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Cancer Stem Cell Phenotype Is Supported by Secretory Phospholipase A₂ in Human Lung Cancer Cells

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Abstract

Background—Lung cancer stem cells (CSCs) are a sub-population of cells that drive growth, invasiveness, and resistance to therapy. Inflammatory eicosanoids are critical to maintain this malignant subpopulation. Secretory phospholipase A₂ group IIa (sPLA₂) is an important mediator of the growth and invasive potential of human lung cancer cells and regulates eicosanoid production. We hypothesized that sPLA₂ plays a role in the maintenance of lung CSCs.

Methods—Cancer stem cells from lung adenocarcinoma cell lines H125 and A549 were isolated using aldehyde dehydrogenase activity and flow cytometry. Protein and mRNA levels for sPLA₂ were compared between sorted cells using Western blotting and quantitative reverse transcriptase–polymerase chain reaction techniques. Chemical inhibition of sPLA₂ and short-hairpin RNA knockdown of sPLA₂ were used to evaluate effects on tumorsphere formation.

Results—Lung CSCs were isolated in $8.9\% \pm 4.1\%$ (mean $\pm$ SD) and $4.1\% \pm 1.6\%$ of H125 and A549 cells respectively. Both sPLA₂ protein and mRNA expression were significantly elevated in the CSC subpopulation of H125 ($p = 0.002$) and A549 ($p = 0.005$; $n = 4$). Knockdown of sPLA₂ significantly reduced tumorsphere formation in H125 ($p = 0.026$) and A549 ($p = 0.001$; $n = 3$). Chemical inhibition of sPLA₂ resulted in dose-dependent reduction in tumorsphere formation in H125 ($p = 0.003$) and A549 ($p = 0.076$; $n = 3$).

Conclusions—Lung CSCs express higher levels of sPLA₂ than the non-stem cell population. Our findings that viral knockdown and chemical inhibition of sPLA₂ reduce tumorsphere formation in lung cancer cells demonstrate for the first time that sPLA₂ plays an important role in CSCs. These findings suggest that sPLA₂ may be an important therapeutic target for human lung cancer.

Lung cancer remains the leading cause of cancer-related death worldwide with an overall 5-year survival of only 16% [1]. This is a result of the advanced local and metastatic disease often present at the time of diagnosis. Clearly, there is a vital need for improved treatment

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options, and although some targeted therapies such as tyrosine kinase inhibitors have shown promising initial results, recurrence rates after treatment remain unacceptably high [2]. Continued work to identify other directed therapies is urgently needed.

Secretory phospholipase A₂ group IIa (sPLA₂) is a secreted enzyme responsible for the release of arachidonic acid in the inflammatory pathway of prostaglandin production [3]. It has been implicated as a factor in the progression of prostate, esophageal, colon, and lung cancer [4–10]. Furthermore, we have shown that this enzyme contributes to tumor growth in vivo and invasion in vitro, specifically in human lung cancer cells [8, 9].

Accruing evidence suggests that mechanisms underlying tumor recurrence and metastases are modulated by means of a self-renewing subpopulation of the overall tumor called cancer stem cells (CSCs). The CSC hypothesis states that this subpopulation of tumor cells is responsible for tumor initiation, growth, and metastases [11]. Additionally, recent findings suggest that these cells are an integral part of the ability of tumors to develop resistance to radiation and chemotherapy, leading to treatment failures caused by metastases [12, 13].

Cancer stem cells have been identified in many solid-organ tumors including breast, head and neck, colon, and lung cancer by an assay measuring aldehyde dehydrogenase (ALDH) activity [14–18]. Aldehyde dehydrogenase serves as an intracellular oxidizer responsible for production of retinoic acid, which regulates many cellular processes including cell differentiation, proliferation, and apoptosis [19]. A validated in vitro assay has been developed to study CSCs [20]. Culturing cancer cells in nonadherent conditions at a low cell density yields tumorspheres that represent CSCs. Maintaining tumorspheres in culture provides a functional assay to reproducibly evaluate CSC activity and allows for investigation into mechanisms that drive the CSC phenotype.

Prostaglandins, a downstream product of sPLA₂, have been implicated in the maintenance of CSCs [21]. Specifically, prostaglandin E₂ has been demonstrated to be an important driver in proliferation of the CSC subpopulation [22]. Given the role of sPLA₂ in prostaglandin production and our previous findings of the importance of sPLA₂ in tumor growth and invasion in lung cancer, we hypothesized that sPLA₂ would play a significant role in maintaining the CSC phenotype and influence the function of CSC in non-small cell lung cancer.

In this study we demonstrate that CSCs, identified by high levels of ALDH activity, contain an increased level of sPLA₂ protein and mRNA expression. Furthermore, short-hairpin RNA (shRNA) knockdown and chemical inhibition effectively decreased the CSC phenotype in non-small cell lung cancer. This is the first report implicating the importance of sPLA₂ in the maintenance and function of the CSC phenotype.

Material and Methods

Cell Culture and Materials

Human non-small cell lung cancer cell lines H125 and A549 were used for all experiments. H125 cells were obtained from the University of Colorado Cancer Center Tissue Culture

Core (Aurora, CO), and A549 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI (Life Sciences, Grand Island, NY) or Ham's F12 (Corning, Manassas, VA), respectively, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. An sPLA₂ inhibitor (S3319) was purchased from Sigma-Aldrich (St. Louis, MO). The sPLA₂ antibody was from Abcam (Cambridge, MA), and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Cell Signaling (Beverly, MA).

For tumorsphere culture, DMEM/F-12 (Corning) medium was supplemented with 2% B27 (Life Sciences), epidermal growth factor 20 ng/mL, and fibroblast growth factor 20 ng/mL (BD Biosciences, Franklin Lakes, NJ). Ultra-low adhesion 6-well plates (Corning) were used for plating cells in tumorsphere assays. The tumorsphere protocol was derived from a previously established method for culturing tumorspheres [20].

Aldefluor Assay

The Aldefluor assay (Stemcell Technologies, Vancouver, Canada) was used to identify CSCs by following the manufacturer's recommended protocol. Briefly, H125 and A549 cells were released from culture flasks, centrifuged at 4°C for 5 minutes at 270g and resuspended at 10⁶ cells per vial in 1 mL of Aldefluor assay buffer. Five microliters of ALDH substrate (BODIPY [boron-dipyrromethene] aminoacetaldehyde) was added to each 1-mL vial. Half of the sample was immediately transferred to a new vial, and 5 µL of diethylaminobenzaldehyde, a potent inhibitor of ALDH, was added, which serves as the internal control. The samples were then incubated at 37°C for 30 minutes followed by centrifugation at 4°C for 5 minutes at 270g. The supernatant was removed, and the pellet was resuspended in 500 µL of cold assay buffer. The samples were kept on ice until evaluation by flow cytometry [15].

The assay uses an uncharged ALDH substrate, which readily crosses the cell membrane. Intracellular ALDH then converts the substrate to a charged state, which is unable to exit the cell. Cells exhibiting higher levels of ALDH activity (ALDH-high) are brighter and represent the CSC subpopulation. The diethylaminobenzaldehyde-treated sample serves as the internal control (ALDH-low). Fluorescent activity was evaluated on a MoFlo XDP 100 (Beckman Coulter, Brea, CA) flow cytometer, and ALDH-high and ALDH-low cell populations were recovered with flow-assisted cell sorting (FACS) for further analysis.

Western Blotting

The ALDH-high and ALDH-low cells recovered from FACS were centrifuged for 5 minutes at 270g, washed once with phosphate-buffered saline solution (PBS), and lysed in Laemmli buffer (Bio-Rad, Hercules, CA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitro-cellulose membranes. Membranes were blocked in 5% nonfat milk dissolved in 1× Tris-buffered saline (TBS), 0.1% Tween-20 (Sigma-Aldrich). Primary antibodies were dissolved in 4% bovine serum albumin (Sigma-Aldrich) in 1× TBS, 0.1% Tween-20. Secondary antibodies were prepared in 5% nonfat milk in 1× TBS, 0.1% Tween-20. Membranes were developed using Pierce ECL Chemiluminescent (Thermo Fisher Scientific, Inc, Rockford, IL). Protein

quantification was performed by densitometric analysis using ImageJ Software (National Institutes of Health, Bethesda, MD). Expression is presented as sPLA₂ relative to GAPDH.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

The ALDH-high and ALDH-low populations recovered from FACS were centrifuged for 5 minutes at 270g and washed once with PBS. Total RNA was isolated using Qiagen RNeasy Mini Kit (Germantown, MD) according to the manufacturer's protocol. Bio-Rad iScript was used to synthesize complementary DNA. Primers for quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) were purchased from Integrated DNA Technologies (Coralville, IA). The primer sequences for sPLA₂ were 5'CCCTCCTACTGTTGGGAGTGA3' (forward) and 5'CCTGTCGTCAACTTGATTCTG3' (reverse). The sequences for GAPDH were 5'AGATCATCAGCAA TGCCTCCT3' (forward) and 5'GGAGTCTGGTCATGAGTCCTT3' (reverse). The RT-PCR was completed with Bio-Rad Sybr Green Master Mix using a Bio-Rad iQ5 thermocycler with the following steps: denaturation (95°C for 15 seconds), annealing (60°C for 30 seconds), and elongation (72°C for 30 seconds). These steps were repeated for 40 cycles. Melt curve analysis was performed at the end of the reaction to ensure specificity of the primer sequences. Data were analyzed by the delta-delta cycle threshold method.

Short-Hairpin RNA Vectors

Puromycin-resistant lentiviral vectors (pLKO.1-puro) containing two distinct sequences targeting sPLA₂ and one nontargeted shRNA sequence were purchased from Sigma-Aldrich. The sequences were as follows: 5'GGCCCATGGGAATTTGGTGAATT and 5'GGTGCTABAAACAAGACGACCTA. The preparation of infectious lentiviral particles were prepared as previously described [7]. Unsorted cells were used for shRNA knockdown experiments.

Tumorsphere Assay

Tumorsphere assay was used to evaluate CSC phenotype in FACS-sorted cells as well as the functional effects of chemical inhibition and shRNA knockdown of sPLA₂. Cells recovered from FACS that were ALDH-high compared with ALDH-low were washed once with PBS, then plated at a density of 10³ cells in 3 mL of tumor-sphere medium per well in ultra-low adhesion plates. After 7 days of culture, cells were assessed for tumor-sphere formation by visually counting spheres on Nikon Eclipse TS-100 (Melville, NY). Tumorspheres greater than 100 mm were considered positive and counted by a blinded observer. Each experiment was completed in duplicate with n = 3 for each cell line.

For shRNA knockdown and sPLA₂ inhibitor experiments, unsorted cells were released from flask by trypsin 0.25% (Life Sciences), washed twice with PBS, and plated at 10³ cells per mL with 3 mL per well in nonadherent conditions. After 7 days of culture, the cells were passaged by centrifugation at 100g for 3 minutes, washed twice with PBS, and disassociated by trypsin 0.05% (Life Sciences) for 10 minutes into a single-cell suspension. Cells were then plated at 10³ per well with 3 mL of medium per well and allowed to grow for 7 days before assessment of tumorsphere formation. Each experiment was completed in duplicate with n = 3 for each experiment.

Statistical Analysis

An unpaired Student's *t* test was used for two-group comparisons. Analysis of variance was used for comparison of more than two groups. No multiple comparisons were performed. StatView V5.0 (1998; SAS institute Inc, Cary, NC) was used for all statistical analysis. A probability value of less than 0.05 was considered statistically significant.

Results

Subpopulation of Cancer Stem Cells Are Identified by Aldefluor Assay

H125 and A549 cell lines were respectively found to have $8.9\% \pm 4.1\%$ and $4.1\% \pm 1.6\%$ of cells positive for ALDH-high subpopulation (Fig 1), representing the CSC subpopulation by flow cytometry analysis. The CSC phenotype was confirmed in the ALDH-high population by tumorsphere assay in which this population demonstrated a twofold ability to form tumorspheres relative to the ALDH-low cells in both H125 ($p = 0.027$; Fig 2A) and A549 ($p = 0.003$; Fig 2B) cell lines. These data demonstrate that CSCs are identifiable in H125 and A549 cell lines by the Aldefluor assay.

The Cancer Stem Cell Subpopulation Demonstrates Increased Secretory Phospholipase A₂ mRNA and Protein Expression

Cells from both the ALDH-high and ALDH-low sub-populations were evaluated for mRNA and protein levels of sPLA₂ by Western blotting and RT-PCR, respectively. Levels of sPLA₂ mRNA expression were significantly elevated in the CSC subpopulation (ALDH-high) of both H125 ($p = 0.002$) and A549 ($p = 0.037$) cell lines (Fig 3A). These data were further confirmed by Western blotting, which also showed significantly elevated sPLA₂ protein levels in H125 ($p = 0.016$) and A549 ($p = 0.005$) CSC subpopulations (Fig 3B). The CSC subpopulation of the H125 cell line also demonstrated a slightly larger elevation in level of sPLA₂ expression at both the protein and mRNA level relative to A549 cells. After identifying elevated levels of sPLA₂ expression in the CSC subpopulation, we proceeded to determine the effect that inhibition of this enzyme would have on the CSC phenotype as evaluated by tumorsphere assay.

Short-Hairpin RNA Knockdown of Secretory Phospholipase A₂-IIa Reduces Tumorsphere Formation in Human Lung Adenocarcinoma Cells

After transfection with pLKO.1-puro, cells were selected by puromycin treatment, which killed nontransfected cells at a concentration of $2 \mu\text{g/mL}$. Superior knockdown of sPLA₂ was established with sequence S1, which was confirmed by Western blotting (Fig 4) for both H125 ($p = 0.002$) and A549 ($p = 0.006$). Using this knockdown cell line and the nontemplate control cell line, the tumorsphere assay was used to evaluate the effects of sPLA₂ knockdown on the CSC phenotype. Figure 5 demonstrates the significantly reduced formation of tumorspheres in sPLA₂ knockdown cells relative to the nontemplate control cells in both H125 ($p = 0.026$) and A549 ($p = 0.001$) cells. The data from this functional in vitro assay demonstrate the importance of sPLA₂ in maintaining the CSC phenotype.

Chemical Inhibition of Secretory Phospholipase A₂ Decreases Tumorsphere Formation in Lung Adenocarcinoma Cells

Unsorted cells growing in standard medium and conditions were released from flasks and plated in nonadherent, tumorsphere conditions with increasing concentrations of sPLA₂ inhibitor using dimethyl sulf-oxide as the vehicle control. The cells were passaged as described and replated with the same concentrations of inhibitor. Treatment groups were performed in duplicate with $n = 3$ for each cell line. A blinded observer then evaluated the number of tumorspheres formed after 7 to 8 days of incubation. The H125 cell line demonstrated a significant, dose-dependent reduction in tumorsphere formation ($p = 0.003$), further confirming the important role of sPLA₂ in the CSC phenotype. The A549 cell line also showed a dose-dependent response, which approached significance ($p = 0.076$; Fig 6).

Comment

Lung CSCs have been shown to be the cells responsible for tumor initiation with subsequent formation of solid tumors, whereas the remaining cells within a tumor lack this function [23]. These cells also exhibit greater invasive capacity than the remaining cells in a tumor population [17]. Cancer stem cells are less susceptible to chemotherapeutic agents, and their mechanisms of resistance are of great research interest [17, 24]. The mechanisms that have been proposed to explain this phenomenon include increased levels of ABC transporters that efflux chemotherapy agents [25], DNA repair mechanisms that are more robust than other cells within a tumor [12], and activity of ALDH within these cells that neutralizes these therapeutic interventions [19]. Recently Li and colleagues [21] demonstrated the importance of the inflammatory cascade, specifically prostaglandin E₂, as a factor strongly relating to the survival ability of these lung CSCs. They showed that tumor-initiating capacity is lost with inhibition of prostaglandin E₂ signaling and completely restored with the addition of prostaglandin E₂ to culture [21]. These findings raised the possibility that factors external to the cells may influence the growth and survival of these lung CSCs.

The results of the present study demonstrate the involvement of sPLA₂ in the CSC phenotype and function of human non-small cell lung cancer cells. Expression of sPLA₂ was significantly elevated in the CSC subpopulation of each cell line at both the protein and mRNA levels. Furthermore, chemical inhibition and shRNA knockdown successfully decreased the CSC population as demonstrated by the reduction in tumorsphere formation, a well-established in vitro surrogate for CSCs [20].

The results of this study support the involvement of sPLA₂ in the maintenance of the CSC phenotype. It also appears that there are other factors responsible for influencing CSCs as we demonstrated that tumorspheres were not completely suppressed with either chemical inhibition or shRNA knockdown of sPLA₂. Some possible explanations of this are that shRNA knockdown was not complete in either of the lung adenocarcinoma cell lines used in this study. Had the knockdown been complete, we may have seen a more robust decrease in tumor-sphere formation. We also found that chemical inhibition of sPLA₂ had a much greater effect on tumorsphere formation in the H125 cell line. As all tumors have different drivers and varying levels of malignancy, this cell line may be more susceptible to loss of sPLA₂. Potentially the A549 cell line has developed a mechanism that leaves it less

dependent on sPLA₂. Also, we have seen that H125 cells express a higher baseline level of sPLA₂ than A549 cells (data not shown), which correlates with the higher percentage of CSCs in this population and may explain the more robust response to inhibition in this cell line. Finally, an in vivo model would have further supported our findings; however, we have previously demonstrated the effectiveness of sPLA₂ shRNA knockdown in reducing tumor growth in nude mice [9].

Alternatively, the incomplete suppression of tumorsphere formation by sPLA₂ inhibition and knockdown could be attributable to other pathways driving the CSC phenotype. Others have shown the Notch pathway contributing to CSC progression by preventing differentiation when constitutively active [26]. Dysregulation of the Wnt/beta-catenin pathway allows for indefinite propagation of progenitor cells, which act as CSCs [27]. Also, upregulation of Hedgehog signaling increases clonogenic growth potential and limits differentiation [28]. Although our data show that the inflammatory sPLA₂ pathway contributes to the CSC phenotype, there are other mechanisms that have also been shown to play a role in these processes.

Our findings support data that others have shown regarding the importance of inflammatory pathways in driving the CSC phenotype. Again, Li and colleagues [21] showed the importance of prostaglandin E₂ in driving the CSC phenotype. Moon and colleagues [29] recently showed that nonsteroidal antiinflammatory agents were capable of reducing CSCs in colorectal cancer cells. Secretory phospholipase A₂ is a key enzyme in this inflammatory pathway that produces prostaglandin E₂ and is inhibited by nonsteroidal antiinflammatory pharmaceuticals.

Secretory phospholipase A₂ is a secreted enzyme that provides a key step in the production of prostaglandins by releasing arachidonic acid from the cell membrane [3]. It has been implicated in the progression of many types of solid-organ tumors [4–10]. The results of this study further demonstrate the importance of sPLA₂ in cancer progression through its role in maintaining CSCs in lung adenocarcinoma cell lines. These CSCs are resistant to established therapies and are responsible for tumor initiation, progression, and metastasis. Secretory phospholipase A₂ has the potential to target these highly malignant CSCs. Here we show that sPLA₂ inhibition reduced the CSC phenotype in vitro. Also, the effect was greater in the H125 cell line, which has a higher baseline level of sPLA₂. Tumors that express greater levels of sPLA₂ may have an improved clinical benefit to sPLA₂ inhibition in clinical practice. Chemotherapeutics targeting sPLA₂, as demonstrated by reduction in invasion, tumor growth, and now CSC formation, have the potential to treat this highly malignant subpopulation of tumor cells.

In summary, the results of the present study demonstrate that sPLA₂ is elevated in the CSC subpopulation of lung adenocarcinoma cell lines. Furthermore, both shRNA knockdown and chemical inhibition of sPLA₂ limit the CSC phenotype in in vitro tumorsphere assays. Given the association of CSCs with growth regulation of tumors, their involvement in resistance to known tumor therapies, and the relationship between eicosanoids and CSCs, sPLA₂ remains a significant potential therapeutic target for human lung adenocarcinoma.

DISCUSSION

DR WAYNE L. HOFSTETTER (Houston, TX): Congratulations on a nice presentation and to your group for a nice set of experiments. My first question relates to the strenuous definition of whether a decrease in tumor sphere formation is actually due to changes in the CSCs (cancer stem cells). What validation did you perform to show that the decrease in tumorsphere formation is actually a decrease in the CSC population? Perhaps there are other quantitative methods used for validation.

DR BENNETT: Unfortunately, there are limited available in vitro assays to assess cancer stem cells. The tumorsphere assay is one of the most widely used and validated to assess in vitro effects on the cancer stem cell phenotype. Each tumorsphere is formed from a single cancer stem cell. Each tumorsphere contains the same composition of cells with the observable difference being a reduction in the number of tumorspheres formed and thus a reduction in cancer stem cells.

DR HOFSTETTER: Would it be possible to do a protein analysis to look for ALDH (aldehyde dehydrogenase) in that group of tumorspheres after you have isolated the tumorspheres?

DR BENNETT: Yes, it would be possible to evaluate ALDH protein levels in the tumorspheres; however, all the tumorspheres contain the same composition. It is unlikely that there would be an observable difference between tumorsphere groups.

DR HOFSTETTER: Were the knockdown experiments performed on an isolated group or the sorted group or overall? It is unclear in the paper.

DR BENNETT: The knockdown experiments were performed on the overall population.

DR HOFSTETTER: Meaning that they were unsorted?

DR BENNETT: Yes, they were unsorted and grown in standard medium conditions.

DR HOFSTETTER: So, again, along those lines, further data to support the assertion that those stem cells such as the ALDH expression would probably be important and of interest to the population as a whole.

My second question is directed to the potential of these findings and steps towards translation. When you looked at the in vivo tumor suppression in your previous studies, was there any confirmation that this was due to reduction in the ALDH-high cell population? You did some previous in vivo studies.

DR BENNETT: Yes, sir.

DR HOFSTETTER: And you showed tumor suppression in your in vivo group. Was there any evidence to show that that reduction in tumor size was due to the decrease in stem cell population?

DR BENNETT: At that time we had not evaluated cancer stem cells, although we are currently developing in vivo models to evaluate that.

DR HOFSTETTER: Exactly. So the follow-up to that would be, would in vivo tumor formation be suppressed by knockdown of PLA₂? So if you were going to do the virus knockdown as an in vivo experiment, would you then see a decrease in the CSCs?

DR BENNETT: Ideally you would see a reduction in the ability of the cells to form in vivo tumors. Specifically, at some dilution the sPLA₂ knockdown cells would most likely not be able to form tumors in an in vivo model whereas the nontransfected cells would be able to form tumors at the same dilution.

DR HOFSTETTER: And then finally, of interest, there was relative resistance to the A549 cell line, one of the two cells lines that you were using, and do you feel that there is perhaps a secondary pathway at work?

DR BENNETT: We found that very interesting. Of note, the baseline level of sPLA₂ expression is much higher in the H125 cells compared to the A549 cells. This partly explains why the A549 cells are less responsive to chemical inhibition and viral knockdown of sPLA₂. These data also support sPLA₂ as a potential therapeutic target in tumors with high levels of sPLA₂ expression. With regards to alternative pathways, sPLA₂ is an upstream regulator of the inflammatory eicosanoid pathway, but it is possible that there are alternative pathways given that drivers of malignancy vary from tumor to tumor.

DR HOFSTETTER: Excellent job. Thank you very much.

Abbreviations and Acronyms

ALDH	aldehyde dehydrogenase
ALDH-high	aldehyde dehydrogenase high cells
ALDH-low	aldehyde dehydrogenase low cells
CSC	cancer stem cell
FACS	flow-assisted cell sorting
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
RT-PCR	reverse transcriptase–polymerase chain reaction
shRNA	short-hairpin RNA
sPLA₂	secretory phospholipase A ₂ –group IIa

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Fig 1. Flow cytometry analysis of aldehyde dehydrogenase (ALDH) activity shows H125 and A549 cells to be $8.9\% \pm 4.07\%$ and $4.1\% \pm 1.6\%$, respectively, positive for cancer stem cells (CSCs) relative to the internal control, DEAB (diethylaminobenzaldehyde). (FL1 = fluorescein.)

Fig 2.

Cancer stem cell phenotype is confirmed by tumorsphere assay with aldehyde dehydrogenase (ALDH)-high populations containing a twofold ability to form tumorspheres relative to the ALDH-low populations in both (A) the H125 ($p = 0.027$) and (B) A549 ($p = 0.003$) cell lines. * $p < 0.05$.

Fig 3.

(A) mRNA expression of secretory phospholipase A₂ (sPLA₂) is significantly elevated in the cancer stem cell (CSC) subpopulation of both H125 ($p = 0.002$) and A549 ($p = 0.037$) cell lines ($n = 4$). (B) Significantly elevated sPLA₂ protein expression is also demonstrated in both H125 ($p = 0.016$) and A549 ($p = 0.005$) cell lines, with representative Western blots below ($n = 3$). * $p < 0.05$. (ALDH = aldehyde dehydrogenase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.)

Fig 4.

Successful short-hairpin RNA knockdown of secretory phospholipase A₂ (sPLA₂) demonstrated by Western blot with sequence S1 having superior effect over S2 in both (A) H125 ($p = 0.002$) and (B) A549 ($p = 0.006$; $n = 3$). * $p < 0.05$. (GAPDH = glyceraldehyde 3-phosphate dehydrogenase; NT = nontemplate; S1 = sequence 1 knockdown; S2 = sequence 2 knockdown; WT = non-transfected.)

Fig 5.

Evaluation of the effect of secretory phospholipase A₂ knockdown on tumorsphere formation in both (A) H125 ($p = 0.026$) and (B) A549 ($p = 0.001$) cell lines show reduced ability to form tumorspheres relative to nontemplate (NT) control ($n = 3$). (S1 = sequence 1 knockdown.)

Fig 6.

(A) H125 cells demonstrate a significant dose-dependent reduction in tumorsphere formation ($p = 0.003$) in the presence of secretory phospholipase A₂ (sPLA₂) inhibitor. (B) A549 cells also showed a dose-dependent response, which approached significance ($p = 0.076$; $n = 3$). (VC = vehicle control.)