Supplementary Data

Materials and Methods

Animal handling

The present study was performed in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (1); it was approved by the Institutional Animal Care and Use Committee of our university (South China Normal University, Guangzhou, China). Six- to eight-week-old BALB/c mice or BALB/c nude mice were obtained from the Laboratory Animal Center (Sun Yat-sen University, Guangzhou, China). Mice were housed four per cage in standard plastic cages, provided food and water ad *libitum*, and maintained on a 12-h light/dark cycle.

In vitro *and* in vivo *reactive oxygen species detection with chemiluminescence*

Reactive oxygen species (ROS) generated by high-fluence, low-power laser irradiation (HF-LPLI) was detected with a chemiluminescence (CL) method as described by Wei *et al.* (2–4). Fluoresceinyl cypridina luciferin analog (FCLA; Tokyo Kasei Kogyo Co., Tokyo, Japan) is a useful ROS CL probe and can selectively detect superoxide anion ($O_2^{-\bullet}$) and singlet oxygen ($^1O_2$) before they change to other species of ROS. FCLA was dissolved in double-distilled water (100 μM) and stored at -80°C. On reaction with either $^1O_2$ or $O_2^{-\bullet}$, the probe produces a 532-nm CL and is at its maximum detection efficiency in biological pH range. For *in vitro* CL measurement, the suspension of samples were stirred in phosphate-buffered saline (PBS) medium at 4°C, transferred to a cell culture dish, and FCLA was subsequently added. After being incubated with FCLA (10 μM) for 30 min in the dark, the samples were transferred into a CL detector instrument and irradiated by the 635-nm CW laser (NL-FBA-2.0-635; nLight Photonics Corporation, Vancouver, WA). The time sequence of the signal acquisition was controlled with the computer program: The laser was interrupted every 30 s for 10 s to collect CL. Emitted photon was counted per 50 ms during irradiation. The irradiation duration was set as 10 min for a fluence of 120 J/cm^2 throughout this study. For the *in vivo* CL measurement, a mouse was restrained inside a custom holder under anesthesia with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). FCLA (10 μM) in 200 μl physiologic saline (0.9%) was injected subcutaneously into the HF-LPLI-CL measurement site of the tumor 1 h before light irradiation to allow adequate absorption. For the HF-LPLI treatment, the tumor was irradiated with the 635-nm CW laser at 500 mW/cm^2 for a fluence of 1200 J/cm^2. The light was delivered in a 30 s/10 s light/dark cycle so that the CL signals could be collected during the dark periods.

Delivery of superoxidase dismutase into cells

Cells were grown in a 6-cm petri dish and 24 h later, subjected to protein delivery assay using PULSin^{TM} (Polyplus Transfection, Illkirch, France). Manganese superoxidase dismutase (MnSOD, 50 U/ml; Sigma-Aldrich, St. Louis, MO) can be delivered into cells and maintained in the cytosol according to the manufacturer's direction of the protein delivery reagent. The delivery efficiency of SOD gives ~70% of total cells detected by flow cytometry analysis. Cells were washed thrice with PBS before addition of the PULSin/protein mixture (4 ml PULSin^{TM} per 1 mg SOD). After 4 h of incubation at 37°C and 5% CO_2, the cells were washed twice with PBS and cultured in fresh medium again.

Construction of respiration-deficient cell line (ρ^0)

The ρ^0 cells were prepared as described by Zhao *et al.* (5). The ρ^0 cells were generated by incubating wild-type cells in ethidium bromide for ~4 weeks in medium supplemented with pyruvate and uridine. The ρ^0 cells were then selected by exposure to the mitochondrial inhibitors rotenone (1 μg/ml; Sigma-Aldrich) and antimycin A (1 μg/ml; Sigma-Aldrich), which were lethal to wild-type cells. The cells were considered ρ^0 cells when there was no cytochrome b when detected by a polymerase chain reaction (Supplementary Fig. S3).

Laser scanning confocal microscopy

The fluorescence emissions from DsRed, tetramethylrhodamine methyl ester (TMRM), 2′,7′-dichlorofluorescein (DCF), MitoTraker Deeper Red 633 (MTR), dihydroethidium (DHE), and MitoSOX^{TM} were observed confocally using a commercial laser scanning microscope (LSM510META) combination system (Zeiss, Jena, Germany) that was equipped with a Plan-Neofluar 40×/1.3 NA oil DIC objective. The excitation wavelengths and detection filter settings for each of the fluorescent indicators were as follows: DsRed (EX: 543 nm; EM: LP560 nm); TMRM (EX: 543 nm; EM: LP600 nm); DCF (EX: 488 nm; EM: BP500–530 nm); MTR (EX: 633 nm; EM: LP650 nm); DHE; and MitoSOX^{TM} (EX: 514 nm; EM: BP565–615 nm). To quantify the results, the average emission intensities of the desired measurement position were processed with Zeiss Rel 3.2 image processing software (Zeiss).

Cytochrome c oxidase RNA interfere

Two different small interfering RNAs (siRNAs) targeting human cytochrome c oxidase (COX) II, and III and negative mismatched siRNA were purchased from RiboBio (Guangzhou, China). The siRNA sequence for COX II were as follows: (i) 5′-CGUUGACA AUCGAGUAGUAdTdT-3′; 3′-dTdTGCAACU GUUAGCUCAUCAU-5′, (ii) 5′-CCAUCGU CCUAGAAUUA AUdTdT-3′; 3′-dTdTGGUAGCAGGAUCUUAAUUA-5′, (iii) 5′-CUACGC AUCCUUUACAUAAdTdT-3′; 3′-dTdTGAUGCG UAGGAAAUGUAUU-5′. The siRNA sequences for COX III were as follows: (i) 5′-CCUAAACACAUCCGUAUUAdT dT-3′; 3′-dT dTGGAUUUGUGUAGGCAUAAU-5′, (ii) 5′-CCAACA CACUAACCAUAUAdT dT-3′; 3′-dT dTGGUUGUGUGAU UGGUAUAU-5′, (iii) 5′-CCACGGACUUCACGUCAUUdT dT-3′; 3′-dT dTGGUGCCUGAAGUGCAGUAA-5′. We set the condition of siRNAs, causing ~60–70% silencing effect with minimal cytotoxicity. Before treatment, the cells were transfected

with 30 n*M* annealed siRNAs targeting human COX II, III, or negative mismatched siRNA using Lipofectamine™ 2000 (Invitrogen Life Technologies, Inc., Grand Island, NY) in antibiotic-free media. After 48 h of transfection, the cells were subjected to further analysis as described in the experimental procedure.

Western blot analysis

Cells were harvested in 300 μl lysis buffer (50 m*M* Tris/HCl, pH 8.0, 150 m*M* NaCl, 50 m*M* b-glycerophosphate, 1% Triton X-100, and 100 m*M* phenylmethanesulfonyl fluoride). The resulting lysates were resolved by 4–12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and transferred to pure nitrocellulose blotting membranes (Bio-Trace NT, Pall Life Science, Pensacola, FL). The membranes were blocked in 10 m*M* Tris/HCl (pH 7.4), 150 m*M* NaCl, and 0.1% Tween-20 containing 5% nonfat milk, and then probed with Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz CA) monoclonal antibodies (MnSOD, 1:1000; Bcl-2, 1:1000). Proteins were detected with an Odyssey two-color infrared imaging system (LI-COR, Lincoln, NE).

Determination of lipid peroxidation, protein carbonyls, and oxidative DNA damage

Lipid peroxidation can be measured by thiobarbituric acid reactive species (TBARS) during an acid-heating reaction. The concentration of TBARS is expressed as malondialdehyde (MDA) production. Oxidation of protein is characterized by protein carbonylation, measured based on the reaction with dinitrophenylhydrazine. Oxidation of DNA is characterized by 8-hydroxydeoxyguanosine (8-OHdG) production. To determine the status of oxidative stress induced by HF-LPLI, we measured the levels of MDA, protein carbonyls, and 8-OHdG production in treated cells using specific commercial kits (Genmed, Shanghai, China) according to the manufacturer's instructions.

Glutathione assay

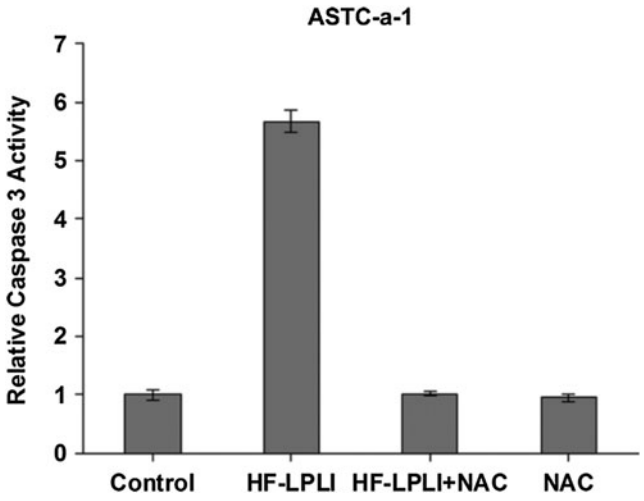
The glutathione (GSH) was determined using the GSH and oxidized glutathione (GSSG) assay detection kit (Genmed) according to the manufacturer's instructions. Briefly, cells were seeded in six-well plates, harvested at 2 h after HF-LPLI treatment for GSH and GSSG analysis. Luminescence signal was measured with an Infinite 2000 plate reader (TECAN, Mönnedorf, Switzerland), and GSH/GSSG ratio was calculated in accordance with the procedure provided.

Caspase-3 activity detection

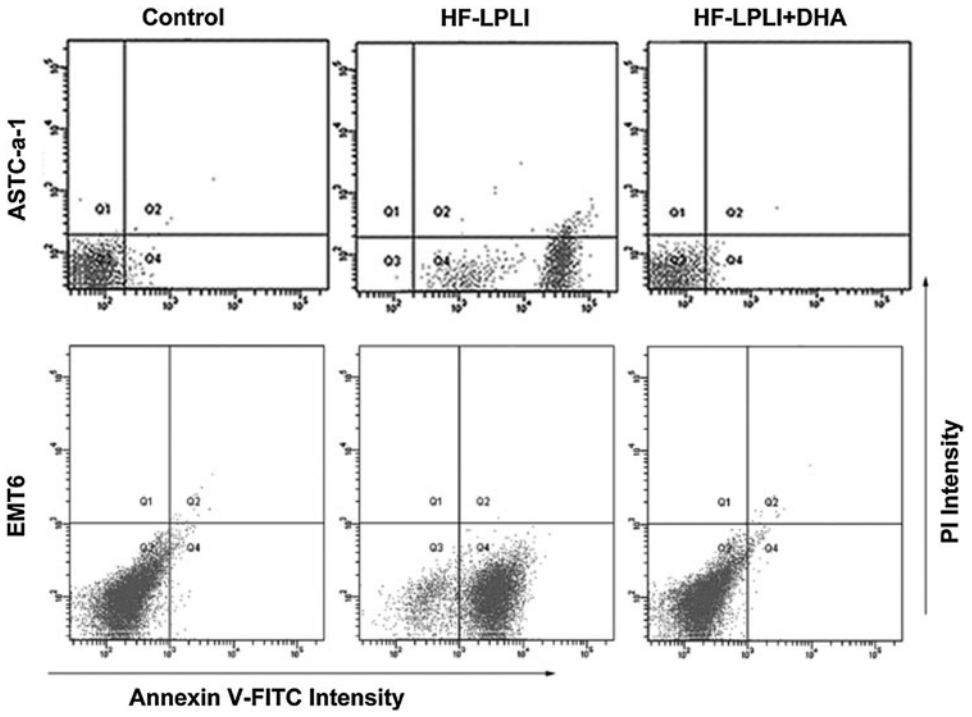
The activity of caspase-3 was determined using the caspase-3 activity assay kit (KeyGEN, Nanjing, China), based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into a yellow formazan product p-nitroaniline (pNA). Lysates were centrifuged at 12,000*g* for 10 min, and protein concentrations were determined by Bradford protein assay. Cellular extracts (30 μg) were incubated in a 96-well plate with 20 ng Ac-DEVD-pNA for 4 h at 37°C. OD405, the absorbance value at 405 nm, was read with an Infinite 2000 plate reader (TECAN). An increase in OD405 indicated the activation of caspase-3.

References

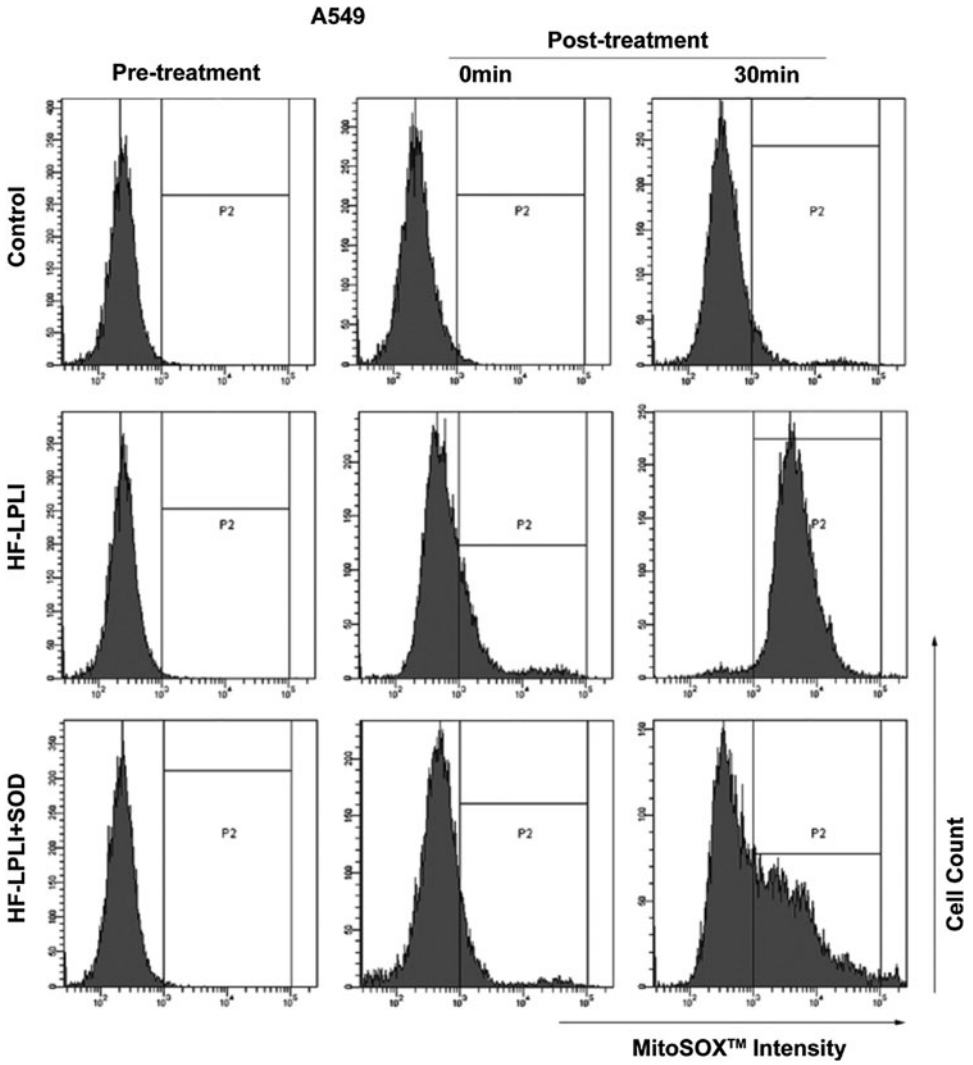
1. Clark JD. "Guide for the care and use of laboratory animals." Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Washington, DC: National Academy Press, 1996.
2. Wei Y, Xing D, Luo S, Xu W, and Chen Q. Monitoring singlet oxygen in situ with delayed chemiluminescence to deduce the effect of photodynamic therapy. *J Biomed Opt* 13: 024023, 2008.
3. Wei Y, Xing D, Luo S, Yang L, and Chen Q. Quantitative measurement of reactive oxygen species in vivo by utilizing a novel method: chemiluminescence with an internal fluorescence as reference. *J Biomed Opt* 15: 027006, 2010.
4. Wei Y, Xing D, and Zhou J. In vivo monitoring of singlet oxygen using delayed chemiluminescence during photodynamic therapy. *J Biomed Opt* 12: 014002, 2007.
5. Zhao H, Xing D, and Chen Q. New insights of mitochondria reactive oxygen species generation and cell apoptosis induced by low dose photodynamic therapy. *Eur J Cancer* 47: 2750–2761, 2011.



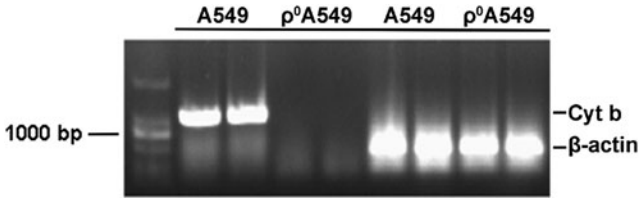
SUPPLEMENTARY FIG. S1. Cell apoptosis analysis was performed by caspase-3 activation detection in ASTC-a-1 cells 10 h after HF-LPLI treatment. Cells were pre-cultured with NAC (250 μM) 1 h before HF-LPLI. The data represent the mean ± SD ($n = 5$). ASTC-a-1, human lung adenocarcinoma cell line; HF-LPLI, high fluence low-power laser irradiation; NAC, *N*-acetyl-L-cysteine; SD, standard deviation.



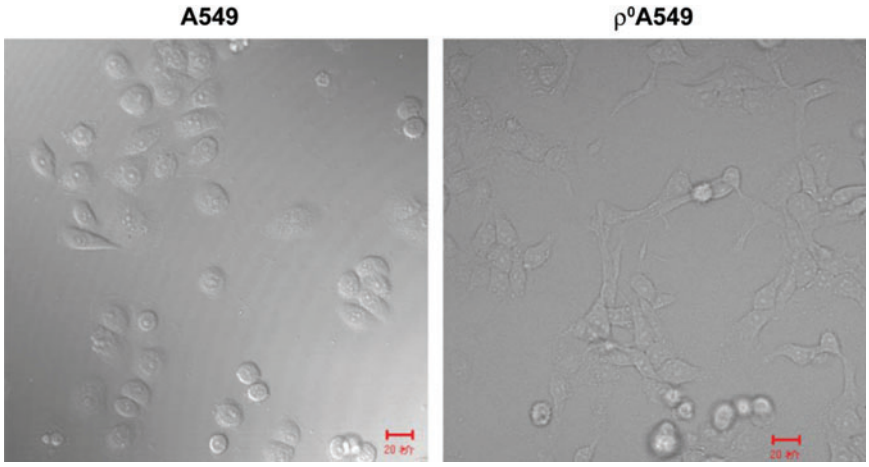
SUPPLEMENTARY FIG. S2. FACS analysis of death with annexin V-FITC/PI double staining in ASTC-a-1 and EMT6 cells 10 h after HF-LPLI treatment (200 mW/cm^2 at 10 min) with or without DHA pre-treatment. DHA, dehydroascorbic acid; EMT6, mouse mammary tumor cell line; FACS, flow cytometry; FITC, fluorescein isothiocyanate; PI, propidium iodide.



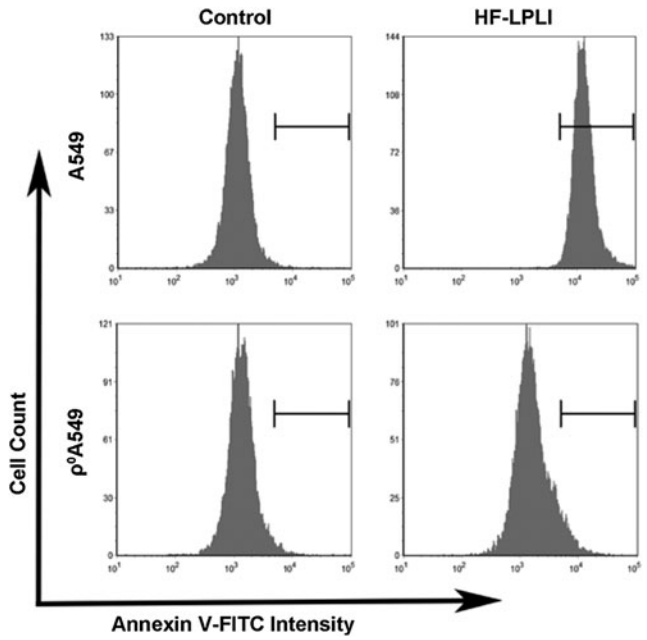
SUPPLEMENTARY FIG. S3. FACS analysis of mitochondrial $O_2^{-\bullet}$ generation in A549 cells before and after HF-LPLI treatment (200 mW/cm^2 at 10 min). The temporal profiles of MitoSOX^{TM} intensities in A549 cells were acquired before and after HF-LPLI treatment. Cells were pre-cultured with SOD (50 U/ml) 1 h before HF-LPLI. A549, human lung adenocarcinoma cell line; $O_2^{-\bullet}$, superoxide anion; SOD, superoxidase dismutase.



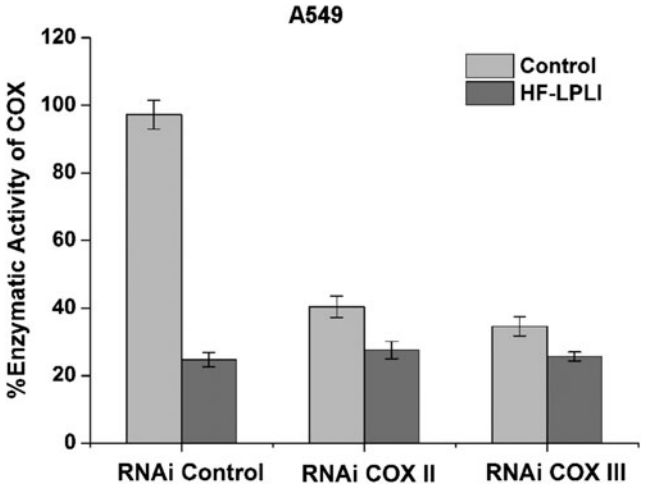
SUPPLEMENTARY FIG. S4. Establishment and analysis of mitochondrial respiration-deficient cell line (ρ^0 cells). Polymerase chain reaction analysis of A549 and ρ^0A549 cells, the latter reveals no detectable cytochrome b (Cyt b).



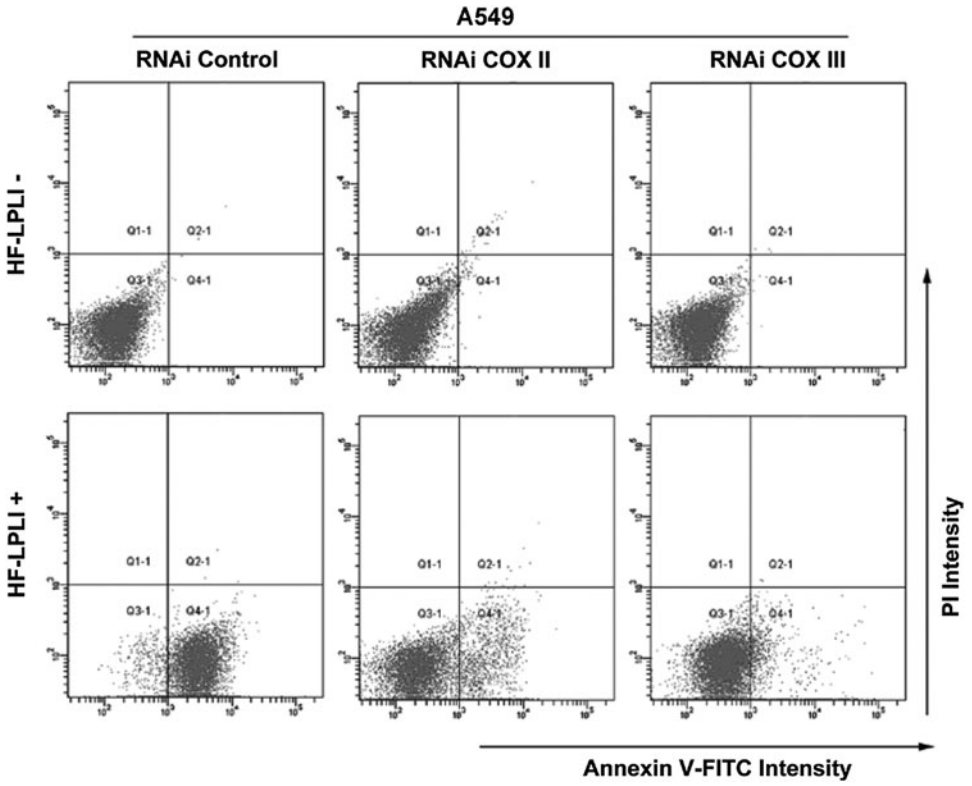
SUPPLEMENTARY FIG. S5. The morphology of A549 and ρ^0A549 cells.



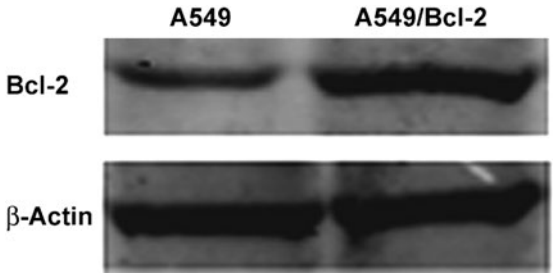
SUPPLEMENTARY FIG. S6. FACS analysis of cell apoptosis with annexin V-FITC staining in A549 and ρ^0A549 cells 10 h after HF-LPLI treatment (200 mW/cm^2 at 10 min).



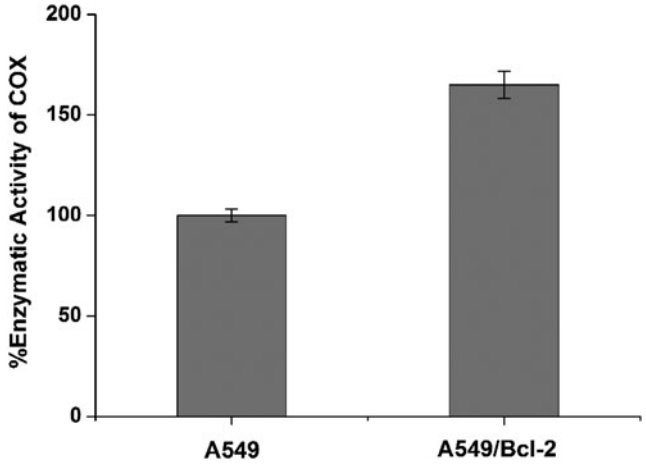
SUPPLEMENTARY FIG. S7. Effect of HF-LPLI (200 mW/cm^2 at 10 min) on COX activity in COX subunit knockdown A549 cells generated with siRNA specifically targeting COX II and COX III. The data represent the mean ± SD ($n = 5$). COX, cytochrome c oxidase; siRNA, small interfering RNA.



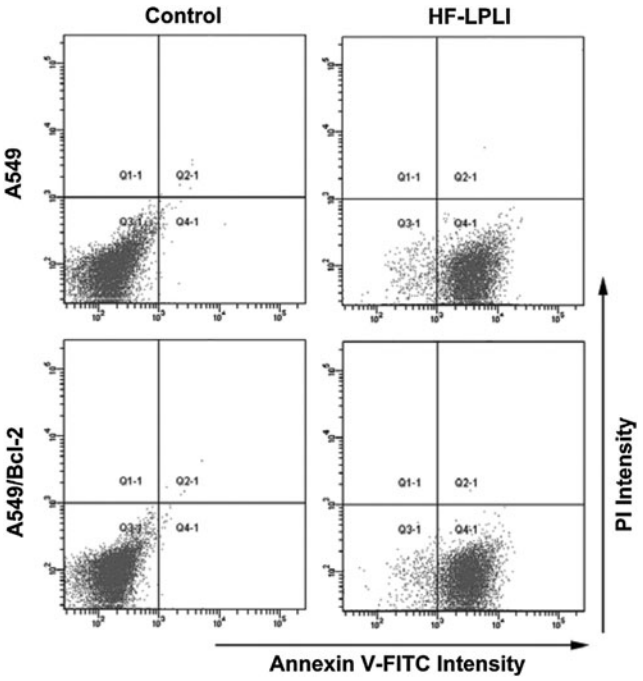
SUPPLEMENTARY FIG. S8. FACS analysis of cell death with annexin V-FITC/PI double staining in COX subunit knockdown A549 cells 10 h after HF-LPLI treatment (200 mW/cm^2 at 10 min).



SUPPLEMENTARY FIG. S9. Overexpression of Bcl-2 was evident in A549/Bcl-2 cells as confirmed by western blot analysis.



SUPPLEMENTARY FIG. S10. Enzymatic activity of COX was assayed by measuring cytochrome c oxidation in A549 and A549/Bcl-2 cells. The data represent the mean ± SD ($n = 5$).



SUPPLEMENTARY FIG. S11. FACS analysis of cell death with annexin V-FITC/PI double staining in A549 and A549/Bcl-2 cells 10 h after HF-LPLI treatment (200 mW/cm^2 at 10 min).