Elevated Heme Synthesis and Uptake Underpin Intensified Oxidative Metabolism and Tumorigenic Functions in Non–Small Cell Lung Cancer Cells

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Abstract

Tumors of human non–small cell lung cancer (NSCLC) are heterogeneous but exhibit elevated glycolysis and glucose oxidation relative to benign lung tissues. Heme is a central molecule for oxidative metabolism and ATP generation via mitochondrial oxidative phosphorylation (OXPHOS). Here, we showed that levels of heme synthesis and uptake, mitochondrial heme, oxygen-utilizing hemoproteins, oxygen consumption, ATP generation, and key mitochondrial biogenesis regulators were enhanced in NSCLC cells relative to nontumorigenic cells. Likewise, proteins and enzymes relating to heme and mitochondrial functions were upregulated in human NSCLC tissues relative to normal tissues. Engineered heme-sequestering peptides (HSP) reduced heme uptake, intracellular heme levels, and tumorigenic functions of NSCLC cells. Addition of heme largely reversed the effect of HSPs on tumorigenic functions. Furthermore, HSP2 significantly suppressed the growth of human NSCLC xenograft tumors in mice. HSP2-treated tumors exhibited reduced oxygen consumption rates (OCR) and ATP levels. To further verify the importance of heme in promoting tumorigenicity, we generated NSCLC cell lines with increased heme synthesis or uptake by overexpressing either the rate-limiting heme synthesis enzyme ALAS1 or uptake protein SLC48A1, respectively. These cells exhibited enhanced migration and invasion and accelerated tumor growth in mice. Notably, tumors formed by cells with increased heme synthesis or uptake also displayed elevated OCRs and ATP levels. These data show that elevated heme flux and function underlie enhanced OXPHOS and tumorigenicity of NSCLC cells. Targeting heme flux and function offers a potential strategy for developing therapies for lung cancer.

Significance: These findings show that elevated heme availability due to increased heme synthesis and uptake causes intensified oxygen consumption and ATP generation, promoting tumorigenic functions and tumor growth in NSCLC.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/79/10/2511/F1.large.jpg.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States (1). About 85% of cases are non–small cell lung cancer (NSCLC). Although several chemotherapeutic and targeted therapeutic agents are approved for treating lung cancer, the 5-year survival rate remains 18%. The effectiveness of targeted therapies for lung cancer is lowered by the presence of multiple driver genes and intratumoral genetic heterogeneity (2). Likewise, three PD-1/PD-L1 checkpoint inhibitors, nivolumab, pembrolizumab, and atezolizumab, generally extend median overall survival by about 3 months for second-line treatment of advanced NSCLC, compared with docetaxel alone (3–5). In the front-line setting, the median progression-free survival extends from 6.0 months with platinum-doublet chemotherapy to 10.3 months with pembrolizumab in patients with untreated NSCLC characterized by a high level of PD-L1 expression (6). Thus, alternative therapeutic strategies are still needed for lung cancer treatment.

Notably, in recent analyses of metabolic pathway flow in human patients, the infusion and analysis of labeled $^{13}$C-glucose
tracers have shown that elevated glycolysis and oxidative metabo-

lism occur concomitantly in lung tumors (7, 8). Although NSCLC
tumors are metabolically heterogeneous, a common feature among
NSCLCs is that pyruvate from elevated glycolysis enters and intensifies
the tricarboxylic acid (TCA) cycle (8). Inten-
sified TCA cycle should provide more TCA intermediates for
biosynthesis and more NADH for ATP generation via oxidative
phosphorylation (OXPHOS). In a follow-up study using
13C-lactate tracers, Faubert and colleagues showed that lactate
also fuels the TCA cycle in molecularly heterogeneous tumors
(9). A separate study using two genetically engineered mouse
models for lung cancer carrying different genetic mutations
(KrasLSL-G12D+/C0 and KrasG12D+/Stk11−/−) also showed that the
contribution of lactate to the TCA cycle is higher than
that of glucose (10). In addition, components of OXPHOS com-
plexes and markers of mitochondrial biogenesis are found to be
highly predictive of reduced overall survival in patients with
NSCLC (11). Likewise, another study showed that the expres-
sion of OXPHOS genes is negatively correlated with the prognosis
of lung adenocarcinoma and that inhibition of OXPHOS
activity impedes the migration and invasion of cisplatin-resistant
cells (12). Strikingly, several recent studies showed that drug-
resistant cells of acute and chronic myeloid leukemia, breast
cancer, lung cancer, and melanoma depend on OXPHOS and
that targeting oxidative metabolism and mitochondrial respira-
tion overcomes drug resistance in these cells (13–15).

Oxidative metabolism involving the TCA cycle and OXPHOS
can be sustained by multiple fuels and requires coordinated
functions of many enzyme subunits. Evidently, despite high
molecular and metabolic heterogeneity, NSCLC cells and other
resistant cancer cells share the common feature of elevated ox-
idative metabolism (13–16). However, the mechanism by which
diverse and heterogeneous cancer cells develop such a common
feature, involving multiple fuels and numerous enzyme subunits,
is not immediately evident. Intriguingly, we previously reported
that several types of human lung tumor xenografts display ele-
vated levels of the rate-limiting heme biosynthetic enzyme ALAS1,
heme transporters, and oxygen-utilizing hemoproteins (16).
Heme is a central metabolic and signaling molecule that regulates
diverse molecular and cellular processes relating to oxygen utili-
ization and metabolism. Heme serves as a prosthetic group in
proteins and enzymes involved in oxygen transport, utilization,
and storage, such as globins and cytochromes (17). One crucial
function of mitochondria is to use oxygen to carry out OXPHOS
for ATP generation. Thus, heme function and mitochondrial respira-
tion are tightly linked. Heme synthesis occurs in mitochon-
dria, and mitochondria cannot function without heme. Multiple
subunits in OXPHOS complexes II–IV contain heme. In addition,
heme acts as a signaling molecule to coordinate the expression
of genes encoding OXPHOS complexes and the assembly of these
complexes (18, 19). Heme binds to and directly regulates the activities
of many proteins controlling processes ranging from
tyrosine kinase signaling to miRNA processing (20–22). Clearly,
heme possesses unique signaling and structural properties that
enable it to coordinate elevated mitochondrial OXPHOS in
diverse cancer cells.

Here, to ascertain whether heme underpins the common fea-
ture of elevated oxidative metabolism in diverse NSCLC cells, we
characterized an array of molecular and cellular characteristics
relating to heme in several types of NSCLC cells. We show that the
levels of the rate-limiting heme biosynthetic enzyme and heme
uptake proteins are highly elevated in several types of NSCLC
cells. Intracellular heme levels in mitochondria are increased in
NSCLC cells. The increased heme levels are accompanied by
elevated levels of oxygen-utilizing hemoproteins. Elevated levels
of heme and hemoproteins correlate strongly with intensified
oxygen consumption rates (OCR) and ATP generation. Converse-
ly, inhibition of heme biosynthesis or uptake diminishes inva-
sion, migration, and colony formation by NSCLC cells. Strikingly,
lung tumor growth in mice is significantly suppressed by inhibi-
tion of cellular heme uptake with a heme-sequestering peptide
(HSP). HSP2. Conversely, overexpression of the heme uptake
protein SLC48A1 strongly enhances tumorigenic functions of
NSCLC cells and tumor growth. These results show that elevated
intracellular heme levels underlie tumorigenic functions of
NSCLC cells.

Materials and Methods
Reagents
Succinyl acetone (SA) was purchased from Sigma-Aldrich (catalog no. D1415-1G). Heme was purchased from Frontier Scientific Inc. (catalog no. H651-9). Zinc (II) protoporphyrin IX (catalog no. Zn 625-9) was purchased from Frontier Scientific Inc. Tin (IV) protoporphyrin was purchased from Porphyrin Products Inc (catalog no. Sn749-9). Deferoxamine mesylate was purchased from Sigma-Aldrich (catalog no. D9533-1G). Ferric chloride was purchased from Sigma-Aldrich (catalog no. 157740-100G). n-Luciferin and the Opal Color HIC Kit were purchased from PerkinElmer. [4-14C]-5-aminolevulinic acid was custom
synthesized by PerkinElmer. Antibodies were purchased from Santa Cruz Biotechnology, Cell Signaling Technology, Novus Biologica, and Abcam. HSP1 and HSP2 were purified with the pET11a expression system. The pET11a expression vector for Yersinia pestis HasA residues 1-193 was kindly provided by Dr. Mario Rivera (University of Kansas, Lawrence, KS; ref. 23). HSP1 contains the Q32H mutation, and HSP2 contains Q32H/Y75M double mutations. The mutations were generated with the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The double mutations were generated with the expression vector for the Q32H mutation. All DNA clones were confirmed by sequencing (Eurofins Genomics LLC). HSP1 and HSP2 were purified from perinElmE, [4-14C]-5-aminolevulinic acid was custom
synthesized by perinElmE. Antibodies were purchased from Santa Cruz Biotechnology, Cell Signaling Technology, Novus Biologica, and Abcam. HSP1 and HSP2 were purified with the pET11a expression system. The pET11a expression vector for Yersinia pestis HasA residues 1-193 was kindly provided by Dr. Iqbal Hamza (University of Maryland, College Park, MD; ref. 24).

Cell culture and analyses of tumorigenic functions
HBEC30KT (RRID:CVCL_AS83) and HCC4017 (RRID:CVCL_V579) cell lines representing normal, nontumorigenic and
NSCLC cells from the same patient (25, 26), respectively, were
provided by Dr. John Minna’s (University of Texas Southwestern
Medical Center, Dallas, TX) lab as a gift. They were developed
from the same patient and were maintained in ACL4 medium
supplemented with 2% heat-inactivated, FBS (25). A pair of
bronchial epithelial cell lines consisting of normal, nontumori-
genic cell line, NL20 (ATCC catalog no. CRL-2503, RRID:
CVCL_3756) and tumorigenic cell line, NL20-TA (ATCC catalog
no. CRL-2504, RRID:CVCL_3757), was purchased from ATCC.
NL20 and NL20-TA cell lines were maintained in Ham’s
F12 medium with 1.5 g/L sodium bicarbonate, 2.7 g/L glucose,
2.0 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 0.005 mg/mL insulin, 10 ng/mL epidermal growth factor, 0.001 mg/mL transferrin, 500 ng/mL hydrocortisone, and 4% FBS. All other NSCLC cell lines, H1299 (ATCC catalog no. CRM-CCL-185, RRID:CVCL_0060), A549 (ATCC catalog no. HTB-177, RRID:CVCL_0459), Calu-3 (ATCC catalog no. HTB-55, RRID:CVCL_0023), H460 (ATCC catalog no. HTB-177, RRID:CVCL_1467) were purchased from ATCC, maintained in RPMI medium, and supplemented with 5% heat-inactivated FBS. All CVCL_0459), Calu-3 (ATCC catalog no. HTB-55, RRID:CVCL_0023), H460 (ATCC catalog no. HTB-177, RRID:CVCL_0060), A549 (ATCC catalog no. CRM-CCL-185, RRID:CVCL_0060), H1395 (ATCC catalog no. CRL-5868, RRID:CVCL_1467) were purchased from ATCC, maintained in RPMI medium, and supplemented with 5% heat-inactivated FBS. All experiments using cells were conducted between passages 3 to 5 from revival of the initial frozen stocks. Cell lines expressing luciferase were generated by infection with lentiviral particles bearing the EF1a-Luciferase (firefly) gene (AMSBIO). Cell lines were authenticated by Genetica and were found to be 96% identical to the standard (authentication requires >80%). Cell lines were tested for Mycoplasma using a MycoFluo Mycoplasma Detection Kit (Molecular Probes), and the results were negative.

For generation of stable overexpression lines overexpressing ALAS1 or SLC48A1, lentiviral vectors expressing ALAS1, SLC48A1, and eGFP (control vector) were purchased from Genecopoeia. The expression vectors for ALAS1 and SLC48A1 also express eGFP, making them comparable with the control and easy for verification of positive clones. All vectors carry the neomycin selectable marker. Lentivirus particles were generated by cotransfecting 293T cells with packaging plasmids pMD2.G (addgene plasmid #12259, RRID:Addgene_12259) and psPAX2 (addgene plasmid #12260, RRID:Addgene_12260) and vector for ALAS1 or SLC48A1 using a MycoFluo Mycoplasma Detection Kit (Molecular Probes), and the results were negative.

For generating stable overexpression cell lines, H1299 and A549 cells (70%–80% confluent) were transduced with virus particles (2.8 × 10^4 units/well) in 48-well tissue culture plates. After series of passes and antibiotic selection, stable clones were selected and verified for overexpression by Western blotting.

Cell proliferation was measured by detecting the luciferase activity in live cells and by using a hemocytometer. Cell migration and invasion assays were carried out with BD Falcon cell culture inserts (Corning Life Sciences) and the manufacturer’s cell migration, chemotaxis, and invasion assay protocols. For the colony formation assay, 5,000 NSCLC cells were seeded in every well in 6-well tissue culture plates in triplicates. Cells were treated with 0.5 mmol/L SA (Sigma-Aldrich), 10 mmol/L HSPs, 50 mmol/L HSO4, 2513 nmol/L ZnPP, was used, as described previously (30, 31). Briefly, 10,000 NSCLC cells were seeded in 96-well plates. Cells were incubated for 3 hours with 60 mmol/L ZnPP in the presence or the absence of 40 mmol/L HSPs. Fluorescence intensity was measured with a Biotek Cytation 5 plate reader. Experiments were conducted in triplicates, and ZnPP uptake was normalized with total cellular proteins. For measuring heme levels in various organelles, we used peroxidase-based reporters, which express peroxidase activity along with a fluorescent marker like mCherry or eGFP in each organelle (24). Heme levels were measured exactly as described, and normalized with the fluorescent signals to correct for variations, such as that in transfection efficiency. Only Calu-3 did not show sufficient fluorescent signals to allow proper measurements (24).

Measurement of oxygen consumption and ATP levels
Oxygen consumption was measured, as described previously (16). Briefly, 10^6 cells (in 350 μL) were introduced into the chamber of an Oxygraph system (Hansatech Instruments), with a Clark-type electrode placed at the bottom of the respiratory chamber. During measurements, the chamber was thermostated at 37°C by a circulating water bath. An electromagnetic stir bar was used to mix the contents of the chamber.

Total ATP was measured with the ATP Determination Kit (Molecular Probes) following the manufacturer’s protocol. Briefly, cultured cells were collected and immediately placed in ice-cold lysis buffer (Cell Signaling Technology) with protease and phosphatase inhibitors. Cell lysates were then centrifuged at 10,000 × g for 10 min. Ten microliter of lysates or 10 μL of ATP standard solution was added to 90 μL of reaction buffer in each well of a 96-well plate. Luminescence was measured using a Biotek Cytation 5 plate reader. All experiments were carried out in triplicate, and the background luminescence was subtracted from the measurement. ATP concentrations were calculated from the ATP standard curve and normalized with the numbers of cells used. To measure OCRs and ATP levels from freshly isolated tumors, subcutaneous tumors were surgically resected from mice and cut into small pieces. Tumors were weighed and homogenized immediately using mechanical homogenizer to gain a homogenous cell suspension. Tissue debris was removed by gentle centrifugation. Cells were suspended in 400 μL of complete medium, and OCRs was measured using a Clark-type electrode. ATP levels were measured with an ATP Determination Kit (Molecular Probes). Both OCRs and ATP levels were normalized with protein amounts.

Preparation of protein extracts and Western blotting
Lung nontumorigenic and tumorigenic cells were maintained (passages 3–5), collected, and lysed by using the RIPA Buffer (Cell Signaling Technology) containing the protease inhibitor cocktail. Protein concentrations were determined by using the Bicinchoninic Acid Assay Kit (Thermo Fisher Scientific). Fifty micrograms of protein from each treatment condition were electrophoresed on 10% SDS–polyacrylamide gels, and then transferred onto the Immuno-Blot Polyvinylidene Difluoride Membrane (Bio-Rad). The membranes were probed with antibodies, followed by detection with a chemiluminescence Western Blotting Kit (Roche Diagnostics). The signals were detected by using a Carestream image station 4000MM Pro, and quantitation was performed by using the Carestream molecular imaging software version 5.0.5.30 (Carestream Health, Inc.). Antibodies used include those to the following proteins: ALAS1 (1:1,000, Novus catalog no. NBP1-91656, RRID:AB_11048622), SLC46A1 (1:1,000, Santa Cruz Biotechnology catalog no. sc-134997, RRID:AB_11142307).
Mice were bred and cared for in a University of Texas at Dallas pathogen-free animal facility in accordance with NIH guidelines. All animal procedures were conducted under protocols approved by Institutional Animal Care and Use Committee at the University of Texas at Dallas. Animals were regularly examined for any signs of stress and euthanized according to preset criteria.

Treatment of human xenograft lung tumors in NOD/SCID mice
To generate mice with NSCLC tumors in the lungs, the 0.75 × 10^6 H1299-luc cells in serum-free medium were injected via tail vein in 6- to 8-week-old female NOD/SCID mice. Alternatively, 0.75 × 10^6 H1299-luc cells (passages 3–5) in serum-free medium containing 50% Matrigel were implanted directly on the lung. Mice were anesthetized with a 2.5% isoflurane and oxygen mixture. H1299-luc cells were injected via tail vein or were injected about 1.5 cm above the lower rib line through the intercostal region. Mice were then placed on a heating pad and observed until they revived from anesthesia. Mice were randomized into three groups (n = 6 per group) that received vehicle (for control) or HSP2 (10 mg/kg, i.v., every 3 days) or HSP2 (25 mg/kg, i.v., every 3 days). Treatments started post cell implantation when lung tumors were detectable using bioluminescence imaging (BLI). Body weights were recorded once every week. Treatments were started only after BLI detected authentic signals (photons/second) to ensure the proper implantation of tumors. When the tumors in the control group reached 1 cm³, mice were euthanized by cervical dislocation. Tumors were resected and weighed.

In vivo BLI
Mice bearing lung tumor xenografts were imaged with an IVIS Lumina III In Vivo Imaging system (Perkin Elmer). Briefly, mice were anesthetized in the isoflurane chamber (2% isoflurane and oxygen), and luciferin (potassium salt; Perkin Elmer; 80 μL of 40 mg/mL) was administered subcutaneously between the scapulae. A BLI time course was acquired over 30 minutes (exposure time, auto; F stop, 1.2; Binning, medium). The images were quantified using Living Image software version 4.5.2 (Perkin Elmer). Regions of interest (ROI) were selected, and bioluminescence signals between 600 to 60,000 counts were accepted as authentic signals. The total bioluminescent signals (photon/sec) from ROIs of mice were calculated as specified by the manufacturer's instructions.

Hematoxylin and eosin staining
Following the final imaging, mice were sacrificed. Lungs were excised and were prepared for histology. Paraffin embedding was performed at Histology core at University of Texas Southwestern Medical Center (Dallas, TX). The paraffin blocks were sectioned to obtain 5-μm sections, which were utilized for hematoxylin and eosin (H&E) staining. For H&E staining, tumor blocks were fixed in 4% formalin, embedded in paraffin, and sectioned (5 μm). Then, sections were stained with H&E. Slides were scanned at a 40 × resolution with an Olympus VS120 slide scanner and quantified using Cell Sens software from Olympus.

IHC
IHC was carried out exactly as described previously (32). Paraffin-embedded tumor tissues from mice described above were used. Six independent sets of human NSCLC grade 2 and 3 tissues and six independent sets of normal human lung tissues in paraffin slides were purchased from US Biomax, Inc. Slides were deparaffinized, hydrated, and washed. After antigen retrieval, slides were blocked with 1× TBS/10% goat serum (16210-072, Gibco). Primary antibodies were diluted in 1× TBS/1%BSA/10% goat serum. The dilutions were 1:200 for ALAS1 (Santa Cruz Biotechnology catalog no. sc-50531, RRID:AB_2225629), 1:200 for SLCA4A1 (Santa Cruz Biotechnology catalog no. sc-101957, RRID:AB_2191218); 1:200 for CYCS (Santa Cruz Biotechnology catalog no. sc-7159, RRID:AB_2090474), 1:200 for PTGS2 (Santa Cruz Biotechnology catalog no. sc-7951, RRID:AB_2084972); 1:200 for TFAM (Cell Signaling Technology catalog no. 8076S, RRID:AB_10949110); COX4I1 1:200, (Santa Cruz Biotechnology catalog no. sc-292052, RRID:AB_10843648), NRF1 (1:1,000, Cell Signaling Technology catalog no. 46743, RRID: AB_2732888), UQCC2 1:100, (Santa Cruz Biotechnology catalog no. sc-90378 RRID:AB_2754980), and COX5A 1:100 (Abcam catalog no. ab110262 RRID:AB_10861723). Sections were incubated with primary antibodies overnight at 4°C and then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (Thermo Fisher Scientific catalog no. 31460, RRID:AB_228341) at a dilution of 1:200 in 1× TBS/1%BSA for 45 minutes at room temperature. Slides were stained with tyramide signal...
amplification (TSA)-conjugated fluorophores, which were diluted 1:100 in 1× Plus Amplification Diluent (NEL810001KT, PerkinElmer). TSA-conjugated fluorophores were aspirated and slides were then washed. DAPI, diluted in TBST, was added to slides and incubated for 5 minutes at RT. Coverslips were mounted over the slides using VECTASHIELD mounting medium (Vector Laboratories), sealed, and stored in darkness at −20°C.

Slides were scanned at a 40× resolution with an Olympus VS120 slide scanner and quantified using cellSens software from Olympus. DAPI was used to visualize nuclei. Multiple ROIs of equal area were drawn over tumor regions. ROIs were selected, so that equal numbers of cells (identified via nuclei) were included in each ROI. The ROIs were positioned evenly throughout tumor regions. ROIs were retested under three filters, FITC, Cy3, and Cy5, to ensure that no artifacts were present. ROIs were repositioned if artifacts were present under one or more filters. Minimum and maximum thresholds were set to avoid any background signal. Mean signal intensity from all ROIs were averaged, and the corresponding negative control average was subtracted to yield the signal intensity for each antigen.

Statistical analyses
Data from different treatment groups of cells, mice, and tissues were compared, and statistical analysis was performed with a Welch two-sample t test. For determining correlation coefficient and statistical significance, we used the following formula:

\[
t(X, Y) = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}
\]

\[
t = \frac{r \sqrt{n - 2}}{\sqrt{1 - r^2}}
\]

Results
Heme synthesis and uptake are elevated to heterogeneous degrees in several types of NSCLC cell lines, leading to elevated mitochondrial heme levels
To gain insights into the degree of elevation and heterogeneity of heme metabolism and flux in lung tumors, we measured heme biosynthesis, uptake, and degradation in several representative types of NSCLC cell lines. These include H1299 (with Nras Q61K p53 null), A549 (with Kras G12S, LKB1 Q377T), H460 (with Kras G12D LKB1 Q377T), Calu-3 (with Kras G13D p53 M237I mutations), and H1395 (with LKB1 deletion). We also used two pairs of cell lines representing normal lung epithelial cells (HBEC30KT and NL20 in Figs. 1 and 2; Supplementary Figs. S1 and S2) and tumorigenic cell lines (NSCLC line HCC4017 and NL20-TA in Figs. 1 and 2; Supplementary Figs. S1 and S2). Clearly, heme biosynthesis (Fig. 1A) and uptake (Fig. 1B) were both increased in NSCLC cell lines and NL20-TA, although the increases varied considerably among different cell lines. When the folds of increase in heme biosynthesis and uptake were added for every cell line (Fig. 1C), they varied from 2- to 8-fold among different lung tumor cell lines. Increases in heme biosynthesis and uptake correlated with increases in the rate-limiting heme biosynthesis enzyme, ALAS1 (Supplementary Fig. S1A; \(r = 0.90, P = 0.0003\)) and the cell membrane heme uptake protein, SLCA6A1 (Supplementary Fig. S1B; \(r = 0.70, P = 0.02\)), respectively.

Heme degradation was also elevated in NSCLC cell lines relative to nontumorigenic cell lines, albeit to a varying degree (Fig. 1D). This increase correlated with the increase in HMOX1 enzyme (Supplementary Fig. S1C; \(r = 0.70; P = 0.02\)). Iron is an essential nutrient and is closely linked to heme (33). Heme synthesis in nonerythroid cells is generally not affected by iron (34). Nonetheless, we detected the levels of transferrin receptor (TFRC), which is responsible for cellular iron uptake from the circulation (35). We found that TFRC levels were increased in some, whereas unaffected or decreased in other NSCLC cell lines (Fig. 1E). This is consistent with the idea that iron availability is not a limiting factor in NSCLC cells.

To determine how elevated heme metabolism in NSCLC cells affects subcellular heme levels, we used a series of previously developed subcellular peroxidase reporters designed to detect subcellular heme levels in mitochondria, cytosol, and other organelles (24). All lung cell lines were efficiently transfected with the reporter plasmids, except for Calu-3, which did not allow efficient transfection of reporter plasmids. Clearly, the mitochondrial heme levels in NSCLC cell lines and the tumorigenic NL20-TA cell line were elevated relative to nontumorigenic cell lines (Fig. 1F). The increase in heme synthesis and uptake was correlated with the increase in intracellular mitochondrial heme levels (\(r = 0.68, P = 0.03\)). Heme levels in other organelles were also increased in some tumor cell lines, but increases were not uniform (Supplementary Fig. S2A–S2E). The increase in heme synthesis and uptake was not correlated significantly with heme levels in other organelles. Mitochondrial heme is crucial for OXPHOS formation and function. Our data suggest that increased heme synthesis and uptake in NSCLC cells lead to elevated mitochondrial heme levels.

Elevated mitochondrial heme levels lead to intensified oxygen consumption and ATP generation in NSCLC cell lines
Next, we measured a series of bioenergetic and tumorigenic parameters. The rates of oxygen consumption (Fig. 2A) and levels of intracellular ATP (Fig. 2B) were elevated in tumorigenic cell lines, except for Calu-3, relative to nontumorigenic cell lines. Elevated oxygen consumption should be accompanied by increased levels of mitochondrial OXPHOS enzymes. Increased expression of mitochondrial proteins should be facilitated by regulators promoting mitochondrial biogenesis, such as NRF1 and TFAM (36). Indeed, the levels of cytochrome c (CYCS) and COX411 (subunits of OXPHOS complexes), as well as the hemoprotein PTGS2, were elevated in NSCLC cell lines relative to nontumorigenic cell lines (Supplementary Fig. S2F–S2H). Notably, two important regulators promoting mitochondrial biogenesis, NRF1 and TFAM (Fig. 2C and D), were also upregulated in tumorigenic cell lines relative to nontumorigenic cell lines. Thus, these observations are consistent with increased OCRs and ATP levels in tumorigenic cell lines.

Measurements of migration (Supplementary Fig. S3A) and invasion (Supplementary Fig. S3B) in NSCLC cell lines showed that they exhibit varying degrees of tumorigenicity. Interestingly, we found that the invasive capabilities of NSCLC cell lines, OCRs, and intracellular ATP levels were well correlated with...
mitochondrial heme levels. The correlation coefficients are as follows: mitochondrial heme and OCRs: \( r = 0.72, P = 0.02 \); mitochondrial heme and ATP levels: \( r = 0.78, P = 0.01 \); and mitochondrial heme and invasion: \( r = 0.71, P = 0.05 \). Together, these results strongly suggest that elevated heme biosynthesis and uptake in NSCLC cell lines lead to elevated levels of mitochondrial heme and OXPHOS subunits, which cause intensified oxygen consumption, ATP generation, and tumorigenic capabilities in NSCLC cells.

We further confirmed the importance of enhanced levels of proteins/enzymes relating to heme function and mitochondrial respiration in lung cancer. Supplementary Figures. S4A and S4B show that the levels of the rate-limiting heme synthetic enzyme, ALAS1, and the heme transporter, SLC48A1, were both significantly enhanced in human NSCLC tissues relative to normal tissues. In the same vein, the heme-containing cytochrome c (Supplementary Fig. S4C) and cyclooxygenase-2 (PTGS2) (Supplementary Fig. S4D) were enhanced in human NSCLC tissues relative to normal tissues. Both cytochrome c (CYCS) and PTGS2 levels have previously been shown to be elevated in NSCLC cell lines and xenograft tumors (16). Notably, the levels of the mitochondrial biogenesis regulator, TFAM, were also enhanced in human NSCLC tissues (Supplementary Fig. S4E), as is the case in NSCLC cell lines (Fig. 2D). Together, data from human NSCLC tissues, NSCLC cell lines, and xenograft tumors show that proteins/enzymes relating to heme

Figure 1.
A, The levels of heme biosynthesis are elevated, though to a varying degree, in NSCLC cell lines. B, The levels of heme uptake are elevated in NSCLC cell lines. C, Elevated heme biosynthesis and uptake can both contribute to increased heme availability to NSCLC cells. D, The levels of heme degradation are elevated in certain NSCLC cell lines. E, The levels of transferrin receptor, TFRC, are not uniformly elevated in NSCLC cell lines. F, Consistent with elevated heme biosynthesis and uptake, the levels of mitochondrial heme are elevated in NSCLC cells. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared with the levels in nontumorigenic cells with a Welch two-sample t test (*, \( P = 0.05 \); **, \( P < 0.005 \)).
function and mitochondrial respiration are upregulated in NSCLC cells and tumors.

Engineered HSPs can inhibit heme uptake in NSCLC cell lines

If elevated heme metabolism is crucial for the tumorigenic functions of NSCLC cells, limiting heme availability may be effective for suppressing lung tumor growth and progression. Previous studies have identified SA as an effective inhibitor of heme biosynthesis, as it inhibits the rate-limiting heme synthesis enzyme, 5-aminolevulic synthase (ALAS1), in nonerythroid cells (37). However, SA is not very effective in suppressing lung tumors in mice (Supplementary Fig. S5A). Therefore, we tried to lower heme availability by taking advantage of bacterial hemophores. We took advantage of the well-characterized Y. pestis hemophore HasA (23). We used structural comparisons of known HasAs and a computational algorithm based on coevolution (38) to identify residues whose mutations may alter but not disrupt heme-binding properties. We designed several HSPs, including HasA Q32H (HSP1) and HasA Q32H Y75M (HSP2; Supplementary Fig. S5B). These two peptides bind to heme strongly (Supplementary Fig. S5C), like the wild-type HasA (23). The changed amino acids in HSP1 and HSP2 are known to coordinate heme well. Thus, the changes are not expected to reduce heme binding. Interestingly, HSP1 and HSP2 have enhanced capabilities to inhibit heme uptake in NSCLC cells (Fig. 3). HSP2 is the most potent in inhibiting heme uptake by NSCLC cell lines, reducing heme uptake by 5-fold in some cell lines (Fig. 3A). Furthermore, the effect of HSPs on heme uptake was reversed if more ZnPP (a heme analogue used for measuring heme uptake) was included (compare 1/C2ZnPP, 2/C2ZnPP, and 2/C2ZnPP + HSP2 in Fig. 3A), indicating that HSP2 does not reduce heme uptake by causing other toxicities. Note that 1/C2ZnPP likely saturated the capabilities of cells to uptake heme/ZnPP so that 2/C2ZnPP did not cause more uptake.

As a bacterial hemophore, HasA is not internalized by human host cells. Thus, HSPs are not expected to be internalized by NSCLC cells. Indeed, HSP2 remained in the medium even after prolonged incubation with NSCLC cells (Supplementary Fig. S5D and S5E). Fluorescent images of NSCLC cells also showed that ZnPP-HSP2 did not enter cells (Supplementary Fig. S5F), whereas ZnPP in the medium without HSP2 entered cells and colocalized with mitotracker (Supplementary Fig. S5G). Furthermore, we detected the effect of HSP2 treatment on mitochondrial heme levels in NSCLC cells, because mitochondrial heme levels are correlated with heme synthesis and uptake, as well as invasion (see above results). Fig. 3B shows that mitochondrial heme levels gradually decreased as the treatment time with HSP2 increased. Together,
HSPs effectively suppress NSCLC cell proliferation and tumorigenic functions

As expected, both HSP1 and HSP2 inhibited NSCLC cell proliferation in various NSCLC cell lines (Fig. 3C) and in a dose-dependent manner (Supplementary Fig. S6). The effects of HSPs on the proliferation of the HBEc30KT cell line representing normal lung epithelial cells were much less severe relative to NSCLC cell lines (Fig. 3C, Supplementary Fig. S6A). This is consistent with the idea that normal cells do not need as much heme as NSCLC cells need. We also tested and compared the efficacies of HSP1, HSP2, and SA at inhibition of tumorigenic functions in NSCLC cells. Evidently, HSP2 was more effective than SA and HSP1 at inhibiting migration of H1299 (Fig. 4A) and A549 cells (Supplementary Fig. S7A). Likewise, HSP2 was more effective than SA and HSP1 at inhibiting invasion by H1299 (Fig. 4B) and A549 cells (Supplementary Fig. S7B). Notably, addition of heme largely reversed the effects of HSP1 and HSP2, like SA, on reducing proliferation, migration, and invasion of NSCLC cells (Figs. 3C, 4A and B, Supplementary Fig. S7A and S7B). The reversal of HSP1 and HSP2 effects by heme addition supports the idea that the effects of HSP1 and HSP2, like SA, on migration and invasion are attributable to their effect on heme uptake. We also found that SA, HSP1, and HSP2 strongly suppressed colony formation in H1299 (see Fig. 4C–E) and A549 (Supplementary Fig. S7C–S7E) cells. Inhibition of heme degradation by SnPP appeared to reduce colony formation in NSCLC H1299 (Fig. 4F) and A549 (Supplementary Fig. S7F) cells.

Addition of heme to cells treated with SA, HSP1, or HSP2 largely reversed the effects of these agents on colony formation (Fig. 4C–E; Supplementary Fig. S7C–S7E), indicating that their effects are attributable to lack of heme. As expected, iron chelator deferoxamine (DFX) also reduced colony formation in NSCLC cells, and addition of iron largely reversed the effect of DFX (Fig. 4C; Supplementary Fig. S7G). Addition of heme to DFX-treated cells partially reversed the effect on colony formation (Fig. 4G; Supplementary Fig. S7G), but addition of iron to SA-, HSP1-, or HSP2-treated cells did not reverse the effects on colony formation (Fig. 4C–E; Supplementary Fig. S7C–S7E). This is consistent with the fact that iron can be obtained via heme. However, extra iron cannot overcome the effect on heme synthesis or uptake, likely because iron is not a limiting factor in NSCLC cells, as in most nonerythroid cells (34). Consistent with this observation of differential effects of iron and heme on colony formation, data in Supplementary Fig. S7H and S7I show that HSP2, unlike DFX, had no significant effects on the levels of transferrin receptor, TRFRC, and ferroportin, SLC40A1.

HSP2 effectively suppresses the growth of human tumor xenografts in mice

To further assess the antitumor activity of HSP2 in vivo, we examined the effects of administering HSP2 on the growth of human xenograft tumors in the lungs of NOD/SCID mice (Fig. 5). Detection of tumor growth and progression with BLI showed that HSP2 significantly suppressed lung tumor growth and progression (Fig. S5A and B). HSP2 did not significantly change the body masses (Fig. 5C). Histologic analysis with H&E staining confirmed that 25 mg/kg of HSP2 nearly eradicated the lung tumors (Fig. 5D). We found that HSP2 was effective at suppressing tumor growth when it was administered to NOD/SCID mice with larger tumor xenografts in the lung (Supplementary Fig. S8A and S8B); when treatments started, the tumors used in Supplementary Fig. S8 showed 10× higher BLI signals than those in Fig. 5). HSP2 did not significantly affect RBC counts (Supplementary Fig. S8C) and hemoglobin levels (Supplementary Fig. S8D) in the blood, as well as liver function shown by ALT activity (Supplementary...
These results show that inhibition of heme uptake by HSP2 can effectively suppress lung tumor growth and progression.

We found that the levels of subunits of OXPHOS complexes, including COX5A (Fig. 5E), COX4I1 (Fig. 5F), UQCRC2 (Fig. 5G), and CYCS (Fig. 5H), were significantly reduced in HSP2-treated tumors, indicating reduced oxygen consumption. To further ascertain the effect of HSP2 on oxygen consumption, we decided to directly detect oxygen consumption and ATP generation in lung tumors in mice. However, we found that it is difficult to isolate sufficient populations of tumor cells from orthotopic lung tumors or do measurements quick enough to collect valid data.
Figure 5.
The effect of HSP2 on the growth and progression of H1299 NSCLC lung tumor xenografts and on the levels of OXPHOS subunits in tumors. **A**, Representative bioluminescence images of mice bearing H1299 lung tumor xenografts treated without (control) or with HSP2 (25 mg/kg) or HSP2 (10 mg/kg; \( n = 6 \) /group). Treatment started at 4.5 weeks after tumor cell implantation when authentic BLI signals (\( > 5 \times 10^5 \) photons/second) were detected from tumors of all tested mice. Treatments were stopped, and mice were sacrificed after the untreated mice with tumors appeared moribund. **B**, The quantified luminescence signals representing tumor volumes. Data are plotted as mean ± SD. For statistical analysis, the levels in treated tumors were compared with the levels in untreated tumors with a Welch two-sample \( t \) test. *, \( P < 0.05 \). **C**, The body masses of mice under each treatment condition. **D**, Representative H&E images of control tumors and tumors treated with HSP2. Tumors are marked with light blue outlines. Montage (scale bar, 2 mm), 10x (scale bar, 200 \( \mu \)m), and 40x (scale bar, 50 \( \mu \)m) images of the H&E sections are shown from left to right. The rectangles in Montage and 10x denote the regions shown in 10x and 40x, respectively. **E**, Representative IHC images of H1299 NSCLC tumor tissue sections and graph showing the levels of COX5A in control and HSP2-treated tumors. **F**, Representative IHC images of H1299 NSCLC tumor tissue sections and graph showing the levels of COX4I1 in control and HSP2-treated tumors. **G**, Representative IHC images of H1299 NSCLC tumor tissue sections and graph showing the levels of UQCRC2 in control and HSP2-treated tumors. **H**, Representative IHC images of H1299 NSCLC tumor tissue sections and graph showing the levels of CYCS in control and HSP2-treated tumors. Shown are montages and 10x images of control and HSP2-treated tumor tissue sections stained with DAPI or antibodies against the indicated protein. The light blue lines in DAPI images outline the tumors in the lung. The white rectangles in DAPI images denote the regions shown in 10x images. The heart was often stained and is marked with "H." Scale bar, montage, 1 mm; 10x, 20 \( \mu \)m. Protein levels were quantified, and data are plotted as mean ± SEM. The values shown in the graphs are averages of signals quantified from three independent IHC experiments. For statistical analysis, the levels in treated tumors were compared with the levels in control tumors with a Welch two-sample \( t \) test (*, \( P < 0.05 \); **, \( P < 0.005 \)).
overcome these difficulties, we used subcutaneously implanted NSCLC tumors. HSP2 was very effective in suppressing subcutaneously implanted NSCLC tumors (Fig. 6A–C). Notably, the OCRs and ATP levels in HSP2-treated tumors were both significantly reduced relative to untreated tumors (populations of cells isolated quickly from tumors; Fig. 6E and F).

Overexpression of ALAS1 or SLC48A1 promotes oxygen consumption, ATP generation, tumorigenic functions of NSCLC cells, and tumor growth

To further ascertain the importance of heme in promoting NSCLC tumors, we generated NSCLC cell lines that overexpress the rate-limiting heme synthesis enzyme, ALAS1, or the heme uptake protein/transporter, SLC48A1 (Fig. 7). We confirmed that relative to control cells, cells overexpressing ALAS1 exhibited elevated heme synthesis (Fig. 7A; Supplementary Fig. S8F) whereas cells overexpressing SLC48A1 exhibited elevated heme uptake (Fig. 7B; Supplementary Fig. S8G). These cells also showed elevated oxygen consumption (Supplementary Fig. S8H). Importantly, these cells overexpressing ALAS1 or SLC48A1 exhibited enhanced migration (Fig. 7C), invasion (Fig. 7D), and colony formation (Supplementary Fig. S8I). When these cells were implanted subcutaneously in NOD/SCID mice, they form bigger tumors than control cells (Fig. 7E and F). Furthermore, tumors overexpressing ALAS1 or SLC48A1 exhibited elevated levels of oxygen consumption (Fig. 7G) and ATP generation (Fig. 7H). Taken together, these results strongly support the idea that increased heme availability resulting from elevated heme synthesis or uptake leads to higher oxygen consumption and ATP generation, which in turn fuels NSCLC cell tumorigenic functions and tumor growth.

Discussion

In the 1920s, Otto Warburg demonstrated that tumor cells metabolize glucose and generate lactate at higher levels than normal cells despite the presence of ample oxygen, a phenomenon called the Warburg effect (39). However, elevated glucose consumption and glycolysis in tumor cells do not necessarily lead to diminished oxidative metabolism and OXPHOS (8, 9). Numerous previous studies have shown that high glycolytic rates occur concomitantly with OXPHOS in cells of most tumors [for a review, see (40)]. More recent studies have demonstrated the importance of mitochondrial OXPHOS in the growth and progression of several types of tumors (41–43). Furthermore, several studies demonstrated that oxidative metabolism and OXPHOS are crucial for conferring drug resistance of cancer cells and cancer stem cells. Farge and colleagues showed that OXPHOS contributes to acute myeloid leukemia resistance to cytarabine (13). Kunz and colleagues showed that targeting mitochondrial OXPHOS eradicates drug-resistant chronic myeloid leukemia stem cells (14). Lee and colleagues showed that MYC and MCL1 confer chemotherapy resistance by increasing mitochondrial OXPHOS in cancer stem cells in triple-negative breast cancer (15).
Heme is a central molecule in mitochondrial OXPHOS and in virtually all processes relating to oxygen transport, storage, detoxification, and utilization (17, 18). Heme serves as an essential prosthetic group or cofactor for many proteins and enzymes that bind and use oxygen, such as cytochrome P450 and nitric oxide synthases, and that detoxify ROSs, such as catalase and peroxidases. Three OXPHOS complexes, II, III, and IV, require heme for proper functioning. Multiple subunits in complexes III and IV require heme as a prosthetic group, and different forms of heme are present (40). Furthermore, heme serves as a signaling molecule that directly regulates diverse processes, including the expression and assembly of OXPHOS complexes (18, 19). Conversely, heme synthesis occurs in mitochondria and requires oxygen (34). Thus, heme and mitochondrial biogenesis are...
linked and are interdependent. Previously, our studies showed that the levels of the rate-limiting heme biosynthetic enzyme ALAS1, heme uptake and transport proteins SLC48A1 and SLC46A1, and oxygen-utilizing hemoproteins, including CYP1B1 and PTGS2, are highly elevated in NSCLC tumors (16, 44). Other studies also showed that the expression of proteins involved in mitochondrial respiration and heme function are elevated in the tumor tissues of with NSCLC (45, 46). In addition, epidemiologic studies indicated a positive association between intake of heme from meat and lung cancer (47).

Here, we show that the levels of heme biosynthesis and uptake, along with the levels of rate-limiting heme biosynthetic enzymes and heme transporters, are upregulated in NSCLC cells relative to nontumorigenic lung cells (Supplementary Fig. S1A and S1B). This elevation causes the elevation of heme biosynthesis and uptake in NSCLC cells (Fig. 1A–C). Increased heme biosynthesis and uptake in turn lead to elevated mitochondrial heme levels (Fig. 1F). On the basis of the levels of heme synthesis in normal medium and medium with depleted heme, we estimate that NSCLC cells obtain about 2/3 of heme via de novo synthesis and about 1/3 via uptake from the medium (Supplementary Fig. S8A). NSCLC cells are known to require serum for growth in culture, whereas normal lung epithelial cells grow better in the absence of serum (25, 48). FBS used to culture NSCLC cells, like human blood, contains approximately 20 μmol/L cell-free heme (49, 50). Thus, both in vitro and in vivo in mice and humans, tumor cells have ample supply of heme from the medium or circulation. Heme degradation is also elevated in some NSCLC cells (Fig. 1D), and the inhibition of heme degradation by SnPP reduced colony formation in NSCLC cells (see Fig. 4F; Supplementary Fig. S7F). SnPP has been shown to be a strong inhibitor for the activities of heme oxygenases (51). This result is consistent with other studies indicating a role of heme degradation in promoting tumorigenesis (52, 53). For example, a previous study showed that inhibition of heme degradation is lethal to hereditary leiomyomatosis and renal cancer cells when fumarate hydratase is deficient (52). Very likely, elevated heme degradation in cancer cells promotes tumorigenic functions by increasing the production of potent antioxidants bilirubin and biliverdin, as well as iron.

Elevated mitochondrial heme levels can potentially influence mitochondrial OXPHOS in two ways: (i) by increasing the pool of heme that is incorporated into OXPHOS complexes and other hemoproteins, and (ii) by upregulating the translocation and assembly of OXPHOS complexes and other enzymes. Therefore, the rates of oxygen consumption and ATP levels are both elevated in NSCLC cells relative to nontumorigenic cells (Fig. 2A and B). Two proteins important for mitochondrial biogenesis, Nrf2 and TFAM, are increased in NSCLC cells relative to nontumorigenic cells (Fig. 2C and D). This is consistent with a previous study showing that loss of TFAM reduces tumorigenesis in an oncogenic Kras-driven mouse model of lung cancer (54). Elevated heme biosynthesis and uptake ultimately lead to enhanced tumorigenic capabilities in NSCLC cells (Fig. 4; Supplementary Fig. S7). Therefore, our data presented here and previous studies all support the idea that heme is a protumorigenic metabolic and signaling molecule. Hemoproteins and enzymes that are required for OXPHOS are also protumorigenic. Interestingly, a recent study from the authors’ lab showed that viable NSCLC tumor cells resistant to the vascular disrupting agent, combretastatin A-4 phosphate, exhibit further elevated levels of hemoproteins and proteins and enzymes involved in heme metabolism (32).

Thus, we expected that inhibitors of heme synthesis and uptake should suppress tumorigenesis and may overcome drug resistance. Indeed, our data presented here show that inhibition of heme synthesis by SA or inhibition of heme uptake by HSPs reduces tumorigenic functions of NSCLC cells (Figs. 3 and 4; Supplementary Figs. S6 and S7). HSP2, which inhibits heme uptake more strongly than HSP1, diminishes tumorigenic functions of NSCLC cells most strongly. This raises the possibility that HSP2 can be a more effective agent against NSCLC cells than SA. Indeed, HSP2 strongly suppressed the growth of both orthotopically implanted NSCLC tumors and subcutaneously implanted tumors (Fig. 5 and 6; Supplementary Fig. S8). Notably, addition of heme largely reverses the effects of SA and HSPs on proliferation, migration, invasion, and colony formation (Figs. 3C and 4; Supplementary Fig. S7). These results strongly support the idea that the effects of SA and HSPs on NSCLC cell tumorigenic functions are attributable to their effects on heme synthesis and uptake, respectively.

The link between heme availability and NSCLC tumorigenesis is strongly supported by data obtained from examining HSP2-treated tumors (Figs. 5 and 6; Supplementary Fig. S8) and tumors formed by NSCLC cells overexpressing the rate-limiting heme synthesis enzyme, ALAS1, or the heme uptake protein/transporter, SLC48A1 (Fig. 7). HSP2-treated tumors (Figs. 5 and 6; Supplementary Fig. S8) show lower levels of OXPHOS complex subunits (Fig. 5E–H), oxygen consumption (Fig. 6E), and ATP generation (Fig. 6F), indicating the effect of inhibited heme uptake on OXPHOS and ATP production. Likewise, our data show that increased levels of ALAS1 or SLC48A1 cause increased heme synthesis (Fig. 7A) or uptake (Fig. 7B), respectively. This increase causes elevated oxygen consumption (Fig. 7G) and ATP generation (Fig. 7H) in NSCLC tumors, which then promotes tumor growth, as shown by increased tumor sizes and masses (Figs. 7E and F).

SA has low toxicity to animals (55, 56). Likewise, our data from mouse studies suggest that HSP2 is not highly toxic to mice (Supplementary Fig. S8C–S8E). HSP2 did not affect RBC count (Supplementary Fig. S8C), hemoglobin levels (Supplementary Fig. S8D), and ALT activity indicative of liver function (Supplementary Fig. S8E). Moreover, HSP2 did not significantly affect the proliferation of HBE320KT cell line representing normal lung epithelial cells in the concentration range that affected proliferation of NSCLC cells (Fig. 3C; Supplementary Fig. S6A). Bacterial hemophore is not internalized by host mammalian cells. Thus, it is expected that HSP2 does not get into NSCLC cells, as indicted in Supplementary Fig. SSSD–SSSF. Notably, our data clearly showed that HSP2 inhibits heme uptake and reduces mitochondrial heme levels in NSCLC cells (Fig. 3A–C). The lack of strong blood toxicity of HSP2 is likely attributable to the lack of the need for heme uptake in normal cells. For example, normal lung cells do not proliferate in the presence of serum, which contains cell-free heme, whereas NSCLC cells need serum for proliferation and tumorigenicity (48). Our data presented here show that the levels of heme synthesis, uptake, oxygen consumption, and ATP are significantly lower in nontumorigenic lung cells relative to NSCLC cells (Figs. 1 and 2). Notably, during erythropoiesis, heme synthesis is induced prior to and is essential for globin synthesis (34, 57). Erythroid heme synthesis is very high and excessive. Previous experimental data suggested that
erythrocytes produce excess heme for export and transport to other organs (57, 58). Thus, heme uptake is not needed for erythropoiesis, and erythrocytes can provide heme for other cells and tissues, including tumors. Thus, it is not surprising that heme sequestration by HSP2 does not cause erythroid toxicity during the treatment periods in mice (Figs. 5 and 6; Supplementary Fig. S8). It is also worth noting that suppression of tumor growth should lower the demand for iron for tumor growth, thereby alleviating potential blood toxicity posed by HSP2.

Heme represents 97% of the functional iron pool in the human body. Iron can contribute to both tumor initiation and progression (59). Indeed, our data show that iron chelator defereroxamine (DFX) inhibited colony formation in NSCLC cells (Fig. 4G; Supplementary Fig. S7G). However, addition of iron does not reverse the effects of SA or HSPs on colony formation (Fig. 4C–E; Supplementary Fig. S7C–S7E), because iron cannot reverse the effects of SA and HSPs on heme synthesis or uptake. Heme and iron are linked: heme synthesis requires iron, and heme degradation releases iron. However, likely due to their respective chemical properties, the main biological functions of heme iron and nonheme iron in living organisms may have become distinct. Because of its unique property for binding oxygen, the primary functions of heme iron are for oxygen utilization, metabolism, and detoxification, particularly in OXPHOS for ATP generation. Nonheme iron, however, often exists in proteins and enzymes as iron–sulfur clusters, and has essential functions in DNA replication, repair, and cell cycle (59, 60). Thus, both iron depletion and heme depletion can have antitumor effects, but the mechanisms largely differ. Iron deficiency causes anemia, because RBCs account for 80% of functional iron needed in humans or mammals (34). In humans and mammals, the need of other cells for iron can presumably be met by taking a small amount of iron from RBCs. Indeed, iron availability affects heme synthesis in erythrocytes, but not nonerythroid cells. Furthermore, heme synthesis in erythroid and nonerythroid cells involves different ALASs and regulatory modes (34, 57). Thus, lowering heme availability should have more selective effects on NSCLC cells. Thus, targeting heme uptake and/or heme synthesis may provide a new and effective strategy for the treatment of NSCLC and perhaps other cancers resistant to existing therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhang

Study supervision: L. Zhang

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