monolayers of cells in a 96-well plate were wounded at the center of the well by a 200-μl micropipette tip. Four random fields of the wound space were examined and imaged under a phase-contrast microscope (Olympus IX51 with DP70) at various time points. Relative cell migration was quantified by dividing the percentage change of the wound space in treated cells to that of the control cells.

Invasion assay. The assay was performed in modified Boyden chambers with 8-μm pore filter inserts in 24-well plates (Corning Life Sciences, Corning, NY). The upper chamber of the inserts was coated with 50 μl of 0.5% Matrigel from BD Biosciences (San Jose, CA), and 3 × 10⁴ cells in serum-free medium were added on top. The lower chamber was filled with RPMI 1640 medium containing 10% FBS as a chemoattractant. After 24 h, the noninvading cells in the upper chamber were removed with a cotton swab, and the invading cells in the lower chamber were fixed with ice-cold methanol for 10 min and stained with 10 μg/ml of Hoechst 33342 for 10 min. The stained cells were then visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

Anoikis assay. Cells were trypsinized into single-cell suspension before being seeded onto Costar six-well ultralow attachment plates (Corning Life Sciences). Suspended cells were incubated at 37°C and were harvested for analysis at various time points. Cell viability was assessed by MTT assay as described above.

Anchorage-independent growth assay. Anchorage-independent cell growth was determined by soft agar colony-formation assay. Soft agar was prepared by using a 1:1 mixture of RPMI 1640 medium containing 10% FBS and 1% agarose. The mixture was allowed to solidify in a 24-well plate to form a bottom layer, after which an upper cellular layer consisting of 3 × 10⁵ cells/ml in the agarose gel with 10% FBS and 0.3% agarose was added. After the upper layer was solidified, RPMI medium containing 10% FBS was added to the system and incubated at 37°C. Colony formation was determined after 2 wk using a phase-contrast microscope (Olympus IX51 with DP70). Relative colony number and diameter were determined by dividing the values of the treated cells by those of the control cells.

Spheroid-formation assay. Spheroids were grown using an adjusted method from Kantara et al. (33). Approximately 5 × 10⁴ cells/well were seeded onto a 12-well ultralow attachment plate using RPMI serum-free medium. Treated cells were cultured every 3 days. Phase-contrast images of formed primary spheroids were taken at day 7 of treatment. Images were analyzed using a phase-contrast microscope (Olympus IX51 with DP70). Primary spheroids were resuspended into single cells, and again 5 × 10³ cells/well were seeded onto a 12-well ultralow attachment plate using RPMI serum-free medium. Secondary spheroids were allowed to form for 30 days.

Western blot analysis. Cells were incubated on ice for 45 min with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 100 mM PMSF, and protease inhibitor mixture from Roche Molecular Biochemicals (Indianapolis, IN). Cell lysates were analyzed for protein content using BCA protein assay kit from Pierce Biotechnology (Rockford, IL). Equal amounts of denatured protein samples (40 μg) were loaded onto 10% SDS-PAGE for ALDH1A1 and Cav-1 analysis or onto 7.5% SDS-PAGE for CD133 analysis before being transferred to 0.45-μm nitrocellulose membranes (Bio-Rad, Hercules, CA). Transferred membranes were blocked with medium (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, and 0.05% Tween 20 (TBST)) containing 5% nonfat dry milk powder for 30 min and incubated overnight with specific primary antibodies against CD133 (Cell Applications, San Diego, CA), ALDH1A1 (Santa Cruz Biotechnology), Cav-1 (Cell Signaling Technology, Beverly, MA), and β-actin (Santa Cruz Biotechnology). Membranes were washed three times with TBST and incubated with the following appropriate horseradish peroxidase-labeled secondary antibodies: anti-rabbit IgG (Cell Signaling Technology), anti-mouse IgG (Cell Signaling Technology), or anti-goat IgG (Santa Cruz Biotechnology), for 2 h at room temperature. The immune complexes were detected by SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology) and exposed to film.
Immunofluorescence. Cells were seeded onto six-well plates at a density of $5 \times 10^3$ cells/well and allowed to adhere for 24 h. The cells were fixed at room temperature for 10 min with 3.7% formaldehyde and blocked for 30 min in a solution containing 0.5% saponin, 1% FBS, and 1.5% goat serum. Cells were then incubated with CD133 (Cell Applications) primary antibody for 2 h before being washed and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Invitrogen) for 1 h. Hoechst 33342 was used to stain the cell nucleus. Immunofluorescence images were acquired via confocal laser-scanning microscopy (Zeiss LSM 510).

Flow-cytometry analysis. Treated and nontreated cells were collected using 1 mM EDTA at day 14 of treatment. Briefly, cells were fixed and blocked, and then incubated on ice with CD133 primary antibody for 1 h, followed by 30-min incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody on ice with light omitted. Fluorescence intensity was scored by flow cytometry using a 488-nm excitation beam and a 519-nm band-pass filter (FLC sorter; Becton Dickinson, Rutherford, NJ). The mean fluorescence intensity was quantified by CellQuest software (Becton Dickinson).

Plasmid and transfection. Transfection of Cav-1 and its shRNA plasmids was performed as previously described (6) with the following modifications. Briefly, subconfluent (70%) monolayers of H292 and H460 cells were transfected with pEX_Cav-1-YFP (ATCC) or shRNA-Cav-1 (Santa Cruz Biotechnology) plasmid in serum-free RPMI 1640 medium using Lipofectamine 2000 reagent, according to the manufacturer's protocol (Invitrogen). After 2 h (H292) or 12 h (H460), the medium was replaced with RPMI 1460 containing 10% FBS. The cells were then cultured and selected for antibiotic resistance for 30 days to obtain stable transfectants. Expression of the targeted proteins was verified by Western blot assay. The cells were cultured in antibiotic-free RPMI 1640 medium for at least two passages before further experiments.

Statistical analysis. All treatment data were normalized to nontreated controls. Data are presented as the means ± SD from at least four independent experiments. Statistical differences were determined using two-way ANOVA and a post hoc test at a significance level of $P < 0.05$.

RESULTS

Effect of DPTA NONOate on human lung cancer cell epithelial-mesenchymal transition. Elevated NO levels have been associated with cancer cell behaviors such as anoikis resistance, increased cell motility, and chemoresistance (8, 60, 67). To test whether NO might affect epithelial-mesenchymal transition (EMT) properties of lung cancer cells, we first determined the appropriate noncytotoxic concentrations of NO donor used in this study. Human lung cancer H292 and H460 cells were treated with various concentrations of DPTA NONOate (0–50 μM), and cell viability was determined after 24 h by MTT assay. DPTA NONOate was relatively nontoxic at the doses below 30 μM in H292 cells and below 15 μM in H460 cells (data not shown). The low-dose DPTA treatments were stained by Hoechst 33342 and PI for apoptotic and necrotic cell death. The lack of DNA condensation/fragmentation and nuclear PI fluorescence indicated that cells do not undergo apoptosis or necrosis (data not shown). To determine the effect of NO donor treatment on cell morphology, H292 and H460 cells were treated with the noncytotoxic concentrations of DPTA NONOate, and cell morphology was examined microscopically. Figure 1, A and B, shows phase-contrast images of the treated cells on days 7 and 14 posttreatment. A gradual but clearly noticeable change in cell morphology toward the mesenchymal stem cell-like (spindle-like) phenotype was evident in both the treated H292 and H460 cells compared with nontreated controls. The cell population lysates were probed for EMT markers, Vimentin and Snail, by Western blotting. Figure 1, C and D, shows that the NO donor induced Vimentin and Snail expression in a dose- and time-dependent manner. Approximately up to fourfold increases in both Vimentin and Snail protein levels were observed for H292 cells treated with 25 μM DPTA for 14 days and approximately threefold for H460 cells treated with 10 μM DPTA for 14 days. We then examined the effect of NO donor on cell migration and invasion by wound-healing assays and Boyden chamber invasion assays, respectively. Not only are EMT cells well known for their metastatic ability, but also previous studies have shown that CSCs possess increased cell motility for more efficient metastasis to secondary sites (10, 19, 29). Figure 1, E and F, shows that, in both cell lines, the motility rate of NO-treated cells was significantly increased relative to the nontreated controls. A 2.5-fold increase in the migration rate was observed in the H292 cells treated with 25 μM DPTA for 14 days (Fig. 1E), and a 2.3-fold increase was observed in the H460 cells treated with 10 μM DPTA for the same amount of time (Fig. 1F). Invasion assay similarly indicated the induction of cell invasivity in both H292 and H460 cells by the NO donor in a dose- and time-dependent manner (Fig. 1, G and H).

NO promotes CSC-like behaviors. Having shown the effect of NO donor treatment on EMT properties, we further investigated whether such treatment also affects CSC-like behaviors. H292 and H460 cells were treated with noncytotoxic concentrations of DPTA NONOate for 7 and 14 days, and cell behaviors including proliferation, anoikis resistance, colony and spheroid formation were examined. Stem cells are known to be slow proliferators. Figure 2, A and B, shows that the DPTA treatment resulted in a significant reduction in the proliferation rate of both H292 and H460 cells.

Resistance to anoikis or detachment-induced apoptosis are hallmarks of CSCs. Figure 2, C and D, illustrates the viability of H292 and H460 cells in response to cell detachment. Cells were first treated with NO donor for 7 and 14 days and analyzed for anoikis response at different time points. The results showed that the treated cells exhibited a significantly reduced anoikis response compared with the nontreated controls, suggesting the ability of NO to induce a CSC-like anoikis-resistant phenotype.

Finally, we tested whether NO can induce colony and spheroid formation, which is a distinguishing feature of CSCs. H292 and H460 cells were similarly treated with the NO donor and analyzed for colony formation on soft agar. Colony number and diameter were determined and expressed as relative values over nontreated control levels. Figure 2E displays the relative colony number and diameter of the treated and nontreated H292 cells. A significant (2.1-fold) increase in the number of colonies formed was recorded for the H292 cells treated with 10 μM DPTA NONOate for 14 days, and a 2.5-fold increase and a 3.4-fold increase were observed for the cells treated for 7 and 14 days, respectively, with 25 μM DPTA NONOate. For H460 cells, a significant increase in the colony number can only be seen at the high treatment dose of 10 μM at day 7 (2.7-fold) and day 14 (3.3-fold) (Fig. 2F). However, both treated cell lines showed no significant difference in the colony diameter compared with controls. Representative images of the H292 and H460 colonies are shown in Fig. 2, G and H, with the circular photograph representing an ×1 image and...
the square representing an ×10 image of the colonies. Figure 2, I and J, displays an ×4 phase-contrast image of day 7 H292 and H460 primary spheroids, respectively. Cells were seeded at low density onto ultralow attach plates, and primary spheroids were allowed to form for 7 days. Control nontreated cells tend to survive through E-cadherin-mediated survival, whereas DPTA NONOate-treated cells can survive on their own as a single cell and slowly proliferate to form dense spheroids. The primary spheroids were then resuspended into single cells, and secondary spheroids were allowed to grow for 30 days in RPMI serum-free medium. By day 30, the control nontreated spheroids were deformed and had already undergone apoptosis, whereas DPTA NONOate-treated spheroids still remained viable and intact (data not shown).

**NO exposure induces CSC marker expression.** We used two well-known CSC markers to verify the CSC-inducing effect of NO in H292 and H460 cells. The cells were cultivated in the presence or absence of DPTA NONOate for 7 and 14 days, and the expression levels of CD133 and ALDH1A1 were determined by Western blotting. Figure 3, A–D, shows that the NO
donor induced CD133 and ALDH1A1 expression in a dose- and time-dependent manner. A 7.1-fold increase in CD133 and a 7.5-fold increase in ALDH1A1 expression were observed in the H292 cells treated with 25 μM DPTA NONOate for 14 days (Fig. 3C). In H460 cells, a 4.8-fold increase and a 5.8-fold increase in expression of the two CSC markers were observed after treatment with 10 μM DPTA NONOate for 14 days (Fig. 3D). To confirm the results, we performed immunofluorescence experiments assessing the expression of the CSC marker CD133 on the cells. H292 and H460 cells were treated with NO donor for 14 days and analyzed for CD133 expression by immunofluorescence staining (Fig. 3, E and F). Consistent with the Western blot results, the immunofluorescence results indicate a dose-dependent increase in CD133 expression in both treated H292 and H460 cells. Furthermore, at day 14 treated and nontreated H292 and H460 cells were analyzed for CD133 cell surface intensity by flow cytometry, as shown in Fig. 3, G and H, respectively. CD133 on the cell surface intensifies dose dependently for both cell types. These results along with the findings on the NO effects on cell morphology and aggressive
behaviors strongly support the role of NO in CSC-like properties of lung cancer cells.

Reversible effect of NO on CSC-like phenotypes. Having shown that NO drives the upregulation of CSC markers and promotes CSC-like behaviors, we next examined whether this effect of NO is reversible. The cells were first treated with 25 \( \mu \)M (H292) or 10 \( \mu \)M (H460) DPTA NONOate for 14 days, after which they were further cultured in the absence of NO donor for an additional 7 days and analyzed for CSC markers and cellular behaviors. Figure 4, A and B, shows that the cells with discontinued NO treatment were less resistant to anoikis, having their viability reverting to nearly the baseline level found in nontreated control cells. These cells also possessed weaker colony-forming activity compared with the normal NO-treated cells (Fig. 4, C and D). Moreover, the expression of CSC markers, CD133 and ALDH1A1, on these cells was significantly reduced after the discontinuation of the NO donor (Fig. 4, E–H). A similar finding was observed in the H460 cells after the treatment and discontinuation of NO donor (Fig. 4H), supporting the generality of the effect of NO on CSC phenotypes.
Effects of NO donor (SNAP) and NO scavenger (PTIO) on CSC-like phenotypes. To confirm the effect of NO on CSC-like phenotypes, another NO donor SNAP and an NO scavenger PTIO were used. Cells were cultured in the presence or absence of NO modulators for 14 days, and CSC-like phenotypes were examined. Figure 5A shows that the SNAP-treated H292 cells displayed a dose-dependent increase in anoikis resistance compared with the nontreated control. In contrast, treatment of the cells with NO scavenger had a reversal effect (Fig. 5A), supporting the role of NO in the resistance process.

Consistent with this finding, a similar effect of NO donor and scavenger was observed in the H460 cells (Fig. 5B). Colony-formation studies also showed increased colony-forming activity of the SNAP-treated cells and decreased activity of the PTIO-treated cells, effects that were observed in both H292 and H460 cells (Fig. 5, C and D). Moreover, the SNAP-treated cells displayed an increased expression of the CSC markers, CD133 and ALDH1A1, whereas the PTIO-treated cells showed a reduced expression of the markers (Fig. 5, E–H). Figure 5, I and J, displays an ×4 phase-contrast image of day

Fig. 4. The effect of NO on CSC-like phenotypes is reversible. After H292 and H460 cells were treated with 25 and 10 μM DPTA NONOate, respectively, for 14 days, the cells were further cultured in the absence of NO donor for 7 days. H292 (A) and H460 (B) cells were detached for 0–24 h. At indicated times, the detached cells were subjected to anoikis assay by measurement of cell viability using MTT assay. H292 (C) and H460 (D) cells were suspended and subjected to colony-formation assay. After 14 days, colony number was analyzed and calculated as relative to the control cells. H292 (E) and H460 (F) cells were collected, and CSC markers, CD133 and ALDH1A1, were analyzed by Western blotting. The blots were reprobed with β-actin to confirm equal loading. The immunoblot signals in H292 (G) and H460 (H) cells were quantified by densitometry, and mean data from independent experiments were normalized to the controls. The bars are means ± SD (n = 4 for A–D, n = 8 for G and H). *P < 0.05 vs. nontreated cells. #P < 0.05 vs. DPTA NONOate-treated cells.

Effects of NO donor (SNAP) and NO scavenger (PTIO) on CSC-like phenotypes. To confirm the effect of NO on CSC-like phenotypes, another NO donor SNAP and an NO scavenger PTIO were used. Cells were cultured in the presence or absence of NO modulators for 14 days, and CSC-like phenotypes were examined. Figure 5A shows that the SNAP-treated H292 cells displayed a dose-dependent increase in anoikis resistance compared with the nontreated control. In contrast, treatment of the cells with NO scavenger had a reversal effect (Fig. 5A), supporting the role of NO in the resistance process.
Fig. 5. Effect of NO donor S-nitroso-N-acetylpenicillamine (SNAP) and NO scavenger 2-(4-carboxy-phenyl)-4,4,5,5 tetramethylimidazoline-1-oxy-3-oxide (PTIO) on CSC-like phenotypes. H292 (A) and H460 (B) cells were either left untreated as control (Ctrl) or treated with SNAP (10–50 μM) or PTIO (20–50 μM) for 14 days and analyzed for anoikis by measurement of the viability of detached cells by MTT assay. The treated and control H292 (C) and H460 (D) cells were suspended and subjected to colony formation assay. The H292 (E) and H460 (F) were suspended and subjected to colony formation assay. The H292 (E) and H460 (F) cells were collected, and CSC markers, CD133 and ALDH1A1, were analyzed by Western blotting. The blots were reprobed with β-actin to confirm equal loading. The bands were normalized to the controls.

Cav-1 regulates aggressive behaviors of CSC-like cells but not CSC markers. Unveiling the key players underlying NO-mediated aggressive behaviors is of importance because of its potential applications in cancer therapy. Cav-1 is known to be involved in various cancer cell behaviors including migration, invasion, and anoikis resistance (11, 27, 28, 30, 42, 45, 59) and is subjected to NO regulation (7, 28). To explore the possible role of Cav-1 in NO-mediated CSC-like behaviors, Cav-1 expression was genetically modulated, and its effect on cellular behaviors in NO-treated cells was examined. Figure 6, A and B, shows that the Cav-1 level was strongly upregulated in the NO-treated cells in a dose- and time-dependent manner, the effect that was found in both treated H292 and H460 cells. To test whether Cav-1 is essential to the effect of NO on cellular behaviors, knockdown by Cav-1 expression or enhancement by stable gene transfection and their effects on NO-induced cellular behaviors in NO-treated cells was examined. Figure 6, C and D, shows the effects of shRNA knockdown and gene overexpression on Cav-1 protein expression. The genetically modified cells were

7 H292 and H460 spheroid formation, respectively. Similar to DPTA NONOate-treated cells, SNAP-treated cells formed dense circular spheroids, whereas control cells and PTIO-treated cells clump together to form irregular-irregular shaped spheroids surviving through E-cadherin-mediated survival. Together, these results indicate the promoting role of NO in CSC-like properties of human lung cancer cells.
CD133 and ALDH1A1 were assessed by Western blotting. Our results showed that the level of these CSC markers did not correlate with the level of Cav-1 (Fig. 6, K and L), suggesting a non-Cav-1-dependent mechanism of CSC marker upregulation in the NO-treated cells.

**DISCUSSION**

The concept of CSCs as a seed of malignant cells has garnered increasing attention and has been a subject of active research in recent years (14, 22, 54). Although detailed knowledge of this cell population remains obscure, the significant impact of this cell population in various forms of cancer has...
inhibits ubiquitin-proteasomal degradation of the protein (7).

NO is a key molecule produced from the crosstalk between cells and inflammation within the tumor microenvironment (13, 17, 20). Several studies have reported increased expression and activity of different forms of NOS in cancers (1, 64, 66). Interestingly, both pro- and antitumorigenic roles of NO have been described; however, it is generally accepted that the effect of NO on tumor progression is concentration dependent (21, 32, 53, 65). Previously, we have reported that NO promotes cell death resistance to Fas ligand (9) and cisplatin (8) in human lung cancer cells. In relevance to metastasis, we also found that NO mediates anoikis resistance of lung cancer cells (7). Together, these findings provide evidence supporting the role of NO in tumorigenesis and metastasis, consistent with the clinical observations showing a correlation between NO level and a high degree of tumor metastasis (45, 60). Several studies have demonstrated that CSCs possess an enhanced ability to migrate, invade, form tumors, and resist anoikis (16, 23, 40, 46, 56, 58, 63). However, the linkage between NO and aggressive cancer phenotypes in the context of CSCs has not been investigated. The present study demonstrated for the first time that NO regulates the stemness and aggressive behaviors of lung cancer cells. The NO donors DPTA NONOate and SNAP upregulate the expression of CSC markers, CD133 and ALDH1A1, and increase aggressive cellular behaviors, whereas the NO scavenger PTIO suppresses the markers and decreases aggressive behaviors (Fig. 5, E–G). These results suggest that NO may mediate its effects by regulating the stemness of cancer cells.

Cav-1 is a scaffold protein and essential constituent of caveolae, a flask-shaped invagination that occupies up to 20% of the cell membrane (41). Several lines of evidence have pointed out that Cav-1 may contribute to the aggressiveness and chemoresistance of human cancer cells, including lung carcinoma, ovarian carcinoma, colon adenocarcinoma, and breast adenocarcinoma cells (31, 37, 38). Cav-1 expression has been linked to increased cell motility and anoikis resistance (11, 12, 27, 28, 30, 42, 45, 59), two important features of metastatic cancer cells. In this study, we provide new evidence that NO increases the motility and resistance to anoikis of human lung cancer cells through a Cav-1-dependent mechanism. Cav-1 is upregulated in the NO-treated cells in a dose- and time-dependent manner (Fig. 6, A and B). Such upregulation is positively associated with the ability of the cells to migrate, invade, form colonies, and resist anoikis. Gene knockdown and overexpression studies confirm the positive regulatory role of Cav-1 in NO-mediated aggressive behaviors of lung cancer cells. This finding is, however, contradictory to the generally regarded role of Cav-1 as a tumor-suppressor protein (44). It is likely that Cav-1 may have multiple functions and may exert both positive and negative roles on cancer cell behaviors depending on the cancer stage, i.e., metastatic or nonmetastatic, tissue of origin, and tumor microenvironment, i.e., presence of nitrosative and oxidative stress. In the environment with a high NO level, Cav-1 is upregulated and prometastatic. Previous studies have also shown that NO stabilizes Cav-1 through a process of S-nitrosylation, which inhibits ubiquitin-proteasomal degradation of the protein (7).

Furthermore, Cav-1 can interact with Mcl-1 and improve its stability, leading to anoikis resistance of lung cancer cells (12).

The effect of NO on aggressive CSC-like behaviors was found to be reversible, which may explain the discrepancy of the NO effect on tumorigenesis. Discontinuation of NO exposure after a 2-wk treatment resulted in a reversal of the CSC-like effects of NO on cell anoikis, colony formation, cell invasion, and migration. It is worth noting that the level of NO in the tumor microenvironment varies depending on the expression of NOS and the activity of local and infiltrating immune cells. Therefore, the effect of NO on CSC-like behaviors may vary depending on the availability of NO and pathological conditions.

In summary, our data provide evidence that NO plays an important role in the regulation of CSC-like phenotypes of human lung cancer cells. NO induces an upregulation of CSC markers, CD133 and ALDH1A1, along with the increase in anoikis resistance, migration, invasion, and colony-formation activities. Such induction of the aggressive CSC-like behaviors is dependent on Cav-1 expression; however, the expression of CSC markers is independent or inversely dependent on the Cav-1 expression. Because increased NO production has been associated with several human cancers, NO may be one of the key regulators of CSCs and metastasis. This novel finding on the role of NO and Cav-1 in CSC regulation may have important implications in cancer chemotherapy and prevention.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.Y. performed experiments; N.Y. and P.C. analyzed data; N.Y., V.P., and P.C. interpreted results of experiments; N.Y. and V.P. prepared figures; N.Y. and P.C. drafted manuscript; N.Y., V.P., A.M., Y.R., and P.C. edited and revised manuscript; N.Y., V.P., A.M., Y.R., and P.C. approved final version of manuscript; P.C. conception and design of research.

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