# Supplementary information

## 1 Materials and methods

### 1.1 Cell cultures and the preparation of conditioned media from osteoclasts , osteoclast precursors, osteoblasts and osteoblast precursors

(1) The murine lymphatic endothelial cell line (LEC) was used in the study by Chen JC et al. (Chen et al., 2012), and the specific methods of LEC source and culture were referenced in previous studies (Wang et al., 2017). (2) For culturing OCs and OCPs, 8-week-old WT C57BL/6 mice (purchased from Henan Skbex Biotechnology, China) were used, and the femur and tibiae were washed and collected for the bone marrow cells. The collected bone marrow cells were lysed with lysis buffer (Solarbio, China) and cultured in modified Eagle's medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 20 ng/mL macrophage colony-stimulating factor (M-CSF, R&D Systems Inc.) at $1 \times 10^4$ or $5 \times 10^4$ cells per well in 96-well or 24-well plates, respectively. Every 2 d, the medium which included 10 ng/mL of nuclear factor-B ligand (RANKL, R&D Systems Inc.) and 20 ng/mL of M-CSF was replaced. Tartrate-resistant acid phosphatase (TRAP) activity was used to identify OCPs and OCs. OCPs were formed after 3 d of culture. Similarly, all cells became fully mature multinucleated OCs, which were TRAP-positive (TRAP+) cells containing ≥3 nuclei after incubation for 6 d. (3) For culturing MC3T3-E1 osteoblast precursors (OBPs) and OBs, OBPs cultured in α-MEM medium (Gibco, USA) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin were obtained from ATCC (California, USA). OBPs were grown in 6-well plates with $1 \times 10^5$ cells per well in osteogenic differentiation induction media, which contains α-MEM medium, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM dexamethasone, and 50 μg/mL ascorbic acid. Alkaline phosphatase (ALP) staining was used to identify osteogenic differentiation, and OBs were formed after 14-21 d of culture. (4) For collecting conditioned media (CM), at the end of culture/induction in OCs, OCPs, OBPs and OBs, serum-containing CM and serum-free CM that were used for migration experiments were harvested. All CM was aliquoted and kept at -80°C for further studies following centrifugation (2,000 rpm) for 10 min at 4°C.

### 1.2 Migration assay and tube formation by LECs in vitro

For our migration tests, we utilized CM that was devoid of serum. Transwell assays were used to measure the ability of chemotactic motility LECs. In the upper chamber of a transwell plate (6.5 mm diameter inserts, 8.0 μm pore size; Corning Inc.), cells ($5\times10^4$ cells/well) were plated and grown for 6-8 h. After that, the cells were stained with 0.1% crystal violet after being fixed with 70% ethanol. We quantified the cells that had migrated through the holes to the bottom surface by counting five random fields per well at 200× magnification. The migration potential of LECs was determined by a test for wound healing. Briefly, cells were planted onto six-well plates and cultivated till 80-90% confluency. To get rid of the suspended cells, plates were scratched with a 200 L micropipette tip and then washed with phosphate-buffered saline. The cells were then incubated in serum-free medium, and images were taken at different time points. Cell migration was assessed by measuring the distance between the opposing sides of the wound with ImageJ (NIH, USA). Data (mean ± SD, n=5) were calculated using the following formula: (distance after an identified culture period/distance at initial scratch). Three independent experiments were performed.

To measure tube formation by LECs in vitro, reduced growth factor Matrigel (Corning Incorporated, USA, #356230) was used according to a previous report (Wang, et al., 2017). Briefly, in a 48-well plate, 150 μL of Matrigel was added, and it was incubated at 37°C for 30 min; tube-like

structures were formed for 4-5 h after the cells ($4\times10^5$) derived from two groups were loaded on polymerized Matrigel at 37°C. The length of cumulative tubes was measured with ImageJ software Angiogenesis Analyzer (NIH, USA).

### 1.3 Construction and packaging of the lentiviral vector encoding Sema3A and generation and transduction of the Sema3A shRNA lentiviruses

For generation and transduction of the Sema3A shRNA lentiviruses, Sema3A and negative control (NC) sequence-specific oligonucleotides by Sangon Biotech Co. (Shanghai, China) were created. The oligonucleotide sequences, NC:TTCTCCGAACGTGTCACGTAA; Sema3A-shRNA_1: CCCAGTGTTTCCTATAAATAA; Sema3A-shRNA_2: GCCTTGGTATATTGGCAATTT. Synthesized shRNAs were inserted into the pLKO.1-Puro vector. pLKO.1-Puro-Sema3A shRNA (LV-shSema3A) or pLKO.1-Puro-NC (LV-shNC) was cotransfected into 293T cells with the packaging plasmids psPAX2 and pMD2. G (Addgene, USA) using Lipofectamine 3000 (Invitrogen, USA). Viral particles were isolated by ultracentrifugation after 48 hours of incubation, aliquoted, and stored at -80°C for subsequent experiments.

### 1.4 Establishment of LECs with stable expression of Sema3A

A total of $5\times10^5$ LECs were seeded in three 10 $cm^2$ tissue culture plates and incubated overnight. The 1st plate contained lentivirus Seme3A supernatant, the 2nd plate contained LV-NC supernatant, and the 3rd plate contained the killing control, where no lentivirus was added. The culture medium was removed 24 h after the cells were incubated. Fresh medium with puromycin was added every two days until all the cells in the control plate were dead. Monoclonal cells were isolated by limited dilution and expanded for culture. The stable cell lines (Sema3A-LEC and NC-LEC) were verified by qPCR and Western blotting and then stored at -80°C for future experiments.

### 1.5 Cell growth assay and flow cytometry analysis

The cell growth tests performed by CCK8 assay (Vazyme, China). In brief, a 96-well cell culture plate was seeded with 1000 cells total in 100 μL of medium. After incubation at 37°C with 5% $CO_2$ overnight, we used serum-containing CM from OCs, OCPs, Obs and OBPs for LEC growth experiments. To determine the effect of Sema3A on the growth of LECs, the culture mixture was replaced with medium containing a variety of quantities of recombinant Sema3A (0, 0.1, 0.5 and 1 μg/mL). 10 μL of CCK-8 reagent was added to each well after cell culture, and the plates were then left to culture for 1-2 h. A spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the optical density (OD) absorbance at a wavelength of 450 nm in order to ascertain the vitality of the cells.

For detection of lymphatic endothelial cells *in vivo*, phosphate buffered saline (PBS) was used to drain out tibial bone marrow cells. After centrifugation and erythrocyte lysis, the cells were collected by repeatedly washing twice with FACS buffer (2% fetal bovine serum in PBS). Up to $10^7$ cells were resuspended and incubated for 30 min on ice in 100 μL FACS buffer with the following fluorochrome-conjugated antibodies: PDPN-PE (Cat#25-5381-82, Invitrogen) and LYVE-488 (Cat#53-0443-82, Invitrogen). Isotype IgG was used as the control.

### 1.6 Total RNA extraction and quantitative real-time PCR (qPCR)

Utilizing the TRIzol reagent (Vazyme, China), total RNA was extracted from the samples, and RNA concentrations were determined by spectrophotometry (Thermo Fisher, USA). HiScript III-RT

SuperMix (Vazyme, China) was used to generate cDNA from mRNA. On a Roche 480 II Real-Time PCR System (Roche, Switzerland), qPCR was carried out using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). The β-actin gene was used as an internal reference. All the primers for qPCR are listed in Supplementary Table S1, and RNA samples from 3 wells were analyzed to determine the mean gene expression. Finally, the fold changes normalized to the housekeeping gene β-actin show the relative levels of expression of the relevant genes (Table S2).

### 1.7 Protein isolation and Western blotting analysis

Using RIPA buffer (Solarbio, China), total proteins were recovered from the cells. Protein (30 μg/lane) was loaded, separated by 10% SDS–PAGE, and transferred to PVDF membranes. Immunoblots were performed with the following primary antibodies: anti-β-actin (1:500, sc-8432, Santa Cruz Biotechnology, USA) and anti-Semaphorin 3A (1:1000, ab199475, Abcam, USA). The proteins were analyzed using Image Lab (Bio-Rad, USA).

### 1.8 Enzyme-linked immunosorbent assay (ELISA)

A Mouse Sema3A Quantikine ELISA Kit (Jiangsu Jingmei Biotechnology, cat# JM11913M2) was used to measure the Sema3A protein levels. A mouse osteocalcin ELISA kit (cat# E4763-100, Biovision) was used to measure the protein level of Osteocalcin (OCN) in mouse bone marrow supernatant. OD (450 nm) values were calculated by the iMark microplate reader (Bio-Rad).

### 1.9 Animal studies

Female C57BL/6 mice were purchased from Henan Skbex Biotechnology Co. Ltd. (Anyang, China, animal certification number was SCXK [YU] 2020–0005). All experiments were performed in 8-week-old mice. All animal experimental procedures and operating procedures in this study were approved by the Laboratory Animal Research Institute of Henan Normal University.

For the intratibial injection of mice, we randomly selected 5 mice in which the right tibia of each mouse was the control and the left tibia was used for each experiment. Three independent experiments were performed in triplicate. Cells or lentivirus were delivered into the bone marrow cavity of the tibia from the tibial crest, epiphysis, and growth plate using 10-μL syringes and 25-gauge needles.

For overexpression of Sema3A-inhibited tibial osteolysis in mice, the same amount of 10 μL ($1\times10^8$ transduction unit/mL) of LV-NC (control) and LV-Sema3A were injected into the right and left tibiae, respectively. Two days before that, 10 μL of $5\times10^5$ LECs that were viable were injected into the right and left tibia. Mice were sacrificed after 14 d.

For overexpression of Sema3A in LECs to suppress osteolysis and local LEC expansion, we injected $5\times10^5$ LECs overexpressing Sema3A (Sema3A-LECs) into the left tibia of mice, and equal amounts of control cells (NC-LECs) administered into the mice's right tibia. After 14 d, mice were slaughtered.

For knockdown of Sema3A in the mouse tibia, we constructed and packaged lentivirus expressing Sema3A shRNA (LV-shSeme3A) and control shRNA (LV-shNC). Ten microliters ($2\times10^8$ transduction units/mL) of LV-shSema3A and LV-shNC were injected into the left and right tibia, respectively. Mice were sacrificed after 14 d.

### 1.10 Microcomputed tomography (μCT) scanning

Tibias were removed from mice, fixed in 4% paraformaldehyde for 24 h and then maintained in 75% ethanol until scanning. The samples were scanned using a Bruker Micro-CT Skyscan 1276

system (Kontich, Belgium) with the following scanning settings: isotropic voxel resolution 6.5 m, 85 kV, 200 A, and exposure time 384 ms. Software called NRecon (version 1.7.4.2, Skyscan, Bruker, Belgium) was used to perform the reconstruction. 3D pictures were produced from contoured 2D photos using methods based on distance modification of the grayscale source images (CTvox; version 3.3.0). Using the program CT Analyzer (version 1.18.8.0, Bruker, Belgium), 3D and 2D analyses were carried out.

### 1.11 Histology, histomorphometric analysis and immunofluorescence (IF) staining

Mice's tibias were removed, fixed in 10% formalin, decalcified in 10% EDTA for 21 d, and then embedded in paraffin for cutting. Stains for general histology and TRAP activity were applied to sections (5 m thick) to help identify OCs. Osteometrics image analysis software (Decatur, GA, USA) was used to assess the number of OCs per mm bone surface.

Each data point was the mean of 3 levels (50 mm apart) from one sample. The whole tissue was selected for imaging by Caseviewer 2.2 scanning software (3DHISTECH, Hungary). After imaging, a complete static bone histomorphometric analysis in long bone was carried out using Image-Pro Plus 6.0 analysis software (Medium Cybemetics, USA).

For immunofluorescence staining, tibias were fixed in 10% formalin, decalcified in 10% EDTA, and embedded in paraffin for sectioning. Individual primary antibodies against mouse osteocalcin (Santa Cruz, sc-365797, 1:50) were incubated overnight at 4°C with bone sections. In the following step, samples were incubated with secondary antibodies that were fluorescently conjugated for 1 hour at room temperature without exposure to light. We counted the number of positively stained cells on trabecular bone and periosteal bone surfaces according to a method previously reported by Xie et al (Xie et al., 2014) and normalized them to the number per millimeter of adjacent bone surface (N/mm) in trabecular bone or per square millimeter of bone marrow area (N/mm2) in trabecular bone.

### 1.12 RNA sequencing (RNA-seq)

RNA-seq and data analysis were conducted by Seqhealth Technology Co., Ltd. (Wuhan, China). OCPs and OCs were rinsed three times with PBS, and total RNA was directly extracted with TRIzol reagent (Invitrogen, cat#15596026). KC-DigitalTM Stranded mRNA Library Prep Kit for Illumina® (cat#DR08502, Wuhan Seqhealth Co., Ltd. China) was used for stranded RNA-seq library preparation following the manufacturer's instructions. Follow-up inspection and data processing were executed according to standard operations. The raw read sequences are deposited in the GEO repository under accession number GSE229371 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE229371).

### 1.13 Statistical analysis

The results were obtained from at least three independent experiments, and all the data are expressed as the mean ± SD. Unpaired, two-tailed Student's t tests were used to assess differences between two groups. One-way ANOVA was used to analyze differences between data from more than two groups, and it was followed by the Tukey's HSD comparison test. Statistical significance was defined as a $P$ value < 0.05, denoted by a '*', a $P$ value < 0.01 was indicated by '**'.

**Table S1 Primer sequences used for qPCR in the study**

| Gene | | Sequence of Primer (5'→3') | Product size (bp) |
|---|---|---|---|
| *Sema3A* | F | CGAAAGCAACGCCGACAAAG | 102 |
| | R | CTCGTGGGTCCTCCTGTTTC | |
| *β-actin* | F | GTCAGGATCTTCATGAGGTAGT | 224 |
| | R | ACCCAGATCATGTTTGAGAC | |
| *Bmp3* | F | ATATCGGCTGGAGCGAATGG | 151 |
| | R | TACTCCTCTACCCCGTGCAA | |
| *Igf1* | F | GCCCCACTGAAGCCTACAAA | 169 |
| | R | GATGTGGCATTTTCTGCTCCG | |
| *Il1b* | F | CTTTCCCGTGGACCTTCCAG | 125 |
| | R | AATGGGAACGTCACACACCA | |
| *Inhba* | F | GGGGAAAAATGGGATGTTACTTT | 228 |
| | R | TGTACAACTCCCTGGCTCCT | |
| *Spp1* | F | CCTCTCACATGAAGAGCGGT | 194 |
| | R | ATCCGACTGATCGGCACTCT | |
| *Tnf* | F | AAACCACCAAGTGGAGGAGC | 154 |
| | R | GCAGCCTTGTCCCTTGAAGA | |
| *Tnn* | F | CTGTCCTGGCGACTGTAGTG | 234 |
| | R | GGTACCCGGACCTGTTTTGT | |
| *Wnt6* | F | CTGGGGGTTCGAGAATGTCA | 114 |
| | R | CACGAAAGCTGTCTCTCGGA | |

**Table S2 Results of *Sema3A, Nrp2, Vegfc, Vegfr3* and several genes which validated by qPCR and RNA sequencing of OCPs and OCs**

| Gene | *P* value (OC-OCP) | $\log_2$(fold change) (OC-OCP) |
|---|---|---|
| *Sema3a* | 0.000850672** | −0.795329208 |
| *Nrp2* | 0.138507328 | 0.087764148 |
| *VEGF-C* | 0.852344239 | −0.020028721 |
| *Vegfr3/Flt4* | | |
| *Bmp3* | 0.001528492** | −1.124322534 |
| *Igf1* | $8.74\times10^{-9}$** | 1.369190102 |
| *Il1b* | $1.94\times10^{-15}$** | 1.65489077 |
| *Inhba* | $1.86\times10^{-8}$** | 1.416283921 |
| *Spp1* | $3.26\times10^{-20}$** | 3.090851178 |
| *Tnf* | $2.40\times10^{-19}$** | 2.376377744 |
| *Tnn* | 0.000670539** | −1.081252131 |
| *Wnt6* | $1.22\times10^{-8}$** | 1.532285801 |

** $P<0.01$

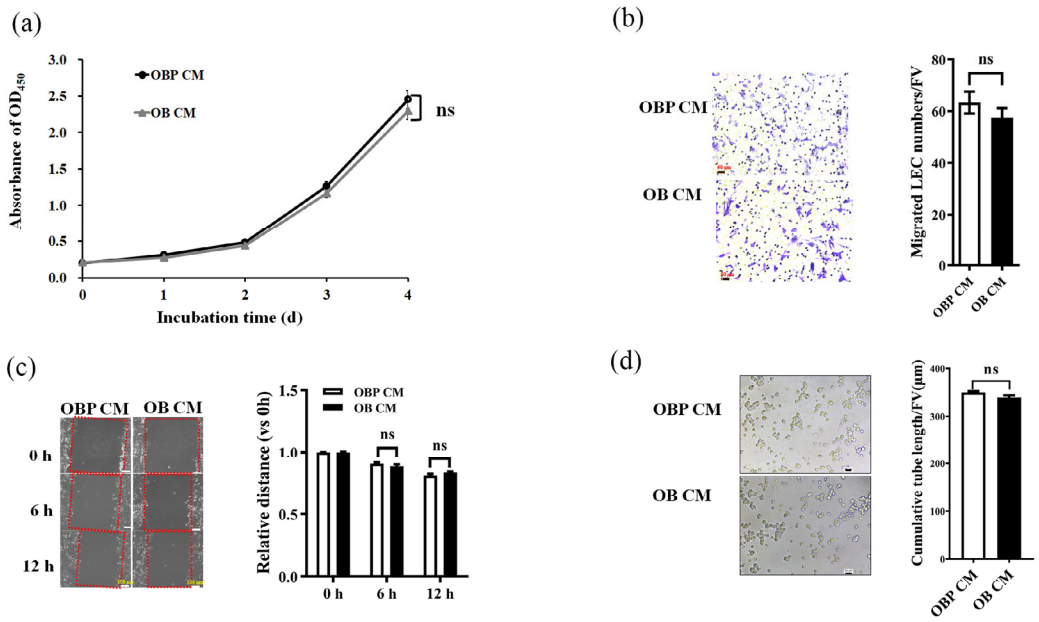


**Fig. S1 Conditioned medium from osteoblasts does not significantly affect LEC growth, migration, or lymphatic vessel formation in vitro. (a) CCK8 assays were performed to evaluate the cellular growth curves of LECs using the conditioned medium of osteoblast precursors (OBP CM) and conditioned medium of osteoblasts (OB CM). (b) Transwell assays for the migration of lymphatic endothelial cells using OBP CM or OB CM were used for quantitative analysis of the migrated cells. field of view (×200 magnification). (c) Representative images showing the migration ability of LECs at 6 h and 12 h by scratch wound assay and quantitative analysis of the migration rate of lymphatic endothelial cells. (d) Representative Matrigel tube formation assay images and quantitative analysis of cumulative tube length with cultures of OBP CM or OB CM. Date are shown as the mean ± SD of three independent experiments. "ns" represents not significant (Student's *t* test).**

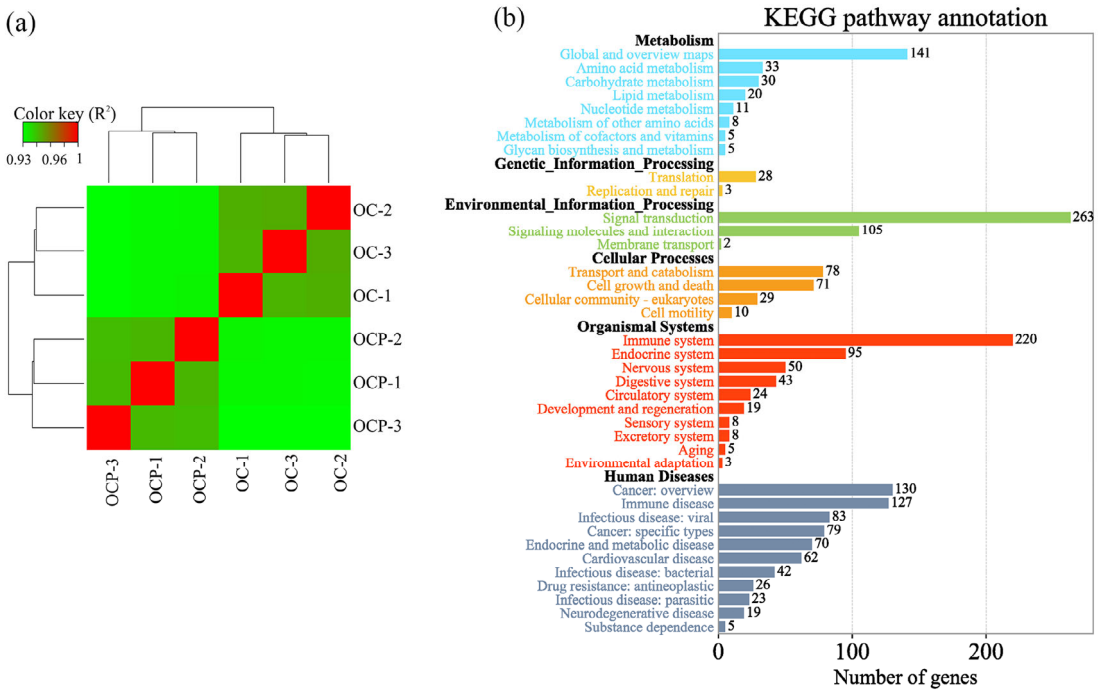


**Fig. S2 Transcriptional differentially expressed gene analysis between the control group (OCP) and the OC group. (a) Hierarchical cluster maps of gene expression levels between OCPs and OCs. (b) KEGG pathways involved in metabolism, organismal systems, cellular processes, genetic information processing, environmental information processing, and human diseases.**

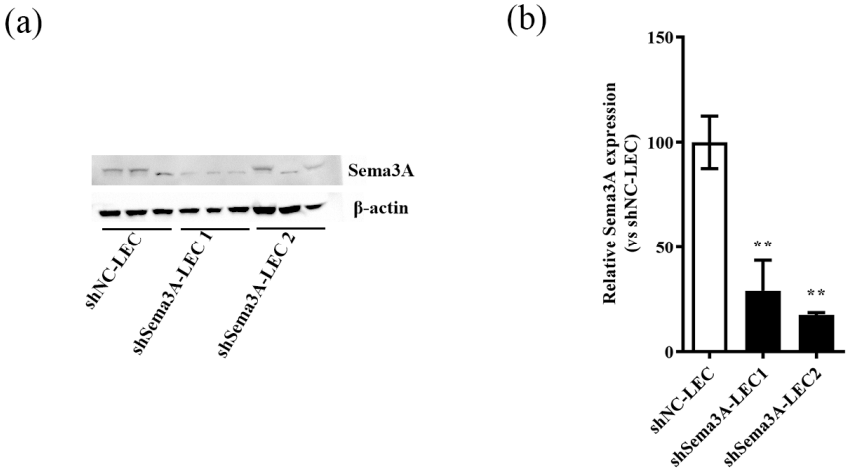


**Fig. S3 Comparison of Sema3A knockdown gene editing activity of two shRNA-expressing plasmids *in vitro*. (a-b) The knockdown efficiency of Sema3A in LECs was detected by WB and grayscale analysis, and the Sema3A knockdown editing activity of shSema3A-LEC1 and shSema3A-LEC2 was 70.97% and 82.46%, respectively. **P*<0.05, ***P*<0.01 (Student's *t* test).**

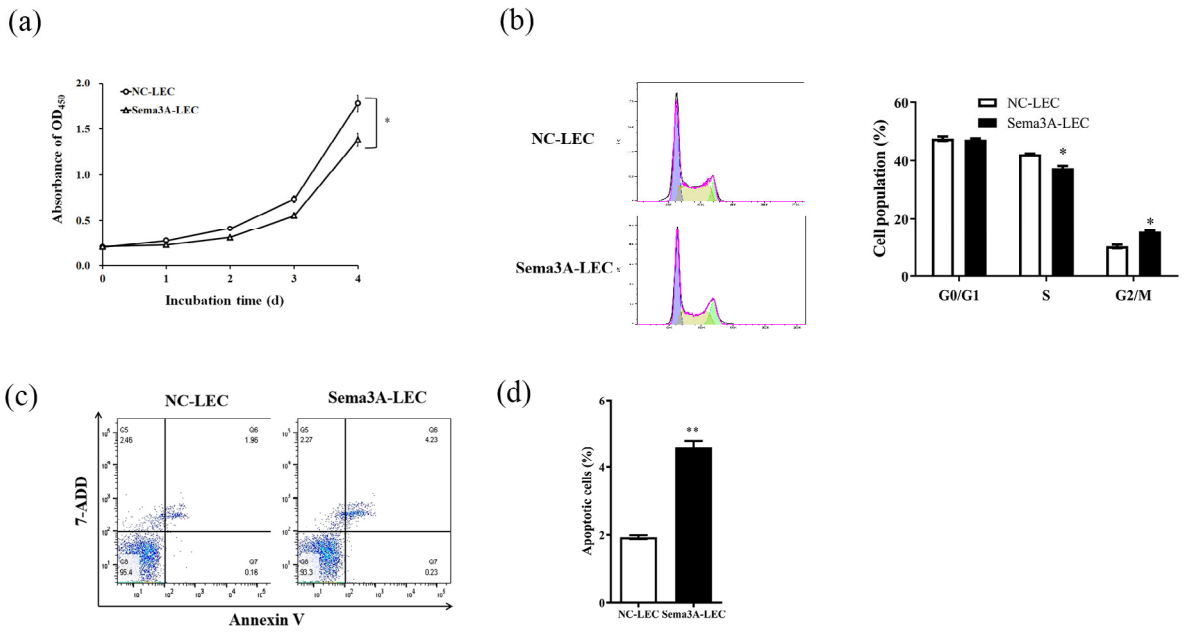


**Fig. S4 Sema3A affects the growth, cell cycle, and apoptosis of LECs. (a) CCK8 assays were performed to evaluate the cellular growth curves of Sema3A-overexpressing LEC cell lines (Sema3A-LEC) and the stable lentivirus strain of LECs (NC-LEC). (b-d) Representative and analysis of the cell cycle diagram and apoptosis diagram. *$P$<0.05, **$P$<0.01 (Student's $t$ test).**