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c-kit$^+$ cells adopt vascular endothelial but not epithelial cell fates during lung maintenance and repair

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Unraveling the fate specification of resident stem cells during lung regeneration is of clinical importance. It has been reported that c-kit$^+$ progenitor cells resident in the human lung regenerate epithelial lineages upon transplantation into injured mouse lung. Here we test the lineage potential of c-kit$^+$ cells by inducible genetic lineage tracing. We find that c-kit$^+$ cells do not contribute to lung epithelium during homeostasis and repair, and instead maintain a vascular endothelial cell fate. These findings call attention to the clinical application of c-kit$^+$ stem cells as lung epithelial progenitors for the treatment of pulmonary disease.

Lung disease is among the leading causes of death in the industrial world. The identification of lung stem cells during homeostasis, repair and regeneration are of clinical importance[1,2]. The lung epithelium is thought to be maintained by lineage-restricted stem cells[3–6], yet a recent study reports that c-kit$^+$ stem cells resident in the human lung are multipotent and regenerate lung epithelial and vascular endothelial lineages upon transplantation into injured mouse lungs[7]. Because transplantation assays may not reveal the actual potency and fate of stem cells under physiological conditions, genetic lineage tracing is required to test the endogenous multipotency of c-kit$^+$ cells during homeostasis and upon injury[8].

To mark and follow the fate of c-kit–expressing cells *in vivo*, we generated a *Kit-CreER* knock-in line by inserting *CreER* gene in frame with the translational start codon of the *Kit* oncogene (**Supplementary Fig. 1a**). CreER is a tamoxifen-inducible fusion protein consisting of Cre and the ligand-binding domain of a mutated estrogen receptor. We then crossed *Kit-CreER* mice with the fluorescent reporter mouse line *Rosa26-RFP* (ref. 9) to generate *Kit-CreER*;*Rosa26-RFP* mice for inducible genetic lineage tracing. To determine which cells are genetically marked by *Kit-CreER*, we analyzed lungs from adult *Kit-CreER*;*Rosa26-RFP* mice at 24–48 h after administration of a single pulse of tamoxifen, or corn oil as control, to assess CreER activity in the absence of tamoxifen treatment (**Supplementary Fig. 1b**,**c**). Co-staining of lung sections with RFP and c-kit–specific antibodies showed that *Kit-CreER* specifically marks c-kit–expressing cells in the alveoli (**Supplementary Fig. 1c**,**d**). By flow cytometry analysis, we verified that *Kit-CreER* genetically marks 19.41 ± 0.82% of c-kit–expressing cells (n = 4; **Supplementary Fig. 1e**,**f**). Additional immunostaining or flow cytometry analysis for the endothelial cell markers PECAM or VE-cadherin (VE-CAD) and the pan-epithelial cell marker E-cadherin (E-CAD) revealed that *Kit-CreER* is expressed in endothelial but not epithelial cells (**Supplementary Fig. 2**), which is consistent with a recent study reporting c-kit expression in human lung endothelium[10].

Administration of four pulses of tamoxifen resulted in efficient labeling of lungs from *Kit-CreER*;*Rosa26-RFP* mice with RFP (**Supplementary Fig. 3a**,**b**). Quantification of labeled cells verified that 85.25 ± 4.34% of c-kit–expressing cells were marked by RFP (n = 4; **Supplementary Fig. 3c**,**d**). By immunostaining for PECAM or VE-CAD, E-CAD, CC10, T1-α and SP-C to mark endothelial, epithelial, club, alveolar type I and type II cells, respectively, we confirmed that *Kit-CreER* genetically marks lung vascular endothelial but not epithelial cells (**Supplementary Fig. 3e**–**h**).

We next studied the fate of c-kit–expressing cells and their descendants during homeostasis (**Supplementary Fig. 4a**). Strong RFP expression was detected in lungs from *Kit-CreER*;*Rosa26-RFP* mice at 4–12 weeks after tamoxifen induction (**Supplementary Fig. 4b**,**c**). Co-staining for RFP, E-CAD, PECAM, T1-α, SP-C and CC10 showed that *Kit-CreER* marks vascular endothelial but not epithelial cells (**Supplementary Fig. 4d**–**g**). These data demonstrate that c-kit$^+$ cells do not contribute to lung epithelium during homeostasis (**Supplementary Fig. 5h**).

Next, we asked whether resident c-kit$^+$ cells are activated and convert to epithelial cells during cryoinjury (CI)[7]. After tamoxifen injection for 2 weeks and CI one week later, we analyzed the fate of c-kit–derived cells in injured lungs (**Fig. 1a**). Expression of RFP was almost undetectable in CI *Kit-CreER*;*Rosa26-RFP* lungs that had not been treated with tamoxifen (**Fig. 1b**), indicating negligible leakiness of *Kit-CreER* upon cryoinjury. In contrast, RFP expression was high in tamoxifen-treated lungs with or without CI (**Fig. 1b**). Immunostaining for epithelial and endothelial markers revealed that a substantial number of endothelial cells, but no epithelial cells, were marked by RFP (**Fig. 1c**–**h**). Although labeled cells were located in close proximity to epithelial cells such as alveolar type I or type II cells, RFP did not colocalize with epithelial

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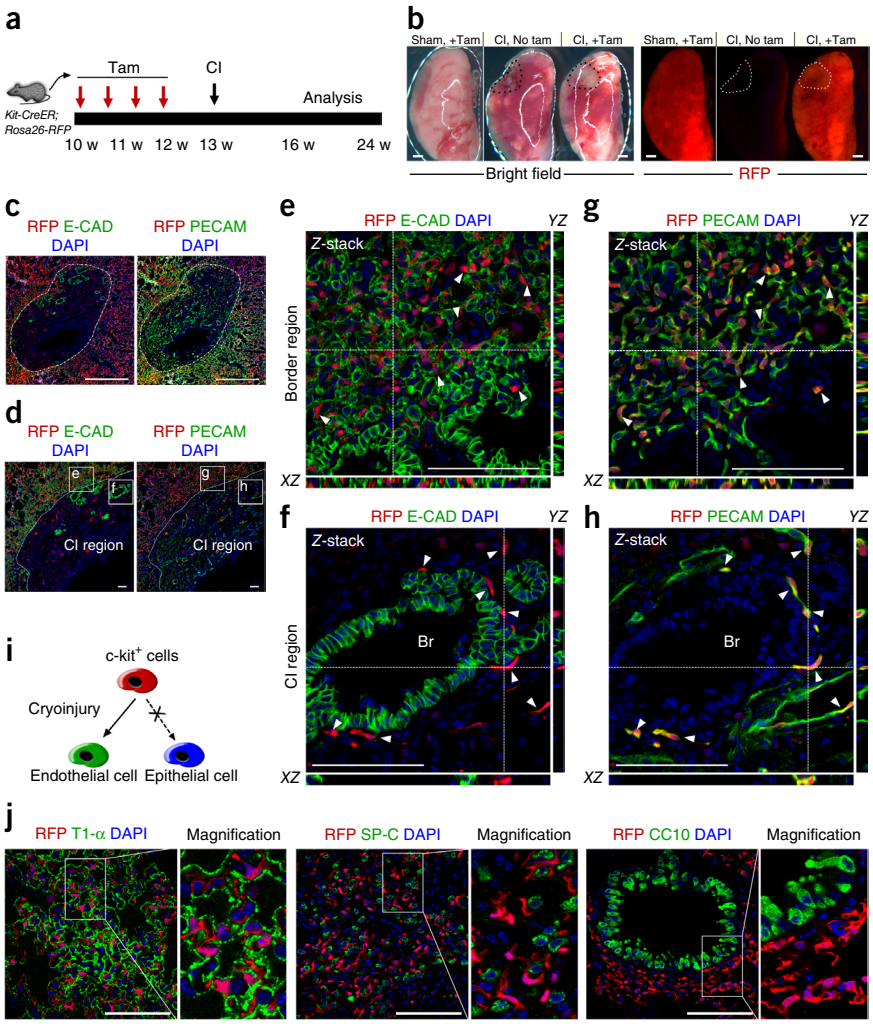

Figure 1 Upon cryoinjury, c-kit$^+$ cells do not generate lung epithelium. (**a**) Schematic diagram showing the strategy for genetic labeling of c-kit$^+$ cells by tamoxifen treatment (Tam) followed by CI. Time is shown in weeks (w). (**b**) Representative whole-mount views ($n = 6$) of lungs from CI or control (sham) *Kit-CreER*;*Rosa26-RFP* mice with or without tamoxifen induction. Dotted lines indicate the CI region. (**c**–**h**) Immunostaining for RFP, E-CAD, PECAM and DAPI on sections of CI lungs. Dotted lines indicate whole CI regions (**c**) and partial CI regions (**d**). Boxed regions in **d** are magnified in **e**–**h**. Arrowheads point to RFP$^+$PECAM$^+$E-CAD$^-$ cells. *XZ* and *YZ* indicate signals from dotted lines on *Z*-stack images in **e**–**h**. (**i**) Schematic figure showing maintenance of endothelial, but not epithelial fate of c-kit$^+$ cells upon lung CI. (**j**) Immunostaining for RFP, T1-α, SP-C, CC10 and DAPI on sections of CI lungs. Scale bars, 1 mm in **b**–**d**; 100 μm in **e**–**h**,**j**.

markers (**Fig. 1c**–**j**). We obtained similar results in regions distant from CI and in sham-operated lungs (**Supplementary Fig. 5**). In addition, more proliferating RFP$^+$ cells were detected in lungs upon injury compared to uninjured lungs (1.69 ± 0.16% in CI versus 0.25 ± 0.10% in sham; $n = 4$; **Supplementary Fig. 6**). In the CI region, a small subset of RFP$^+$ cells expressed the macrophage marker CD11b and fibroblast-specific protein 1 (FSP1) (**Supplementary Fig. 7**). These data suggested that c-kit$^+$ cells do not generate lung epithelium upon cryoinjury but mainly adopt a vascular endothelial cell fate.

To test whether c-kit$^+$ cells generate epithelial cells in alternative lung injury models, we performed left-sided pneumonectomy (PNX) in adult *Kit-CreER*;*Rosa26-RFP* mice. We adopted a similar tamoxifen induction regime as for cryoinjury, and we analyzed lung tissue at 3–11 weeks after PNX (**Fig. 2a**). We found that the right cranial, middle and caudal lobes were efficiently labeled by RFP (**Fig. 2b**). Immunostaining for RFP, E-CAD, PECAM or VE-CAD on tissue sections showed that RFP marks 55.63 ± 1.52% endothelial cells, but not epithelial cells ($n = 6$; **Fig. 2c**,**d** and **Supplementary Fig. 8**). We also found more *Kit-CreER*–marked endothelial cells after PNX (**Supplementary Fig. 9**). In addition, more proliferating RFP$^+$ cells were detected after PNX compared to sham (1.88 ± 0.16% in PNX versus 0.19 ± 0.06% in sham; $n = 4$; **Supplementary Fig. 10**). We did not observe any contribution of c-kit–derived cells to T1-α$^+$ alveolar type I cells, SP-C$^+$ alveolar type II cells or CC10$^+$ club cells (**Fig. 2e**). Taken together, these data provided evidence that c-kit$^+$ cells do not contribute to lung epithelium during injury, but instead maintain a vascular endothelial cell fate.

Cell transplantation studies report contribution of bone marrow–derived c-kit$^+$ cells to infarcted myocardium[11]. These results led to clinical investigations on the use of stem cells for treatment of myocardial infarction[12]. However, lineage tracing reveals a minimal contribution of c-kit$^+$ cells to cardiomyocytes[13–15], indicating that conclusions on the potency of cells made on the basis of transplantation assays should be taken with caution.

A recent study reports that c-kit$^+$ human lung stem cells are multipotent and generate epithelial cells in injured mouse lungs[7]. In contrast, our lineage-tracing experiments show that c-kit$^+$ cells do not give rise to lung epithelium during homeostasis and repair. A possible explanation for this difference is that the *in vitro*–expanded human lung c-kit$^+$ cells may acquire multipotency during culture and adopt an epithelial cell fate after engraftment. Moreover, although c-kit$^+$ stem cells have previously been reported to be rare in lung tissue (1 stem cell per 24,000 cells in the lung)[7], our study shows that c-kit$^+$ cells constitute a large proportion of vascular endothelial cells in the normal lung. Our genetic lineage-tracing data do not support c-kit as a marker for lung epithelial stem cells, and our work calls attention to the clinical application of c-kit$^+$ stem cells as lung epithelial progenitors for the treatment of pulmonary disease.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

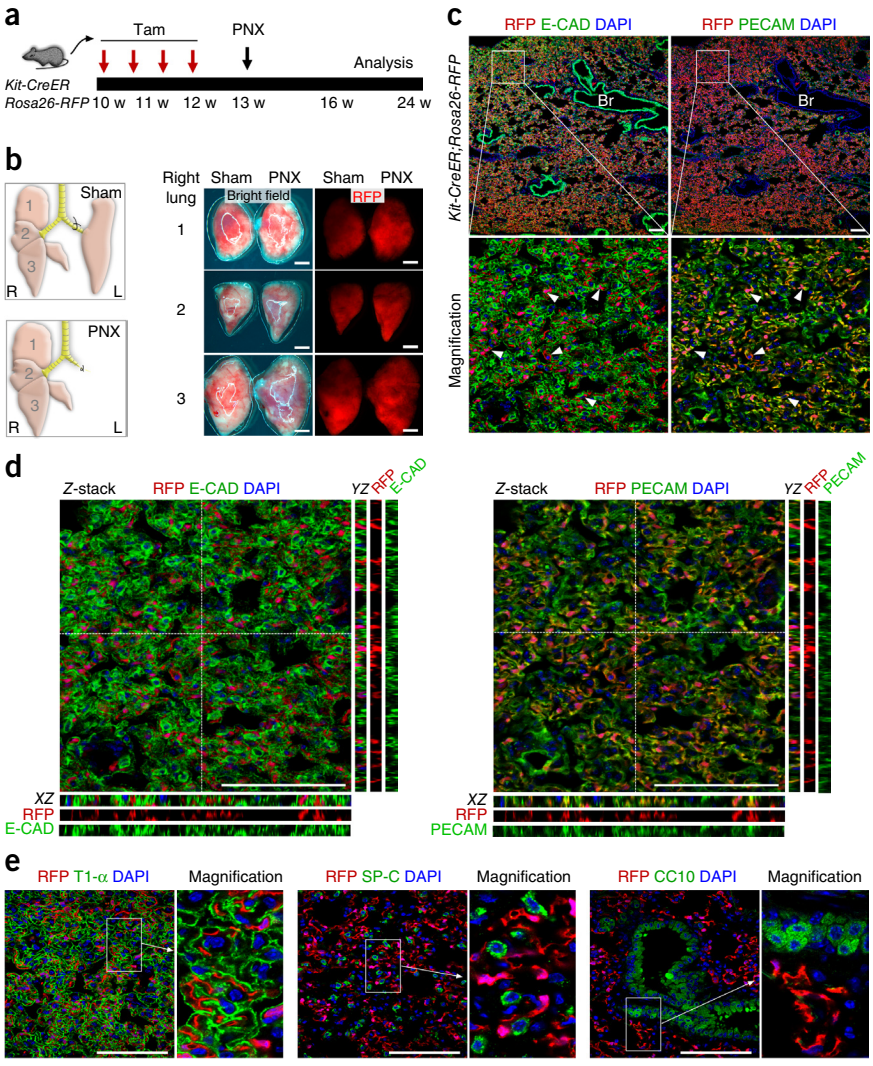

Figure 2 Upon pneumonectomy, c-kit$^+$ cells do not contribute to lung epithelium. (**a**) Schematic diagram showing the strategy for tamoxifen induction and left-sided PNX. Time is shown in weeks (w). (**b**) Left, sham or PNX model on lung left lobe; and 1–3 in the diagram indicate the cranial, middle and caudal lobes of the right lung, respectively. Right, representative whole-mount views ($n = 6$) of lungs from sham- and PNX-operated *Kit-CreER;Rosa26-RFP* mice. R, right; L, left. (**c**) Immunostaining for RFP, E-CAD and PECAM on sections of lungs from *Kit-CreER*;*Rosa26-RFP* mice. Arrowheads point to RFP$^+$PECAM$^+$E-CAD$^-$ cells. Br, branchiole. (**d**) Magnified *Z*-stack images (of the boxed areas in **c**) with *XZ* and *YZ* sections (dotted lines) shown. RFP$^+$ cells are PECAM$^+$ endothelial cells that are located in proximity to E-CAD$^+$ epithelial cells. (**e**) Immunostaining for RFP, T1-α, SP-C, CC10 and DAPI on the sections of right lung of PNX mice. Scale bars, 2 mm in **b**; 100 μm in **c**–**e**.

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AUTHOR CONTRIBUTIONS

Q.L. conducted experiments and analyzed the data. X.H., H.Z., X.T., L.H., R.Y., Y.Y. and Q.-D.W. bred mice, performed experiments or provided discussion. A.G. provided valuable suggestions and comments, interpreted data and edited the manuscript. B.Z. supervised the study, interpreted the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All animals were used according to the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. *Kit-CreER* mice were crossed with *Rosa26-RFP* (Ai9) and *Rosa26-GFP* (Ai47) mice to generate *Kit-CreER;Rosa26-RFP* and *Kit-CreER;Rosa26-GFP* for genetic lineage tracing of c-kit$^+$ cells. Tamoxifen (Sigma, T5648) was dissolved in corn oil (20 mg/ml) and administered by gavage at the indicated time (0.1–0.15 mg tamoxifen per gram of mouse body weight)[16]. To assess the leakiness of inducible Cre in the absence of tamoxifen, we administered corn oil by gavage to *Kit-CreER;Rosa26-RFP* mice in parallel. Both male and female mice were used in our experiments and randomized in allocation into different treatment groups. The researchers were not blinded to the mice of sham, CI or PNX operations. We generated a *Kit-CreER* knock-in mouse line by inserting a cDNA encoding Cre recombinase fused with a mutant form of the estrogen receptor hormone–binding domain (CreER^{T2}) into frame with the translational start codon of the *Kit* gene as previously described[17]. Briefly, A strain-129 mouse BAC clone containing the complete mouse c-*Kit* gene was obtained from Sanger Institute (UK). We constructed the targeting vector containing the following cassettes: *CreER* cDNA, SV40 polyA sequence and *Frt* site-flanked PGK-EM7-Neo resistance gene. Two homologous arms on the 5′ and 3′ ends of first coding exon of *c-Kit* were generated on targeting vectors by recombination from BAC. The targeting vector was digested with I-CeuI for linearization, and linearized DNA was electroporated into mouse embryonic stem (ES) cells. The targeting vector containing the aforementioned cassettes was knocked into the *c-Kit* locus for endogenous expression of *CreER*. After G418 selection, over 200 clones were selected for retrieval of genomic DNA and screening of positive clones. Neomycin-resistant clones were tested for correct gene targeting by long PCR assays with primer pairs spanning the targeting vector and flanking genomic DNA. After verification of correct targeting and karyotype, we expanded three positive ES clones and injected ES cells into blastocyst for mice generation. The obtained chimeric mouse lines were crossed to C57B/6 lines for germ-line transmission. We designed PCR primers spanning the genomic DNA and inserted cassette (forward: 5′-GCCTTCTATCG CCTTCTTGACG-3′, reverse: 5′-CAGTCGGCACAAAAGCATCAC-3′) to test the correct targeted allele. The established *Kit-CreER* mouse lines were maintained on a C57B6/C129 background.

Lung cryoinjury (CI) model. We performed a lung CI model as described previously to test the multipotency of c-kit$^+$ cells *in vivo*[7]. Adult mice were anesthetized with isoflurane in an airtight induction chamber. When there was no response to stimuli, we removed the hair of the chest with a hair clipper and disinfected the skin with iodine. Mice were ventilated and anesthetized via tracheotomy, with the cannula connected to a respiratory machine (Harvard Apparatus) and an anesthetic gas machine (Harvard Apparatus). The left lung was exposed by thoracotomy and damaged by touching with a copper probe precooled in liquid nitrogen for about 10 s. The control mice were anesthetized and chest was opened using the same procedures as in the CI model but without cryoinjury. The mice were then ventilated with oxygen for another 5 min and kept warm until they awakened. We collected mice lungs at 3–11 weeks after cryoinjury for analysis of c-kit$^+$ cell fates after lung injury.

Lung pneumonectomy model (PNX). We performed the mice lung PNX model according to the protocol described previously[18,19]. Mice were anesthetized, ventilated and secured as described above. After mice were secured on a heating pad in a supine position by adhesive tape, an incision was made on the chest and left lung was exposed by thoracotomy with a retractor to ensure wide field of view. We ligated the lung blood vessel at the hilum tightly and removed the complete left lung. We closed each mouse's chest by bringing together the ribs around the incision with one 5-0 suture. The muscle and skin layers were stitched, respectively, and disinfected with iodine again. The sham-operated mice were anesthetized for thoracotomy using the same procedures as in PNX without left lung removal. After mice restored autonomous respiration, we removed the trachea cannula, and mice were then kept on a warm pad for better recovery. We collected mice lungs at 3–11 weeks after PNX and the right lung had restored the lost lung volume.

Immunostaining. We performed immunostaining according to the previous protocols[20]. Briefly, we collected mice lungs and washed them in cold PBS for several times. Then the lung tissue was fixed in 4% PFA for ~1 h and dehydrated in 30% sucrose overnight at 4 °C. After soaking the tissue in OCT (Sakura) for 1 h at 4 °C, we embedded the tissue in block and froze it at −80 °C until we were ready to section. Frozen sections of 10 μm in thickness were collected on slides and blocked in blocking solution (5% normal donkey serum in PBS with 0.1% Triton X-100) for 30 min at room temperature and incubated in primary antibodies overnight at 4 °C. After fully washing out the primary antibodies, slides were then developed with Alexa Fluor fluorescent antibodies (Invitrogen) and nuclei were stained with DAPI. For weak signals, we used horseradish peroxidase– or biotin-conjugated antibodies (Jackson ImmunoResearch) with a tyramide signal amplification kit (PerkinElmer). We mounted the slides with fluorescence-protecting mounting medium (Vector Lab) and took pictures using Zeiss or Olympus confocal microscopy system. Primary antibodies and dilution ratios are listed below: c-kit (R&D, AF1356, 1:50); RFP (Rockland, 600-401-379,1:500); VE-Cadherin (R&D, AF1002, 1:100); PECAM (BD Pharmingen, 553370,1:200); E-cadherin (Cell Signaling Technology, 3195, 1:100); Pdpn or T1-α (DSHB, 8.1.1, 1:200); Surfactant proteins C, SP-C (Millipore, Ab3786, 1:500); CC10 (Santa Cruz, SC-9772, 1:100), Fibroblast specific protein 1, FSP1 (Dako, A5114, 1:100), CD11b (BD Pharmingen, 550282, 1:100). Images were acquired by a Zeiss confocal microscope (LSM510, LSM710) and an Olympus confocal microscope (FV1000), a Leica stereomicroscope (M165 FC) and an Olympus microscope (BX53). To provide *Z*-stack confocal images, we scanned 5–7 consecutive *XY* images on the *z* axis with a Zeiss confocal microscope (LSM710). The obtained image data was analyzed by ImageJ (NIH) software. The images were merged using the Image color-merge channels function, and then stacks were done using *Z*-projects and average intensity projection. In the stack, orthogonal view was done to reveal the signals on the *XZ* and *YZ* axes. Merged signals and split channels were shown to delineate the signals on single cell resolution.

Lung cell isolation. Lung cell isolation was performed as previously described[21]. We euthanized mice and perfused the lungs with 10 ml of cold PBS through the right ventricle. To inflate the lung, we injected 2 ml of PBS through the trachea. The digestion protease was composed of 500 U/ml collagenase I, 4 U/ml elastase, 5 U/ml dispase and 0.33 U/ml DNase I in DMEM/F12 solution. 1.5 ml of digestion protease was injected through the trachea to inflate the lung first, and then the lung was cut into small pieces (<2 mm^2). The lung pieces were incubated in 2.5 ml of protease for 25 min at 37 °C with agitation. We added DMEM/F12 containing 10% FBS and disrupted the tissue by gently pipetting. After washing with DMEM/F12, the tissue was then incubated at 37 °C for 20 min in 2 ml of 0.1% Trypsin-EDTA + 0.325 mg DNase I with intermittent agitation. We then added equal volume of DMEM/F12 containing 10% FBS and filtered the dissociated cells through a 100-μm strainer, followed by washing with 5 ml of DMEM/F12. The lung cells were incubated at room temperature for 5 min in 2 ml of red blood cell lysis buffer. The cells were then filtered through a 40-μm strainer, centrifuged and resuspended in DMEM/F12.

Lung cell staining for flow cytometry analysis. The dissociated lung cells were centrifuged and the supernatant was discarded. We added 50 μl of Violet dye (Invitrogen L34955, 1:1,000 in PBS) to resuspend the cell pellet, and then incubate at 4 °C for 30 min. 500 μl of isolation buffer (2 mM EDTA and 0.5% BSA in PBS) was added to wash cells, followed by centrifugation at 4,600*g* for 3 min. Supernatant was discarded and Fc block solution (BD Pharmingen 553141, 1:100 in Isolation buffer) and antibody (25 μl for each) were added to re-suspend the cell pellet. The FACS antibodies used were c-kit (553353, BD Pharmingen, dilution, 1:20), E-cadherin (13-3249-80, eBioscience, dilution, 1:50), PECAM (12-0311, eBioscience, dilution, 1:20). The cells were incubated with antibodies at 4 °C for 30 min. After washing with 500 μl of isolation buffer, cells were centrifuge at 4,600*g* for 3 min and the supernatant was discarded. If a secondary antibody was used, we performed the same procedure for staining of the secondary antibody and washing step as above. Finally, the cells were resuspended with 300 μl of isolation buffer. We analyzed stained cells on a BDAria IIu Cytometer by Flowjo software (TreeStar).

Statistical analysis. All data were determined from 4–6 independent experiments, as indicated in each figure legend, and presented as mean values ± s.e.m.

Statistical comparisons between data sets were made with analysis of normality and variance, followed by a two-sided unpaired Student's *t*-test for comparing differences between two groups. $P < 0.05$ was considered to be statistically significant. All mice were randomly assigned to different experimental groups. The investigators were not blinded to mice allocation during experiments and analysis. No statistical method was used to predetermine sample size.

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