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Supplementary Materials for

Mechanoresponsive stem cells to target cancer metastases through biophysical cues

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Published 26 July 2017, *Sci. Transl. Med.* **9**, eaan2966 (2017)
DOI: 10.1126/scitranslmed.aan2966

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Supplementary Materials:

Materials and Methods

Cell lines and cell culture

Human bone marrow MSCs were obtained from the Texas A&M Health Science Center and were expanded to passages 3-6 for further use. The cells were routinely maintained in Minimum Essential Medium α (MEM α, Life Technologies) supplemented with 15% fetal bovine serum (FBS, Atlanta Biologicals) and 1% penicillin-streptomycin (PenStrep, 100 U/ml, Life Technologies) at 37°C in a humidified incubator containing 5% CO_2. The human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC). These cells were grown in Leibovitz's L-15 medium containing L-glutamine (Corning), and supplemented with 10% FBS and 1 U/ml PenStrep at 37°C in a humidified incubator without CO_2. The 293T-LV cell line (Gen Target) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% FBS, non-essential amino acids (NEAA, 1X, 100 U/ml, Life Technologies) and 1 U/ml PenStrep at 37°C in a humidified incubator containing 5% CO_2.

Generation of lentiviral vectors and lentiviral transduction

The following lentiviral vectors were used in this study: LV-*CMV*::eGFP, LV-*CMV*::Luc-RFP, LV-*CMV*::CD, LV-MRCS-eGFP, LV-MRCS-Luc, and LV-MRCS-CD. The sequences of interest from pUCBB-eGFP (a gift from Claudia Schmidt-Dannert (*57*), Addgene #32548), pcDNA3.1(+)/Luc2=tdT (a gift from Christopher Contag (*58*), Addgene #32904), pSelect-zeo-*Fcy*::*Fur* (InvivoGen) and 8xGTIIC-luciferase (a gift from Stefano Piccolo (*32*), Addgene #34615) were cloned into the promoterless lentiviral transfer vector LV-PL4 (GenTarget). All

MSCs and breast cancer cells were transduced as previously described (*42*). Briefly, all lentiviral constructs were packaged (gifts from Didier Trono, pMD2.G, Addgene #12259; pRSV-Rev, Addgene #12253; pMDLg/pRRE, Addgene #12251) as lentiviral (LV) vectors in 293T-LV cells using Lipofectamine LTX and PLUS Reagents (Life Technologies) (*42*). Cells were transduced with LVs by incubating virions in a culture medium containing 100 μg/ml protamine sulfate (Sigma). Cells transduced with LVs containing empty vectors (EV) were used as a control. After selection with medium containing 10 μg/ml puromycin (MP Biomedicals), fluorescent protein-expressing cells were visualized for protein expression using fluorescence microscopy (Eclipse Ti, Nikon).

Hydrogel synthesis and immunocytochemistry (in vitro)

Polyacrylamide tunable hydrogels coated with collagen were synthesized as previously described (*59*). The stiffness of hydrogels was attuned by adjusting the ratio of acrylamide and bis-acrylamide (*59*). Cells were seeded in droplets to evenly spread onto the hydrogels and harvested for further assays 24-48 hours later, according to previous literature (*32*). To test if the MRCS was stiffness-dependent, in some experiments, 50 μM (−) blebbistatin (Sigma), 10 μM ML-7 (Sigma), or 20 μM PF 573228 (PF228, Sigma) were added to the MRCS after attachment. Cells were briefly fixed in 4% paraformaldehyde (PFA, Amresco) and permeabilized in 0.1% IGEPAL CA-630 (Sigma). Primary antibodies (mouse anti-YAP 1: 100; sheep anti-CD 1: 200; chicken anti-eGFP 1: 500) were incubated overnight in phosphate buffered saline (PBS, Lonza) with 0.1% Triton X-100 (Sigma) and 2% goat (Thermo Fisher Scientific) or donkey serum (Sigma) for double-staining. Secondary antibodies were diluted 1: 500 and were applied for 30 minutes at room temperature. Slides were washed in PBS and mounted with Fluoromount-G

(Southern Biotech). DAPI (50 μg/ml, Life Technologies) in PBS was added onto samples before mounting. All the antibodies used in this experiment are listed in table S1 (primary antibodies) and table S2 (secondary antibodies). Protein expression was quantified by normalizing the average of fluorescence intensity within the cells to the glass control using the NIS-Elements AR software (Nikon) after background subtraction. Triplicate samples were used for the analysis.

For calculating the ratio of MRCS which was activated with the stiffness-sensing promoter, a threshold was set using mean + 2 x SD of fluorescent signals of MRCS seeded on ~ 1 kPa hydrogels. The same threshold was applied to all cells seeded on hydrogels with different stiffness, and all cells with fluorescent signal higher than this threshold were defined as activated.

Quantitative reverse transcriptase PCR (RT qPCR)

Hydrogels were synthesized and cells were seeded as above. To test the expression of mRNAs regulated by mechano-cues in vitro, cells were harvested from hydrogels, glass coverslips, or tissue culture plates in TRIzol (Invitrogen) for total RNA extraction with DNase I (Thermo Fisher Scientific) treatment to remove DNA contamination according to the manufacturer's protocol. cDNA synthesis was performed with Oligo(dT) (Invitrogen) primed SuperScript III RNase H Reverse Transcriptase (Invitrogen) and Power SYBR Master Mix (Life Technologies). RT qPCR was performed in quadruplicates on an Applied Biosystems ViiA 7 Real-Time PCR System, and data were analyzed with ViiA^{M} 7 Software v1.2. Relative gene expression was normalized to the endogenous gene *GAPDH.* Sequences of primers (*32, 60, 61*) used in this study [Integrated DNA Technologies (IDT)] are provided in table S3.

In vitro bioluminescence assays and XTT cell viability assays

LV-*CMV*::Luc MSCs (Luc-MSC) expressing firefly luciferase (Luc) and LV-MRCS-Luc MSCs (MRCS-Luc) were seeded onto collagen-coated hydrogels with different stiffness as described (*32*). After the cells were washed with PBS, D-luciferin (150 μg/ml in PBS, Perkin Elmer) was added, and the activity of Luc was then measured. MSCs [LV-MRCS-CD MSCs (MRCS-CD), LV-*CMV*::CD MSCs (CD-MSC) or native MSCs (N-MSC)] were seeded onto hydrogels with MDA-MB-231 breast cancer cells with a ratio of 2: 1 (231: MSCs). Prodrug 5-fluorocytosine (5-FC, 800 μg/ml in MSC growth medium, Sigma) was added after attachment. Reagents from XTT kit (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5 Carboxanilide, ATCC) were mixed and added to cells after 5 days of co-culture. The color absorbance was measured after 2 hours of incubation at 37°C in a humidified incubator. Bioluminescent signals and color absorbance were measured with a plate reader (BioTek). All samples above were measured at least in triplicate.

Sample preparation and ultra high performance liquid chromatography-electrospray ionization tandem quadrupole mass spectrometry (UPLC-MS/MS)

MSCs (MRCS-CD, CD-MSC, or N-MSC) were seeded at a density of $10^4/cm^2$ on the hydrogels with varying stiffness or cover slides. Cells were allowed to grow for 24 hours in MSC growth medium and were then treated with 800 μg/ml 5-FC (Day 0). On Days 1, 2, and 5, conditioned medium was collected for extraction. 200 μl of the conditioned medium from each condition was extracted using 1000 μl of ethyl acetate: isopropanol = 1: 1 (v:v) (Sigma). For in vivo experiments, whole lungs were collected from NSG mice 1 day after treatment with 5-FC (500 mg/kg in DPBS). The tissue was homogenized using Bel-Art Micro-Tube Homogenizer (Bel-

Art) and extracted using 500 µl of ethyl acetate: isopropanol: acetic acid = 84: 15: 1 (v:v:v) (Sigma). Organic phase was collected after centrifugation at 3,000 *g* for 10 minutes. Additional protein precipitation was done by adding 80 µl of saturated ammonium sulfate solution. After centrifuging at 3,000 *g* for 10 minutes, the organic layer was transferred into a new microcentrifuge tube and dried using a centrifugal vacuum concentrator (Speedvac, Thermo Fisher Scientific). The extracted compounds were reconstituted in 250 µl water containing 20% acetonitrile, and 100 µl was used for the UPLC-MS/MS.

Culture medium spiked with 400 µg/ml 5-FC and 5-FU was used to determine the extraction yield. A 6-point 5-FU standard curve (⅓ dilution starting from 10 µg/ml) with a $R^2 >$ 0.98 was used to quantify the 5-FU in the samples. 5-FU standard solutions were prepared in water with 20% acetonitrile. 10 µl was injected into the UPLC system for analysis, and then eluted on an Acquity UPLC BEH C18 1.7 µm column (Waters). UPLC was performed using the Acquity UPLC system (Waters) with a mobile phase gradient starting with 98% of a mobile phase composed of 98% water, 2% acetonitrile, and 0.2% acetic acid, which progressively went up to 95% of the second mobile phase (100% acetonitrile and 0.2% acetic acid) for the elution of the samples. Then, the samples were injected into the triple quad mass spectrometer (Waters Micromass Quattro Premier XE Tandem Quadrupole Mass Spectrometer, Waters) for the mass analysis. The electrospray ionization was performed using the negative ion mode, which generates a precursor to product ion transition of m/z 129 > 42 for the 5-FU. After training, the cone voltage (CV) and the collision cell energy (CE) were optimized at 20V and 30V, respectively. Dwelling time was 0.285 seconds, and total run time per sample was 3 minutes. MassLynx software was used for data acquisition, and QuantLynx software for the data analysis and quantification.

Transwell co-culture assays

MSCs (MRCS-CD or N-MSC) were plated on type I collagen (BD Biosciences)-coated 6.5 mm transwell culture inserts with pore size of 0.45 μm (Corning Life Sciences), while Luc-RFP-231 cancer cells were plated on the lower chamber. After cell seeding, both of them were allowed to grow for 24 hours in MSC growth medium separately before the transwell got assembled. Then the MSCs and cancer cells started to be co-cultured with 800 μg/ml of 5-FC in MSC growth medium (Day 0). On Days 0, 1, 2, 5, 7, and 9, XTT assays were performed to measure the cell viability. Procedures were similar to the above XTT section, except XTT reagents were collected after the incubation of the reagents, and redistributed to a new 96-well plate for measurement. XTT reagent was washed off with sterile PBS and MSC growth medium after each measurement. Cells were then re-incubated in growth medium until the next time point of measurement. All values were normalized to that of cancer cells alone without co-culture at each time point. In an independent experiment, inserts with MSCs were removed on Days 1, 2, 7, and 9, and an in vitro proliferation luciferase assay was performed on Day 9 to quantify the living Luc-RFP-231 cells. All values were normalized to that of cancer cells co-cultured with N-MSC.

Breast cancer lung metastasis animal models

0.5 x 10^6 (2.5 x 10^6/ml in Dulbecco's Phosphate-Buffered Saline (DPBS, Lonza)) LV-*CMV*::Luc-RFP MDA-MB-231 (Luc-RFP-231), or LV-*CMV*::eGFP MDA-MB-231 (eGFP-231) breast cancer cells were infused intravenously (*i.v.*) into immunocompromised female nude mice (8 weeks, #088, Charles River Laboratories) or NOD-SCID gamma (NSG) mice (8 weeks, #005557, The Jackson Laboratory). For the spontaneous metastasis model, 1 x 10^6 Luc-RFP-231

cells in Matrigel were implanted subcutaneously (*s.c.*) into the fat pads of female NSG mice (8 weeks). All animals injected with cancer cells or DPBS as healthy controls were picked randomly. All animal experiments and procedures were performed after the approval from the University of California Irvine (UCI) Institution of Animal Care and Use Committee (IACUC protocol number 2012-3062) and conducted according to the Animal Welfare Assurance (#A3416.01).

MSC transplantation and prodrug treatment

Four (NSG *i.v.* model) or six (nude *i.v.* model or NSG *s.c.* model) weeks after Luc-RFP-231 cell transplantation, 1 x 10^6 LV-*CMV*::eGFP MSCs co-transduced with MRCS-CD (eGFP-MRCS-CD), LV-MRCS-eGFP MSCs (MRCS-eGFP), CD-MSC, MRCS-CD, N-MSC (5 x 10^6 /ml in DPBS), or DPBS were *i.v.* infused into the mice harboring breast cancer cells and into tumor-free control mice (Day 0) through the tail vein. For second harmonic generation (SHG) imaging and immunohistochemistry (IHC) (ex vivo), animals infused with eGFP-MRCS-CD or MRCS-eGFP were euthanized *(n* = 3 for each group) 24 hours later (Day 1) and lungs were harvested. For the cancer treatment experiment, Luc-RFP-231 tumor-bearing or tumor-free mice infused with CD-MSC, MRCS-CD, N-MSC, or DPBS were intraperitoneally (*i.p.*) treated with 5-FC (500 mg/kg in DPBS) for 7 days (two doses/day for Day 1-Day 5 and one dose/day for Day 6-Day 7). Representative mice (*n* = 3 for each group) were euthanized on Day 1 and Day 9 for ex vivo assays. For the survival experiment, the endpoint for mice was defined as "found dead" or euthanasia criteria stated in UCI IACUC protocol 2012-3062. For the survival experiment, the endpoint for mice was defined as "found dead" or euthanasia criteria stated in UCI IACUC protocol 2012-3062: that is, a condition scoring system was used to monitor if the mouse has

body weight loss (0 to 3), abnormalities on physical appearance (0 to 3), unprovoked behavior (0 to 3), and/or abnormal behavioral responses to external stimuli (0 to 3), with 0 being healthy. Any mouse was to be euthanized if 1) the total score was ≥ 5 or 2) it scored a "3" for any variable regardless of the total score.

In vivo and ex vivo bioluminescence imaging

Four (NSG *i.v.* model) or six (nude *i.v.* model or NSG *s.c.* model) weeks after Luc-RFP-231 cell transplantation, in vivo Luc activity from Luc-RFP-231 cells was measured (Day 0) as previously described (*42*). Briefly, in vivo Luc signal was imaged with IVIS Lumina (Caliper LifeSciences) 10 minutes after intraperitoneal (*i.p.*) injection of D-luciferin (150 mg/kg in DPBS) into mice. Mice were anesthetized with 2-3% of isoflurane (Western Medical Supply), and in vivo Luc activity was measured at the indicated time points. Before cancer treatment, nude mice with Luc signals in the lungs were imaged and grouped randomly. Minimal adjustment was performed to keep the differences between "week 0" groups (red spots) not statistically significant. After the cancer treatment experiments, in vivo Luc activity from Luc-RFP-231 cells was measured on Day 9 and 6 weeks after treatment.

Four (NSG) or six (nude) weeks after eGFP-231 cell transplantation, 1 x 10^6 Luc-MSC, MRCS-Luc, N-MSC (5 x 10^6 /ml in DPBS), or DPBS were systemically infused into the mice harboring breast cancer cells and into tumor-free control mice (Day 0). In vivo Luc activity was measured at the indicated time points.

Six weeks after *s.c.* Luc-RFP-231 cell transplantation, mice were sacrificed and lungs were harvested. 150 μg/ml D-luciferin was added onto the lungs, and ex vivo Luc activity from Luc-RFP-231 cells in the lungs was measured with IVIS Lumina.

Tissue processing, histology, and TUNEL assays

Lungs, livers, and brains were collected from tumor-bearing or tumor-free nude mice (Day 0 or Day 9). After overnight fixation in 4% PFA, followed by overnight incubation in 30% sucrose solution (Amresco), samples were flash frozen in Tissue-Tek O.C.T Compound (Sakura Finetek). Frozen sections 8 µm thick were taken using a Reichert-Jung Cryocut 1800 microtome (Leica Instruments) onto UltraClear positively charged slides (Denville, 25 x 75 x 1 mm). Bones were fixed overnight in 4% PFA, then decalcified for 10 days in a 14% EDTA solution with 0.2% PFA in PBS before embedding in paraffin and sectioning with a Leica microtome.

Masson's trichrome staining was performed to assess extent of tissue crosslinking and fibrosis (Day 0). Slides were fixed in Bouin's solution (Sigma) overnight at room temperature, then briefly rinsed in tap water before further washing in water on a shaker for 20 minutes. Slides were stained in a working solution of Weigert's hematoxylin (1: 1 ratio of solution A and solution B, Sigma) for 8 minutes and washed thoroughly in running tap water. Slides were then stained in Biebrich scarlet-acid fuchsin solution (Sigma) for 5 minutes and washed in water. Slides were differentiated in a 1: 1 phosphomolybdic-phosphotungstic acid solution (Sigma) for 5 minutes. Slides were then stained in aniline blue solution (Sigma) for 5 minutes and differentiated in a 1% acetic acid (Sigma) solution for 2 minutes. Finally, slides were rinsed in water, then dehydrated with a few dips each in 70%, 90%, and 100% ethanol and cleared for 1 minute in Histoclear (Thermo Fisher Scientific) before mounting with Permount (Thermo Fisher Scientific).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to further assess tissue damage (Day 0 and Day 9). The ApoBrdU-IHC DNA

Fragmentation Assay Kit (Biovision) was used with the included protocol. Data were analyzed with ImageJ (http://imagej.nih.gov/, NIH). For each picture, the areas of TUNEL-positive cells (stained brown, [T+]) and those of TUNEL-negative cells (stained blue, [T-]) were extracted with ImageJ. Then the percentage of TUNEL-positive cells was calculated as X = [T+] / ([T+] + [T-]) x 100%. 10 representative pictures per group were included in the analysis.

Hematoxylin and eosin (H&E) staining was performed to assess systemic tissue damage in lung, liver, brain, and bone. Slides were stained with Harris hematoxylin (Sigma) for 10 minutes, followed by acid alcohol (1% HCl in 70% ethanol), Scott's bluing reagent (Sigma) for 1 minute, and Eosin Y (Sigma) for 5 minutes. Slides were dehydrated and mounted as described above.

Ex vivo immunohistochemistry

Lung tissues were harvested from tumor-bearing or tumor-free nude or NSG mice (Day 0 for LOX staining, Day 1 for MRCS homing and activation, and Day 9 for Annexin V) and processed as mentioned above. Frozen slides (8 µm) were thawed and rehydrated in dH_2O for 5 minutes, then fixed in chilled acetone (Thermo Fisher Scientific) at -20°C for 10 minutes, permeabilized in 0.1% Triton X-100 for 10 minutes, and blocked in 0.1% Triton X-100 with 5% normal donkey serum for 1 hour. Primary antibodies (table S1) were diluted 1: 100 from the stock solution and applied overnight at 4°C. Slides were washed in 1X PBS. Then, secondary antibodies (table S2) were diluted 1: 500 from the stock solution and applied for 30 minutes at room temperature. Slides were stained for nuclei with DAPI (1 µg/ml), then washed in PBS and mounted with Fluoromount-G. Slides were imaged with Nikon Eclipse Ti inverted microscope.

Second Harmonic Generation (SHG) Imaging

For SHG imaging, frozen 40 μm sections of fixed (for LOX-SHG co-localization) or unfixed (for MRCS-SHG co-localization and SHG quantification), OCT-mounted mouse lungs (Day 0 for LOX-SHG co-localization and Day 1 for MRCS-SHG co-localization) were obtained as mentioned above and dried overnight, in the dark, at room temperature on slides. Slides were then prepared as described in the "Ex vivo Immunohistochemistry" session. The fluorescent signals from antibody-stained slides were imaged using a Zeiss LSM710 multiphoton/confocal microscope (Zeiss) with a 40x W1.2NA objective (Zeiss). Slides were sequentially imaged in order of increasing fluorescent wavelength to reduce photo bleaching. SHG was performed at an 840 nm excitation wavelength and narrow bandpass detection, with a spectral window of 420/20 nm (*62-64*). Images were processed with ImageJ and Matlab (MathWorks Inc.). For MRCS-SHG co-localization and representative images for SHG quantification, multiple high quality pictures were generated per sample and tiled into a larger image, from which the representative pictures were selected. For SHG quantification, collagen fibers imaged by SHG were automatically or manually selected, and the ratio of displacement and length of the collagen fibers were analyzed and plotted. To minimize artifacts caused by collagen bundles close to blood vessels, the fibrillar length and curvature of collagen were extracted and adjusted by quasi-manual selection of fibrillar structures. CT-FIRE was used to automatically select and quantify the curvature of collagen fibers (*65, 66*). Regions of the lung tissue defined as "cancer region" or "non-cancer region" were manually selected.

For SHG-PARP p85 co-localization, large lung tissue scans of fluorescent channels 594 nm (for cancer) and 640 nm (for PARP p85) were taken on a fluorescent microscope (Eclipse Ti-E, Nikon) with 7-color Spectra X Light Engine (Lumencor) and Zyla sCMOS camera (Andor) at

20x magnification. The sample was then scanned in the same approximate area using Zeiss LSM710. The SHG scan was then overlaid upon the fluorescent scan using ImageJ to get a high-definition fluorescence and SHG combined image for analysis. The overlaid large scans were then divided into sections using a grid of approximately 500 x 500 μm. Each section of the grid was treated as a separate data point for SHG-PARP p85 co-localization.

Bone marrow flushing and flow cytometry

1 day, 2 days, 7 days, or 9 days after MSC infusion with 5-FC treatment as mentioned above, nude or NSG mice were sacrificed, and bones, specifically femurs from both legs, were harvested, with muscle tissue removed carefully. Bone marrow was then flushed with PBS, dissociated, and filtered using a 70 μm cell strainer (Corning). The red blood cells were then lysed using ammoniumchloride potassium buffer (Lonza) for 3 minutes at 37°C. The remaining cells were washed twice with PBS and diluted to 1 x 10^6 cells/ml. 300 μl of cells in suspension were then stained with either Annexin V-FITC (BioLegend) in Annexin V binding buffer (BioLegend) and 7-Aminoactinomycin D dye (7-AAD, Thermo Fisher Scientific) or anti-B220 APC (CD45R), anti-CD11b FITC, and anti-Gr-1-PE-Cy7 (Ly6G) (table S1) according to the manufacturer's instructions. Compensation and unstained controls were also prepared for the analysis. For samples requiring multiple stains, a compensation coefficient was first estimated from controls using unstained cells or only a single stain and inputted into the analysis software to correct for fluorescent signal spillover from other channels. Finally, the cells were analyzed with a BD Accuri C6 flow cytometry machine (BD Biosciences). The different populations were analyzed as follows: monocytes/macrophages ($CD11b^+$, $Gr\text{-}1^{neg/low}$), granulocytes ($CD11b^+$, $Gr\text{-}1^{high}$), and B cells ($B220^{low}$ and $B220^{high}$).

Atomic force microscopy (AFM)

Six weeks after *i.v.* infusion of Luc-RFP-231 cancer cells or DPBS administration for the tumor-free control, nude mice (Day 0) were euthanized and lungs were immediately harvested and kept in ice-cold Ringer solution (Thermo Fisher Scientific). All the AFM assays were performed within 48 hours after mouse euthanasia. Lung samples were cut into 500 µm-thick slices using a rat heart slicer matrix (Zivic Instruments) and mounted to a 60 mm plastic petri dish using epoxy glue (Devcon). Samples were measured using Bruker BioScope Catalyst Atomic Force/ Zeiss LSM5 Confocal Fluorescence Microscope (Bruker/ Zeiss) using contact mode in fluid with MLCT C Triangular cantilevers (spring constant = 0.01 N/m, Bruker). Data points were taken 5 µm apart on 50 x 50 µm scans. Data were analyzed with Nanoscope Analysis v1.5 software, using a Sneddon conical model with Poisson ratio 0.4 (*67, 68*).

Supplementary Figures

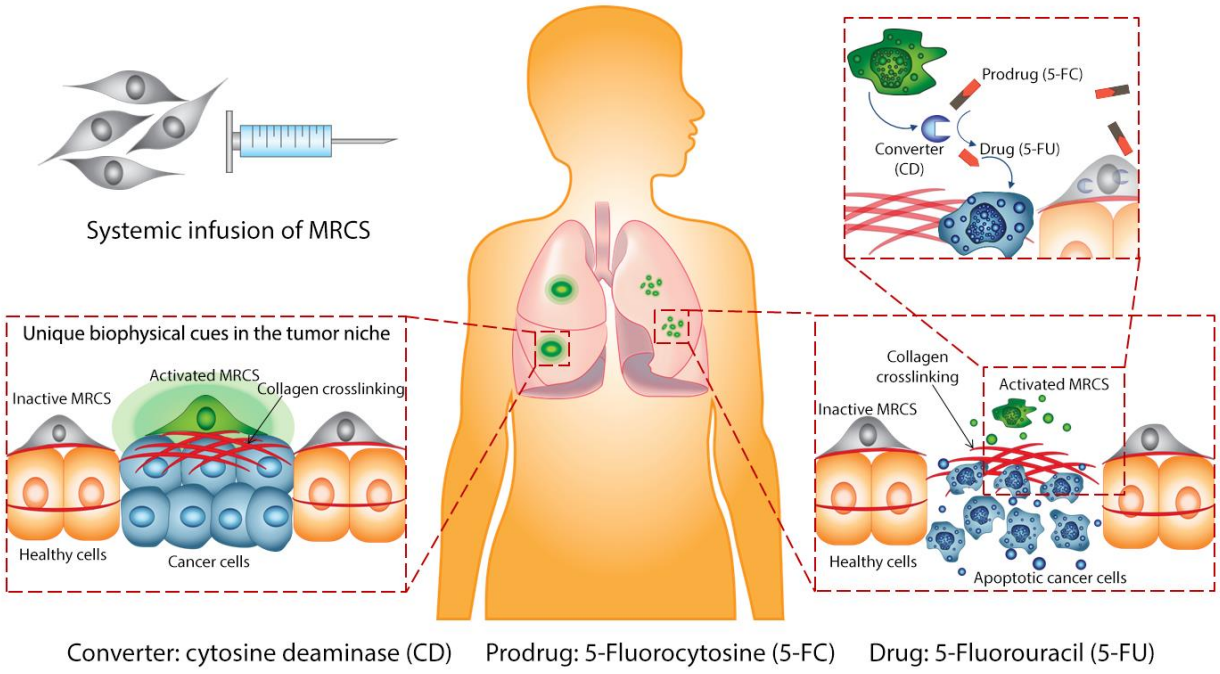


Fig. S1. Concept of MRCS for targeting breast cancer metastases in the lung.

The tumor-homing MRCS are locally activated by specific ranges of stiffness linked to collagen crosslinking found at the metastatic niche (red crosshatching), thereby expressing or secreting reporters and therapeutics (left side) and selectively treating cancer metastases using converter enzyme CD and prodrug 5-FC (right side).

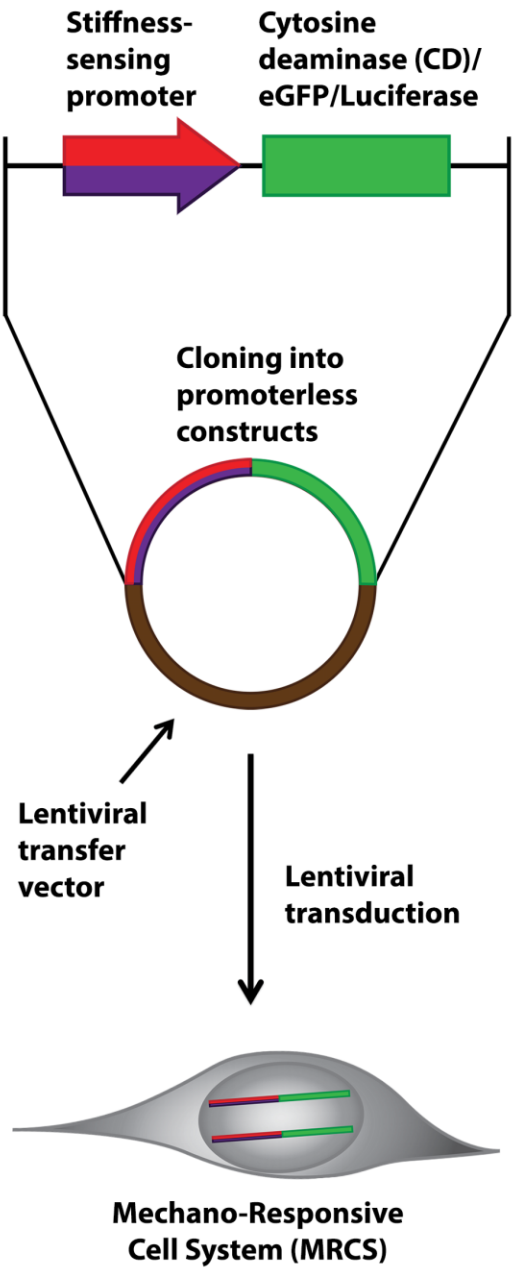


Fig. S2. Construction of MRCS.

The scheme of cell engineering. Promoters of genes responsive to specific ranges of stiffness are cloned into promoterless vectors to drive expression of eGFP, luciferase, and/or CD. Then the constructs are transduced into mesenchymal stem cells (MSCs) to produce stable engineered MSCs (MRCS).

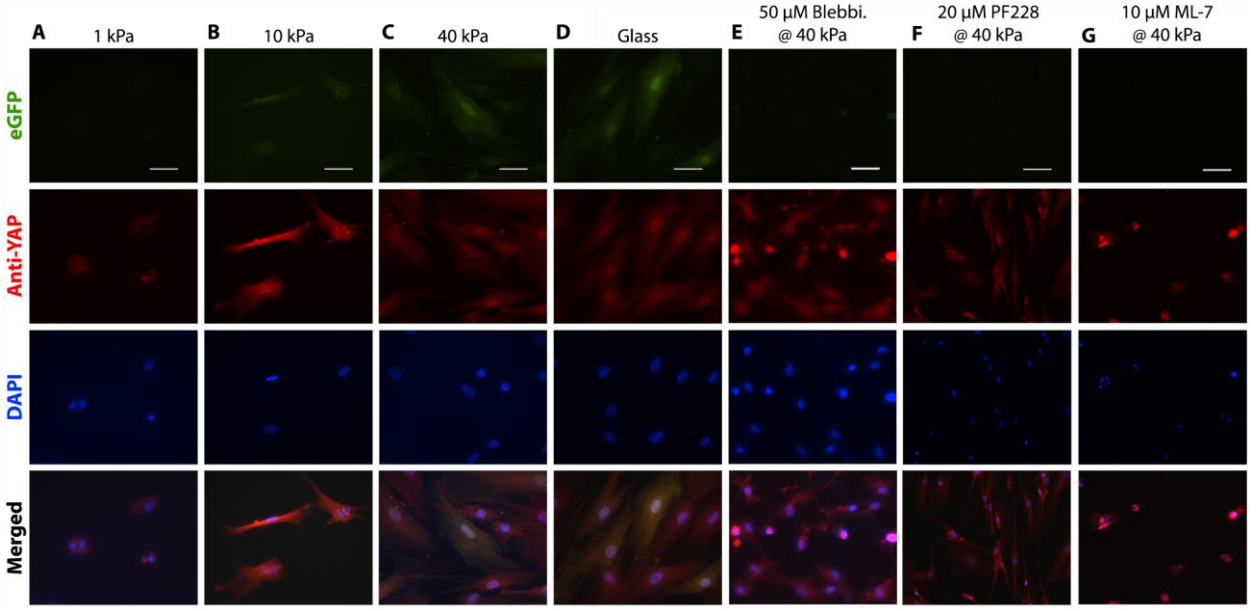


Fig. S3. MRCS-eGFP activation in response to substrate stiffness in vitro.

MRCS-eGFP were plated on (**A**) soft (~ 1 kPa), (**B**) medium (~ 10 kPa), or (**C**) firm (~ 40 kPa) polyacrylamide gel or (**D**) glass. eGFP (green, no staining) was turned on in response to higher stiffness (≥ 10 kPa). YAP (red, stained with anti-YAP) relocalization is also regulated by stiffness, and concentrated in nuclei on stiffer substrates (≥ 10 kPa). When MRCS-eGFP on firm (~ 40 kPa) substrates were treated with (**E**) 50 μM blebbistatin, (**F**) 10 μM ML-7, or (**G**) 20 μM PF228, eGFP (green, no staining) was turned off and YAP (red, stained with anti-YAP) was localized in the cytoplasm. DAPI (blue, nuclear counterstain) is also displayed. Scale bar = 100 μm.

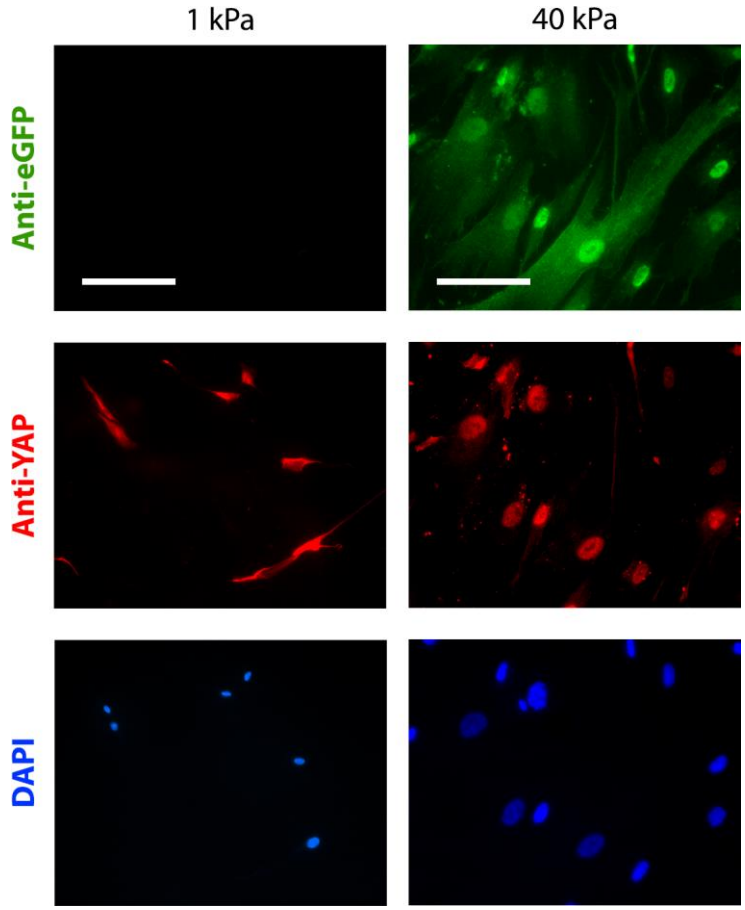


Fig. S4. MRCS-eGFP in vitro validation with immunostaining.

Representative images of MRCS-eGFP plated on soft (~ 1 kPa) and firm (~ 40 kPa) polyacrylamide gels. eGFP (green, stained with anti-eGFP) was turned on responding to higher stiffness. YAP (red, stained with anti-YAP) was concentrated in nuclei on stiffer substrates. DAPI (blue, nuclear counterstain) is displayed. Scale bar = 100 μm.

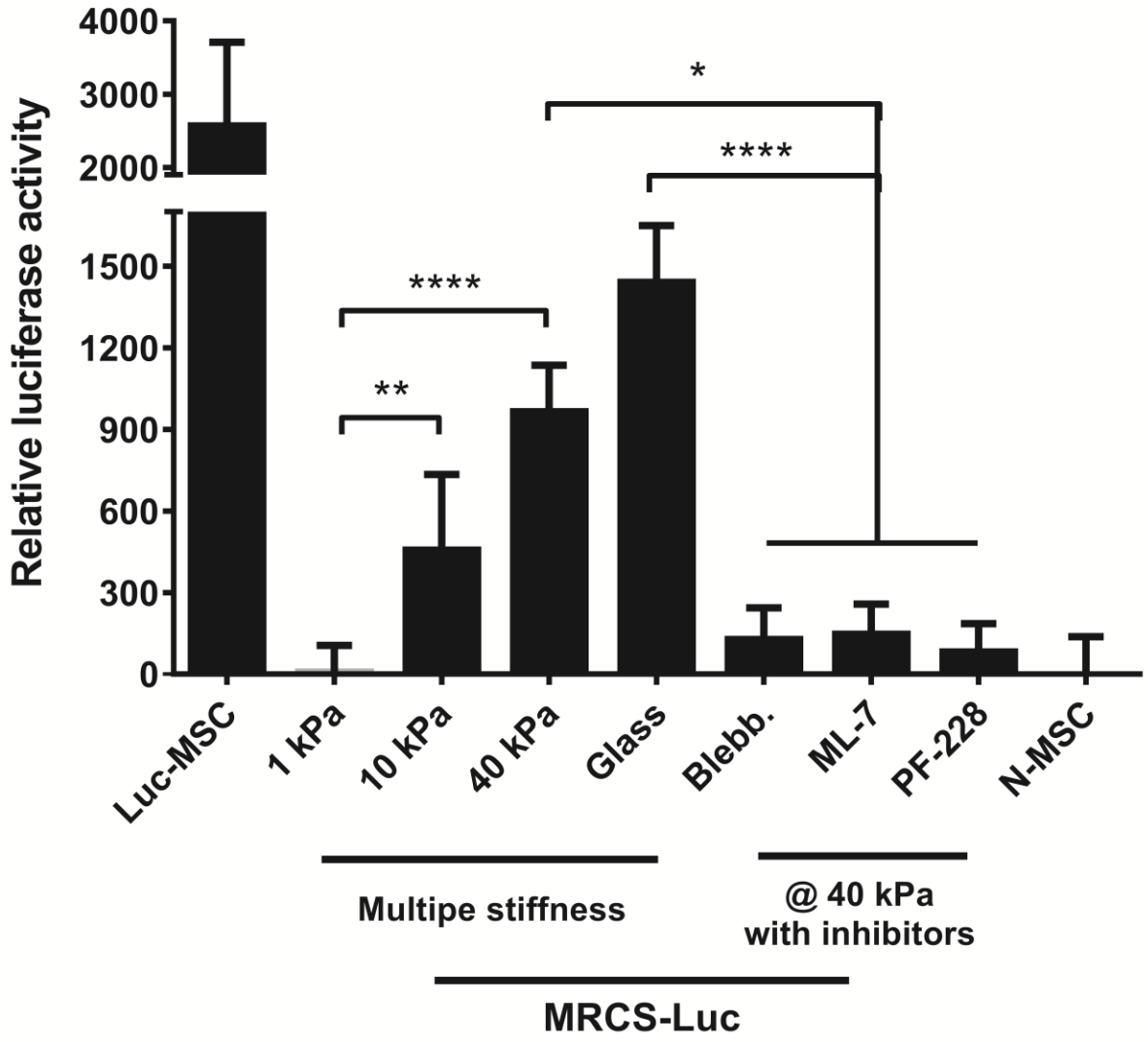


Fig. S5. Further MRCS-Luc in vitro validation.

MRCS-Luc were seeded on substrates with different stiffness or on firm (~ 40 kPa) substrates treated with inhibitors as above in fig. S3. Luciferase activity was upregulated on stiff substrate and downregulated on soft substrate or with mechano-sensing inhibitors. *P < 0.05, **P < 0.01, and ****P < 0.0001. Triplicate samples were used for the analysis. Data shown as mean ± SD.

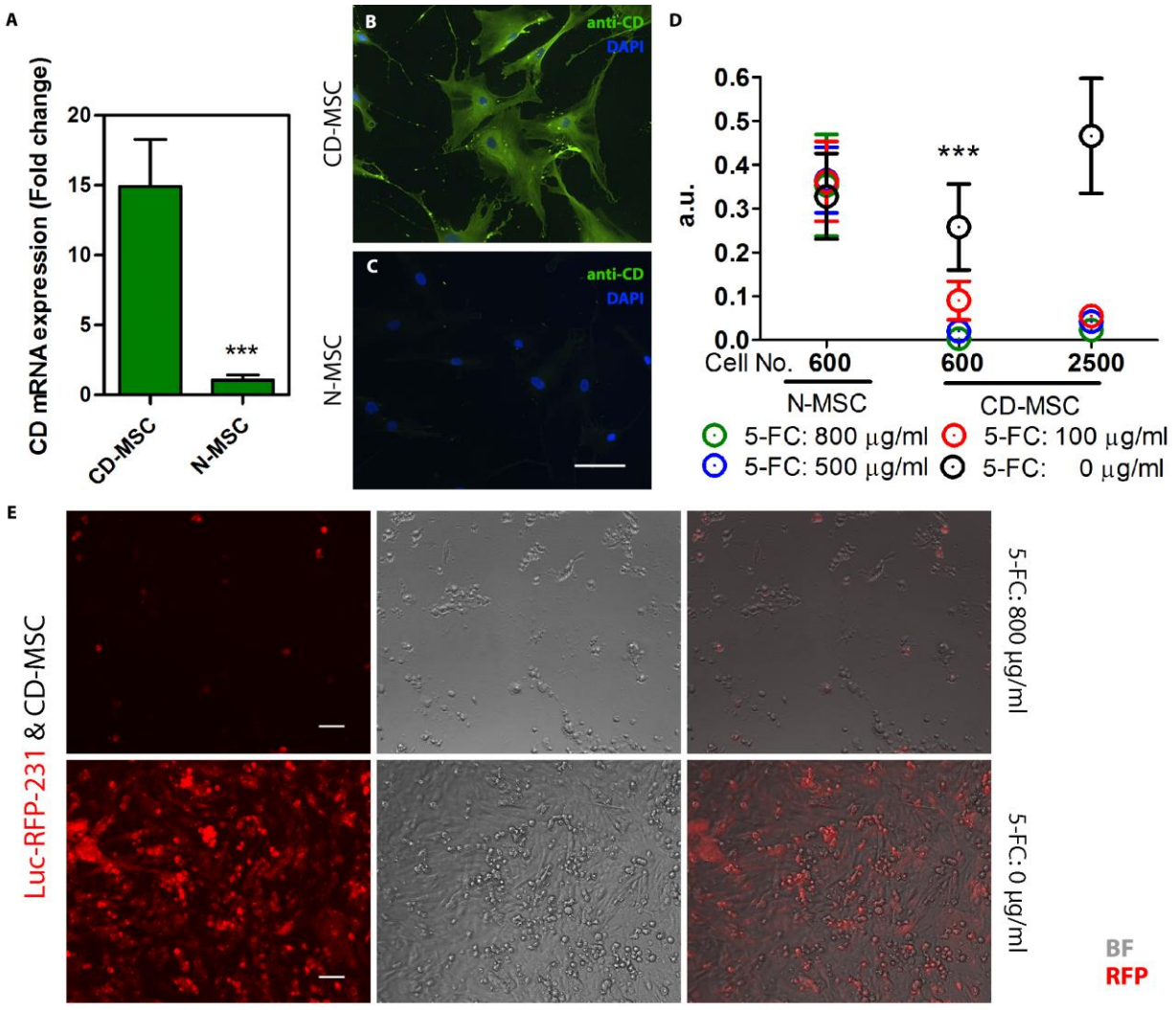


Fig. S6. CD-MSC able to kill cancer cells in the presence of 5-FC in vitro.

The expression of CD was validated by (**A**) RT qPCR and (**B**) immunofluorescent staining. CD (green); DAPI (blue, nuclear counterstain). N-MSC were included as control in (**A**) and (**C**). In (**A**), CD mRNA expression of N-MSC was normalized to 1. N-MSC do not express CD. (**D**) XTT assay was performed to show that CD-expressing MSCs are suicide agents in the presence of 5-FC at various concentrations. MSC proliferation was highly decreased only with both CD-expressing MSCs and prodrug 5-FC. (**E**) Co-culture experiment was conducted with CD-MSC and RFP-expressing MDA-MB-231 breast cancer cells (231: MSC=2:1) with or without 800 μg/ml 5-FC. About 95% of breast

cancer cells were killed and rest were undergoing apoptosis, whereas control without 5-FC had a high confluency. RFP (red) and bright field (BF) images are displayed. Triplicate samples were used for the analysis. Scale bar = 100 μm. Data shown as mean ± SD. ***$P < 0.001$.

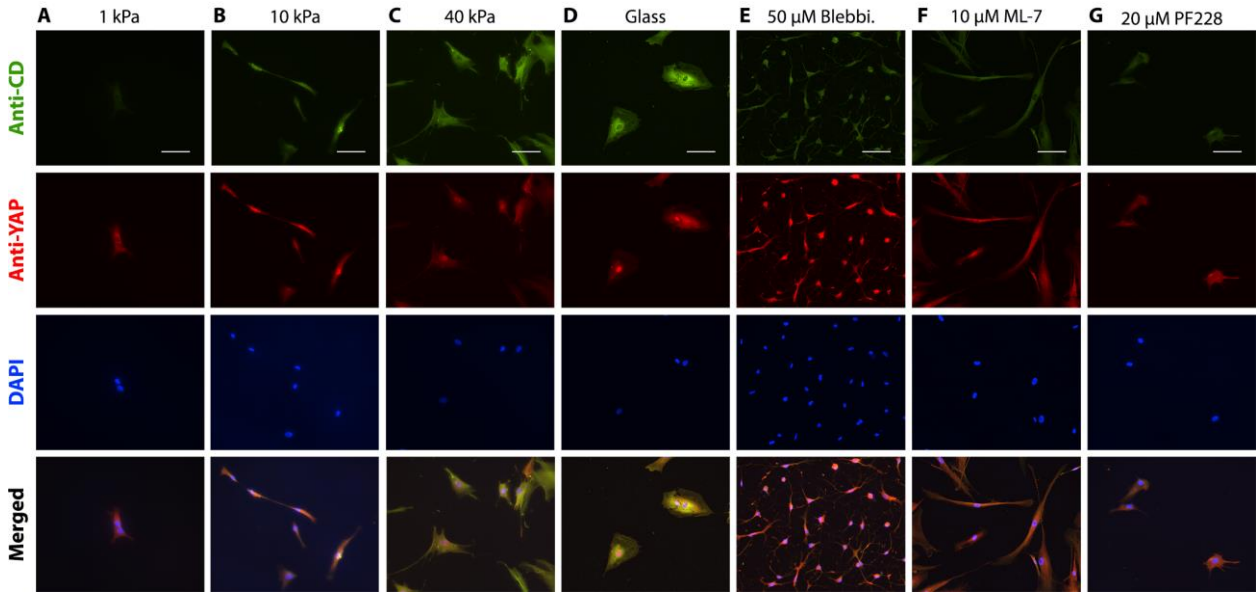


Fig. S7. MRCS-CD responding to matrix stiffness in vitro.

MRCS-CD were plated on (**A**) soft (~ 1 kPa), (**B**) medium (~ 10 kPa), or (**C**) firm (~ 40 kPa) polyacrylamide gel or (**D**) glass. CD (green) was turned on responding to high stiffness (≥ 10 kPa). YAP (red) relocalization was also regulated by stiffness. When MRCS-CD plated on firm (~ 40 kPa) polyacrylamide gel were treated with (**E**) 50 μM blebbistatin, (**F**) 10 μM ML-7, or (**G**) 20 μM PF228, CD (green) was turned off and YAP (red) was localized in cytoplasm. DAPI (blue, nuclear counterstain) is displayed. Scale bar = 100 μm.

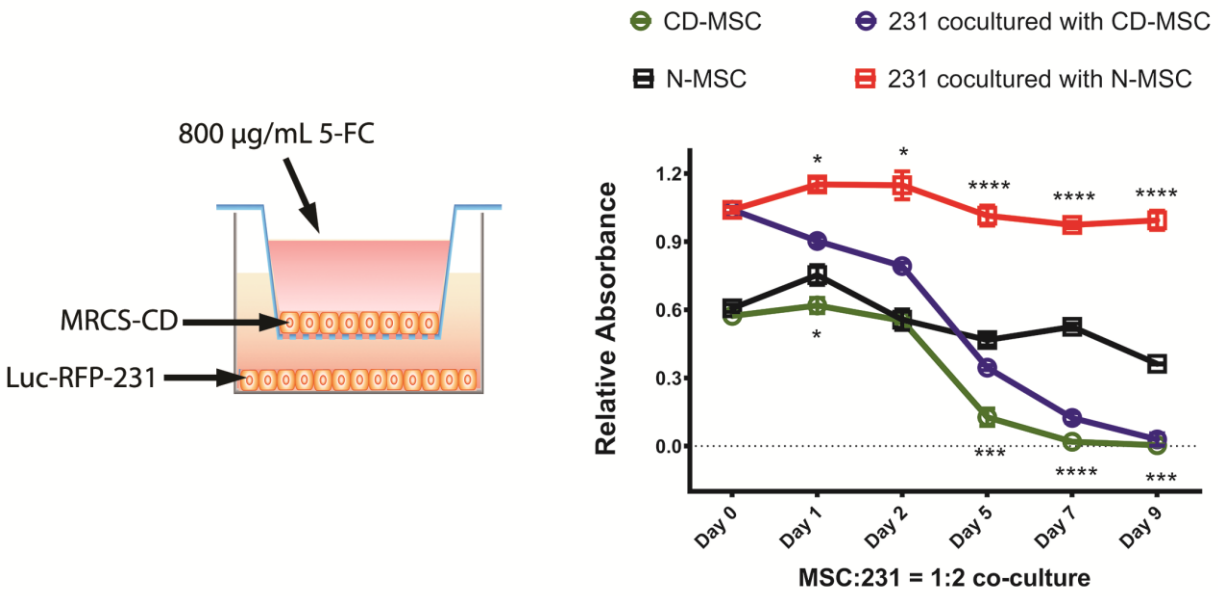


Fig. S8. Bystander effect from MRCS-CD starting at 24 hours in vitro on stiff substrate.

Co-culture of MRCS-CD (upper chamber) and Luc-RFP-231 (bottom chamber) was performed using transwell (MSC: 231=1:2) in the presence of 800 µg/ml 5-FC. On day 0 (cell seeding), day 1, day 2, day 5, day 7, and day 9, XTT assay was performed to measure the proliferation of cells. Triplicate samples were used for the analysis. Data shown as mean ± SD. *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$.

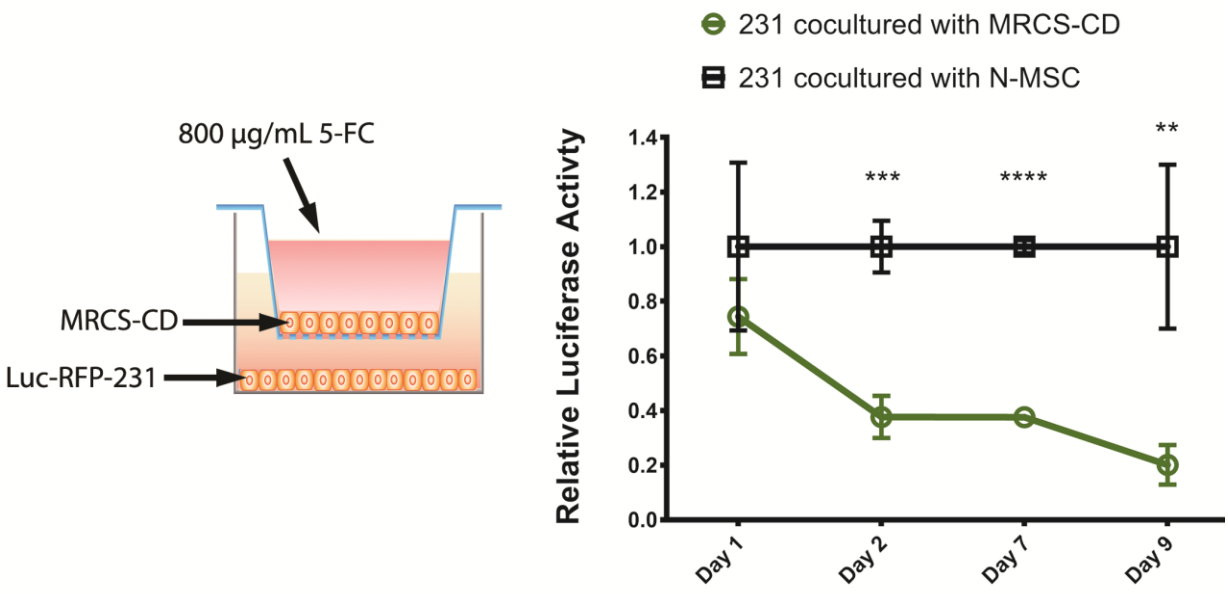


Fig. S9. Bystander effect from MRCS-CD lasting after MSC removal in vitro on stiff substrate.

Co-culture of MRCS-CD (upper chamber) and Luc-RFP-231 (bottom chamber) was performed using transwell (MSC: 231=1:2) in the presence of 800 μg/ml 5-FC. MSCs were removed on day 1, day 2, day 7, or day 9, and in vitro luciferase proliferation assay was performed on day 9 to measure the signals from Luc-RFP-231. n.s., not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Triplicate samples were used for the analysis. Data shown as mean ± SD.

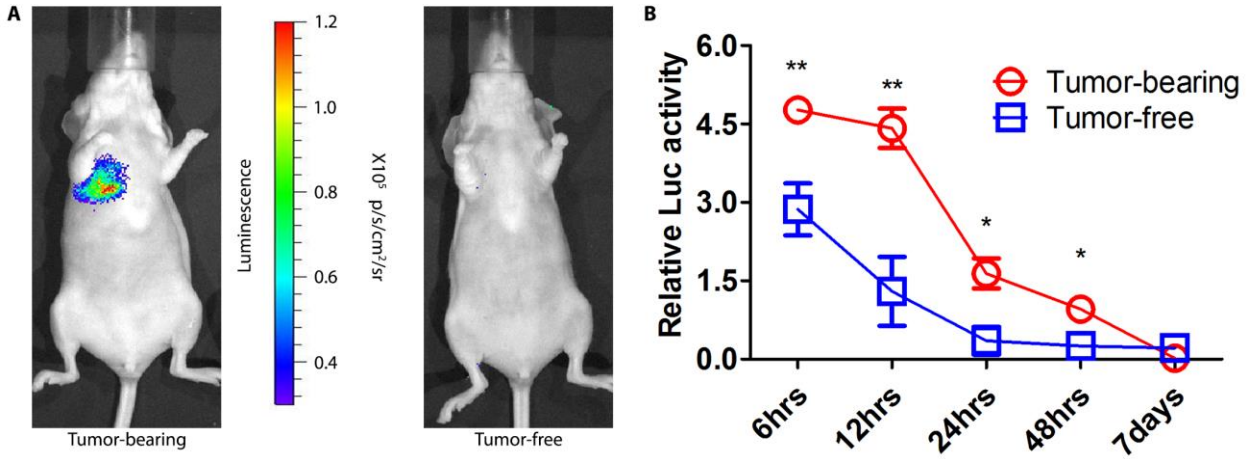


Fig. S10. Luc-MSC homing to the metastatic niche in vivo.

(**A**) Representative pictures of in vivo luciferase imaging of systemically infused Luc-MSC 12 hours after MSC infusion. (**B**) Quantification of luciferase activity of Luc-MSC in the lungs of eGFP-231 tumor-bearing and tumor-free nude mice at different time points after systemic infusion. MSCs persisted longer in tumor-bearing mice than in tumor-free mice but were cleared out in approximately 1 week. Relative Luc Activity = Log_2 [(luciferase read of the tested mouse infused with Luc-MSC) / (luciferase read of control mice average injected with DPBS)], such that the RLA of mice injected with DPBS = 0. n = 4 for tumor-bearing and n = 3 for tumor-free nude mice. Data shown as mean ± SEM. * P <0.05, ** P <0.01.

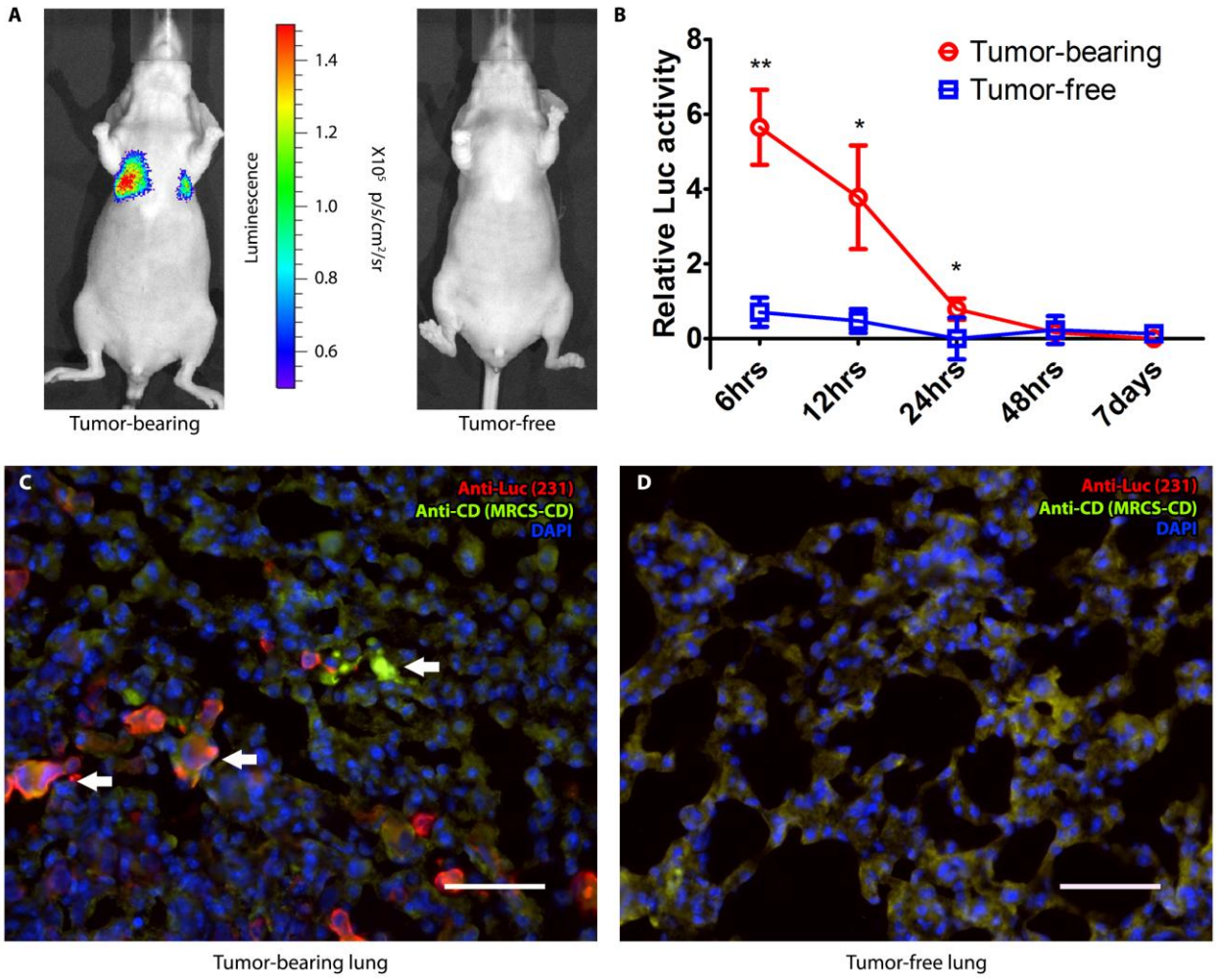


Fig. S11. MRCS homing and specific activation in response to the metastatic niche in vivo.

(**A**) Representative pictures of in vivo luciferase imaging of systemically infused MRCS-Luc 12 hours after infusion. (**B**) Systemically infused MRCS-Luc were turned on in the lungs of eGFP-231 tumor-bearing nude mice but not tumor-free mice. Relative Luc Activity (RLA) = Log_2 [(luciferase read of the mouse infused with MRCS-Luc) / (luciferase read of control mice average injected with DPBS)], such that the RLA of mice injected with DPBS = 0. RLA were measured and plotted for tumor-bearing and tumor-free mice at different time points after systemic infusion of MRCS-Luc. $n = 4$ for tumor-bearing and $n = 3$ for tumor-free nude mice. Data shown as mean ± SEM. * $P < 0.05$ and

** $P < 0.01$. (**C** and **D**) Frozen sections of lungs of Luc-RFP-231 tumor-bearing NSG mice and tumor-free NSG mice sacrificed 24 hours after MRCS-CD infusion were stained with anti-Luc (red) for lung metastasis, anti-CD (green) for cytosine deaminase expressed by MRCS-CD, and DAPI (blue). White arrows indicate the co-localization of lung metastatic sites and MRCS-CD expressing CD (turned on). Scale bar = 50 μm.

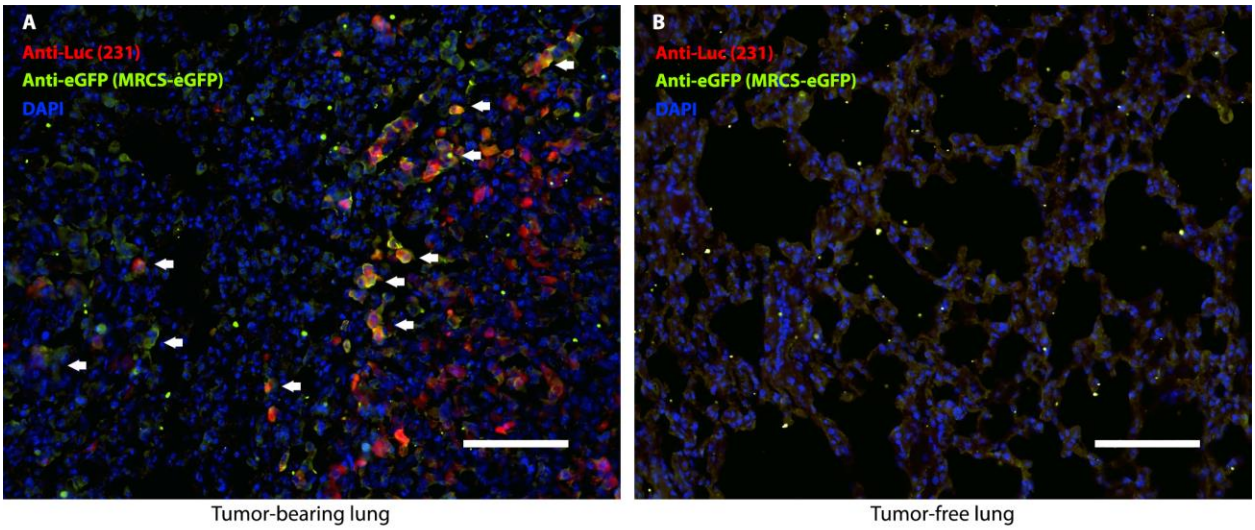


Fig. S12. Specific activation of MRCS-eGFP in response to the metastatic niche in vivo.

Frozen sections of lungs of (**A**) Luc-RFP-231 tumor-bearing mice and (**B**) tumor-free mice sacrificed 24 hours after MRCS-eGFP infusion were stained with anti-Luc (red) for lung metastasis, anti-eGFP for eGFP expressed by MRCS-eGFP (green), and DAPI (blue). White arrows indicate the co-localization of lung metastatic sites and MRCS-eGFP expressing eGFP (turned on). Scale bar = 100 μm.

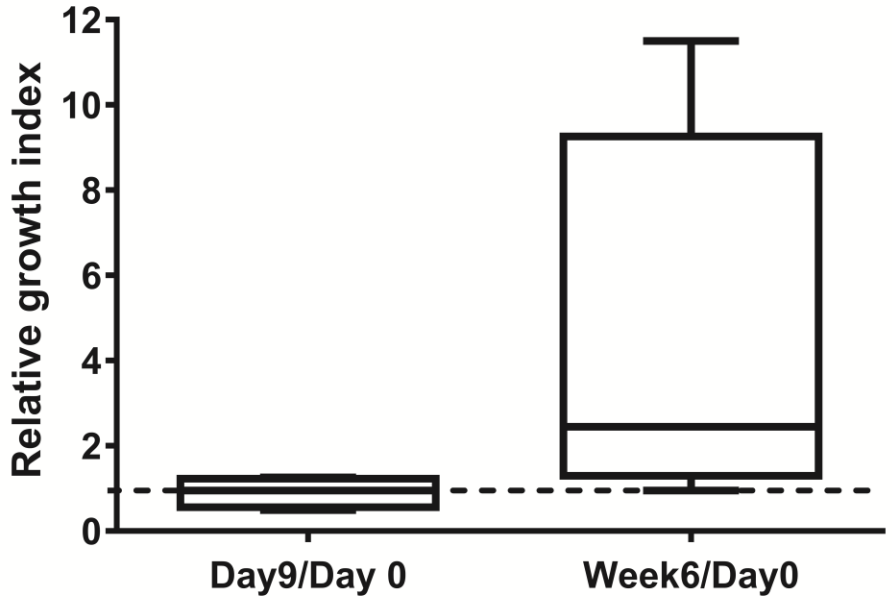


Fig. S13. MRCS-CD unable to attenuate cancer growth in the absence of 5-FC in vivo.

Six weeks after Luc-RFP-231 were seeded i.v. into nude mice, 10^6 MRCS-CD were administered systemically into tumor-bearing mice without 5-FC injection. In vivo luciferase activity was measured at different time points [before (Day 0), 9 days (Day 9) and 6 weeks (Week 6) after MRCS infusion]. Relative Growth Index (RGI) = luciferase read on Day 9 or Week 6 (after) / luciferase read on Day 0 (before). *n* = 4 for each group. The data are shown as box and whisker plots, with the box indicating 25% to 75% percentiles and the center line being the median. The error bars on either side indicate min and max of the data set.

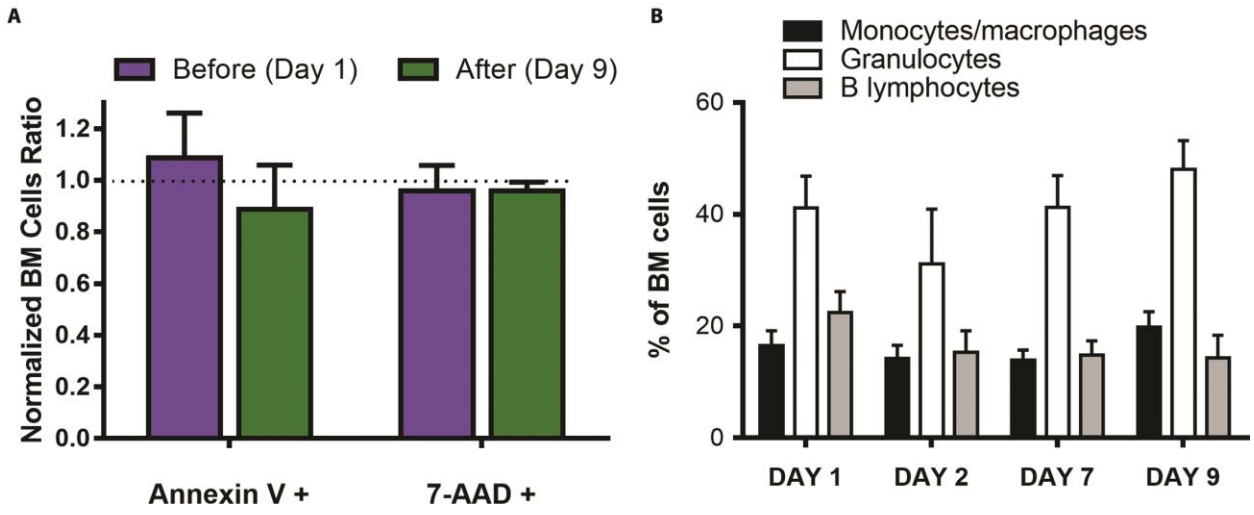


Fig. S14. No detectable side effects in bone marrow cell populations after systemic treatment with MRCS-CD.

(**A**) Luc-RFP-231 tumor-bearing and tumor-free nude mice treated by MRCS-CD were sacrificed before (Day 1) or after (Day 9) 5-FC injections. Bones were harvested, and bone marrow (BM) was flushed and stained with Annexin V and 7-AAD for FACS assay. Normalized BM cells = BM cells treated with MRCS-CD / BM cells treated with native MSCs, thus if ratio = 1, it would meant that MRCS-CD cause similar damage to the BM as native MSCs. (**B**) FACS assay shows no significant difference in bone marrow cell population during MRCS-CD treatment in the presence of 5-FC (Day 1, Day 2, Day 7, and Day 9 as indicated). Triplicate samples were used for the analysis. Data shown as mean ± SD.

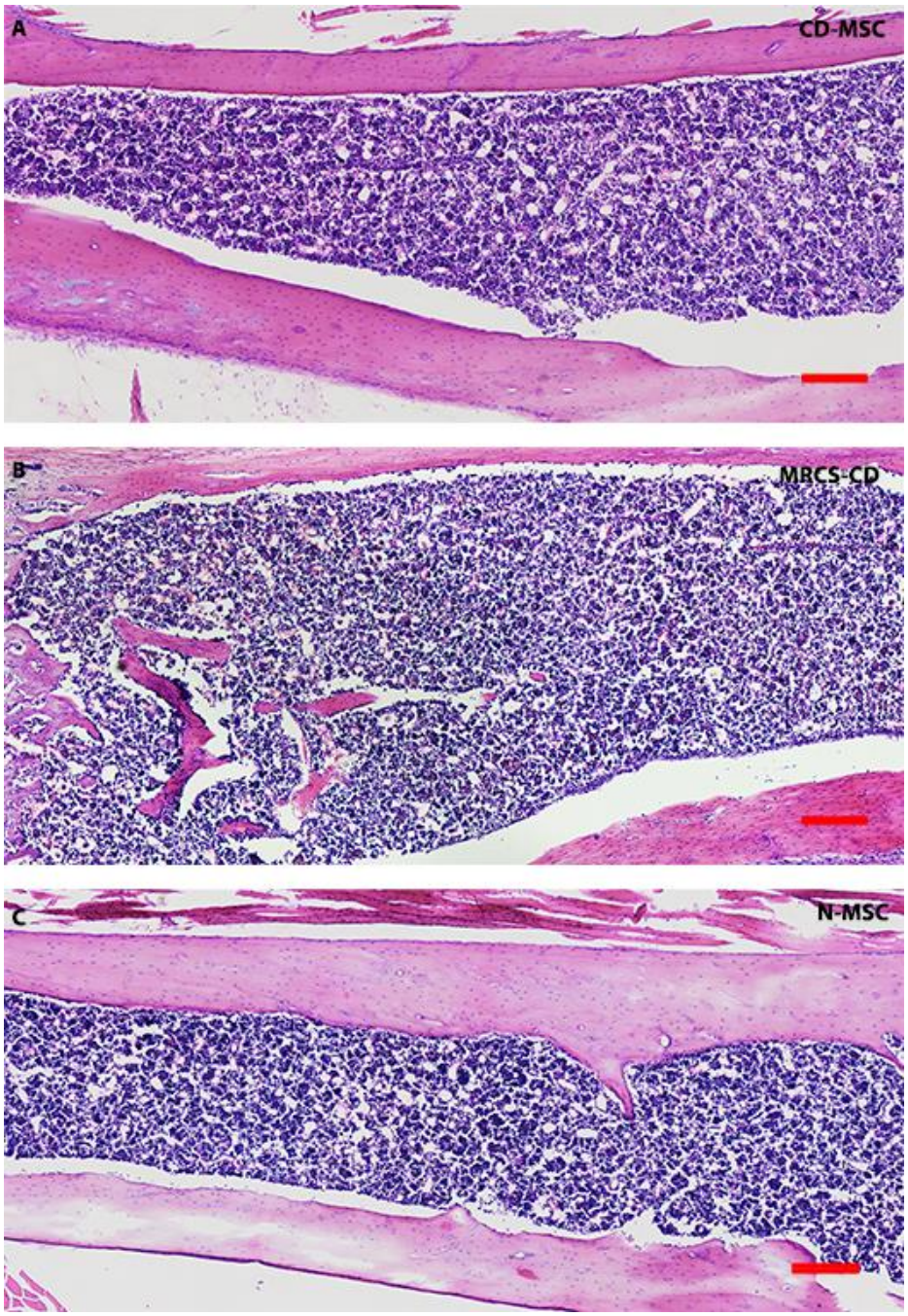


Fig. S15. MRCS-CD causing no detectable side effects in vivo in bone marrow.

Representative images of sectioned bones with H&E staining indicate no detectable tissue damage in bone marrow of tumor-bearing mice treated with (**A**) CD-MSC, (**B**) MRCS-CD, or (**C**) native MSCs, accompanied by 5-FC treatment. Scale bar = 200 μm.

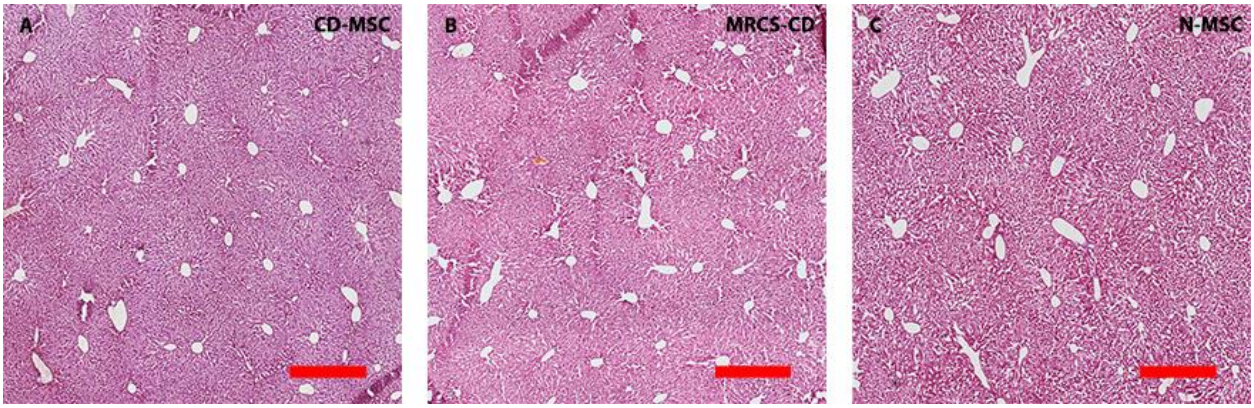


Fig. S16. MRCS-CD causing no detectable side effects in vivo in livers.

Representative images of sectioned livers with H&E staining indicate no detectable tissue damage in livers of tumor-bearing mice treated with (**A**) CD-MSC, (**B**) MRCS-CD, or (**C**) native MSCs, accompanied by 5-FC treatment. Scale bar = 200 μm.

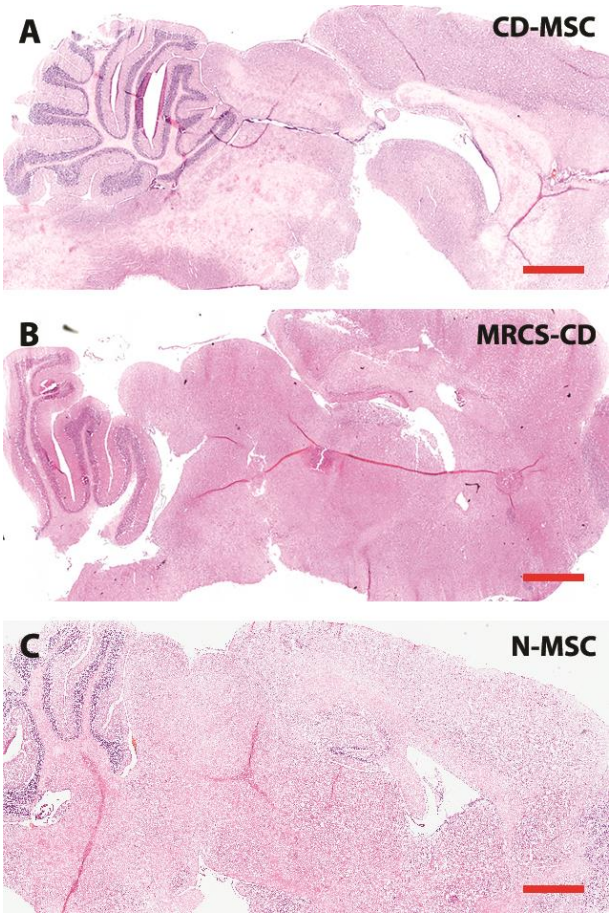
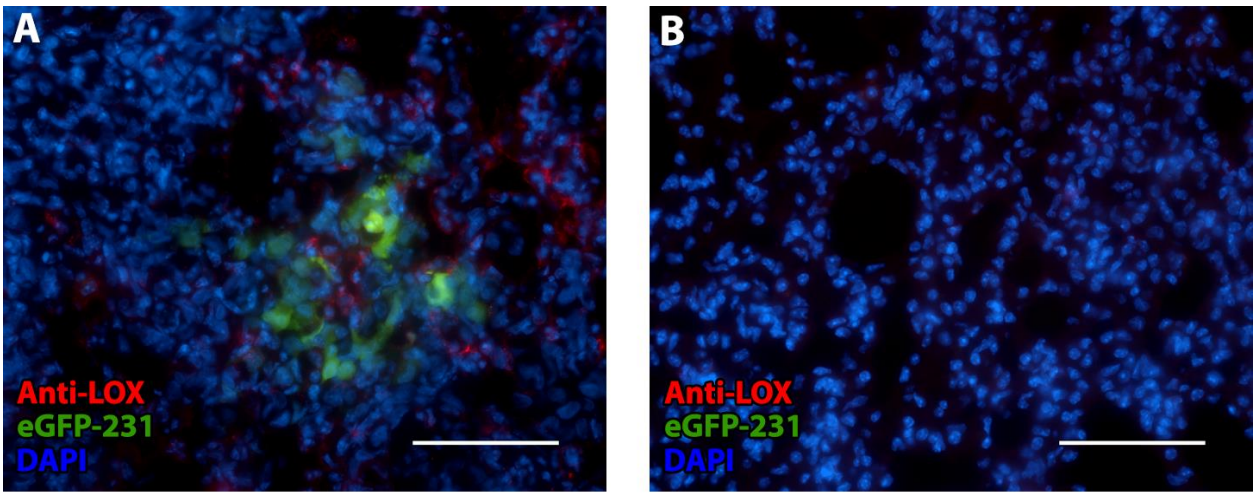


Fig. S17. MRCS-CD causing no detectable side effects in vivo in brains.

Representative images of sectioned brains with H&E staining indicate no detectable tissue damage in brains of tumor-bearing mice treated with (**A**) CD-MSC, (**B**) MRCS-CD, or (**C**) native MSCs, accompanied by 5-FC treatment. Scale bar = 200 μm.



Tumor-bearing lung

Tumor-free lung

Fig. S18. Up-regulation and colocalization of LOX expression with tumor in tumor-bearing lungs.

Representative frozen sections of lungs of (**A**) eGFP-231 tumor-bearing NSG mice and (**B**) tumor-free NSG mice sacrificed before MRCS infusion (Day 0) were stained with anti-LOX (red) and DAPI (blue). eGFP (green) indicates lung metastasis. Scale bar = 50 μm.

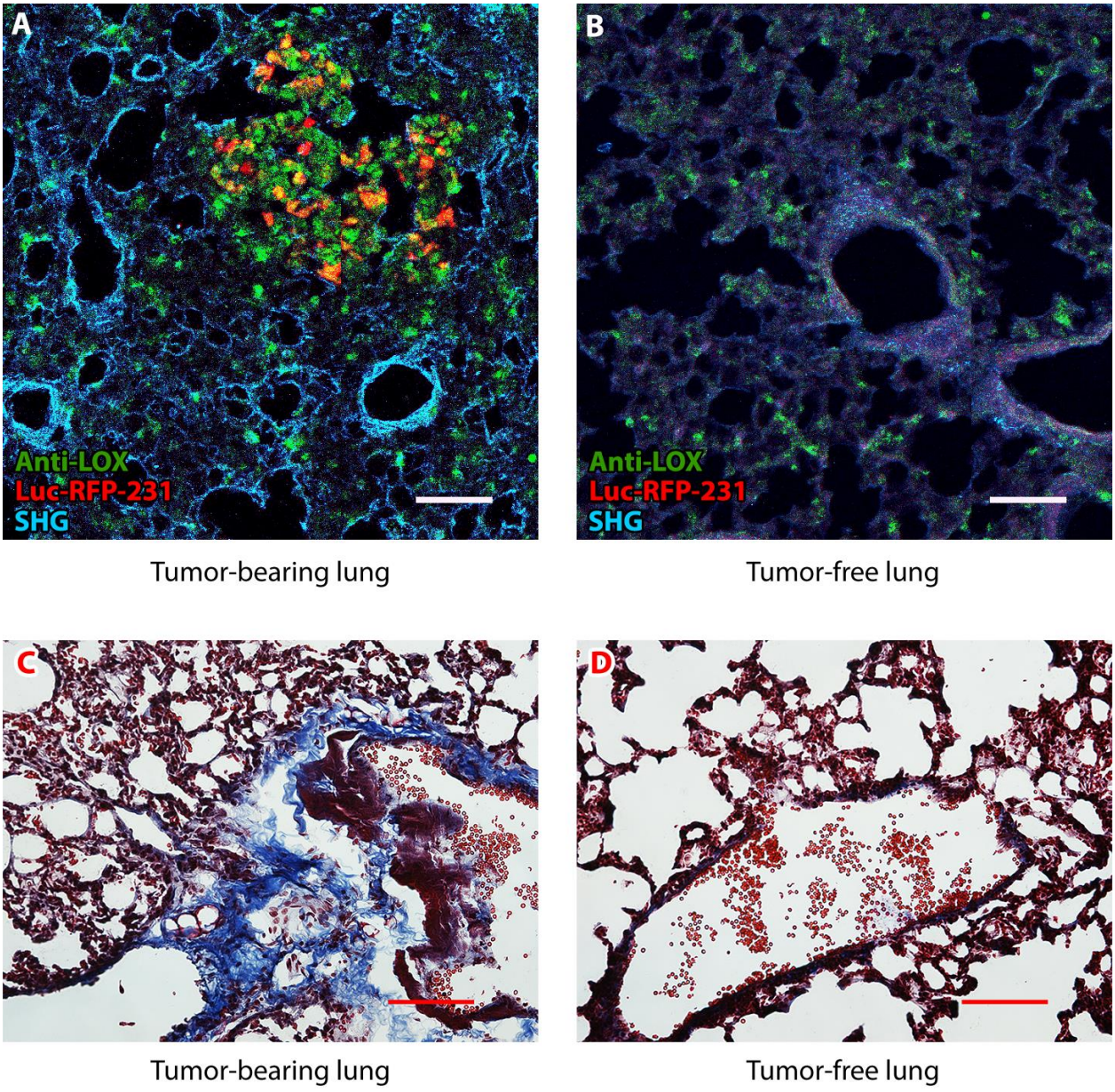


Fig. S19. LOX expression up-regulated with increased collagen expression in the metastatic niche.

Representative frozen sections of lungs of (**A**) Luc-RFP-231 tumor-bearing NSG mice and (**B**) tumor-free NSG mice sacrificed before MRCS infusion, showing staining with anti-LOX (green), lung metastases (RFP, red), and SHG imaging of collagen networks (cyan). Scale bar = 100 μm. Trichrome staining on representative frozen sections of lungs

from (**C**) tumor-bearing and (**D**) tumor-free mice shows the degree of collagen expression in the lungs of tumor-bearing and tumor-free nude mice. Scale bar = 100 μm.

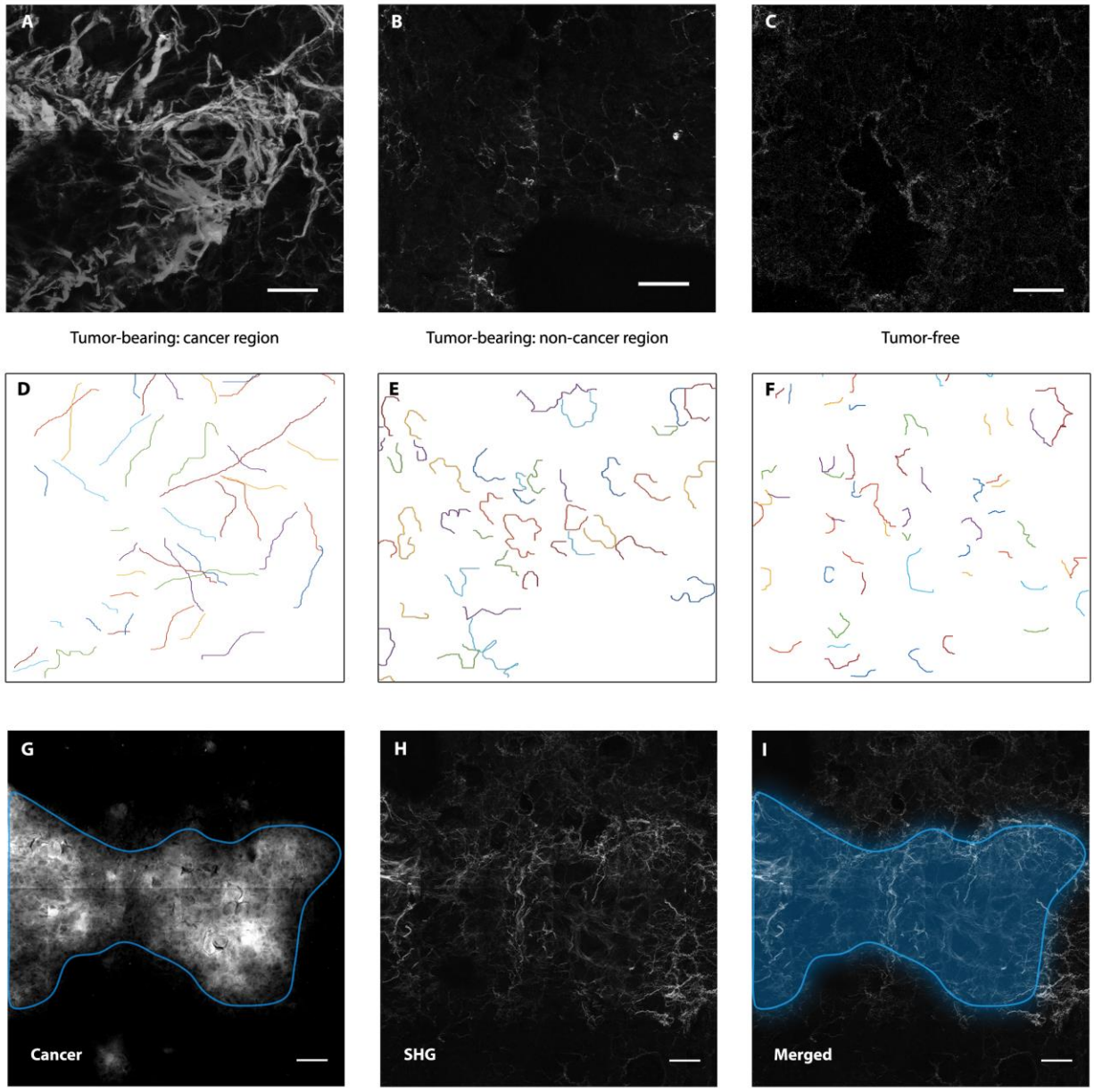


Fig. S20. SHG imaging showing up-regulated and more linearized collagen in tumor-bearing lungs.

Representative SHG images of (**A**) cancer (Luc-RFP-231) region and (**B**) non-cancer region of tumor-bearing lungs as well as (**C**) tumor-free lungs of NSG mice show that collagen is upregulated and more linearized in cancer regions. Scale bar = 50 μm. (**D**, **E**, and **F**) are corresponding images of selected fibrillar structures of (**A**, **B**, and **C**),

respectively. They were used for quantification of collagen linearization. (**G**) The regions of cancer expressing RFP were imaged with confocal microscopy and highlighted with a blue outline. (**H**) SHG imaging of the corresponding area of panel **G**. (**I**) Co-localization of cancer signal and collagen network by merging panels **G** and **H**. The highlighted areas are defined as “cancer regions.” Scale bar = 50 μm.

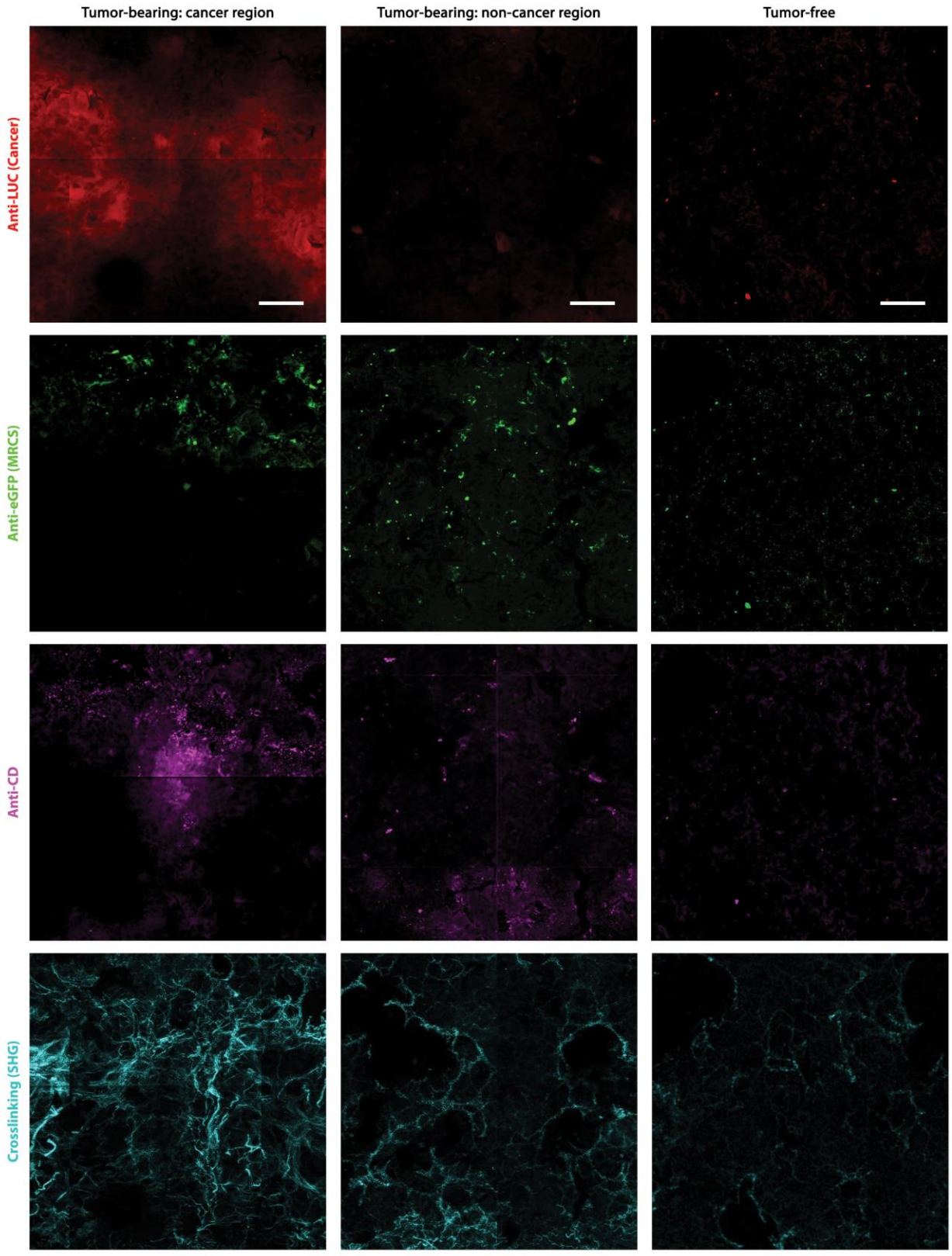


Fig. S21. Split-channel views of MRCS activation in the metastatic niche in vivo.

Frozen sections of lungs of Luc-RFP-231 tumor-bearing NSG mice (cancer region and non-cancer region) and tumor-free NSG mice sacrificed 24 hours after infusion of MRCS-CD co-transfected with eGFP were stained with anti-Luc (red) to detect lung metastasis, anti-CD (magenta) for CD expressed by MRCS-CD, and anti-eGFP (green) for MRCS-CD tracking. Second harmonic generation (SHG) imaging of collagen networks (cyan) was also presented and overlaid on IHC imaging in Fig. 5. The data indicate that the MRCS-CD and its specific activation were co-localized with lung metastatic sites and collagen crosslinking and linearized networks. Scale bar = 50 μm.

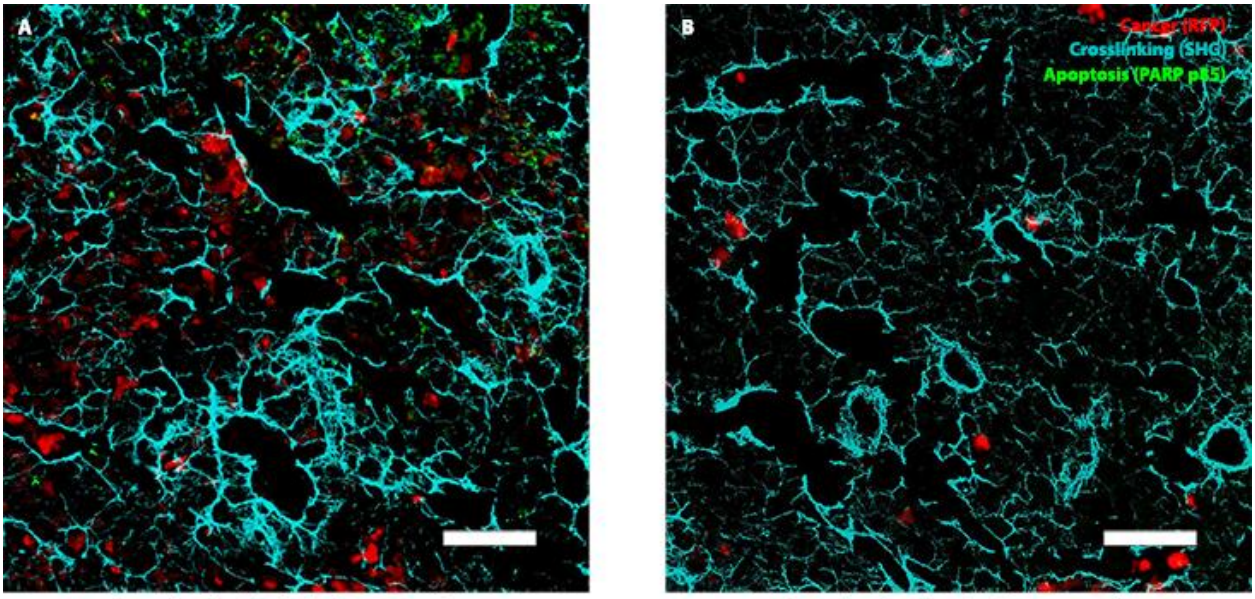


Fig. S22. Cross-linking–specific tissue damage by MRCS in response to mechano-cues in the metastatic niche in vivo.

Frozen sections of lungs of Luc-RFP-231 tumor-bearing NSG mice sacrificed after MRCS-CD infusion and 5-FC treatment (Day 9) were stained with anti-PARP p85 (green) for tissue apoptosis. RFP signal (red) indicates the presence of lung metastasis. SHG imaging of collagen networks (cyan) was overlaid with IHC imaging. (**A**) Tissue damage was only observed in lung metastatic sites with crosslinked collagen network but not in less crosslinked (**B**) regions, indicating that the MRCS-CD and its specific tissue damage were co-localized with lung metastatic sites and collagen crosslinking and linearized networks. Scale bar = 100 μm.

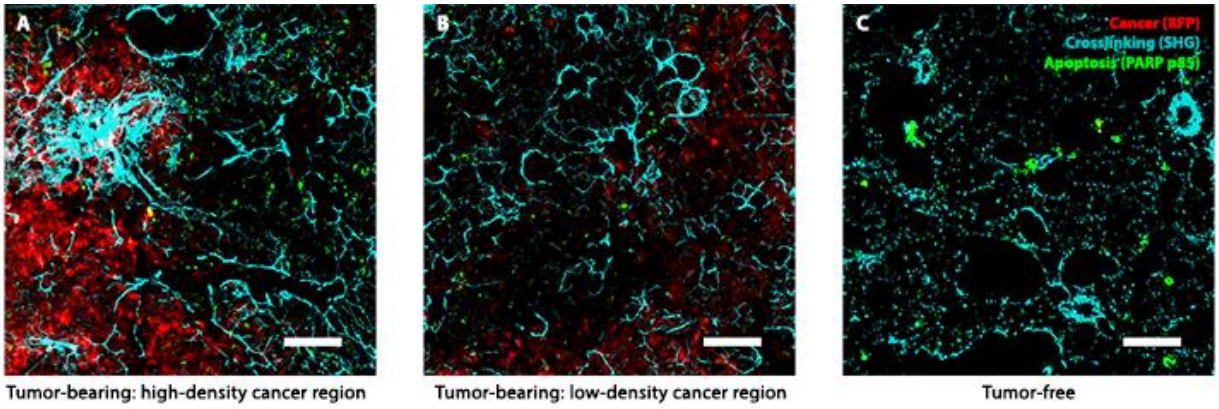


Fig. S23. Constitutively CD-expressing MSCs causing nonspecific tissue damage in vivo.

Frozen sections of lungs of Luc-RFP-231 tumor-bearing NSG mice and tumor-free NSG mice sacrificed after CD-MSC infusion and 5-FC treatment (Day 9) were stained with anti-PARP p85 (green) for tissue apoptosis. RFP signal (red) indicates the presence of lung metastasis. SHG imaging of collagen networks (cyan) was overlaid with IHC imaging. Non-specific tissue damage was observed in both (**A** and **B**) tumor bearing and (**C**) tumor free lungs regardless of the extent of collagen crosslinking. Scale bar = 100 μm.

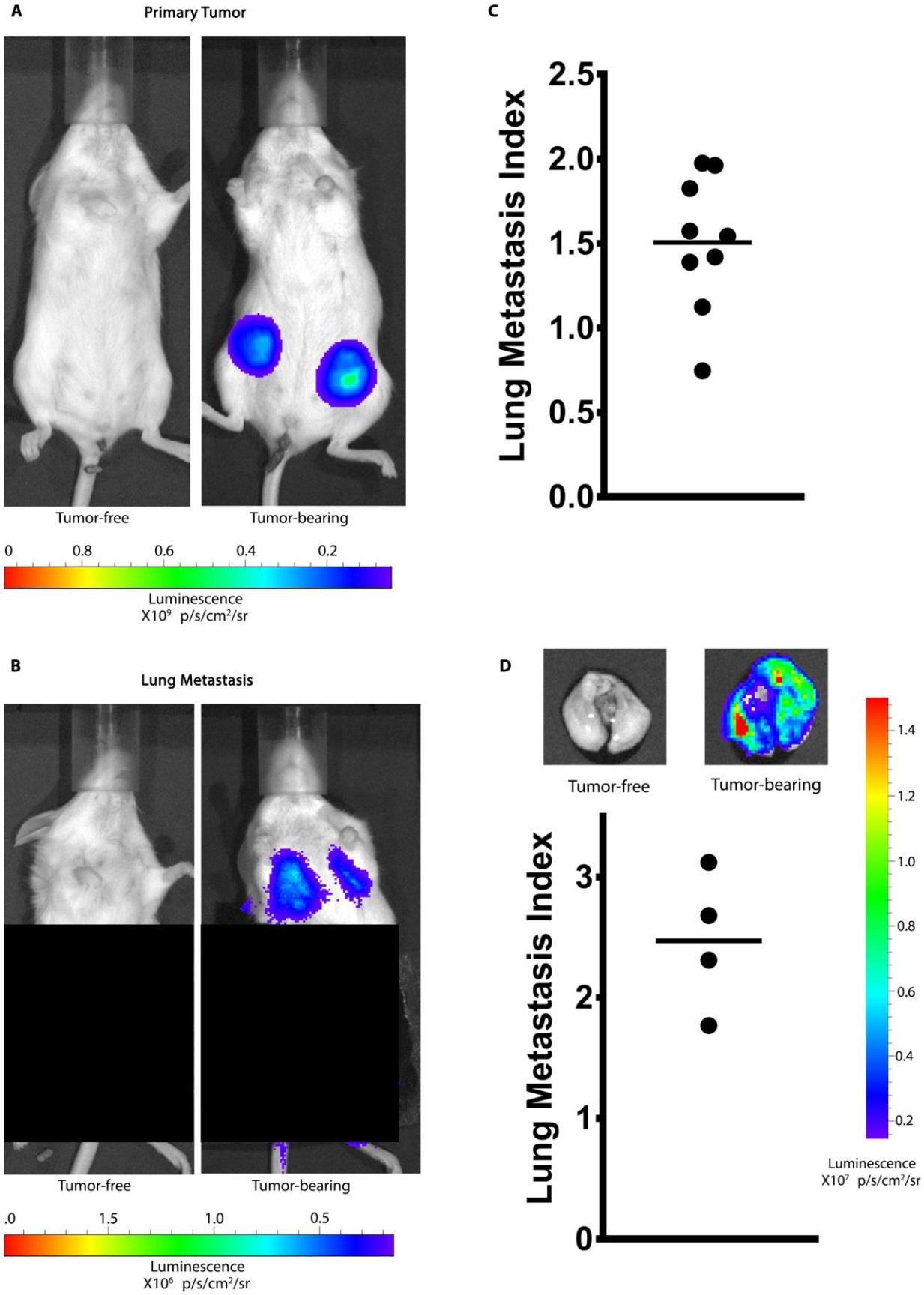

A
Primary Tumor
Tumor-free
Tumor-bearing
0
0.8
0.6
0.4
0.2
Luminescence
X10⁹ p/s/cm²/sr
B
Lung Metastasis
Tumor-free
Tumor-bearing
.0
1.5
1.0
0.5
Luminescence
X10⁶ p/s/cm²/sr
C
Lung Metastasis Index
2.5
2.0
1.5
1.0
0.5
0.0
D
Tumor-free
Tumor-bearing
Lung Metastasis Index
3
2
1
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Luminescence
X10⁷ p/s/cm²/sr

Fig. S24. Spontaneous lung metastasis model establishment.

(**A** to **C**) 6 weeks after subcutaneous implantation of Luc-RFP-231 into fat pads of female NSG mice, in vivo luciferase imaging was performed with an IVIS Lumina. Representative pictures of in vivo luciferase imaging of (**A**) primary tumors and (**B**) lung metastasis. Primary tumors were covered with black opaque tape during imaging to prevent oversaturation. Black squares were added for consistency. (**C**) Quantification of luciferase activity of Luc-RFP-231 in the lungs of tumor-bearing NSG mice 6 weeks after Luc-RFP-231 implantation. $n = 9$ (**D**) 6 weeks after Luc-RFP-231 implantation, tumor-bearing NSG mice were sacrificed, and lungs were imaged with an IVIS Lumina. $n = 4$. Lung Metastasis Index = Log_{10}[(luciferase read of the tested tumor-bearing mouse/lung) / (luciferase read of tumor-free mice/lungs average)], such that the LMI of tumor-free mice or lungs = 0.

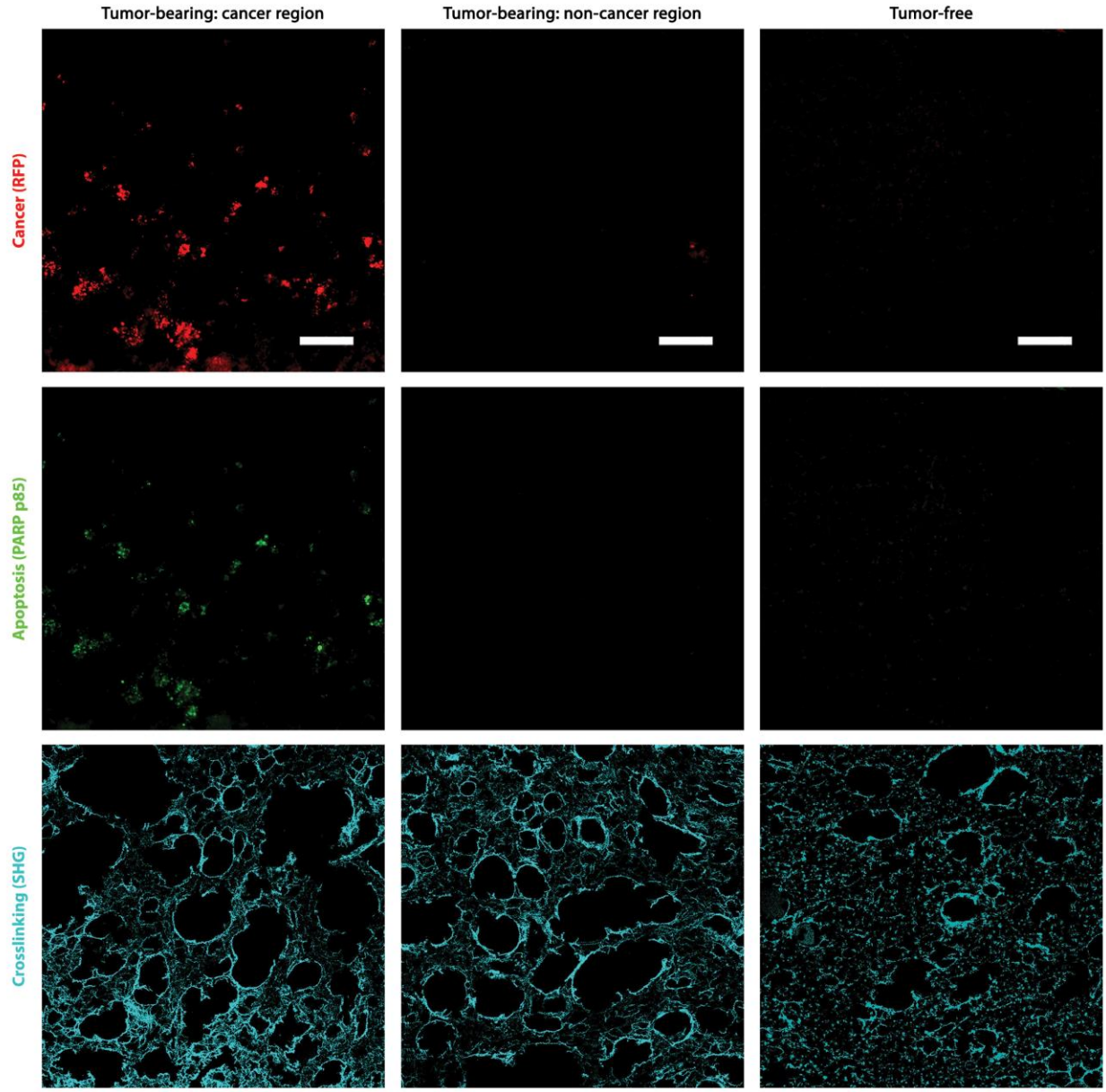


Fig. S25. Split-channel views of cross-linking–specific tissue damage by MRCS in the metastatic niche in vivo in spontaneous lung metastasis model.

Frozen sections of lungs of tumor-bearing NSG mice with Luc-RFP-231 spontaneous lung metastasis from primary tumors (cancer region and non-cancer region) and tumor-free NSG mice sacrificed after MRCS-CD infusion and 5-FC treatment as indicated (Day

9) were stained with anti-PARP p85 (green) for tissue apoptosis. RFP signal (red) indicates the presence of lung metastasis. SHG imaging of collagen networks is also shown (cyan).

Table S1. Primary antibodies.

Antigen	Company	Catalog #	Dilution	Notes
Annexin V	Bioss Antibodies	bs-0398R	1: 100	
Cytosine deaminase	Bioss Antibodies	bs-2950R	1: 100	Fig.5 & S11
Cytosine deaminase	Thermo Fisher	PA185365	1: 200	Fig.S6 & S7
eGFP	Abcam	ab111258	1: 100	Fig.S12
eGFP	Abcam	ab13970	1: 500	Fig.1 & S4
eGFP	Thermo Fisher	OSE00001G	1: 100	Fig.5
Firefly luciferase	Abcam	ab21176	1: 100	Fig.S12
Firefly luciferase	Abcam	ab181640	1: 100	Fig.5 & S11
Lysyl oxidase (LOX)	Abcam	ab31238	1: 100	
PARP p85 fragment	Promega	G7341	1: 100	
YAP	Santa Cruz Biotechnology	sc-101199	1: 100	Ref. 32
CD45R/B220	BioLegend	103212	N/A	APC
CD11b	BioLegend	101206	N/A	FITC
Ly-6G (Gr-1)	TONBO Biosciences	60-5931	N/A	PE-Cy7

Table S2. Secondary antibodies.

All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories except for ab63507.

Tag	**Species**	**Catalog #**	**Dilution**
Alexa Fluor 488	Donkey α chicken	ab63507 (Abcam)	1: 1000
Alexa Fluor 488	Donkey α goat	705-545-147	1: 500
Alexa Fluor 488	Donkey α rabbit	711-545-152	1: 500
Alexa Fluor 488	Donkey α sheep	713-545-003	1: 500
Rhodamine (TRITC)	Donkey α goat	705-025-147	1: 500
Rhodamine (TRITC)	Donkey α rabbit	711-025-152	1: 500
Alexa Fluor 594	Goat α mouse	115-585-062	1: 500
Alexa Fluor 647	Donkey α rabbit	711-605-152	1: 500

Table S3. Primers used in qPCR.

All primers were purchased from Integrated DNA Technologies (IDT).

Name	**Sequence**	**Notes**
GAPDH-C-F1	5’ – CTC CTG CAC CAC CAA CTG CT – 3’	Ref. 32
GAPDH-C-R2	5’ – GGG CCA TCC ACA GTC TTC TG – 3’	
ANKRDI-C-F	5’ – AGT AGA GGA ACT GGT CAC TGG – 3’	
ANKRDI-C-R	5’ – TGG GCT AGA AT GTC TTC AGA T – 3’	
CTGF-C-F	5’ – AGG AGT GGG TGT GTG ACG A – 3’	
CTGF-C-R	5’ – CCA GGC AGT TGG CTC TAA TC – 3’	
GFP-F	5’ – CTG CTG CCC GAC AAC CAC – 3’	Ref. 60
GFP-R	5’ – ACC ATG TGA TCG CGC TTC TC – 3’	
CDy-F	5’ – ACC ATG GTC ACA GGA GGC AT – 3’	Ref. 61
CDy-R	5’ – TTC TCC AGG GTG CTG ATC TC – 3’	