

In Vitro Tracking of Intracellular Metabolism-Derived Cancer Volatiles via Isotope Labeling

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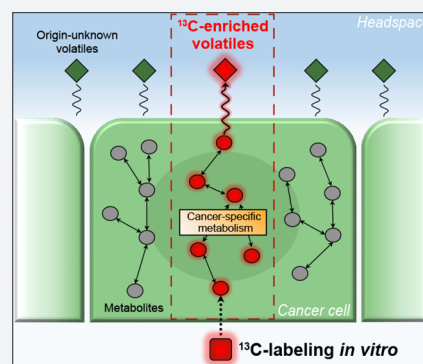
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S Supporting Information

ABSTRACT: Cancer detection relying on the release of volatile biomarkers has been extensively studied, but the individual biochemical processes of the cells from which biogenic volatiles originate have not been thoroughly elucidated to date. Inadequate determination of the metabolic origin of the volatile biomarkers has limited the progress of the scientific and practical applications of volatile biomarkers. To overcome the current limitations, we developed a metabolism tracking approach combining stable isotope labeling and flux analysis of volatiles to trace the intracellular metabolism-derived volatiles and to reveal their relation to cancer metabolic pathways. Specifically, after the ¹³C labeling of lung cancer cell, the isotopic ratio of whole cellular carbon was measured by nanoscale secondary ion mass spectrometry-based imaging. The kinetic modeling with the time-dependent isotopic ratio determined the period during which cancer cells reach the metabolic steady state, at which time all of the potential volatiles derived from intracellular metabolism were fully enriched isotopically. By measuring the isotopic enrichment of volatiles at the end-stage of isotopic flux, we found that 2-pentadecanone appeared to be derived from the metabolic cascade starting from glucose to fatty acid synthesis. Furthermore, this biosynthetic pathway was determined to be distinct in cancer, as it was upregulated in colon, breast, and pancreatic cancer cells but not in normal cells. The investigation of the metabolic footprint of 2-pentadecanone demonstrates that our novel approach could be applied to trace the metabolic origin of biogenic volatile organic compounds. This analytical strategy represents a potential cutting-edge tool in elucidating the biochemical authenticity of cancer volatiles and further expanding our understanding of the metabolic network of airborne metabolites *in vitro*.



INTRODUCTION

A wide variety of airborne-released metabolites, also known as biogenic volatile organic compounds (BVOCs), have attracted interest from researchers as potential diagnostic biomarkers of cancer.^{1–3} Since the first discovery of the potential of volatile-based diagnosis in the middle of the 20th century,⁴ the application of this type of diagnostic tool for the detection of cancer has received considerable attention.⁴ Furthermore, in recent years, the advances in quantitative and qualitative techniques to analyze BVOCs have enabled researchers to assess the volatile biomarkers specific to cancer.⁵ Along with the potential for the volatile-based diagnosis, a volatile compound analysis combining a variety of extraction methods and detectors has been developed.^{6–8} Thanks to these techniques, cancer researchers revealed the composition, dynamics, or alterations of cancer volatiles emitted in the breath, tissues, fluids, and tumor cells of cancer patients.^{9,10} For example, human lung cancer and gastric cancer, which are of particular interest because these organs could come into contact with the exhaled breath, appeared to release distinctive

cancer volatiles.^{11–16} Furthermore, *in vitro* experiments using cancer cells, the smallest unit of cancer, were also conducted, and a variety of volatiles, including aldehydes and hydrocarbons, were identified as cancer volatiles.^{9,17} In summary of the previous discoveries, a wide variety of BVOCs released specifically from various types of cancer were identified as discriminative BVOCs for cancer.¹⁸

However, previous studies utilized conventional methods that could not determine whether the volatiles known as cancer biomarkers are biochemically emitted by and derived from the intracellular metabolism of cancer cells. Most of the studies using cancer cells have been limited to just comparing the amounts of BVOCs in normal and cancer cells. The problem is that this approach cannot clearly establish their cancer-specific biochemical origin. To date, there is no robust evidence regarding the association between BVOCs and their biosynthetic pathways, and only theoretical estimates of their

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biochemical origin, based on the classes of each compound, have been studied.^{1,3} To dispel the uncertainty regarding the origins of the cancer volatiles, it is necessary to precisely examine the cancer-specific intracellular metabolic pathways from which volatiles arise.

In this study, a method for tracking intracellular metabolism that generates volatiles was developed as a breakthrough in solving the aforementioned issues. We applied stable isotope-assisted metabolic labeling to elucidate cancer metabolism-specific volatiles.^{19,20} Specifically, we used [U-¹³C]glucose, which is a major source of carbon via the Warburg effect of cancer cells, as a labeling agent.²¹ To determine when ¹³C from glucose was enriched completely in every volatile candidate, we defined the moment when the cells reached the isotopic steady state using nanoscale secondary ion mass spectrometry (nanoSIMS), as it offers an outstanding spatial resolution adequate for cell samples.^{22,23} At the steady state, cancer metabolism-derived volatiles were sorted from uncertain volatiles by isotopic influx, which was detected by headspace–solid phase microextraction (HS–SPME) and gas chromatography–mass spectrometry (GC–MS).^{24,25}

RESULTS AND DISCUSSION

Mass Spectrometry-Based Cellular Imaging Measures the Intracellular ¹³C Flux. A platform for tracking intracellular metabolism-derived volatiles is shown in Figure 1a,b.

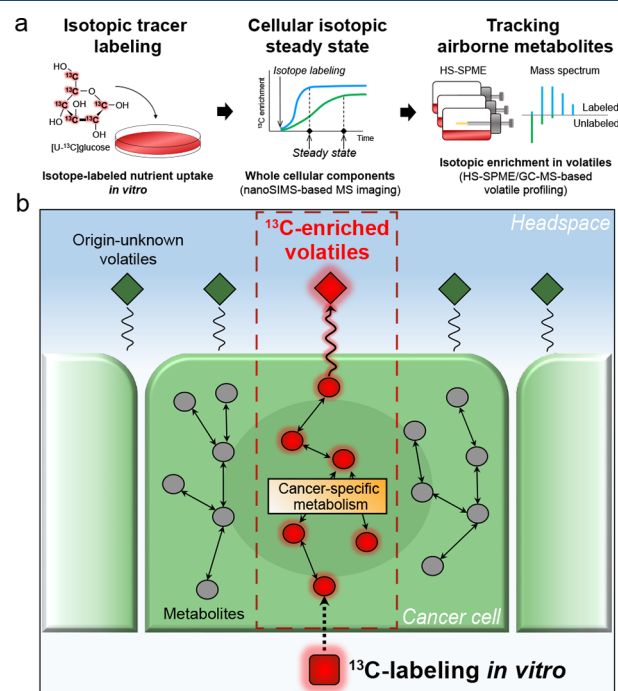


Figure 1. (a) Platform for tracing intracellular metabolism-derived volatile compounds. (b) Schematic illustration of finding cancer volatiles produced by cancer-specific metabolism. Red-highlighted features indicate isotopically enriched compounds.

The main purpose of this platform is to trace all the volatiles synthesized metabolically, starting from the first isotope-labeled metabolite. Because we sought to observe unknown isotopic flux into volatiles and their related pathways, it was necessary to verify that the isotopic distribution into all of the possible volatiles was complete.^{23,26} It is worth mentioning that the metabolic fluxes represent a relatively narrow range of

carbon metabolism, which makes metabolites insufficient to account for whole cellular carbon located in a complex biochemical network. The isotopic fluxes measured at the metabolite level rapidly changed compared to the results of the intracellular carbon discussed later in this section (Note S1 and Table S1). Accordingly, we determined the time for reaching an isotopic steady state of whole cellular carbon using nanoSIMS. At first, we selected CN as a detected ion because this ion could selectively detect cell-specific carbon and had a lower background noise (Figure S1). By measuring ¹³C¹⁴N (27.05 *m/z*) and ¹²C¹⁴N (26.03 *m/z*), we observed time-dependent ¹³C/¹²C above the natural ratio after labeling ¹³C-glucose for 14 days (Figure 2a). These cellular images at various times indicated that the rate of ¹³C enrichment in cellular components slowed down slightly between 4 and 7 days.

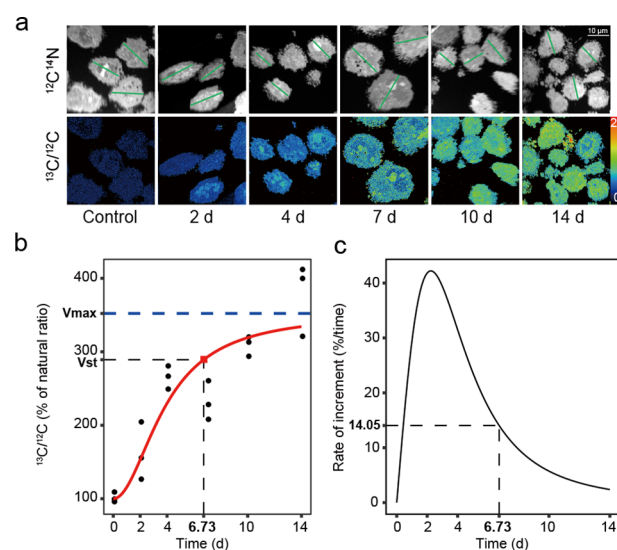


Figure 2. Cellular imaging by nanoSIMS for measuring carbon flux and steady state in whole cellular components. (a) Cellular shapes (¹²C¹⁴N) and corresponding ¹³C/¹²C ratios after isotope labeling. The rainbow scale indicates the ¹³C/¹²C ratio ranging from blue (0) to red (20). The intensity of pixels designated as green bars was measured for calculating the average ¹³C/¹²C ratio of each cell. Scale bars, 10 μ m. (b) Determination of isotopic pseudosteady state (PSS) of the cellular carbon. The average ¹³C/¹²C ratio of each cell measured in Figure 2a (*n* = 3 for each time point) is plotted as % above the natural ratio (black dots). The red curve for ¹³C flux was obtained by fitting 18 data points to the kinetic model. Time to 6.73 days, where the increment of ¹³C/¹²C ratio was reached, was selected as a PSS. (c) Rate of increment of ¹³C at each time point. The first derivative of the curve in Figure 2b is plotted.

Kinetic Modeling for Estimation of Isotopic Pseudosteady State Determines the End-Stage of ¹³C Enrichment. We further quantified the average ¹³C/¹²C ratio of the cells (Figure 2a and Figure S2), and an isotopic pseudosteady state (PSS) of the cellular carbon was assumed over a nonlinear least-squares method modeled by a modified Michaelis–Menten kinetics equation as two substrate reactions (Figure 2b).²⁷ We utilize the nonlinear least-squares method to examine a trend that properly explains the fluctuation in the data. Specifically, we aim to investigate a point of steady state in a carbon flux. Throughout the following discussion, we assume the true model is increasing without loss of generality, as seen in Figure 2b since the decline can be accounted for

after being reflected by a horizontal axis. Our model equation is written as $y = (Vx^2)/(K + x^2) + c$, where x and y denote time (in days or hours) and the increment of carbon flux, respectively. V represents the total variation of y which can be explained by the model, and K is the constant that determines the time point when reaching $0.5V + c$. The last two values are parameters of the model to be estimated from data points. It is noted that c is a prespecified constant required to set the baseline on y . For example, c is set to 100 in Figure 2 since we convert all values to a relative scale (in percent) based on the value at day 0. Also, c represents the minimum value of the function while $V_{\max} = V + c$ indicates the maximum value we can achieve. To estimate the coefficients K and V , we implement the Gauss–Newton algorithm using the “nls” function in R language. In this kinetic model, the model equation with estimated parameters $\hat{V} = 252.18$ and $\hat{K} = 15.06$ is given by $y = (252.18x^2)/(15.06 + x^2) + 100$ where c is set to 100. For a measurement of the goodness-of-fit of our model, the coefficient of determination R^2 is used here, which is defined as

$$R^2 = 1 - \frac{\sum_{i=1}^{18} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{18} (y_i - \bar{y})^2}$$

where $\hat{y}_i$ is the fitted value of i th data, and $\bar{y}$ is the average of y_i 's.²⁸ As we obtain $R^2 = 0.80$, it can be said that approximately 80% of our data are accounted for by the nonlinear function, which implies the high reliability of the fitting. It is noted that our model equation implicitly postulates that the rate of increment gradually decreases. Therefore, we define the PSS as the point when an increment from the reference level becomes 0.75 of the whole variation V (V_{st} in Figure 2b). The red line in Figure 2b represents the fitted model based on 18 data points (black dots), of which values were derived from the average intensity of pixels along the green bars in Figure 2a, and $V_{\max} = \hat{V} + c = 352.18$ and $V_{st} = 0.75\hat{V} + c = 289.14$. In Figure 2c, the first derivative (instantaneous rate of change) of the function is drawn, which shows how rapidly y grows; when it is larger, y increases more rapidly. It reveals that the maximum rate of increment is approximately 42.20 (%/day) at 2.8 days; thereafter, the rate begins to decline, reaching one-third (14.05) of the maximum at the steady state. It implies that y tends to increase as much as 42.20% in 1 day at 2.8 days, but only 14.05% per day at 6.73 days. Observation of a gradual change in y after 6.73 days (Figure 2b) underlies that this time point nears the steady state. This result reflects the PSS, the minimal time for analyzing volatiles by ensuring the near completion of the isotopic enrichment. The PSS at 6.73 days indicated that every volatile potentially produced from glucose metabolism could be labeled completely if A549 cells were cultured for 7 days.

Isotopic Flux Analyses Trace the Intracellular Metabolism-Derived Volatiles. In PSS, we identified intracellular metabolism-derived airborne metabolites. Because there was no evidence that potential volatiles arose from intracellular metabolism, nontargeted volatile profiling *in vitro* using HS–SPME coupled with GC–MS was performed using optimal extraction parameters (Figure S3 and Note S2).²⁹ The optimized method using divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber with a 24 h extraction time detected 99 volatile and semivolatile compound features released from A549 lung cancer cells, out of which 40 compounds were identified (Table S2). As we observed the

¹³C enrichment, noticeably, only 2-pentadecanone showed an increased heavy isotope ratio in the mass spectrum (Figure 3a). This finding indicated that 2-pentadecanone may be the

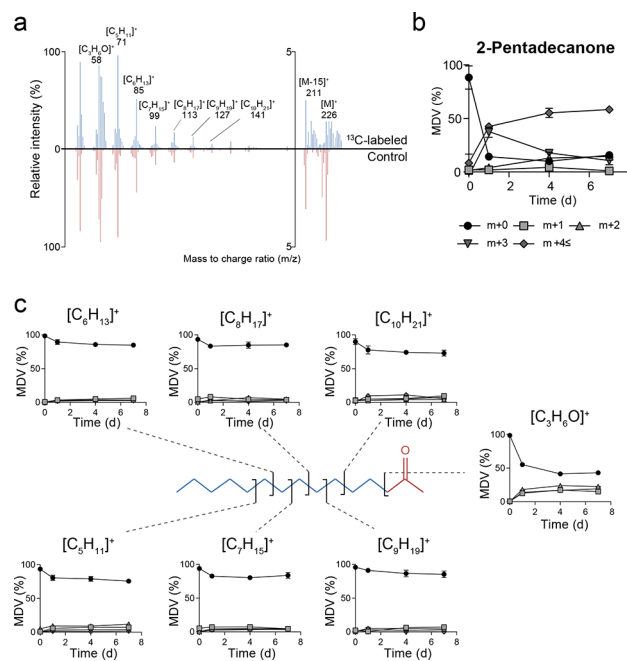


Figure 3. The 2-pentadecanone is identified as the only volatile derived from glucose metabolic cascade in cancer cells. (a) ¹³C-labeled (blue) and unlabeled (red) spectrum of 2-pentadecanone and its fragment ions. (b) Mass distribution vector (MDV, %) of 2-pentadecanone (precursor ion, 226 m/z). $m+0$ indicates the relative abundance of monoisotopic mass, and $m+1$ to $m+4$ stand for the abundance of molecules with one to four isotopes. (c) MDV (%) of seven fragment ions corresponding to each molecular part. In parts b and c, x -axis indicates the time after start of ¹³C labeling. Data are presented as mean and SEM.

only molecule derived from the cellular metabolism of glucose. Other volatiles were not significantly enriched by ¹³C-glucose labeling (Figure S4). The mass distribution vector (MDV) of 2-pentadecanone during isotope labeling revealed that cancer cells produce this compound from at least two types of substrates (Figure 3b and Table S3). $m+0$, the monoisotopic mass of molecules, dramatically decreased at 1 day, estimated to reach the PSS at 0.21 days, and was maintained at that ratio until 7 days. However, $m+3$, which includes three isotopes in the molecule, did not follow the adjusted kinetic model. After a period of rapid increase at 1 day (up to 37.2%), $m+3$ exhibited a decreasing trend. The decreased proportion of $m+3$ was possibly due to the induction of the isotopologues over $m+3$ ($\geq m+4$) from 2 days. These data indicated the rapid uptake of three ¹³C from one substrate and the slower uptake of other carbons from the other substrates, which in turn demonstrated that 2-pentadecanone was synthesized from two types of isotope-labeled metabolites. Moreover, the positional influx of ¹³C was verified by inspecting the isotopologues of multiple fragment ions from 2-pentadecanone (Figure 3a and Table S3).³⁰ Many fragments consisting of only carbons and hydrogens (six C_nH_{2n+1}) appeared to show a slight decrease of $m+0$ at 1 day, but C_3H_6O containing a carbonyl group with three carbons underwent a sharp decrease by 43.3% (Figure 3c). Accordingly, the three-carbon uptake as explained above occurred on the side of the carbonyl group among the 15

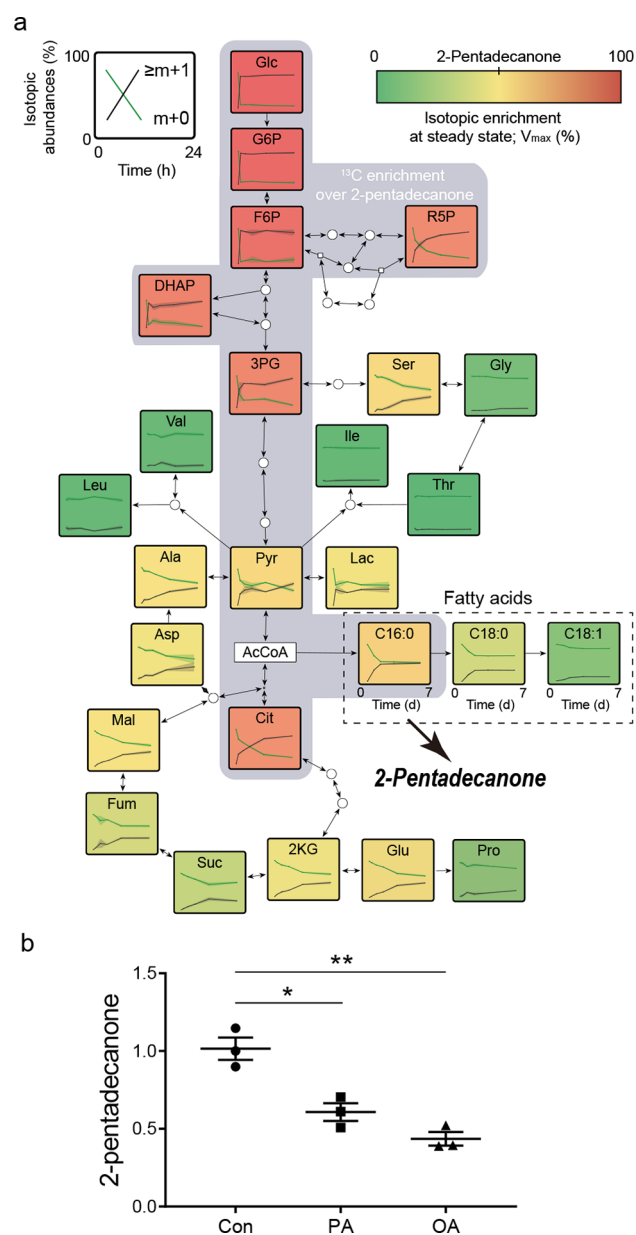


Figure 4. The 2-pentadecanone is related to fatty acid synthesis. (a) Measurement of isotopic flux of metabolites in central carbon metabolism. A decrease of monoisotopic peak ($m+0$, green line) reflects a time-dependent ^{13}C flux of metabolites. Each box is colored on the basis of the percentage of ^{13}C enrichment (V_{max} from the adjusted kinetic model). Data are presented as mean and SEM. (b) Release of 2-pentadecanone after treatment with the fatty acid synthesis inhibitors, palmitic acid (PA) and oleic acid (OA). Data are shown as mean and SEM. * $p < 0.05$, ** $p < 0.01$ by Student's t test. Abbreviations are included in Note S1.

carbons of 2-pentadecanone. The different influx ratios of these three carbons and others may suggest that 2-pentadecanone is derived from two different intracellular metabolites. However, the nature of the specific substrates is unknown.

2-Pentadecanone is a Cancer-Specific Volatile Modulated by Fatty Acid Synthesis. We found 2-pentadecanone to be produced during fatty acid synthesis, the most common characteristic of the abnormal proliferation of cancer cells.³¹ In fact, it can be easily assumed that 2-pentadecanone with a long hydrocarbon chain is structurally similar to fatty acids.

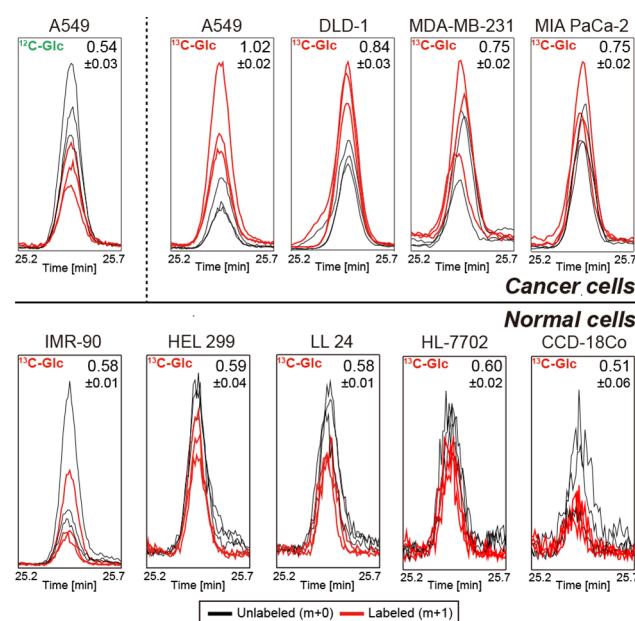


Figure 5. Isotope abundance of 2-pentadecanone released by normal cells (lung, IMR-90, HEL 299, and LL 24; colon, CCD-18Co; liver, HL-7702) and cancer cells [control (^{12}C -Glc) and ^{13}C -glucose-treated (^{13}C -Glc) A549, lung cancer; DLD-1, colon cancer; MDA-MB-231, breast cancer; and MIA PaCa-2, pancreatic cancer]. $m+0$ and $m+1$ indicate the unlabeled (black) and labeled (red) forms, respectively. Average labeled/unlabeled ratio with SEM is shown on top right.

Nevertheless, it has not been investigated whether ^{13}C from glucose is integrated into this volatile via fatty acid synthesis. Therefore, we first observed the metabolic flux in central carbon metabolism including glucose and fatty acids. Specifically, metabolites that have a higher isotopic enrichment than 2-pentadecanone were specified, assuming the upstream metabolic source should have a higher enrichment than a product (Figure 4a and Table S1). Most metabolites in the pathway, from glucose to palmitic acid (C16:0 fatty acid), exhibited higher levels of ^{13}C enrichment than 2-pentadecanone. These data indicated that upstream substrates may be involved in the synthesis of 2-pentadecanone. Regarding palmitic acid, we inhibited fatty acid synthesis by treating A549 cells with two feedback inhibitors, palmitic and oleic acid, to determine if the synthesis of 2-pentadecanone was altered (Figure 4b).³² As a result, the abundance of 2-pentadecanone was decreased by both inhibitors compared with the control.

In addition, the release of 2-pentadecanone was highly dependent upon cancer cells. Among the investigated volatiles, only the release of 2-pentadecanone showed a clear linear correlation with the cell population, with a large coefficient (r ; 0.994) and a significant p -value for the null hypothesis test of regression slope ($p < 0.0001$) (Figure S5, Table S2, and Note S3). In addition, the incorporation of ^{13}C into this compound was highly enriched in other types of cancer cells including breast cancer (MDA-MB-231), colon cancer (DLD-1), and pancreatic cancer (MIA-PaCa-2) cells, but not in normal lung (IMR-90, HEL 299 and LL 24), normal colon (CCD-18Co), and normal liver (HL-7702) cells (Figure 5). After labeling for 7 days, the cancer groups had increased ratios of labeled to unlabeled form. On the contrary, the normal cells had an approximately equal labeled/unlabeled ratio as compared to

that of the control (without ^{13}C -glucose), and this observation held true in HEL 299, LL 24, HL-7702, and CCD-18Co cells, wherein the unpaired t test between cell lines with ^{13}C (four cancer cells and five normal cells) and ^{12}C -glucose (A549) clearly showed the significant alteration of the ratio in cancer cells only (Figure S6). These results support the notion that 2-pentadecanone is strongly associated with fatty acid synthesis beginning from glucose in cancer cells.

CONCLUSION

In this paper, we applied a novel analytical platform to trace intracellular metabolism-derived volatiles and established the relationship between volatiles and cancer-specific metabolism. At the cellular steady state determined by nanoSIMS-based imaging and kinetic modeling, we successfully elucidated the isotopic enrichment into 2-pentadecanone, the only volatile that originated from the metabolic cascade beginning with ^{13}C -glucose. This phenomenon was found to be regulated by fatty acid synthesis and to be a universal feature of various types of cancer cells. The novel insight into this previously unexamined compound in cancer research can expand our understanding of new regulatory mechanisms regarding the emissions of volatile compounds at the cellular level. Future studies are warranted to provide better insights into the biochemical origin of a wide range of volatile compounds using this approach as a solution to address the bottleneck in biogenic volatile compound studies.

MATERIALS AND METHODS

Chemicals and Reagents. [$\text{U-}^{13}\text{C}$]glucose was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). ATCC-modified RPMI1640 medium with phenol red, Dulbecco's phosphate-buffered saline, fetal bovine serum, trypsin-EDTA solution, and antibiotic-antimycotic solutions were from Life Technologies (Gaithersburg, MD). Acetone, methanol, 2-propanol, and water (HPLC grade) were from J.T. Baker (Center Valley, PA). Reagents for cell fixation, epoxy embedding, derivatization of metabolites, and all standards for metabolite identification were from Sigma-Aldrich (St. Louis, MO).

Cell Culture Conditions and Stable Isotope Labeling. A549, DLD-1, MIA-PaCa-2, MDA-MB-231, IMR-90, HEL 299, and LL 24 cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). All cell lines were maintained in ATCC-modified RPMI1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. The cells were maintained in T-75 culture flasks (Life Technologies) at 37 °C and 5% CO_2 . Isotope labeling was performed with the same concentration (4.5 g/L) of [$\text{U-}^{13}\text{C}$]glucose in glucose-free RPMI1640. Before immersing the cell lines in the labeled medium, we cultured all cell lines simultaneously in unlabeled medium until they reached a metabolic steady state. After labeling began, we changed the media daily to sustain an adequate concentration of ^{13}C to fully enrich volatile compounds. At 24 h before volatile compound analysis, we transferred 1×10^6 cells into glass bottles.

Nanoscale Secondary Ion Mass Spectrometry-Based Cellular Imaging. The preparation of cell samples for nanoSIMS began by fixation in a 2.5% glutaraldehyde and 4% paraformaldehyde mixture in 0.1 M phosphate buffer for 3 h at room temperature. Then, 1% osmium tetroxide was added as a secondary fixative, and cells were incubated for 2 h at 4 °C.

They were washed three times with distilled water and dehydrated with increasing concentrations of acetone (30%, 60%, 90%, and 100% with 2 repeats). After dehydration, gradually increasing concentrations of epoxy embedding solution on acetone were added to the dehydrated cells (50%, 75%, and 100% embedding ingredient in acetone). Epoxy resins were polymerized at 45 °C for 12 h, followed by 24 h at 60 °C. Embedded samples were sliced using an ultramicrotome (EM UC7; Leica, Wetzlar, Germany) and were deposited onto silicon wafers (6 × 6 mm, 0.5 mm thick; Sigma-Aldrich). MS-based cell imaging was performed using a NanoSIMS 50 (Cameca, Courbevoie, France) at the Korea Basic Science Institute (KBSI). The primary Cs^+ ion set to 16 keV collision energy was used for presputtering at 160 pA for 30 min and sputtering at 0.9 pA for 33 min. An ion beam was focused onto a 50 nm nominal spot on the sample surface during the analysis. The beam was rastered over a square region of 40 × 40 μm with a scanning resolution of 256 × 256 pixels. To measure carbon isotope abundances, we choose $^{13}\text{C}^{14}\text{N}$ and $^{12}\text{C}^{14}\text{N}$ as detected ions for calculating the $^{13}\text{C}/^{12}\text{C}$ ratio (Figure S1). The mass resolving power (MRP) with a coaxial path of primary and secondary ion beam was greater than 5000 to differentiate the $^{13}\text{C}^{14}\text{N}$ ion from $^{12}\text{C}^{15}\text{N}$, which is 0.007 m/z heavier.

Analysis of Volatile Organic Compounds (VOCs). VOC extraction *in vitro* employed a headspace–SPME technique as previously described.²⁹ Polyacrylate (PA) 85 μm , polydimethylsiloxane (PDMS) 100 μm , carboxen/polydimethylsiloxane (CAR/PDMS) 75 μm , polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 μm , and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm fibers (Supelco, Bellefonte, PA) were conditioned at temperatures and times recommended by the manufacturer. After the cells were transferred into a glass bottle 24 h before the end of each ^{13}C labeling period (0–7 days), VOC extraction using conditioned fibers was performed for 24 h. VOC-adsorbed fibers were analyzed by GC–MS (GCMS-QP2010, Shimadzu, Tokyo, Japan) equipped with a DB-5 ms capillary column (30 m × 0.25 mm, 0.25 μm ; Agilent Technologies, Palo Alto, CA). VOCs adsorbed on fibers were injected at the same temperature for conditioning in splitless mode (sampling time 30 s). High-purity helium (1 mL/min constant flow) was used as a carrier gas with the following column temperature program: an initial temperature at 70 °C, an increase to 150 °C at 4 °C/min, a hold at 150 °C for 1 min, an increase to 280 °C at 5 °C/min, and a hold for 3 min, for a total of 50 min. The compounds were then ionized using electron impact (EI) mode with a 70 eV filament at a 200 °C ion source temperature. The mass detection range was 40–500 m/z , with a scan rate of 2500 s^{-1} . A retention index (RI) was calculated using alkane mixture (C7–C40). VOC identification used RI (± 50) and mass spectrum (similarity index over 80) for comparison to the NIST library (NIST08, Gaithersburg, MD); 2-pentadecanone was confirmed using standard data from a reference compound.

Metabolites Extraction. Intracellular metabolites, including fatty acid and polar metabolites, were extracted using a biphasic extraction with direct solvent scraping method as previously described.³³ Cells for metabolite extraction were prepared without any media change. After removing media with ice-cold water three times, quenching and extraction were simultaneously achieved by adding 2 mL of 80% methanol at –70 °C with internal standards [5 $\mu\text{g}/\text{mL}$ nonadecanoic acid

(C19:0 fatty acid) for fatty acids; 5 $\mu\text{g/mL}$ glycine- d_3 , succinic acid- d_4 , and citric acid- d_4 for polar metabolites]. The collected cell pellets were fully disrupted by three freeze/thaw cycles in liquid N_2 followed by ice-cold freezer. After the final cycle, 1 mL of chloroform was added to perform a liquid–liquid extraction (LLE). Samples were vortexed for 10 s and centrifuged at 13 000g for 5 min, and the aqueous and organic phases were transferred to Eppendorf tubes and glass vials, respectively. LLE was repeated two more times. Collected organic phases were evaporated using a SpeedVac instrument (Savant AES2010, Thermo Fisher Scientific, Waltham, MA), and the aqueous phases were evaporated with a nitrogen purge.

Polar Metabolite Profiling. Polar metabolites were derivatized using a method previously described.³⁴ A methoxyamine hydrochloride solution (100 μL of 20 mg/mL in pyridine) was added to the polar metabolite extracts, and the reaction proceeded for 90 min at 37 $^\circ\text{C}$. The residues were trimethylsilylated using the same volume of *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) solution with 1% trimethylchlorosilane (TMCS). Derivatized samples were transferred to crimp vials and analyzed using the same GC–MS used for VOC analysis, with some parameter modification. The injection temperature was 300 $^\circ\text{C}$, and the instrument was operated in split mode (1:2). The column temperature program was slightly changed as follows: an initial temperature of 80 $^\circ\text{C}$ for 2 min, an increase to 100 $^\circ\text{C}$ at 4 $^\circ\text{C}/\text{min}$, a hold for 3 min, an increase to 200 $^\circ\text{C}$ at the same rate, a hold for 1 min, an increase to 300 $^\circ\text{C}$ at 8 $^\circ\text{C}/\text{min}$, and a hold for 2 min, for a total of 50.5 min. All metabolites were identified using the same method in VOC analysis, and the sugars and sugar phosphates were identified using standard compounds.

Fatty Acid Profiling. Fatty acids were derivatized using acidic methanolysis as previously described.³⁵ A 1 mL portion of methanol/37% hydrochloric acid solution (4:1) was added in a glass vial. Methylation was performed at 100 $^\circ\text{C}$ for 120 min. After cooling to room temperature, 1 mL of hexane was added for liquid–liquid extraction of methylated fatty acids and repeated twice. Hexane layers were transferred to the glass vial and reconstituted into 200 μL of hexane. GC–MS detection and the fatty acid identification protocol were the same as for the polar metabolites, and the column temperature program was modified as follows: an initial temperature of 70 $^\circ\text{C}$ for 1 min, an increase to 150 $^\circ\text{C}$ at 20 $^\circ\text{C}/\text{min}$, an increase to 180 $^\circ\text{C}$ at 6 $^\circ\text{C}/\text{min}$, an increase to 220 $^\circ\text{C}$ at 20 $^\circ\text{C}/\text{min}$, a hold for 1 min, an increase to 240 $^\circ\text{C}$ at 4 $^\circ\text{C}/\text{min}$, and a hold for 17 min, for a total of 35 min.

Data Processing and Statistical Analysis. All GC–MS data were aligned using MetAlign 3.0 software.³⁶ The extracted areas of isotopic m/z (mass to charge ratio) of each compound were chosen to measure the abundance of labeled isotope. MDV, a corrected natural abundance of each isotope (^{13}C , 1.07%; ^{15}N , 0.368%; ^2H , 0.0115%; ^{17}O , 0.038%; ^{18}O , 0.205%; ^{29}Si , 4.6832%; ^{30}Si , 3.0872%), was calculated using IsoCor, an MDV correction tool.³⁷ The corrected MDV of intracellular metabolites was mapped onto the central carbon metabolism pathway using VANTED³⁸ connected to SBGN plugin.³⁹ NanoSIMS data were analyzed using WinImage software (Cameca) to obtain $^{13}\text{C}^{14}\text{N}/^{12}\text{C}^{14}\text{N}$ and $^{12}\text{C}^{14}\text{N}$ images and ImageJ⁴⁰ with openMIMS plugin⁴¹ software (MIMS, Harvard University, Cambridge, MA; <https://nano.bwh.harvard.edu/openMIMS>) to extract the values of each detected ion. Linear regression analysis and Pearson's correlation coefficient for VOCs, and Michaelis–Menten equation for calculating

isotopic steady state were performed using Graphpad Prism 7.0 software (Graphpad Software, Inc., San Diego, CA). To estimate the coefficients K and V for every flux, we used the Gauss–Newton algorithm using the “nls” function in R language.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00296.

Flux analysis of intracellular metabolites; optimization of HS–SPME; cell population-dependent abundance of volatiles; determination of detected ions by nanoSIMS; ^{13}C measurement in cancer cells; searching for isotope-enriched volatiles; statistical analysis of labeled/unlabeled ratio in each cell line; and mass isotopologues of metabolites and fragment ions of 2-pentadecanone (PDF)

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Author Contributions

D.-K.L. and S.W.K. conceived and designed the study. D.-K.L. performed mass spectrometry-based cellular imaging. D.-K.L. and E.N. carried out profiling of polar metabolites and fatty acids. S.P. and J.L. undertook the kinetic modeling for estimation of isotopic steady state. D.-K.L., S.P., J.H.P., and S.W.K. wrote the manuscript. All authors commented on and accepted the final version.

Notes

The authors declare no competing financial interest.

Safety statement: no unexpected or unusually high safety hazards were encountered.

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