Supporting Information

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SI Materials and Methods

Cell Culture. All of the cells were stored in liquid nitrogen. Fresh aliquots thawed from liquid nitrogen were used in less than 3 mo. All of the cells were cultured in DMEM supplemented with 10% FBS (Invitrogen) in 1:100 dilution of an antibiotic-antimycotic solution (Invitrogen) at 37 °C in a 5% CO_2 incubator. Cultured fibroblasts up to 10 passages were used. Serum-starved Hs68 foreskin fibroblasts (HsFb) were prepared by washing Hs68 cells and incubating them in serum-free medium for 24 h. Proliferative HsFb were prepared by washing the serum-starved HsFb and incubating them in medium containing 2.5% FBS for 24 h.

Materials. Phorbol 12-myriste 13-acetate (PMA), 5-hydroxytryptophan (5-HTP), 5-methoxytryptophan (5-MTP), and L-tryptophan were from Sigma-Aldrich. Stock 5-MTP was dissolved in 0.3 N HCl with further dilution with double-deionized water. Cells were pretreated with the tryptophan-related compounds for 30 min before addition of PMA for 4 h.

Western Blot Analysis. In brief, whole-cell lysates were prepared in cold RIPA lysis buffer (Upstate) containing 0.5 M Tris·HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% Nonidet P-40, 10 mM EDTA, and a complete EDTA-free protease inhibitor mixture (Roche) and centrifuged at 4 °C (10 min, 13,000 × *g*). The protein concentration of the supernatants was determined at 595 nm using the Bio-Rad protein assay. Twenty micrograms of proteins diluted in sample buffer were denatured at 95 °C for 10 min and electrophoretically separated on 10% SDS polyacrylamide minigel (Bio-Rad). Proteins were blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech) for 1 h. After blocking in 0.5 M Tris-Base (pH 7.4) containing 5% milk powder, 1.5 M NaCl, and 0.05% Tween 20, the membranes were incubated with polyclonal rabbit anti–cyclooxygenase-2 (anti–COX-2) (1:1,000; Cell Signaling Technology) and monoclonal mouse anti–β-actin (1:10,000; Sigma-Aldrich). The protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) and analyzed by densitometry.

Cell Migration and Invasion Assays. A549 cells (10^4/well) were seeded on the upper chamber (6.5-mm insert, 8-μm polycarbonate membrane; Corning Inc.), which was placed in each well of a 24-well plate. The lower chamber was filled with 700 μL DMEM supplemented with 10% FBS. For assessment of in vitro invasion, cells were seeded onto the upper chamber insert coated with Matrigel (BD Biosciences) under the same conditions as the Transwell migration assays. At the indicated time, nonmigrated cells that remained at the top surface of the insert were removed with a cotton swab. Cells that migrated to the lower membrane surface and cells that invaded through the matrigel gel to the underside of the membrane were fixed, stained with 0.1% crystal violet for 20 min, and counted under light microscopy. All of the assays were done in triplicate with excellent reproducibility.

siRNA Transfection. siRNA or scramble control RNA (scRNA) plasmids (1 μg DNA in 10 μL) and transfection reagent (5 μL) were incubated in 200 μL serum-free medium for 20 min at room temperature. The mixture was added dropwise to each well and incubated for 7 h. The cells were then washed twice with PBS and maintained in culture medium for 24 h.

Analysis of COX-2 Promoter Activity. A promoter region of human COX-2 gene (−891 to +9) was constructed into a luciferase reporter vector pGL3 as previously described (1). In brief, 4 μg of the promoter vector or control pCL vector was mixed with 10 μL of lipofectamine 2000 (Invitrogen). The mixture was slowly added to cells in a six-well plate and incubated for 24 h. After treatment, cells were lysed, and luciferase activity was measured using an assay kit from Promega in a luminometer (TD 20/20). The cells were washed and incubated in serum-free medium for 24 h before evaluating the COX-2 promoter activity.

Methylthiazole Tetrazolium Assay for Cell Proliferation. A549 cells (1×10^4) were added to each well in a 96-well plate, pretreated with 5-MTP followed by PMA for 4 h or 24 h at 37 °C. Cells were incubated with methylthiazole tetrazolium (MTT) [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) at a final concentration of 1mg/mL for 2h at 37 °C. Yellow MTT solution was removed and formazan was solubilized by adding 100 μL dimethyl sulfoxide. The sample was mixed thoroughly until formazan crystals were dissolved completely. This mixture was applied to an ELISA reader (TECAN, Infinite 200PRO, NanoQuant) with absorbance at 570 nm and a reference wavelength at 690 nm.

Preparation of CMF2. Fibroblasts or cancer cells at 60–70% confluency were cultured in T175 (30 flasks per cell type) or a 15-cm dish (50 dishes per cell type) containing serum-free DMEM. After 24 h incubation, cells were washed and incubated in DMEM containing 2.5% FBS for 24 h. The cultured medium was collected and subjected to ultrafiltration through a 10-kDa membrane. The <10-kDa fraction with positive COX-2 inhibitory activity was collected, lysophilized, and extracted by methanol. The extract was again lysophilized and suspended in di-deionized water. The reconstituted extract was chromatographed on a Superdex 30 column by FPLC and eluted with PBS (pH 7.4). Five peaks were detected at 220 nm. Fractions corresponding to each peak were collected, concentrated, and reconstituted with cultured media. Their COX-2 inhibitory activity was determined by Western blotting. Fractions corresponding to peak 2 (CMF2) prepared from HsFb conditioned medium possessed anti–COX-2 activity.

Metabolomic Analyses. For ultraperformance liquid chromatography (UPLC), a 1.7-μm (2.1 × 100 mm) C18 column (Acquity UPLC System; Waters Corporation) was used. The liquid chromatography separation was carried out at 40 °C with a flow rate of 0.2 mL/min using the following gradient for the analysis: 0–2 min 2% B, 2–6 min from 2% B to 10% B, 6–10 min from 10% B to 30% B, 10–16 min 100% B and 16–20 min 100% A [solvent system A: water/formic acid (100:0.1, vol/vol); B: acetonitrile/formic acid (100:0.1, vol/vol)]. The quardrupole-time of flight (QTof) mass spectrometry (MS) system (QTof-MS) was operated in positive electrospray ionization mode with a mass resolution larger than 10,000. Data were acquired by MassLynx software (Waters Corporation) in uncentroided format over a mass range of *m/z* 100–800 with scan duration of 0.5 s and an interscan delay of 0.1 s. The capillary and the cone voltage were maintained at 3,200 and 40 V and the desolvation and source temperature at 300 °C and 80 °C, respectively. Nitrogen was used as cone (25 L/h) and desolvation gas (800 L/h). For accurate mass measurement, the QTof-MS was calibrated with formic acid 0.1% (vol/vol) in acetonitrile/water (50:50, vol/vol), and the dynamic range enhancement mode was used for data recording. All analyses were monitored by using leucine-enkephaline ($[M+H]^+$ ion at 556.2771 or $[M-H]^-$ ion at 554.2615) (Sigma-

Aldrich) as lock spray reference compound at a concentration of 0.2 μg/mL in acetonitrile water (50:50, vol/vol) and a flow rate of 10 μL/min. The raw mass spectrometry data of all samples were processed using the MassLynx software (Waters Corporation).

5-MTP Enzyme-Immunoassay. 5-MTP was measured by a modified sandwich enzyme-immunoassay (EIA) in a 96-well microtiter plate coated with polyclonal rabbit anti-MTP antibodies (Abcam) using a coated buffer, 0.05 M carbonate-bicarbonate (pH 9.6) at 4 °C overnight. After washing and treatment with blocking buffer, 5-MTP standards or samples (conditioned medium) were added to the wells and incubated at room temperature for 1 h. The wells were washed and treated with a polyclonal rabbit anti-5-MTP antibody (Abcam) which is highly selective for 5-MTP with very low cross-reactivity with the following metabolites: cross-reactivity ratio was 1:>100,000 with 5-HTP; 1:5,800 with Try and 1:100,000 with 5-methoxytryptamine followed by biotinylated anti-rabbit IgG (Abcam). Streptavidin-conjugated horseradish peroxidase (Upstate) and tetramethylbeuzidine (Upstate) substrate were added, and the product was analyzed at 450 and 570 nm. The calibration curve was established by using pure 5-MTP at concentrations of 10–3,000 ng/mL. The curve is linear up to 2,000 ng/mL (Fig. S5).

Bioluminescent Imaging. The bioluminescent imaging was monitored weekly be IVIS Spectrum imaging system (Caliper Life Sciences), which consists a cooled CCD camera mounted in a light-tight imaging chamber and XGI-8 gas anesthesia system. Imaging and quantification of signals were controlled by the acquisition and analysis of Living Imaging 4.0 software (Caliper Life Sciences). The mice were intraperitoneally injected with 150 mg luciferin/kg body weight 10 min before imaging, anesthetized by 2–3% isoflurane in an induction chamber, and then placed in the imaging chamber under continuous exposure to 1–2% isoflurane through nose cones. Five mice were imaged together, and an image was taken for ~2 s. Bioluminescence intensity was quantified as the sum of all detected photons/second within the region of interest after subtraction of background luminescence.

1. Deng WG, Zhu Y, Wu KK (2004) Role of p300 and PCAF in regulating cyclooxygenase-2 promoter activation by inflammatory mediators. *Blood* 103:2135–2142.

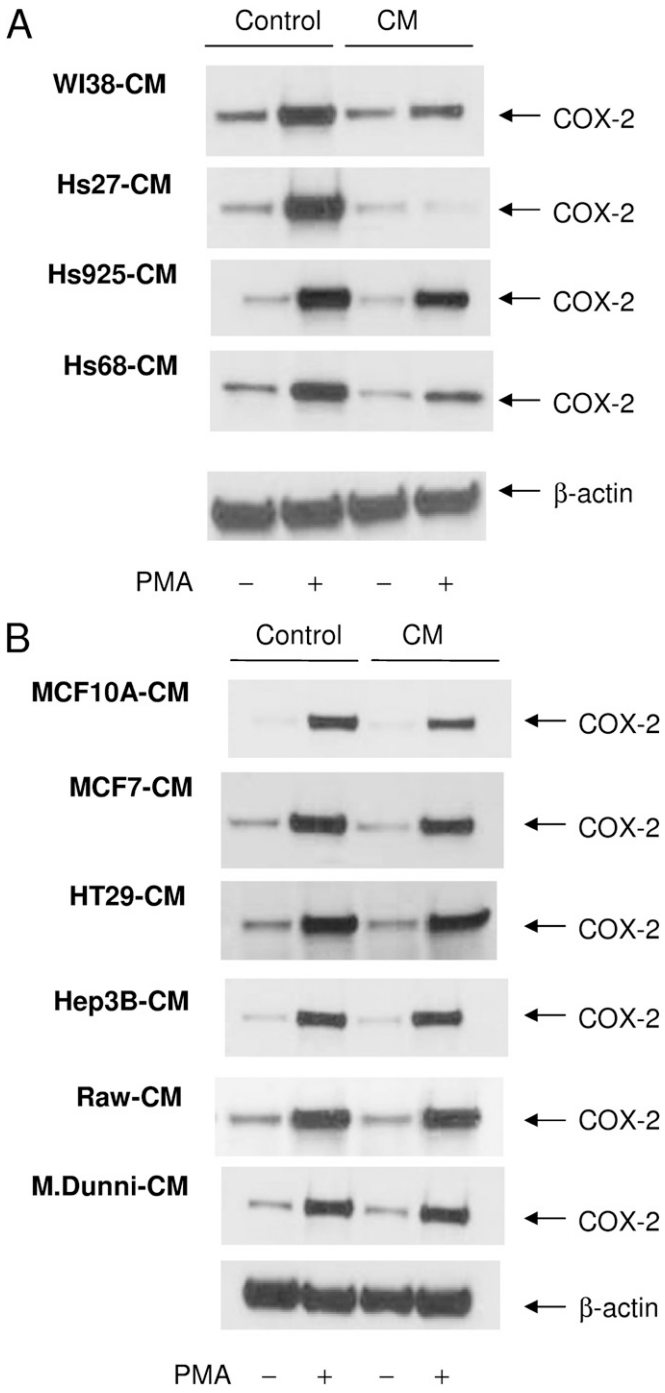

Fig. S1. Influence of conditioned medium (CM) on COX-2 expression. (*A*) CM of various types of fibroblasts inhibits PMA-induced COX-2 expression in A549 cells. WI38 is a human lung fibroblast; Hs27 and Hs68, foreskin fibroblasts; and Hs925sk, adult human skin fibroblasts. All experiments were performed at least twice with consistent results. (*B*) CM of cancer cells does not inhibit PMA-induced COX-2 expression. MCF10A, a human breast epithelial cell, was included as a reference for MCF7, a breast cancer cell line. HT29 and Hep3B are colorectal and hepatocellular cancer cells, respectively. RAW (RAW264.7) and M. Dunni are transformed murine macrophage and skin cell lines, respectively.

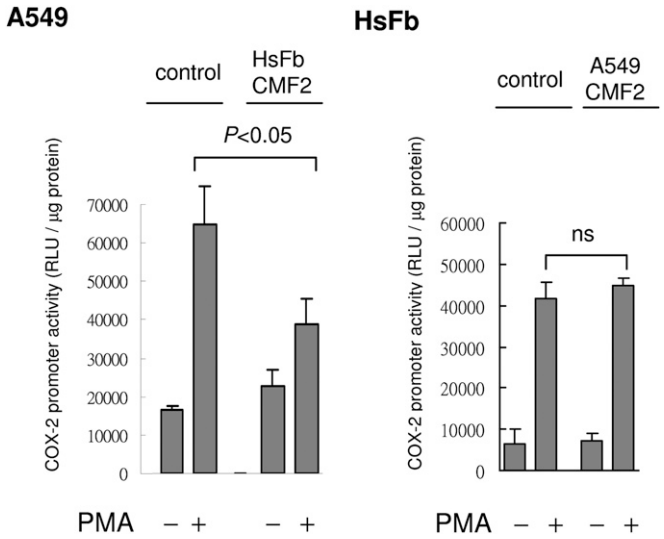

Fig. S2. HsFb factors inhibit PMA-induced COX-2 promoter activity in A549. CMF2 was prepared from the CM of HsFb or A549 as described in *SI Materials and Methods*. HsFb CMF2 inhibited A549 COX-2 expression whereas A549 CMF2 had no effect on HsFb COX-2 expression induced by PMA. Error bars denote mean ± SEM (n = 3). ns, statistically nonsignificant.

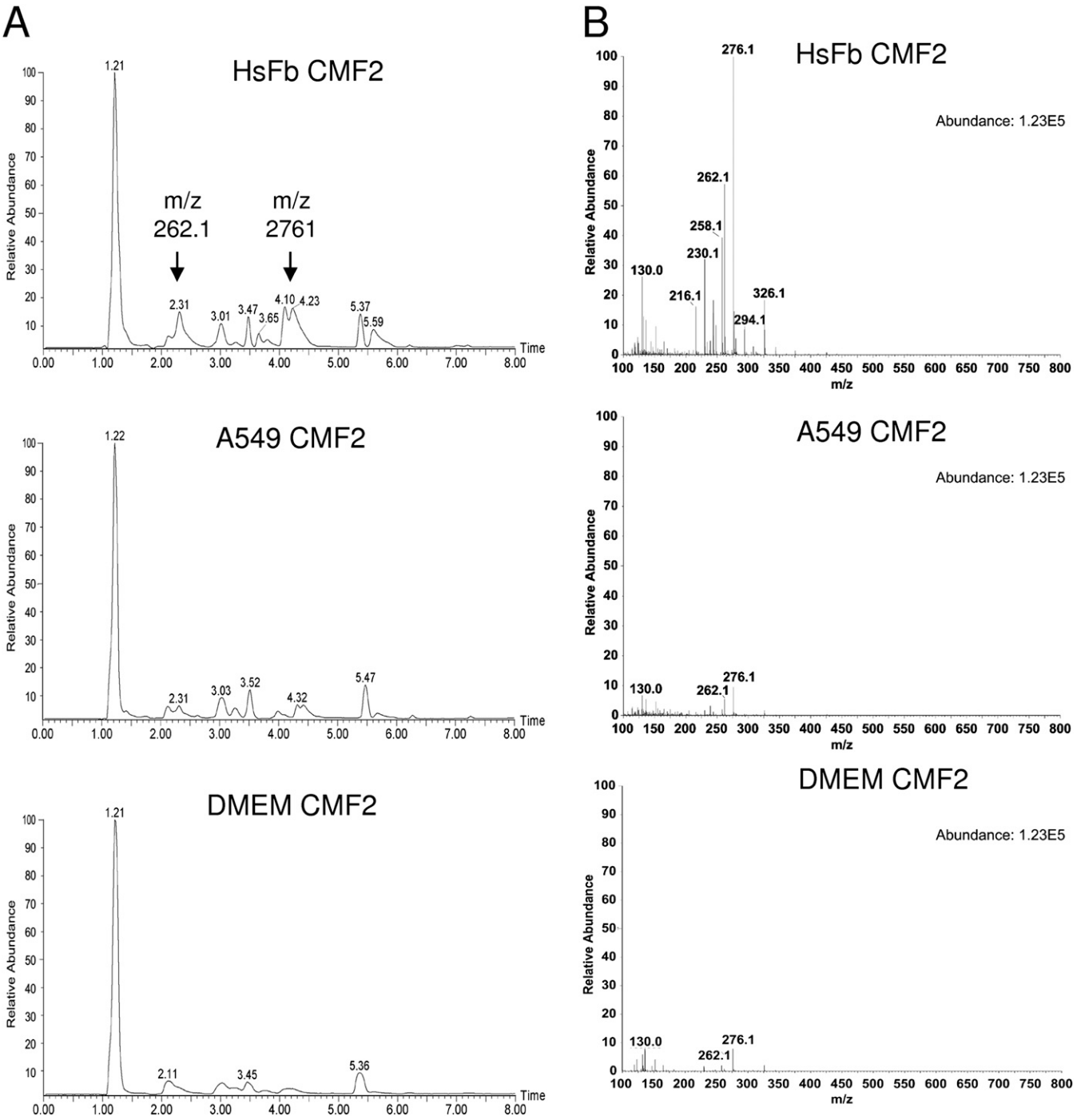

Fig. S3. Metabolomic analysis of CMF2 by UPLC-QTof mass spectrometry. (*A*) UPLC chromatography. DMEM CMF2 refers to medium control. The arrows in the *Top* panel denote the indicated *m/z* as demonstrated by mass spectrometric analysis of the specific chromatograph peaks. (*B*) Mass spectra show elevation of several peaks between *m/z* 100 and 350, notably *m/z* 276.1 and 262.1 in the CMF2 of HsFb compared with that of A549. The mass spectra of A549 CMF2 closely resemble the control medium.

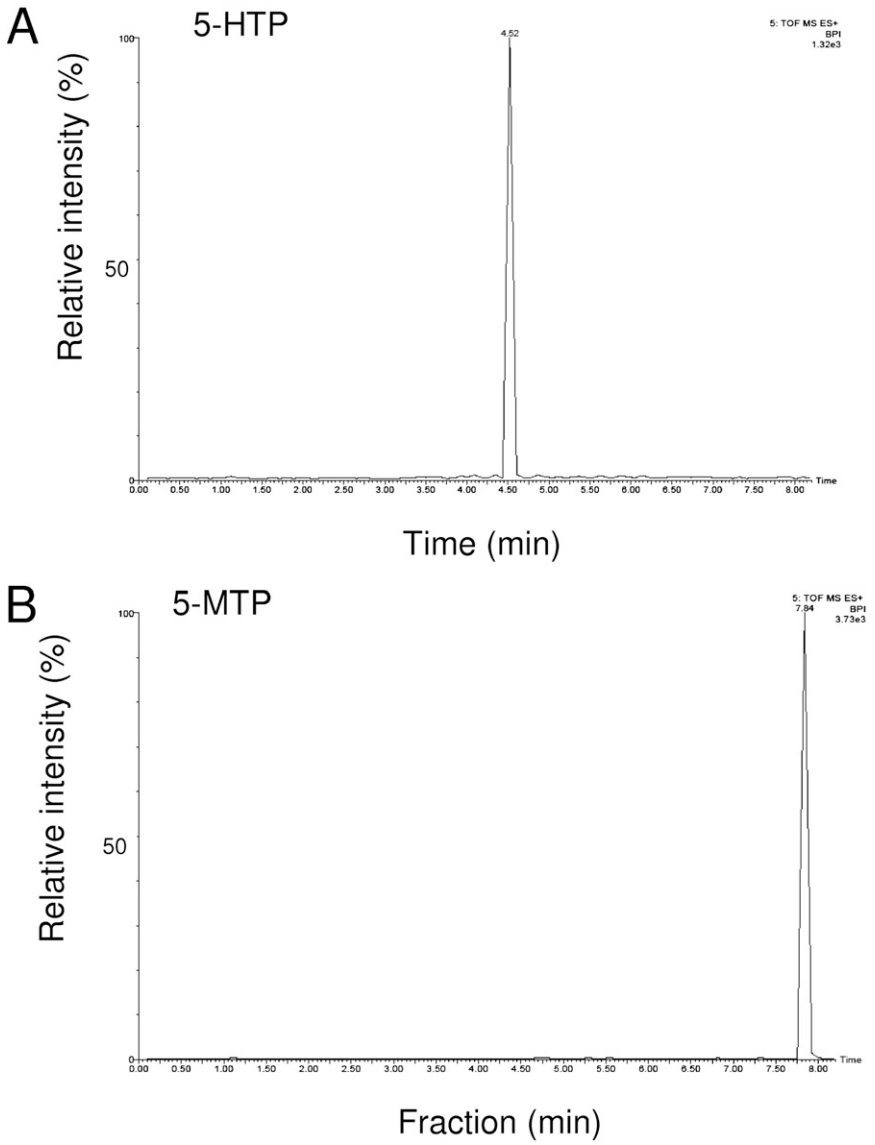

Fig. S4. Validation of purity of 5-HTP and 5-MTP preparations. (*A*) 5-HTP (10 μM) or (*B*) 5-MTP (10 μM) was analyzed using UPLC-QTof mass spectrometry. A single peak was detected.

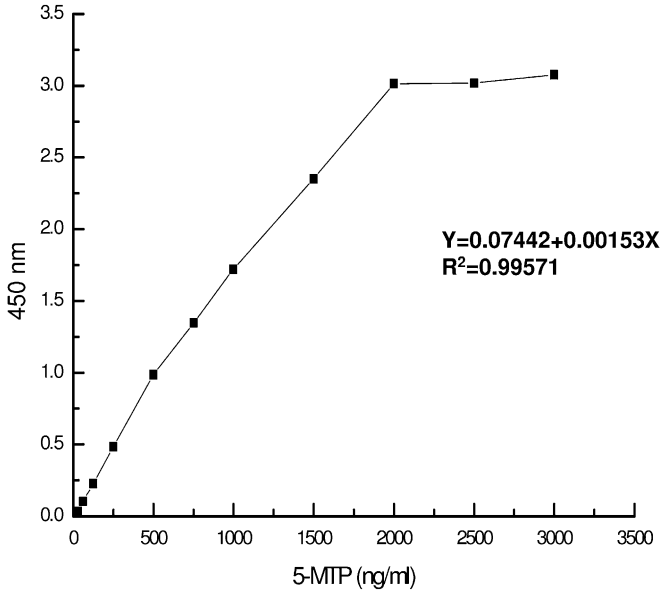

Fig. S5. A representative calibration curve for measuring 5-MTP by EIA. 5-MTP EIA was performed as described in *SI Materials and Methods*. The calibration curve was linear from 0 to 2,000 ng/mL.

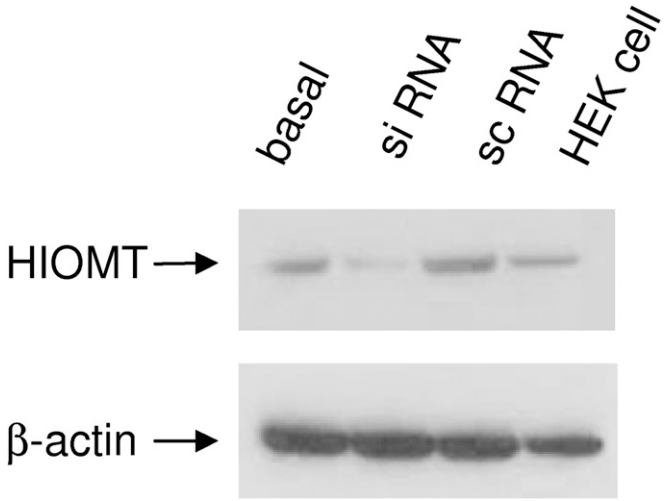

Fig. S6. Silencing of hydroxyindole O-methyltransferase (HIOMT) expression by siRNA. HsFb were transfected with HIOMT siRNA or control scRNA. HIOMT proteins were analyzed by Western blotting. HEK cell lysates were analyzed to serve as positive control.

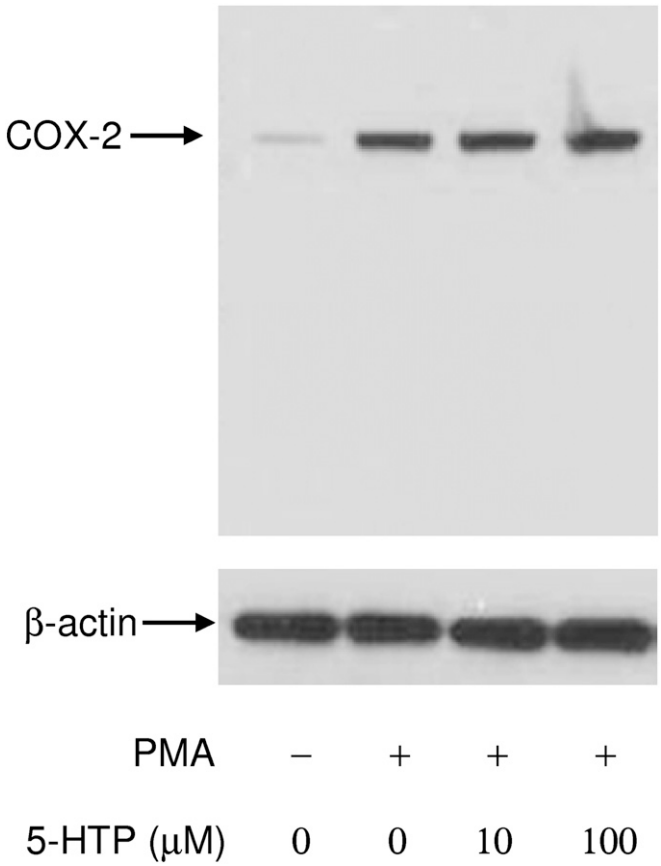

Fig. S7. 5-HTP does not inhibit PMA-induced COX-2 proteins in A549 cells. A549 cells were pretreated with 5-HTP at the indicated concentration for 2 h followed by PMA for 4 h. COX-2 proteins were analyzed by Western blotting.

Vehicle group	No. of histology-confirmed metastatic nodules
C31	6
C33	28
C35	12
C36	13
C37	7
5-MTP group	
M44	7

Fig. S8. Analysis of metastatic lung nodules. Ten mice in the 5-MTP–treated group and 10 in the vehicle control group were euthanized on day 52 after drug administration. Lungs were isolated and inspected for nodules. Sections were made and stained with H&E. Metastatic cells were examined under microscopy. Each code number listed under vehicle group or 5-MTP group refers to an individual mouse.

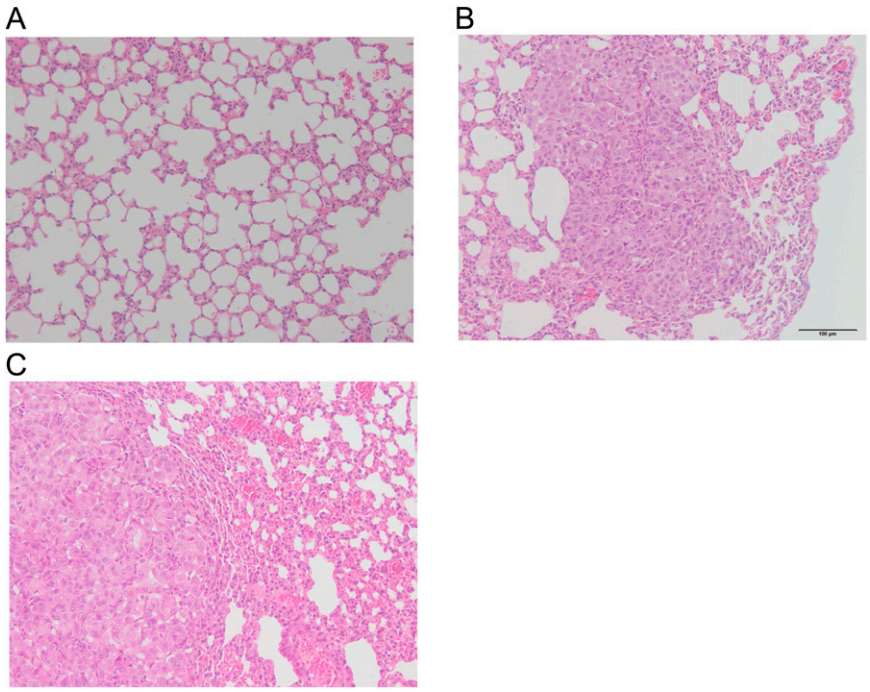

Fig. S9. Representative photomicrographs of normal lung tissues and metastatic nodules. Lung tissues of the murine xenograft tumor model were resected and stained with H&E. (*A*) Normal lung tissue. (*B*) Metastatic lung cancer cells in vehicle-treated mouse C37. (*C*) Metastatic lung cancer cells in the only 5-MTP–treated mouse (M44).