Site-1 protease controls osteoclastogenesis by mediating LC3 transcription

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INTRODUCTION: Site-1 protease (S1P) is a Golgi-located protein that activates unique membrane-bound latent transcription factors, sush as ATF6, SREBPs and GNPTAB, and it plays an indispensable role in endoplasmic reticulum stress, lipid metabolism, inflammatory response and lysosome function. S1P knockout caused skeletal dysplasia or lethality in mice and zebrafish. A patient with S1P mutation exhibits severe skeletal dysplasia with kyphoscoliosis, dysmorphic facial features and pectus carinatum. However, whether S1P regulates bone remodeling by affecting osteoclastogenesis remains elusive.

METHODS: In this study, Western blot, immunofluorescence staining, qPCR, TRAP staining and bone resorption assay were used to explore the role of S1P in the process of osteoclast differentiation. Database comparison, miRNA inhibitor and mimic, luciferase reporter assay were performed to determine the upstream mechanism of regulating S1P. We used CHIP, co-immunoprecipitation, molecular docking and luciferase reporter assay to prove how S1P controlled osteoclast differentiation. Furthermore, we constructed Lysm-Cre-S1P$^{f/f}$ and CTSK-Cre-S1P$^{f/f}$ conditional knockout mice to study the effect of S1P deficiency in osteoclasts on bone mass. The ovariectomy- and LPS-induced bone loss models were constructed in wild type and S1P knockout mice to explore the influence of S1P deficiency on pathological osteoporosis. Meanwhile, specific S1P inhibitor (PF429242) was injected into mice following ovariectomy or LPS injection to study whether it could protect mice from osteoporosis. All animal studies were approved by the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine.

RESULTS SECTION: In the process of osteoclast differentiation, S1P mRNA expression showed no significant change but the protein increased in a time-dependent manner. More expression and colocalization of S1P and ACP5 were found in the OVX group by immunofluorescence staining. S1P knockout or pharmacologically S1P inhibitor impeded osteoclast differentiation and bone resorption which could be promoted by S1P overexpression. S1P ablation in mice led to significant osteosclerosis and TRAP staining showed that LysM-Cre-S1P$^{f/f}$ mice had fewer osteoclasts on trabecular surfaces of the femur compared with wild-type littermates. Mechanistically, S1P showed upregulated during osteoclastogenesis and was identified as a direct target of miR-9-5p which showed gradually decreased in a time-dependent manner. S1P deletion in bone marrow monocytes inhibited ATF6 and SREBP2 maturation, which subsequently impeded CHOP/SREBP2-complex-induced LC3 expression and autophagy flux. Overexpression of LC3 promoted wild-type, and rescued LysM-Cre-S1P$^{f/f}$ BMMs' osteoclastogenesis. Also, we identified the C3 region (bZIP) of CHOP and the S5 region (LZIP) of SREBP2 are the crucial interaction regions by co-immunoprecipitation and molecular docking. Furthermore, S1P deletion or inhibitor efficaciously rescued ovariectomy- and LPS-induced bone loss in vivo.

DISCUSSION: We show that S1P regulates osteoclast differentiation via ATF6/CHOP/LC3 and SREBP2/LC3 axis in a LC3-dependent manner. S1P deletion or inhibitors can effectively rescue ovariectomy- and LPS-induced bone loss in vivo, and so is a potential therapy target for osteoporosis. Nevertheless, the osteoclast-targeted drug delivery system should be utilized to ensure security and specific-targeting in further studies.

SIGNIFICANCE/CLINICAL RELEVANCE: (1-2 sentences): Our study is the first to clarify how S1P controls osteoclastogenesis by affecting LC3 transcription via the ATF6/CHOP/LC3 and SREBP2/LC3 pathways. The rescue experiment in vivo implies that S1P can be a potential target for treatment of osteoporosis.

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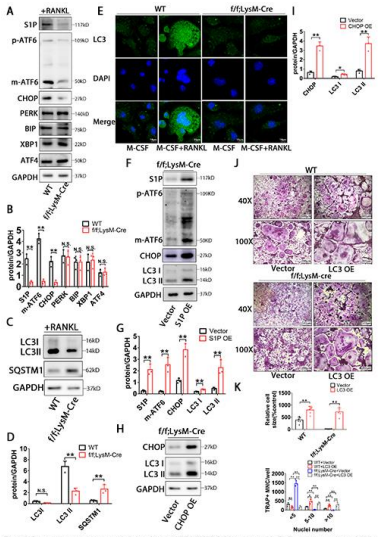

Fig.1 S1P controls osteoclastogenesis by mediating ATF6/CHOP/LC3 pathway. (A, B) Protein expression of S1P, ATF6 precursor (p-ATF6), ATF6 maturation (m-ATF6), CHOP, PERK, BIP, XBP1 and ATF4 in BMMs of WT and LysM-Cre-S1P$^{f/f}$ mice. (C, D) Protein expression of LC3I, LC3II and SQSTM1 in BMMs of WT and Ly-sM-Cre-S1P$^{f/f}$mice. (E) Confocal images of LC3 immunofluorescence of BMMs of WT and LysM-Cre-S1P$^{f/f}$mice ; Scale bars, 10μm. (F, G) Protein expression of S1P, p-ATF6, m-ATF6, CHOP, LC3I and LC3II in BMMs of Ly-sM-Cre-S1P$^{f/f}$mice following the lentivirus transduction of pLenti-EF1a-S1P or the empty vector. (H, I) Protein expression of CHOP, LC3I and LC3II in BMMs of LysM-Cre-S1P$^{f/f}$mice following the lentivirus transduction of pLenti-EF1a-CHOP or the empty vector. (J) TRAP staining to detect osteoclastogenesis of BMMs of WT or Ly-sM-Cre-S1P$^{f/f}$ mice following the lentivirus transduction of pLenti-EF1a-LC3 or the empty vector; 40X, Scale bars, 500um; 100X, Scale bars, 200μm. (K) Quantification of size and nuclei numbers of TRAP-positive multinu-clear cells. Data are presented as mean ± SD. *P < 0.05, **P < 0.01.

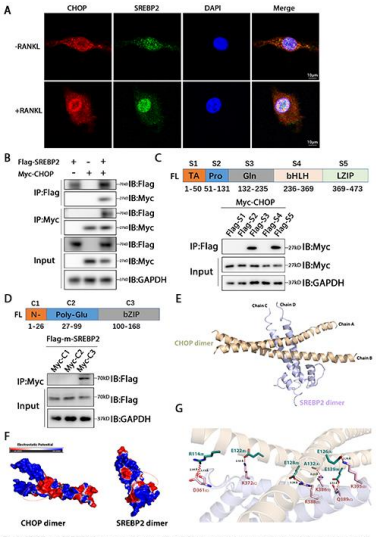

Fig.2. CHOP and SREBP2 form a complex to regulate LC3 transcription synergistically. (A) Confocal images of CHOP (red) and SREBP2 (green) immunofluorescence of BMMs; (B) HEK293T cells were transfected with Flag-SREBP2, Myc-CHOP or Flag-SREBP2 and Myc-CHOP. Flag immunoprecipitates and Myc immunoprecipi-tates were analyzed by immunoblotting. (C) Full-length and various truncated fragments of SREBP2. S1 to S5 and CHOP plasmids were co-transfected in HEK293T cells. (D) Full-length and various truncated fragments of CHOP. C1 to C3 and m-SREBP2 plasmids were co-transfected in HEK293T cells. (E) Overall structure of CHOP dimer bound to SREBP2 dimer in cartoon view. CHOP and SREBP2 are colored in wheat, light blue respectively, and the chain identifiers of each protein are labeled. (F) Computed electrostatic potentials of CHOP dimer and SREBP2 dimer. (G) Detailed interaction network between CHOP dimer and SREBP2 dimer. Key residues of CHOP (deep teal) and SREBP2 (pink) are displayed as sticks and the chain identifiers of residues are showed. H-bonds are displayed in red dash lines and the distances (acceptor to donor heavy atom) of H-bonds are labeled.

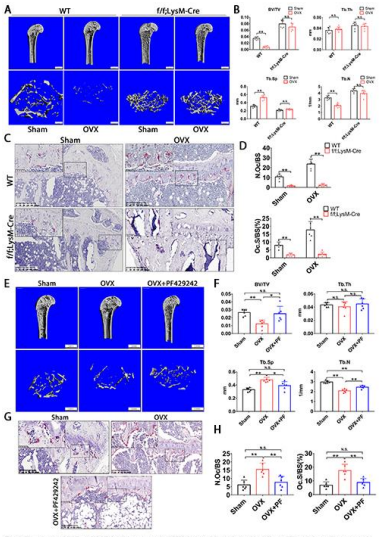

Fig.3 Knockout of S1P and S1P inhibitor prevent OVX-induced bone loss. (A) Micro-CT and three-dimensional reconstructed images of distal femurs from WT or LysM-Cre-S1P$^{f/f}$mice that underwent either sham or OVX op-eration.Scale bar, 1mm. (B) Quantification of BV/TV,Tb.N, Tb.Sp,Tb.Th. (C) TRAP staining of WT or Ly-sM-Cre-S1P$^{f/f}$ mice femurs that underwent either sham or OVX operation; Scale bars, 200μm. (D) Quantification of N.Oc/BS and Oc.S/BS(%) . (E) Micro-CT and three-dimensional reconstructed images of distal femurs from sham and OVX mice injected with PBS or PF429242 for 2 months; Scale bar, 1mm. (F) BV/TV, Tb.N, Tb.Sp and Tb.Th. (G) TRAP staining of the three groups mice femurs; Scale bars, 200μm. (H) Quantification of N.Oc/BS and tOc.S/BS(%). Data are presented as mean ± SD. *P < 0.05, **P < 0.01.