Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2024 25(1):38-50
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Research Article

https://doi.org/10.1631/jzus.B2300180

Reduced expression of semaphorin 3A in osteoclasts causes lymphatic expansion in a Gorham-Stout disease (GSD) mouse model

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Abstract: Gorham-Stout disease (GSD) is a sporadic chronic disease characterized by progressive bone dissolution, absorption, and disappearance along with lymphatic vessel infiltration in bone-marrow cavities. Although the osteolytic mechanism of GSD has been widely studied, the cause of lymphatic hyperplasia in GSD is rarely investigated. In this study, by comparing the RNA expression profile of osteoclasts (OCs) with that of OC precursors (OCPs) by RNA sequencing, we identified a new factor, semaphorin 3A (Sema3A), which is an osteoprotective factor involved in the lymphatic expansion of GSD. Compared to OCPs, OCs enhanced the growth, migration, and tube formation of lymphatic endothelial cells (LECs), in which the expression of Sema3A is low compared to that in OCPs. In the presence of recombinant Sema3A, the growth, migration, and tube formation of LECs were inhibited, further confirming the inhibitory effect of Sema3A on LECs in vitro. Using an LEC-induced GSD mouse model, the effect of Sema3A was examined by injecting lentivirus-expressing Sema3A into the tibiae in vivo. We found that the overexpression of Sema3A in tibiae suppressed the expansion of LECs and alleviated bone loss, whereas the injection of lentivirus expressing Sema3A short hairpin RNA (shRNA) into the tibiae caused GSD-like phenotypes. Histological staining further demonstrated that OCs decreased and osteocalcin increased after Sema3A lentiviral treatment, compared with the control. Based on the above results, we propose that reduced Sema3A in OCs is one of the mechanisms contributing to the pathogeneses of GSD and that expressing Sema3A represents a new approach for the treatment of GSD.

Key words: Semaphorin 3A; Gorham-Stout disease; Osteoclast; Osteolysis; Lymphatic endothelial cell

1 Introduction

Gorham-Stout disease (GSD) is a rare primary idiopathic syndrome, also known as disappearing bone disease (Gorham and Stout, 1955; Bruch-Gerharz et al., 2007; Páez Codeso et al., 2017). It is mainly characterized by osteolysis with excessive production of lymphatic vessels in bone tissue (Franco-Barrera et al., 2017). To date, only approximately 300 cases of GSD have been reported (Dellinger et al., 2014). Since GSD is rare and sporadic, the etiology and pathogenesis are poorly understood. Much evidence strongly supports the hypothesis that increased numbers or activity of osteoclasts (OCs), as found in pathological samples, may be the cause of osteolysis; this idea was demonstrated by several different groups (Möller et al., 1999; Hominick et al., 2018). Recent studies have identified a somatic activating hotspot mutation in Kirsten rat sarcoma viral oncogene homolog (KRAS) in tissues from a patient with GSD and demonstrated that expression of an active form of KRAS (p.G12D) in murine lymphatics led to the development of lymphatics in bone, suggesting that mutations in KRAS are involved in the pathogenesis of GSD

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Received Mar. 16, 2023; Revision accepted May 17, 2023; Crosschecked Dec. 21, 2023

(Nozawa et al., 2020; Homayun-Sepehr et al., 2021). It has also been demonstrated that vascular endothelial growth factor-C (VEGF-C) stimulated by receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) in OCs and OC precursors (OCPs) enhanced bone resorption by OCs, but not OC differentiation (Zhang et al., 2008). Hominick et al. (2018) found that overexpression of VEGF-C in VEGF-C conditionally transgenic mice resulted in the development of lymphatic vessels in bone and osteolysis, which resembles GSD; but VEGF-C was not found to have a direct effect on OC formation. However, under normal conditions, the level of VEGF-C is not sufficient for lymphangiogenesis in bone marrow, suggesting that there are other factors involved in bone loss in GSD (Hu et al., 2022; Li et al., 2022). Although the above studies partially revealed the mechanism by which lymphatic endothelial cells (LECs) cause bone erosion, whether bone cells impact LEC properties such as growth or survival remains unclear; this information is crucial for understanding the pathogenesis of GSD.

Currently, there are no targeted pharmacological therapeutics or standardized effective treatments for GSD (Dellinger and McCormack, 2020; Monroy et al., 2020). Commonly used clinical therapies include interferon therapy, calcitonin and bisphosphonate antiabsorption therapy, and radiation therapy. Liu et al. (2018a, 2018b) found that treatment by bone cement reconstruction combined with interferon-α-2b, zoledronic acid, and calcitriol in the spine and femur could continuously relieve pain in GSD and stabilize the spine and femur. Qu et al. (2018) treated GSD with alendronate, which significantly improved the condition. Zoledronic acid combined with thalidomide has also been reported to have significant efficacy in treating GSD (Mao et al., 2018). In addition, Sirolimus is used as a long-term treatment agent for GSD (Ricci et al., 2019; al Baroudi et al., 2020). Although these various agents have certain therapeutic effects on GSD, they have less effect on the recovery of eroded bone. Thus, it is necessary to explore novel treatments for GSD.

Semaphorin 3A (Sema3A), a secreted protein, was originally identified as being involved in axonal guidance in neurogenesis (Lee et al., 2017; van der Klaauw et al., 2019). Recent studies have shown that Sema3A has osteoprotective effects. The direct interaction between OCs and osteoblasts (OBs) allows for bidirectional transduction of activation signals through Sema3A-neuropilin 1 (Nrp1) to stimulate OB bone formation and suppress osteoclastic bone resorption. Furthermore, administration of recombinant Sema3A suppresses bone loss in mouse models of osteoporosis (Hayashi et al., 2012; Fukuda et al., 2013; Hayashi et al., 2019; Kim et al., 2020). Sema3A is also involved in angiogenesis and lymphangiogenesis, although it is dispensable for vascular development in mice. Sema3A or Sema3A-Nrp1 signaling contributes to vascular remodeling (Gu et al., 2003; Serini et al., 2003; Raimondi and Ruhrberg, 2013). Sema3A prevents tumor-induced angiogenesis in vivo and modulates endothelial-cell migration and survival in vitro (Guttmann-Raviv et al., 2007; Maione et al., 2009); furthermore, overexpression of Sema3A inhibits vascular smooth-muscle-cell proliferation and migration and suppresses neointimal hyperplasia after vascular injury in vivo (Wu et al., 2019). Vascular-endothelial-cell (VEC)-derived Sema3A also inhibits filo podia formation in vascular tip cells (Ochsenbein et al., 2016). Blocking the interaction of Sema3A and Nrp1 leads to aberrant lymphatic vessels and valve morphology and reduces drainage of lymphatic vessels (Jurisic et al., 2012; Ochsenbein et al., 2014). VEGF-C/VEGF receptor 3 (VEGFR3)-mediated lymph angiogenesis is blocked by inhibiting the coreceptor Nrp2 of Sema3A and Sema3F (Bussolino et al., 2013). Therefore, Sema3A has a wide range of effects in both bone-associated cells and endothelial cells, but the effects of bone-cell-derived Sema3A on LECs have not been discussed.

In this study, we investigated the effects of OCs and OBs on LECs and found that expression of Seme3A was decreased in OCs, which enhanced the growth, migration, and tube formation of LECs in vitro and provided a new direction for the treatment of GSD. Overexpressing Sema3A in bone marrow alleviated osteolysis induced by LECs and inhibited the growth of LECs. Our results indicate that Sema3A is a potential target for GSD treatment.

2 Results

2.1 Effects of OC, but not OCP, conditioned media on LEC function

We treated LECs with conditioned media (CM) from OBs or OCs and examined the effects of the

media on the growth, migration, and tube formation of LECs. We found that OC CM significantly promoted LEC growth compared with OCP CM (Fig. 1a). The LEC migration assay showed that LEC migration roughly doubled under OC CM treatment compared with OCP CM treatment through the basement membrane, indicating that OC CM also promoted LEC migration (Fig. 1b). This result was verified by a wound-healing assay. After 12-h of OC CM treatment, the scratch distance in cells treated with OC CM was only approximately 30%–40% of that for OCP CM treatment at the 12-h time point (Fig. 1c). OC CM also promoted LEC tube formation compared with OCP CM (Fig. 1d). In contrast to OC CM, OB CM had no significant impact on the growth, migration, or tube formation of LECs (Fig. S1). These results indicate that OCs either produce LEC stimulators or lack LEC inhibitors.

2.2 Lower Sema3A levels expressed by OCs than by OCPs

To determine the factors by which OCs act differently on LECs than OCPs, we performed RNA sequencing (RNA-seq) of OCPs and OCs, along with a correlation analysis that tested the reliability of the samples. The sample selection was reasonable (correlation coefficient (R^2)>0.92 indicates good reliability) (Fig. S2a). Moreover, there were 2950 differentially expressed genes (DEGs) in OCs compared to OCPs, including 1911 upregulated and 1039 downregulated DEGs ($|\log_2(\text{fold change})|>0.585$ and P-value<0.05) (Fig. 2a). Among these, expression of *Sema3A* was significantly reduced in OCs, while expression levels of *Nrp2*, *VEGF-C*, and *VEGFR3* genes that are closely related to Sema3A function were not significantly changed (Table S1). Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the 2950 DEGs showed that the DEGs were involved in metabolism, organismal systems, cellular processes, genetic information processing, environmental information processing, and human diseases (Fig. S2b). The top 30 KEGG pathway results indicated that enriched pathways played important roles in the immune system, such as antigen processing and presentation and hematopoietic cell lineage, while five main signal-transduction pathways, including hypoxia-inducible factor-1 (HIF-1), NF-κB, Toll-like receptor, interleukin-17 (IL-17), and tumor necrosis factor (TNF) signaling pathways, were

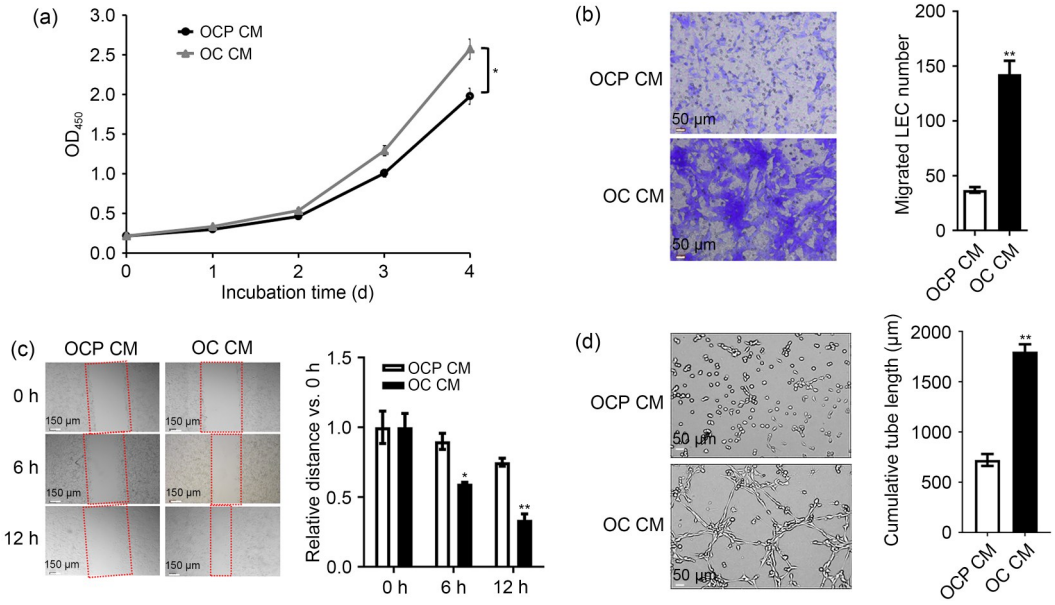

Fig. 1 Effects of OC (but not OCP) CM on LEC growth, migration, and lymphatic vessel formation in vitro. (a) CCK-8 assays were performed to evaluate the cellular growth curves of LECs using OCP CM and OC CM. (b) Transwell assays for the migration of LECs using OCP CM or OC CM were used for quantitative analysis of the cells. (c) Representative images showing the migration ability of LECs at 6 and 12 h by wound-healing assay and quantitative analysis of the migration rate of LECs. (d) Representative Matrigel tube-formation assay images and quantitative analysis of cumulative tube length with cultures of OCP CM or OC CM. Data are shown as mean±standard deviation (SD) of three independent experiments performed in triplicate. * P<0.05, ** P<0.01, vs. OCP CM. OC: osteoclast; OCP: OC precursor; CM: conditioned media; LEC: lymphatic endothelial cell; CCK-8: cell counting kit-8; OD_{450}: optical density at 450 nm.

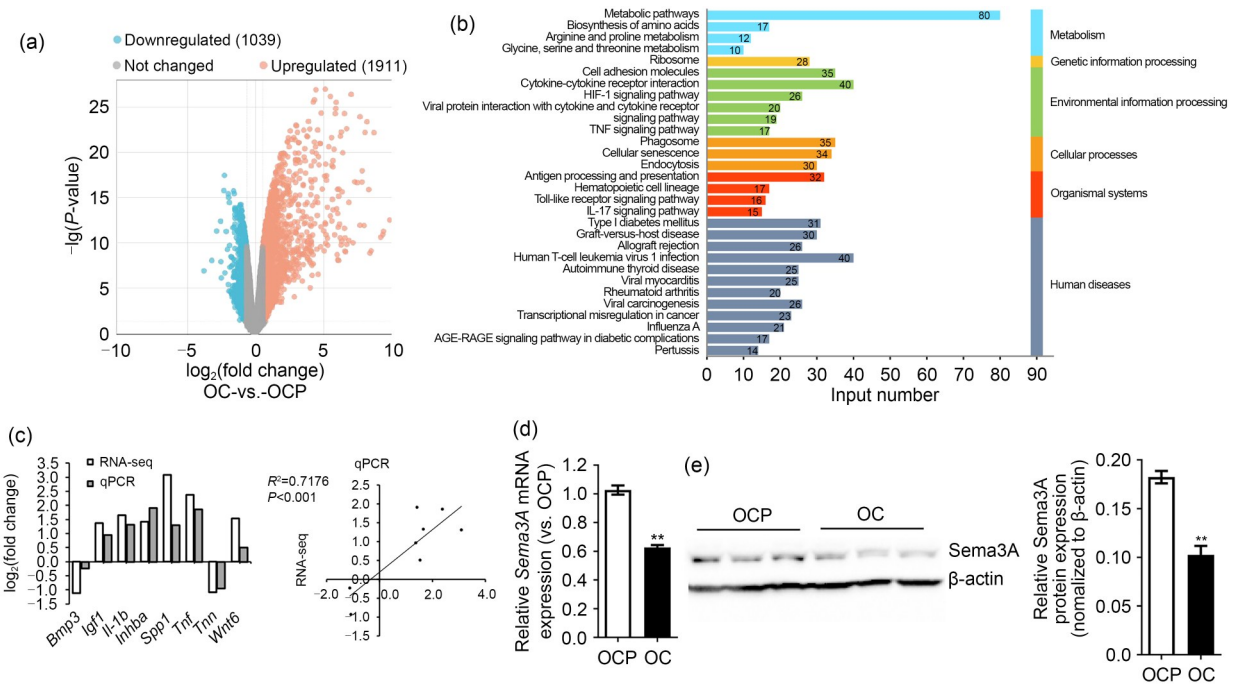

Fig. 2 Decreased expression of Sema3A in OCs than in OCPs by RNA sequencing (RNA-seq). (a) Volcano plot of DEGs (|$\log_2$(fold change)|>0.585 and *P*-value<0.05). (b) The 30 most significantly enriched KEGG pathways. (c) Comparison of RNA-seq data with qPCR results for *Bmp3*, *Igf1*, *Il-1b*, *Inhba*, *Spp1*, *Tnf*, *Tnn*, and *Wnt6* between OCPs and OCs. Correlation analysis of the above eight DEGs by qPCR and RNA-seq showed that the qPCR results were positively correlated with the RNA-seq results (R^2=0.7176). (d, e) Expression of Sema3A in OCs and OCPs was measured by qPCR and western blotting. Sema3A expression is lower in OCs than in OCPs. Data are shown as mean±standard deviation (SD) of three independent experiments. ** *P*<0.01, vs. OCP. Sema3A: semaphorin 3A; OC: osteoclast; OCP: OC precursor; DEG: differentially expressed gene; KEGG: Kyoto Encyclopedia of Genes and Genomes; qPCR: quantitative real-time polymerase chain reaction; *Bmp3*: bone morphogenetic protein 3; *Igf1*: insulin like growth factor 1; *Il-1b*: interleukin-1β; *Inhba*: inhibin subunit beta A; *Spp1*: secreted phosphoprotein 1; *Tnf*: tumor necrosis factor; *Tnn*: tenascin N; *Wnt6*: Wnt family member 6.

also identified as KEGG-enrichment pathways (Fig. 2b). The expression patterns of eight DEGs that were randomly selected were confirmed by quantitative real-time polymerase chain reaction (qPCR) (Fig. 2c). Pearson's bivariate correlation analysis revealed a high correlation between qPCR and RNA-seq data (R^2=0.7176, P<0.001), demonstrating the accuracy and reliability of the RNA-seq results obtained in this study (Fig. 2c, Table S1). qPCR and western blotting showed that Sema3A expression in OCs was much lower than that in OCPs (Figs. 2d and 2e). These data reveal that Sema3A may represent as an OC-produced LEC inhibitor.

2.3 Inhibition of LEC growth, migration, and tube formation by Sema3A

To determine the effect of Sema3A on LECs, we examined the effect of recombinant Sema3A (0, 0.1, 0.5, and 1.0 μg/mL) on the growth of LECs with a cell counting kit-8 (CCK-8) assay, and found that Sema3A dose-dependently inhibited LEC growth (Fig. 3a). We also performed wound-healing and Transwell assays to examine the effect of Sema3A on LEC migration. In the presence of Sema3A, gap healing was much slower, and the number of LECs migrating through the Transwell membrane was obviously lower than that without Sema3A (Figs. 3b and 3c). Furthermore, Sema3A was shown to suppress tube formation compared with the samples without Sema3A (Fig. 3d). Therefore, the data indicate that Sema3A acts as an LEC inhibitor.

2.4 Sema3A expression in GSD mice, and effects of Sema3A on GSD bone lesions and LECs

To determine whether Sema3A expression is altered in osteolytic bone, we examined Seme3A protein levels in bone on Day 14 after LEC injection by enzyme-linked immunosorbent assay (ELISA). The

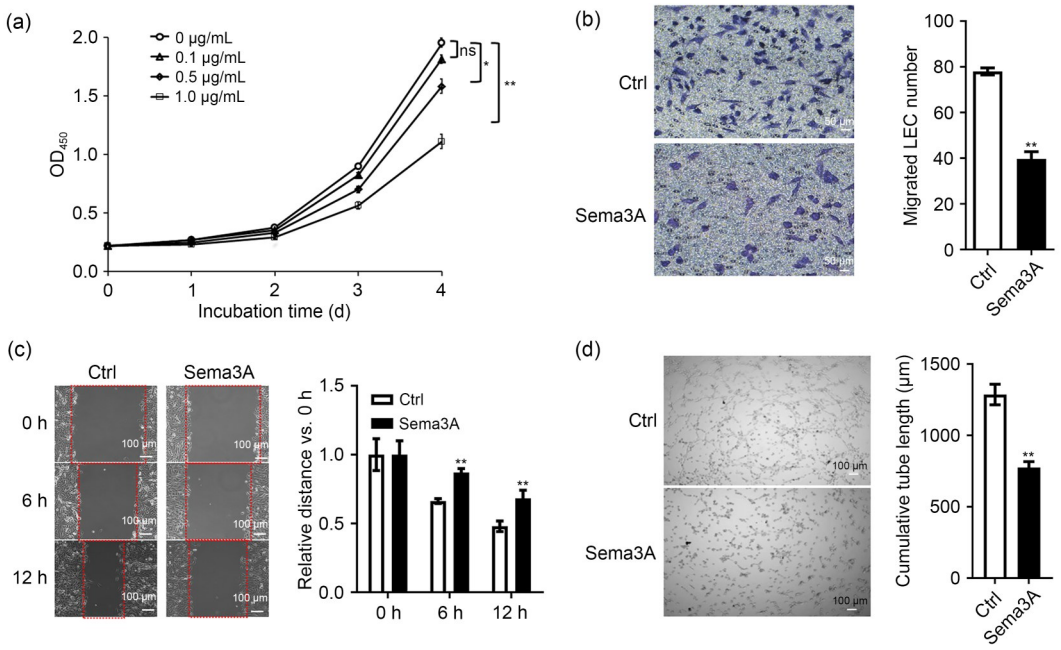

Fig. 3 Suppression of the growth, migration, and tube formation of LECs by Sema3A. (a) After treatment with 0, 0.1, 0.5, and 1.0 μg/mL Sema3A, CCK-8 assays were performed to evaluate LEC growth. (b) Transwell assays for migration of LECs in the presence of 1.0 μg/mL Sema3A and quantitative analysis of the migrated cells. PBS was used as a control. (c) Representative images showing the migration ability of LECs in the control and Sema3A groups at 6 and 12 h by wound-healing assay and quantitative analysis of the migration rate of LECs. (d) Representative Matrigel tube-formation assay images and quantitative analysis of cumulative tube length in the control and Sema3A groups. Data are shown as mean±standard deviation (SD) of three independent experiments. * P<0.05, ** P<0.01, vs. Ctrl. Sema3A: semaphorin 3A; LEC: lymphatic endothelial cell; CCK-8: cell counting kit-8; PBS: phosphate-buffered saline; OD_{450}: optical density at 450 nm; ns: not significant; Ctrl: control.

results showed that levels of Sema3A were lower in bone marrow extracted from osteolytic bone (Fig. 4a). To determine whether Sema3A suppresses osteolytic lesions, we administered lentivirus expressing Sema3A (LV-Sema3A) or empty lentivirus (packaged vector without insert as a negative control, LV-NC) 2 d after mice received intratibial injection of LECs. The mice were euthanized after 14 d, and tibial samples were analyzed by micro-computed tomography (micro-CT) and histology. Micro-CT scans showed less bone destruction in mice that received LV-Sema3A than in mice that received LV-NC (Fig. 4b). The analysis of bone parameters revealed that LV-Sema3A increased the trabecular bone volume fraction (Tb. BV/TV) and trabecular number (Tb. N), while reducing trabecular separation (Tb. Sp); LV-Sema3A had no effect on trabecular thickness (Tb. Th) (Fig. 4c). Hematoxylin-eosin (H&E)- and tartrate-resistant acid phosphatase (TRAP)-stained paraffin sections showed that osteolysis and $TRAP^+$ OCs were decreased in the tibia after LV-Sema3A injection (Figs. 4d and 4e). Osteocalcin (OCN) is a protein secreted by OBs. Immunostaining of OCN from tibial sections showed that LV-Sema3A significantly increased OCN in trabecular bone and cortical bone (Fig. 4f). ELISA showed that LV-Sema3A significantly increased the protein levels of OCN in the bone-marrow supernatant (Fig. 4g). The Sema3A levels in the tibia injected with LV-Sema3A also increased (Fig. 4h). Flow cytometry showed that the ratio of LECs in the bone cavity with LV-Sema3A was lower than that in the LV-NC group (Fig. 4i, Q1+Q2+Q3). These data suggest that intratibial administration of Sema3A virus reduces GSD bone pathogenesis, which is associated with decreased OCs, increased OBs, and reduced LEC expansion.

2.5 Effects of Sema3A shRNA on GSD bone lesions and LEC expansion

The above results suggested that Sema3A effectively alleviated the occurrence of GSD-like osteolysis and LEC expansion, so we proposed the hypothesis that a reduction in Sema3A would aggravate osteolysis

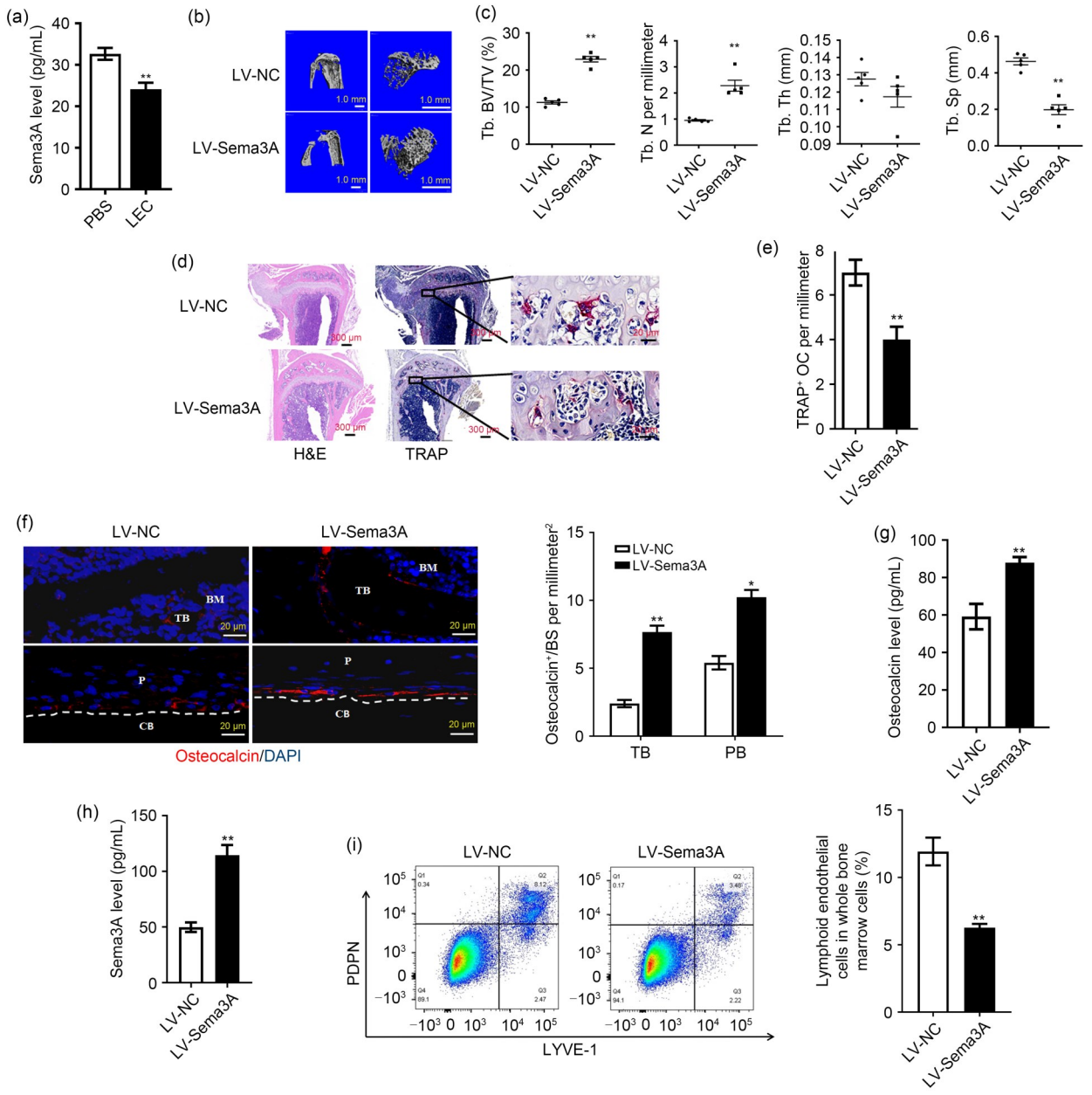

Fig. 4 Sema3A expression in GSD mice, and the effects of Sema3A on GSD bone lesions and LECs. Eight-week-old C57BL/6 mice received intratibial injection of LECs to establish the GSD mouse model. (a) Sema3A levels in bone marrow (BM) extracted 14 d post LEC injection by ELISA. (b, c) GSD mice 2 d post LEC injection received intratibial administration of negative control lentivirus (LV-NC, control group) and lentivirus expressing Sema3A (LV-Sema3A, experimental group). Mice were examined 14 d post injection. Representative micro-CT images and quantification of trabecular bone volume fraction (Tb. BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular separation (Tb. Sp). (d, e) Representative images of H&E- and TRAP-stained images and quantification analyses of the number of TRAP$^+$ osteoclast cells (TRAP$^+$ OC No.) per bone. (f) Representative images of immunostaining of osteocalcin (red) and quantification of osteocalcin$^+$ cell numbers on trabecular bone (TB) and periosteal bone (PB) surfaces. Dashed lines outline the bone surface. DAPI was used to stain nuclei (blue). CB, cortical bone; P, periosteum; BM, bone marrow. (g) Osteocalcin concentrations in BM extracted by ELISA. (h) Sema3A levels in BM extracted by ELISA. (i) PDPN$^+$ (up area) and LYVE-1$^+$ (right area) cells in tibial BM as shown by flow cytometry. Data are shown as mean±standard deviation (SD) of three independent experiments (n=5 per group). * P<0.05, ** P<0.01, vs. PBS or LV-NC. Sema3A: semaphorin 3A; GSD: Gorham-Stout disease; LEC: lymphatic endothelial cell; ELISA: enzyme-linked immunosorbent assay; Micro-CT: micro-computed tomography; H&E: hematoxylin-eosin; TRAP: tartrate-resistant acid phosphatase; DAPI: 4, 6-diamino-2-phenyl indole; PDPN: podoplanin; LYVE-1: lymphatic vessel endothelial receptor-1.

and lymphangiogenesis. To confirm this, we constructed and packaged LV-Sema3A short hairpin RNA (shRNA) (LV-shSema3A) and control shRNA (LV-shNC). We first used specific small interfering RNAs (siRNAs) to knockdown Sema3A expression in vivo. LV-shSema3A and LV-shNC were injected into the left and right tibia, respectively. The in vivo ELISA analyses showed that the protein levels of Sema3A were significantly decreased (Fig. 5a), and the same was true for western blotting analyses of LECs (Fig. S3). The change in the tibia was examined by micro-CT and histology. Micro-CT scanning and analysis showed that LV-shSema3A caused decreased trabecular bone volume and trabecular number and an increase in trabecular bone separation, although no change in trabecular bone thickness was observed (Figs. 5b and 5c). Paraffin section staining with H&E showed substantial bone loss and cortical bone erosion in the tibia after LV-shSema3A injection, along with an increased number of TRAP$^+$ OCs (Figs. 5d and 5e). Podoplanin-positive (PDPN$^+$) and lymphatic vessel endothelial receptor-1-positive (LYVE-1$^+$) cells in bone marrow were analyzed by flow cytometry, and the results showed that PDPN$^+$ and LYVE-1$^+$ cells in bone marrow were higher in the LV-shSema3A group than in the LV-shNC group (Fig. 5f, Q1+Q2+Q3). The results indicate that intratibial administration of Sema3A shRNA virus promotes GSD bone pathogenesis, which is associated with increased OCs and LEC expansion in vivo.

2.6 Reduction of GSD bone phenotype in LECs by the overexpression of Sema3A

Both the gain- and loss-of-function approaches described in Figs. 4 and 5 indicate that bone local delivery of Sema3A has a beneficial role in GSD pathology, while Sema3A inhibition has the opposite effect. However, since these experiments used a nonspecific

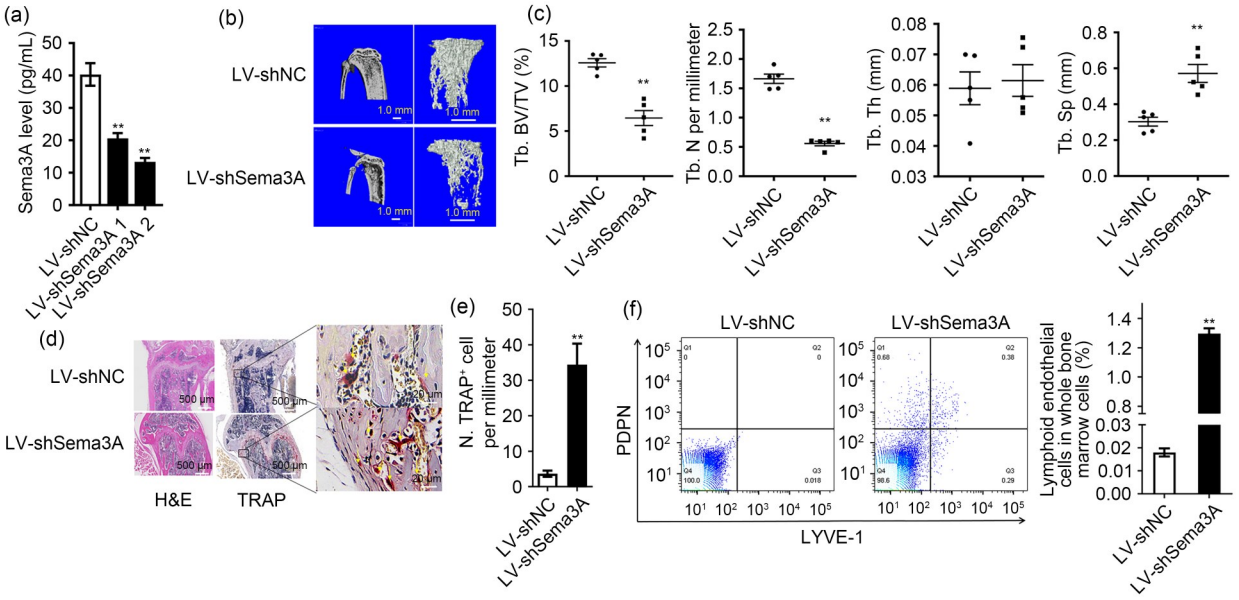

Fig. 5 Effects of Sema3A shRNA on GSD bone lesions and LEC expansion. A lentiviral vector expressing Sema3A shRNA was constructed (LV-shSema3A) and packaged into the virus along with an empty vector (LV-shNC). LV-shNC and LV-shSema3A lentivirus were injected into the right and left tibiae of eight-week-old mice, respectively, *n*=5 per group. (a) ELISA was used to detect the protein level of Sema3A in mice, and the Sema3A knockdown editing activity of LV-shSema3A 1 and LV-shSema3A 2 in vivo was 48.87% and 66.93%, respectively. (b) Representative reconstituted micro-CT images showing bone loss in an LV-shSema3A-injected tibia. (c) Quantification of trabecular bone volume fraction (Tb. BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular separation (Tb. Sp). (d) H&E- and TRAP-stained images showing bone erosion in an LV-shSema3A-injected tibia associated with increased TRAP$^+$ OCs (yellow arrows). (e) Quantification analyses of the number of TRAP$^+$ OCs (N. TRAP$^+$) in bone. (f) PDPN$^+$/LYVE-1$^+$ cells in tibial bone marrow were detected by flow cytometry. Data are shown as mean±standard deviation (SD) of four independent experiments performed in triplicate. ** *P*<0.01, vs. LV-shNC. Sema3A: semaphorin 3A; shRNA: short hairpin RNA; GSD: Gorham-Stout disease; LEC: lymphatic endothelial cell; ELISA: enzyme-linked immunosorbent assay; H&E: hematoxylin-eosin; TRAP: tartrate-resistant acid phosphatase; OC: osteoclast; PDPN: podoplanin; LYVE-1: lymphatic vessel endothelial receptor-1; Micro-CT: micro-computed tomography.

delivery method and Sema3A can act on all cell types in the bone marrow, it is important to determine specific cell types that may mediate the effect of Sema3A on GSD pathogenesis. Because Sema3A inhibits LEC function (Fig. 3), we hypothesize that overexpression of Sema3A in LECs will reduce their capacity to cause GSD lesions following intratibial administration. Thus, we generated LEC cell lines overexpressing Sema3A (Sema3A-LEC) and control cell lines (NC-LEC) (Fig. 6a). Sema3A affects the growth, cell cycle, and apoptosis of LECs in vitro (Fig. S4). Tb. Sp was significantly decreased in the tibias injected with Sema3A-LECs compared with those injected with NC-LECs, but there was no significant decrease in Tb. Th (Figs. 6b and 6c). H&E- and TRAP-stained paraffin sections showed that $TRAP^+$ OCs in the Sema3A-LEC group were decreased compared with those in the NC-LEC group (Figs. 6d and 6e). Flow cytometry analysis indicated that $LYVE\text{-}1^+$ and $PDPN^+$ cells in bone marrow were much lower in the Sema3A-LEC group than in the NC-LEC group (Fig. 6f, Q1+Q2+Q3). These results suggest that overexpression of Sema3A in LECs has the capacity to alleviate GSD lesions.

3 Discussion

Lymphatic hyperplasia associated with osteolysis is an important pathological feature of GSD (Dellinger et al., 2014; Franco-Barrera et al., 2017). Although lymphatic vessels were not present in normal bones in the past (Edwards et al., 2008), it has recently been reported that lymphatic vessels exist in normal bone (Biswas et al., 2023). We proposed before beginning our study that LECs interact with bone cells. Most previous studies have focused on the mechanism of osteolysis in GSD. Several cytokines, such as IL-1β, IL-6, macrophage colony-stimulating factor (M-CSF), and receptor activator of NF-κB ligand (RANL), have been reported to be related to GSD because they stimulate OC formation (Hirayama et al., 2001; Dellinger et al., 2014). Our group previously established a GSD mouse model by injecting LECs into the bone-marrow cavity and found that LECs significantly promoted OC formation in vitro and in vivo. We demonstrated that LECs enhanced osteolysis by stimulating OC formation through secreting M-CSF (Wang et al., 2017), but it is still unclear whether bone cells affect the expansion of lymphatic vessels. In this study, we explored the effects of bone cells such as OBs and OCs on LECs and found that OC CM promoted LEC growth, migration, and tube formation (Fig. 1), although OB CM had little effect on LECs (Fig. S1); further studies revealed that this effect was related to reduced Sema3A expression in OCs (Fig. 2). It has been demonstrated that OB lineage cell-derived Sema3A is involved in reducing osteoclastic bone resorption and increasing osteoblastic bone formation (Hayashi et al., 2012; Yamashita et al., 2022). However, although the function of Sema3A in maintaining bone metabolism balance has been well studied, the effect of Sema3A on LECs has not been explored. In this study, we found that either recombinant or overexpressed Sema3A inhibited the growth, migration, and tube formation of LECs in vitro (Figs. 3 and 6). Injection of Sema3A-expressing lentiviruses into the tibia also inhibited LEC expansion in vivo. These results indicate that Sema3A plays a role in LECs, one similar to their role in VECs. Defects in Sema3A signaling impacted vascular and lymphatic vessels during development (Bussolino et al., 2013). Previous studies showed that Sema3A inhibited tumor growth by suppressing angiogenesis and contributed to vascular regression in noncancer diseases such as early diabetic retinopathy (Casazza et al., 2011; Joyal et al., 2011). It has been demonstrated that proliferation of LECs in bone leads to bone erosion by stimulating OC formation (Wang et al., 2017), so we propose that low expression of Sema3A in OCs enhances OC formation by stimulating LEC expansion, thereby resulting in the occurrence of GSD.

We also investigated the mechanism by which Sema3A inhibits LEC growth. Maione et al. (2009) analyzed the effect of Sema3A in inhibiting tumor progression and found that Sema3A blocked tumor growth by inducing VEC apoptosis. Here, we analyzed the means by which Sema3A inhibited the growth of LECs. Our data showed that the effect of Sema3A on the cell cycle was minor, although there was a significant change. The apoptosis induced by Sema3A led to more cell death (Fig. S4). Therefore, apoptosis appears to be one of the major mechanisms by which Sema3A inhibits LEC growth.

In addition to Sema3A, VEGF-C, which appears to be increased in the serum of GSD patients, also

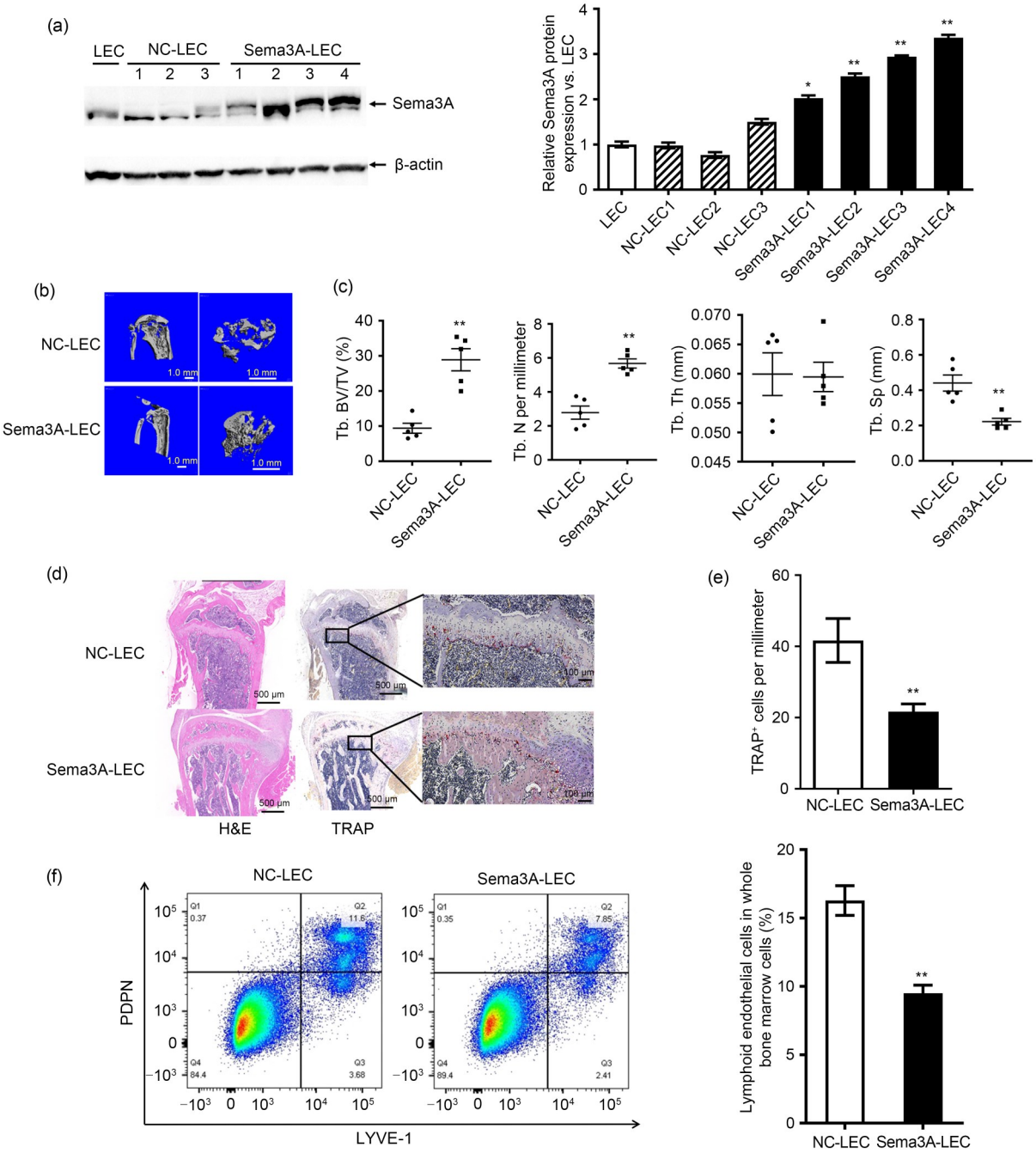

Fig. 6 Reduction of GSD bone phenotype in LECs by the overexpression of Sema3A. (a) Sema3A-overexpressing LEC cell lines (Sema3A-LEC) were established by infecting LECs with lentivirus expressing Sema3A. The stable lentivirus strain of LECs (NC-LECs) was used as the control group. The expression levels of Sema3A were determined by western blotting and quantified by ImageJ software. (b, c) The 5×10^5 viable NC-LECs and Sema3A-LECs were injected into the right and left tibiae, respectively. Mice were examined two weeks post injection. Representative micro-CT images and quantification of trabecular bone volume fraction (Tb. BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular separation (Tb. Sp). (d, e) Representative images of H&E- and TRAP-stained images and quantification analyses of the number of $TRAP^+$ osteoclast cells per respective bone. (f) $PDPN^+/LYVE\text{-}1^+$ cells in tibial bone marrow were detected by flow cytometry. Data are shown as mean±standard deviation (SD) of three independent experiments. For (b)−(f), five mice were used for each experiment. * $P<0.05$, ** $P<0.01$, vs. LEC for (a) and vs. NC-LEC for (c, e, f) . Sema3A: semaphorin 3A; GSD: Gorham-Stout disease; LEC: lymphatic endothelial cell; H&E: hematoxylin-eosin; TRAP: tartrate-resistant acid phosphatase; OC: osteoclast; PDPN: podoplanin; LYVE-1: lymphatic vessel endothelial receptor-1; Micro-CT: micro-computed tomography.

stimulates lymphangiogenesis. Although we found no difference in VEGF-C expression between OCPs and OCs by RNA-seq, this may be due to differences in the mouse model established by LEC injection compared to other models. Hominick et al. (2018) showed that mice conditionally overexpressing VEGF-C developed GSD-like pathological features. High expression of VEGF-C is considered to be the cause of GSD. However, VEGF-C does not stimulate OC formation, so there must be other factors responsible for enhancing OC formation. It has been reported that Sema3A, which binds to Nrp1 as a competitor of VEGF-A165, inhibits endothelial cell migration and sprouting. Thus, we propose that excessive VEGF-C blocks the Sema3A signal pathway through competitive binding with Nrp1 to promote OC formation. Another receptor of Sema3A, Nrp2, interacts with VEGF-A and VEGF-C to promote endothelial cell survival and migration (Favier et al., 2006). Therefore, Sema3A is thought to compete for Nrp2 binding with VEGF-C, thus decreasing LEC survival and migration (Staton, 2011; Sakurai et al., 2012; Toledano et al., 2019; Jiao et al., 2021). It has also been reported that excessive Sema3A does not affect binding of VEGF-C to Nrp2 but rather reduces binding of VEGF-C to VEGFR3, which would indicate that inhibition of LEC growth by Sema3A may occur through regulation of the VEGFR3 signaling pathway (Bernatchez et al., 2002). The relationship between Sema3A and VEGF-C in GSD needs to be further studied.

Traditional treatment regimens for GSD primarily involve bisphosphonates and interferon-α (INF-α), though there are other options such as VEGF-A antibodies and hormones (Dellinger et al., 2014). Because VEGF-C is involved in the pathogenesis of GSD, suppressing VEGF-C expression or using VEGFR3 antibodies has been shown to effectively inhibit GSD progression and partially restore osteolysis (Hominick et al., 2018). Because of its action in promoting OB differentiation and inhibiting OC formation, Sema3A has been demonstrated to alleviate osteoporosis in a mouse model (Hayashi et al., 2012; Yang et al., 2018). Here, we tried to inhibit or alleviate GSD development with Sema3A. Consistent with our expectations, overexpression of Sema3A significantly inhibited osteolysis induced by LECs and suppressed LEC expansion in vitro and in vivo. Sema3A thus provides a new therapeutic approach for GSD.

The rarity of GSD makes research on the disease difficult, and establishing a GSD mouse model can be of great assistance with this. In this study, we found that the tibia of mice injected with LV-shSema3A clearly exhibited osteolysis and LEC expansion in vivo. Sema3A knockdown clearly resulted in GSD-like phenotypes. Knockdown of a gene can cause major defects compared with the knockout of the gene (Wilkinson, 2019). We think that reducing Sema3A in bone is another approach to establishing a mouse model of GSD. The level of Sema3A in our GSD mouse model was significantly decreased (Figs. 4a and 5a). Interestingly, Hayashi et al. (2019) showed that serum levels of Sema3A decreased with age or after menopause in humans. However, whether Sema3A is involved in GSD needs to be verified in humans as well.

In conclusion, we demonstrated that LEC expansion is caused by reduced Sema3A expression in OCs. The occurrence of GSD is a result of positive feedback between LECs and OCs. Expansion of intraosseous LECs stimulates OC formation by secreting M-CSF, disrupting the balance between OCs and OBs and leading to osteolysis, while increased OC reduces Sema3A levels, weakening control of LECs and leading to their excessive growth. Overexpression of Sema3A effectively alleviates the development of osteolysis, inhibits LEC expansion, and provides a new treatment option for GSD.

4 Conclusions

Our study revealed that reduced Sema3A in OCs enhances LEC growth, migration, and tube formation. Overexpression of Sema3A effectively alleviates development of osteolysis and inhibits LEC expansion, which may offer a new therapeutic target for the treatment of GSD. In addition, knockdown of Sema3A in bone marrow is a new approach for establishing a GSD mouse model.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 81974321), the Discipline Innovation and Talent Introduction Program to Universities from Ministry of Education of China (Project 111), the Tackling Project for Science and Technology of Xinxiang City (No. GG2019003), the Natural Science Foundation of Henan Province of China (No. 212300410173), the State Administration of Traditional Chinese Medicine Young Qi Huang Scholar, and the Innovation Team Project of Scientific Research of Traditional Chinese Medicine of Shanghai Health Committee (No. 2022CX001), China. We thank Dr. Peipei ZHANG (Henan Normal University, Xinxiang, China) and Mr. Yongjian ZHAO (Shanghai University of Traditional Chinese Medicine, Shanghai, China) for micro-CT scanning.

Author contributions

Wensheng WANG: conceptualization, supervision, funding acquisition, methodology, project administration, and writing – review and editing. Qianqian LIANG: conceptualization, supervision, project administration, writing – original draft, and writing – review and editing. Dongfang ZHANG: writing – original draft, formal analysis, investigation, and visualization. Hao XU, Chi QIN, Kangming CAI, Jing ZHANG, and Xinqiu XIA: formal analysis, investigation, and visualization. Jingwen BI: visualization. Li ZHANG: funding acquisition, writing – original draft, and writing – review and editing. Lianping XING: methodology and writing – review and editing. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Dongfang ZHANG, Hao XU, Chi QIN, Kangming CAI, Jing ZHANG, Xinqiu XIA, Jingwen BI, Li ZHANG, Lianping XING, Qianqian LIANG, and Wensheng WANG declare that they have no conflicts of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. The animal study protocol was approved by the Ethics Committee of Henan Normal University (No. HNSD-2019-12-02).

References

al Baroudi S, Jabre NA, Dunn E, et al., 2020. A 15-year-old boy with dyspnea and vanishing bones. *Am J Respir Crit Care Med*, 202(3):451-452. https://doi.org/10.1164/rccm.201907-1339IM

Bernatchez PN, Rollin S, Soker S, et al., 2002. Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration and PAF synthesis: role of neuropilin-1. *J Cell Biochem*, 85(3):629-639. https://doi.org/10.1002/jcb.10155

Biswas L, Chen JY, de Angelis J, et al., 2023. Lymphatic vessels in bone support regeneration after injury. *Cell*, 186(2): 382-397.e24. https://doi.org/10.1016/j.cell.2022.12.031

Bruch-Gerharz D, Gerharz CD, Stege H, et al., 2007. Cutaneous lymphatic malformations in disappearing bone (Gorham-Stout) disease: a novel clue to the pathogenesis of a rare syndrome. *J Am Acad Dermatol*, 56(S2):S21-S25. https://doi.org/10.1016/j.jaad.2006.01.063

Bussolino F, Giraudo E, Serini G, 2013. Class 3 semaphorin in angiogenesis and lymphangiogenesis. *In*: S. Karger AG (Ed.), Angiogenesis, Lymphangiogenesis and Clinical Implications. Karger, Basel, p.71-88. https://doi.org/10.1159/000353315

Casazza A, Fu X, Johansson I, et al., 2011. Systemic and targeted delivery of semaphorin 3A inhibits tumor angiogenesis and progression in mouse tumor models. *Arterioscler Thromb Vasc Biol*, 31(4):741-749. https://doi.org/10.1161/atvbaha.110.211920

Dellinger MT, McCormack FX, 2020. The emergence of targetable MEKanisms in sporadic lymphatic disorders. *EMBO Mol Med*, 12(10):e12822. https://doi.org/10.15252/emmm.202012822

Dellinger MT, Garg N, Olsen BR, 2014. Viewpoints on vessels and vanishing bones in Gorham-Stout disease. *Bone*, 63:47-52. https://doi.org/10.1016/j.bone.2014.02.011

Edwards JR, Williams K, Kindblom LG, et al., 2008. Lymphatics and bone. *Hum Pathol*, 39(1):49-55. https://doi.org/10.1016/j.humpath.2007.04.022

Favier B, Alam A, Barron P, et al., 2006. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood*, 108(4): 1243-1250. https://doi.org/10.1182/blood-2005-11-4447

Franco-Barrera MJ, Zavala-Cerna MG, Aguilar-Portillo G, et al., 2017. Gorham-Stout disease: a clinical case report and immunological mechanisms in bone erosion. *Clin Rev Allergy Immunol*, 52:125-132. https://doi.org/10.1007/s12016-016-8594-z

Fukuda T, Takeda S, Xu R, et al., 2013. Sema3A regulates bone-mass accrual through sensory innervations. *Nature*, 497(7450):490-493. https://doi.org/10.1038/nature12115

Gorham LW, Stout AP, 1955. Massive osteolysis (acute spontaneous absorption of bone, phantom bone, disappearing bone): its relation to hemangiomatosis. *J Bone Joint Surg Am*, 37-A(5):985-1004.

Gu CH, Rodriguez ER, Reimert DV, et al., 2003. Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell*, 5(1):45-57. https://doi.org/10.1016/s1534-5807(03)00169-2

Guttmann-Raviv N, Shraga-Heled N, Varshavsky A, et al., 2007. Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. *J Biol Chem*, 282(36):26294-26305. https://doi.org/10.1074/jbc.M609711200

Hayashi M, Nakashima T, Taniguchi M, et al., 2012. Osteoprotection by semaphorin 3A. *Nature*, 485(7396):69-74. https://doi.org/10.1038/nature11000

Hayashi M, Nakashima T, Yoshimura N, et al., 2019. Autoregulation of osteocyte Sema3A orchestrates estrogen action and

counteracts bone aging. *Cell Metab*, 29(3):627-637.e5. https://doi.org/10.1016/j.cmet.2018.12.021

Hu LH, Wu W, Zou J, 2022. Circular RNAs: typical biomarkers for bone-related diseases. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 23(12):975-988. https://doi.org/10.1631/jzus.B2200211

Hirayama T, Sabokbar A, Itonaga I, et al., 2001. Cellular and humoral mechanisms of osteoclast formation and bone resorption in Gorham-Stout disease. *J Pathol*, 195(5): 624-630. https://doi.org/10.1002/path.989

Homayun-Sepehr N, Mccarter AL, Helaers R, et al., 2021. *KRAS*-driven model of Gorham-Stout disease effectively treated with trametinib. *JCI Insight*, 6(15):e149831. https://doi.org/10.1172/jci.insight.149831

Hominick D, Silva A, Khurana N, et al., 2018. VEGF-C promotes the development of lymphatics in bone and bone loss. *Elife*, 7:e34323. https://doi.org/10.7554/eLife.34323

Jiao B, Liu SY, Tan X, et al., 2021. Class-3 semaphorins: potent multifunctional modulators for angiogenesis-associated diseases. *Biomed Pharmacother*, 137:111329. https://doi.org/10.1016/j.biopha.2021.111329

Joyal JS, Sitaras N, Binet F, et al., 2011. Ischemic neurons prevent vascular regeneration of neural tissue by secreting semaphorin 3A. *Blood*, 117(22):6024-6035. https://doi.org/10.1182/blood-2010-10-311589

Jurisic G, Maby-El Hajjami H, Karaman S, et al., 2012. An unexpected role of semaphorin3A–neuropilin-1 signaling in lymphatic vessel maturation and valve formation. *Circ Res*, 111(4):426-436. https://doi.org/10.1161/circresaha.112.269399

Kim JM, Lin CJ, Stavre Z, et al., 2020. Osteoblast-osteoclast communication and bone homeostasis. *Cells*, 9(9):2073. https://doi.org/10.3390/cells9092073

Lee H, Macpherson LJ, Parada CA, et al., 2017. Rewiring the taste system. *Nature*, 548(7667):330-333. https://doi.org/10.1038/nature23299

Li TT, Zhang SH, Yang YX, et al., 2022. Co-regulation of circadian clock genes and microRNAs in bone metabolism. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 23(7): 529-546. https://doi.org/10.1631/jzus.B2100958

Liu SZ, Zhou X, Song A, et al., 2018a. A rare case of Gorham-Stout syndrome of femur treated with cement augmentation. *Chin Med J (Engl)*, 131(13):1628-1629. https://doi.org/10.4103/0366-6999.235121

Liu SZ, Zhou X, Song A, et al., 2018b. Successful treatment of Gorham-Stout syndrome in the spine by vertebroplasty with cement augmentation: a case report and literature review. *Medicine (Baltimore)*, 97(29):e11555. https://doi.org/10.1097/md.0000000000011555

Maione F, Molla F, Meda C, et al., 2009. Semaphorin 3A is an endogenous angiogenesis inhibitor that blocks tumor growth and normalizes tumor vasculature in transgenic mouse models. *J Clin Invest*, 119(11):3356-3372. https://doi.org/10.1172/jci36308

Mao TJ, Xu L, Yu YM, et al., 2018. Use of zoledronic acid combined with thalidomide in the treatment of Gorham-Stout disease. *Rheumatology (Oxford)*, 57(6):1122-1124. https://doi.org/10.1093/rheumatology/key055

Möller G, Priemel M, Amling M, et al., 1999. The Gorham-Stout syndrome (Gorham's massive osteolysis). A report of six cases with histopathological findings. *J Bone Joint Surg Br*, 81(3):501-506. https://doi.org/10.1302/0301-620x.81b3.9468

Monroy M, Mccarter AL, Hominick D, et al., 2020. Lymphatics in bone arise from pre-existing lymphatics. *Development*, 147(21):dev184291. https://doi.org/10.1242/dev.184291

Nozawa A, Ozeki M, Niihori T, et al., 2020. A somatic activating *KRAS* variant identified in an affected lesion of a patient with Gorham-Stout disease. *J Hum Genet*, 65(11): 995-1001. https://doi.org/10.1038/s10038-020-0794-y

Ochsenbein AM, Karaman S, Jurisic G, et al., 2014. The role of neuropilin-1/semaphorin 3A signaling in lymphatic vessel development and maturation. *In*: Kiefer F, Schulte-Merker S (Eds.), Developmental Aspects of the Lymphatic Vascular System. Springer, Vienna, p.143-152. https://doi.org/10.1007/978-3-7091-1646-3_11

Ochsenbein AM, Karaman S, Proulx ST, et al., 2016. Endothelial cell-derived semaphorin 3A inhibits filopodia formation by blood vascular tip cells. *Development*, 143(4): 589-594. https://doi.org/10.1242/dev.127670

Páez Codeso FM, Morillo Domínguez MC, Dorado Galindo A, 2017. A rare case of chylothorax. Gorham-Stout syndrome. *Arch Bronconeumol*, 53(11):640. https://doi.org/10.1016/j.arbres.2017.04.010

Qu LY, Cai XY, Wang BL, 2018. Diagnosis and treatment of Gorham-Stout disease in maxillofacial regions. *J Craniofac Surg*, 29(2):460-461. https://doi.org/10.1097/scs.0000000000004188

Raimondi C, Ruhrberg C, 2013. Neuropilin signalling in vessels, neurons and tumours. *Semin Cell Dev Biol*, 24(3): 172-178. https://doi.org/10.1016/j.semcdb.2013.01.001

Ricci KW, Hammill AM, Mobberley-Schuman P, et al., 2019. Efficacy of systemic sirolimus in the treatment of generalized lymphatic anomaly and Gorham-Stout disease. *Pediatr Blood Cancer*, 66(5):e27614. https://doi.org/10.1002/pbc.27614

Sakurai A, Doçi CL, Gutkind JS, 2012. Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. *Cell Res*, 22:23-32. https://doi.org/10.1038/cr.2011.198

Serini G, Valdembri D, Zanivan S, et al., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature*, 424(6947):391-397. https://doi.org/10.1038/nature01784

Staton CA, 2011. Class 3 semaphorins and their receptors in physiological and pathological angiogenesis. *Biochem Soc Trans*, 39(6):1565-1570.

https://doi.org/10.1042/bst20110654

Toledano S, Nir-Zvi I, Engelman R, et al., 2019. Class-3 semaphorins and their receptors: potent multifunctional modulators of tumor progression. *Int J Mol Sci*, 20(3):556. https://doi.org/10.3390/ijms20030556

van der Klaauw AA, Croizier S, de Oliveira EM, et al., 2019. Human semaphorin 3 variants link melanocortin circuit development and energy balance. *Cell*, 176(4):729-742.e18. https://doi.org/10.1016/j.cell.2018.12.009

Wang WS, Wang H, Zhou XC, et al., 2017. Lymphatic endothelial cells produce M-CSF, causing massive bone loss in mice. *J Bone Miner Res*, 32(5):939-950. https://doi.org/10.1002/jbmr.3077

Wilkinson MF, 2019. Genetic paradox explained by nonsense. *Nature*, 568(7751):179-180. https://doi.org/10.1038/d41586-019-00823-5

Wu JH, Zhou YF, Hong CD, et al., 2019. Semaphorin-3A protects against neointimal hyperplasia after vascular injury. *EBioMedicine*, 39:95-108. https://doi.org/10.1016/j.ebiom.2018.12.023

Yamashita Y, Hayashi M, Saito M, et al., 2022. Osteoblast lineage cell-derived Sema3A regulates bone homeostasis independently of androgens. *Endocrinology*, 163(10): bqac126. https://doi.org/10.1210/endocr/bqac126

Yang K, Miron RJ, Bian Z, et al., 2018. A bone-targeting drug-delivery system based on semaphorin 3A gene therapy ameliorates bone loss in osteoporotic ovariectomized mice. *Bone*, 114:40-49. https://doi.org/10.1016/j.bone.2018.06.003

Zhang Q, Guo RL, Lu Y, et al., 2008. VEGF-C, a lymphatic growth factor, is a RANKL target gene in osteoclasts that enhances osteoclastic bone resorption through an autocrine mechanism. *J Biol Chem*, 283(19):13491-13499. https://doi.org/10.1074/jbc.M708055200

Supplementary information

Materials and methods; Table S1; Figs. S1–S4