Krüppel-like factor 3 inhibition by mutated lncRNA Reg1cp results in human high bone mass syndrome

Mi Yang1, Qi Guo1,3, Hui Peng1,3, Yu-Zhong Xiao1,3, Ye Xiao1, Yan Huang1, Chang-Jun Li2, Tian Su1, Yun-Lin Zhang2, Min-Xiang Lei1, Hui-Ling Chen1, Tie-Jian Jiang1, and Xiang-Hang Luo1,

High bone mass (HBM) is usually caused by gene mutations, and its mechanism remains unclear. In the present study, we identified a novel mutation in the long noncoding RNA Reg1cp that is associated with HBM. Subsequent analysis in 1,465 Chinese subjects revealed that heterozygous Reg1cp individuals had higher bone density compared with subjects with WT Reg1cp. Mutant Reg1cp increased the formation of the CD31hiEMCNhi endothelium in the bone marrow, which stimulated angiogenesis during osteogenesis. Mechanistically, mutant Reg1cp directly binds to Krüppel-like factor 3 (KLF3) to inhibit its activity. Mice depleted of Klf3 in endothelial cells showed a high abundance of CD31hiEMCNhi vessels and increased bone mass. Notably, we identified a natural compound, Ophiopogonin D, which functions as a KLF3 inhibitor. Administration of Ophiopogonin D decreased the abundance of CD31hiEMCNhi vessels and bone formation. Our findings revealed a specific mutation in lncRNA Reg1cp that is involved in the pathogenesis of HBM and provides a new target to treat osteoporosis.

Introduction

The skeleton is one of the most complex tissues in mammals and undergoes continuous shaping, remodeling, and repair throughout adulthood to provide protection of the vital organs and rigid support for the whole body (Long and Ornitz, 2013; Riddle and Clemens, 2017). The structure and function of bone is maintained by the balance between bone resorption and formation (Long, 2011; Niedźwiedzki and Filipowska, 2015; Croucher et al., 2016). Osteoporosis, characterized by increased fragility in skeletal tissue, typically reflects an imbalance of bone remodeling in which bone resorption exceeds bone formation (Rachner et al., 2011). With the progressive aging of the general population, osteoporosis has emerged as a medical and socioeconomic problem. However, most current treatment options for osteoporosis have limitations and side effects that affect their long-term administration and patient adherence (Rachner et al., 2011; Jaleel et al., 2018). Disturbance of osteoblast or osteoclast regulation would also lead to abnormal accumulation of bone, such as osteosclerosis, which is classified according to its causative factor as either acquired or hereditary. Hereditary osteosclerosis includes osteopetrosis and high bone mass (HBM). Osteopetrosis is a rare inherited disorder involving decreased bone resorption. HBM results in increased bone formation that leads to an abnormal elevation in bone density (Boyd et al., 2002; Bonewald, 2011). Numerous studies have shown that mutations in the regulators of bone metabolism are the genetic determinants of HBM (Johnson et al., 1997; Boyd et al., 2002; Leupin et al., 2011). Boyd et al. (2002) performed genetic and biochemical analyses in a kindred with HBM and detected gain-of-function mutations in the gene encoding low-density lipoprotein receptor-related protein 5 (LRP5). Identifying the pathway(s) that affect the imbalance in bone remodeling during HBM pathogenesis might lead to the identification of therapeutic targets for osteoporosis.

Specialized vessels formed in tissues participate in the formation of a specific microenvironment that decides the fate of progenitor cells (Jabalee and Franz-Öndenaa, 2015; Ramsamy et al., 2015, 2016; Rafii et al., 2016). CD31hiEMCNhi vessels (CD31, also known as PECAM1 [platelet and endothelial cell adhesion molecule 1]; EMCN, endomucin), which are located in the metaphysis and endosteam of postnatal long bones and stain strongly for CD31 and EMCN, were identified as specific vessels in the skeletal system that couple angiogenesis and osteogenesis (Kusumbe et al., 2014; Ramsamy et al., 2014). However, the abundance of CD31hiEMCNhi vessels declines markedly during aging (Kusumbe et al., 2014; Wang et al., 2017; Yang et al., 2017). In our previous study, we demonstrated that inducing CD31hiEMCNhi vessels could prevent bone loss in osteoporosis (Xie et al., 2014; Yang et al., 2017). Angiogenesis coupled with...
osteogenesis plays an important role in bone metabolism and could be a new target to treat low bone mass diseases.

In the present study, we identified a novel mutation in a long noncoding RNA (lncRNA) gene, Reg1cp, which is associated with HBM. The homozygous mutant of Reg1cp binds directly to Krüppel-like factor 3 (KL3), a transcription factor, to regulate angiogenesis. Endothelial-specific klf3 knockout mice showed increased CD31\(^{hi}\)EMCN\(^{hi}\) vessels and bone formation. Notably, we identified a natural compound as a KLF3 inhibitor, which could increase the CD31\(^{hi}\)EMCN\(^{hi}\) endothelium and promote bone formation in aged mice. Taken together, our study identified a potential therapeutic target to treat osteoporosis.

Results

Reg1cp is a new HBM-associated gene

During clinical screening, we identified one patient who had extremely high bone density. This patient was female, 20 yr old, with a date of birth of April 22, 1997. She was of normal height (163.5 cm) and weight (60 kg). Clinical examination was not remarkable, except for HBM, a bone mineral density (BMD) of 1.266 g/cm\(^2\) in the hip (Z-score = +4.0), 1.169 g/cm\(^2\) in the femoral neck (Z-score = +3.9), and 1.191 g/cm\(^2\) in the lumbar spine (Z-score = +2.9). Radiographical examination at several skeletal sites showed normal skeletal morphology, except for a significant thickening of the cortical bones (Fig. 1 A). The circulating levels of type I procollagen amino-terminal propertide (PINP), indicated that the level of bone formation was slightly increased; however, the circulating levels of β-isomerized type I collagen (COL 1) C-telopeptide breakdown products (β-CTX), which reflect the level of bone resorption, were normal (Table 1). This patient also had normal levels of bone-related hormones, such as parathyroid hormone (PTH), human chorionic throphorin (hCT), and 25-hydroxy vitamin D (25-OHVD; Table 1).

After excluding a unique dietary lifestyle or conditions affecting bone metabolism, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause. To investigate the potential genetic variation among the genes known to cause hyperostosis, we hypothesized that the HBM phenotype had a genetic cause.

Reg1cp is involved in the regulation of CD31\(^{hi}\)EMCN\(^{hi}\) endothelial formation

LncRNA Reg1cp belongs to the Reg family and is encoded as a pseudogene that is mainly expressed in the pancreas (Charib et al., 1993). Reg1cp has little reported association with bone metabolism regulation. To determine the role of Reg1cp and its mutant in bone metabolism, we examined the expression of Reg1cp in isolated bone marrow endothelial progenitor cells, bone marrow mesenchymal stem cells (BMSCs), osteoblasts, and osteoclast precursor cells from human bone samples. Reg1cp was mainly expressed in bone marrow endothelial progenitor cells in skeletal tissue (Fig. 2 A). The expression level of Reg1cp was similar in Reg1cp\(^{+/+}\) individuals and age-matched Reg1cp\(^{+/−}\) controls (Fig. 2 B). However, structure prediction suggested that the mutation led to a large change in the structure of Reg1cp (Fig. 2 C and Fig. S2).

The CD31\(^{hi}\)EMCN\(^{hi}\) endothelium couples angiogenesis with osteogenesis. We observed that CD31, EMCN, and vessel growth factors (VEGFA, VEGFB, PDGFA, and PDGFB) transcripts were expressed at higher levels in endothelial cells (ECs) isolated from the Reg1cp\(^{+/+}\) individuals than in cells from age-matched Reg1cp\(^{+/−}\) controls (Fig. 2 E and F). We also found one Reg1cp\(^{+/−}\) subject that had pronounced induction of CD31\(^{hi}\)EMCN\(^{hi}\) ECs in the bone marrow compared with that in aged-matched Reg1cp\(^{+/−}\) controls (Fig. 2 D). To clarify whether the increased levels of growth factors are related to mutation of Reg1cp, we transfected plasmids encoding mutant Reg1cp (Reg1cp-mut) or WT Reg1cp (Reg1cp-wt) into ECs isolated from Reg1cp\(^{+/−}\) subjects. Quantitative real-time RT-PCR (qRT-PCR) confirmed the successful transfection (Fig. 2 G). The delivery of the exogenous Reg1cp-mut plasmid significantly induced the transcription of CD31, EMCN, VEGFA, and VEGFB (Fig. 2, H and I). However, the expression levels of these genes did not change after delivery of the exogenous Reg1cp-wt plasmid (Fig. 2, H and I). Reg1cp was expressed in BMSCs (Fig. 2 A). To investigate whether Reg1cp-mut could affect osteogenic differentiation, we transfected Reg1cp-mut or Reg1cp-wt plasmids into BMSCs undergoing osteogenic induction. Alizarin Red staining showed no difference between the Reg1cp-mut and Reg1cp-wt groups (Fig. 2 J). The expression level of the osteoblast transcription factors SP7 and RUNX2 remained unchanged (Fig. 2, K and L). This result
indicated that the mutant Reg1cp was involved in the regulation of angiogenesis in ECs.

**Mutant Reg1cp directly binds to KLF3 and affects its binding to downstream genes in ECs**

To identify the downstream molecules of Reg1cp that mediate angiogenesis, we conducted mass spectrometry (MS) of proteins after RNA pulldown using WT Reg1cp or mutant Reg1cp in human microvascular ECs (HMECs). Among the identified proteins, KLF3 showed the largest difference (Fig. 3 A). Mutant Reg1cp (Mut-Reg1cp), but not WT Reg1cp (WT-Reg1cp), specifically retrieved KLF3 (Fig. 3 B). Phylogenetic analysis of the C-terminal DNA-binding domains divides the KLF family into three subgroups. KLF3 is a member of group 2, which also contains KLF8 and KLF12 (Pearson et al., 2011). However, neither KLF8 nor KLF12 was retrieved by Mut-Reg1cp (Fig. S3 A). To further validate the interaction between KLF3 and Mut-Reg1cp, we transfected HMECs with the Reg1cp-mut or Reg1cp-wt plasmids; qRT-PCR confirmed the successful transfection (Fig. 3 C). We then conducted RNA immunoprecipitation of HMECs transfected with the Reg1cp plasmids. The anti-KLF3 antibody pulled down significantly more Reg1cp than did the IgG control (Fig. 3 D).
Further distinguish which Reg1cp (Mut-Reg1cp or WT-Reg1cp) bound to KLF3, the pulled down transcripts were reverse transcribed into cDNA and subjected to Sanger sequencing. The sequencing result showed that KLF3 pulled down Mut-Reg1cp, but not WT-Reg1cp (Fig. 3 E). Thus, these results indicated that Mut-Reg1cp binds directly to KLF3 in ECs.

KLF3 acts as a transcriptional repressor that mediates transcriptional silencing via recruiting the corepressor C-terminal binding protein (CTBP; Pearson et al., 2011; Dewi et al., 2015). To investigate whether the binding of Mut-Reg1cp affected the function of KLF3, we transfected Hek293T cells with the Reg1cp-mut plasmid, the Reg1cp-wt plasmid, or both. Transfection of the Reg1cp plasmids did not affect the expression of KLF3 (Fig. 3, F and G). JUNB (encoding JunB proto-oncogene, AP-1 transcription factor subunit) is one of KLF3’s downstream genes. We conducted an electrophoretic mobility shift assay (EMSA) using the nuclear extracts from the Hek293T cells and an oligonucleotide probe designed based on the predicted KLF3 binding site on the promoter of JUNB. The EMSA results demonstrated that KLF3 could specifically bind to the promoter of JUNB (Fig. 3 H). KLF3 binding was reduced after Reg1cp-mut plasmid transfection; however, there was no difference between the group transfected with the Reg1cp-mut plasmid and the group transfected with both plasmids, which suggested that the mutant Reg1cp does not compete with the WT form (Fig. 3, I and J). Transfection with Reg1cp-mut plasmid did not affect the recruitment of corepressors CTBP1 and CTBP2 to KLF3 (Fig. S3, C and D). These results indicated that Mut-Reg1cp binds directly to KLF3 and affects the binding of KLF3 to its downstream genes.

**Mutant Reg1cp abolishes the role of KLF3 in CD31hiEMCNhi vessel formation**

KLF3 is a potent transcriptional repressor with widespread roles in differentiation (Pearson et al., 2011). Therefore, we investigated how the binding of Mut-Reg1cp to KLF3 affected CD31hiEMCNhi vessel formation. We performed chromatin immunoprecipitation (ChiP)–PCR assays to assess the binding of KLF3 at the reported downstream genes involved in angiogenesis (Isley et al., 2017). Among them, JUNB has been reported as a critical independent regulator of VEGFA transcription (Schmidt et al., 2007). ChiP-PCR assays showed strong KLF3 binding to the promoter of JUNB (Fig. 4, A and B). Luciferase reporter constructs containing the WT or mutant predicted KLF3-binding site of JUNB (WT-pGL3-JunB and MUT-pGL3-JunB) were generated. We transfected WT-pGL3-JunB or MUT-pGL3-JunB with the KLF3 plasmid or empty vector into HMECs and measured luciferase fluorescence. KLF3 repressed the luciferase activity of the WT-pGL3-JunB reporter gene, but not that of MUT-pGL3-JunB (Fig. 4 C). We also transfected HMECs with a KLF3 siRNA to silence the KLF3 gene. Western blotting analysis showed significantly lower KLF3 but higher JUNB protein levels after transfection of siRNA-KLF3 (Fig. 4, D and E). The level of VEGFA was elevated along with JUNB (Fig. 4, D and E). These results confirmed the direct repression of JUNB transcription by KLF3.

To examine whether Mut-Reg1cp regulates angiogenesis by directly affecting the function of KLF3 in the transcription of JUNB, we performed the same ChiP-PCR assays in HMECs transfected with the Reg1cp-mut or Reg1cp-wt plasmids. Mutant Reg1cp abolished the binding of KLF3 to the promoter region of JUNB (Fig. 4, F and G). In addition, the HMECs transfected with the Reg1cp-mut plasmid showed significantly increased expression of JUNB and VEGFA, without affecting KLF3 expression (Fig. 4, H and I). To further investigate how the heterozygous mutation could have a dominant effect, we obtained an HMEC cell line with a heterozygous (Het) or homozygous (Hom) mutation of the Reg1cp gene using CRISPR/Cas9-mediated genome engineering. Sanger sequencing confirmed the successful construction (Fig. S3 B). The HMECs with the heterozygous mutation affected the binding of KLF3 to the JUNB promoter (Fig. 4, J and K), and further increased the protein level of JUNB and VEGFA in HMECs (Fig. 4, L and M), similar to the homozygous mutation. Both the heterozygous and homozygous mutation could increase the migration and tube formation ability of HMECs, and the homozygous mutation was more effective (Fig. 4, N–Q). The HMECs with the Reg1cp mutation showed increased VEGF signaling compared with that in the WT controls under hypoxic conditions (Fig. 4, R and S).

These results indicated that KLF3 represses the expression of JUNB in ECs; however, the mutated Reg1cp directly binds to KLF3 and abolished this process.

**Endothelial-specific Klf3 knockout mice show increased CD31hiEMCNhi vessels and bone formation**

KLF3 was broadly expressed in bone marrow, and there were no regional differences among the diaphysis, metaphysis, or endosteme in KLF3 expression in the bone vasculature (Fig. S4, A and B). To investigate the role of Klf3 in ECs in vivo, we crossed Cdh5 (PAC)-Cre transgenic mice with Klf3floxed/floxed mice to specifically knock out Klf3 in ECs (Klf3floxed/+. As expected, the expression level of Klf3 was significant decreased in the ECs of Klf3floxed/+ mice (Fig. 5 A). Specific knockout of Klf3 in ECs did not affect the body weight of the mice (Fig. S4 J). The expression levels of JUNB and Vegfa increased significantly in the ECs of Klf3floxed/+/ mice compared with that in their Klf3floxed/+/ littermates (Fig. 5, C and D). Flow cytometry confirmed a significant increase in CD31hiEMCNhi ECs in the bone marrow of...
Reg1cp is involved in the regulation of CD31hiEMCNhi endothelium formation. (A) Reg1cp expression levels in different cells isolated from human bone marrow. (B) Reg1cp expression levels in ECs. (C) The predicted secondary structure of Reg1cp and mutant Reg1cp around the mutant site (RNAfold Webserver, http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). (D) FACS analysis dot plot and quantitation of CD31hiEMCNhi ECs (Type H ECs) of bone samples from one 37-yr-old male Reg1cp+/mut subject and five age-matched Reg1cp+/+ controls. (E) qRT-PCR analysis of CD31 and EMCN expression levels in ECs. (F) qRT-PCR analysis of VEGFA, VEGFB, PDGFA, PDFB, TGFβ, and FGF1 expression level in ECs. (G) Reg1cp expression level in human ECs transfected with Reg1cp-mut or Reg1cp-wt plasmid. (H and I) CD31 and EMCN expression levels (H) and VEGFA, VEGFB, PDGFA, PDFB, TGFβ, and FGF1 expression levels (I) in ECs. (J–L) Representative images of Alizarin Red S staining (J) and qRT-PCR analysis of the levels of SP7 and RUNX2 expression (K and L) in human BMSCs transfected with Reg1cp-mut or Reg1cp-wt plasmid with osteogenic induction. Scale bar, 0.5 cm. In A, B, E–I, K, and L, n = 5 in each group from three independent experiments. Data are shown as the mean ± SD. *, P < 0.05; **, P < 0.01; N.S, no significance; Student’s t test (B, E, and F) and ANOVA (A, G–I, K, and L). BMEPC, bone marrow endothelial progenitor cell; OB, osteoblast; PRE-OC, osteoclast precursor cell.
Figure 3. Mutant Reg1cp binds directly to KLF3 and affects its binding to downstream genes in ECs. (A) KLF3’s interaction with Mut-Reg1cp was identified by MS. The b ions and y ions indicate peptide backbone fragment containing the N and C termini, respectively (y1+, y2+, b3+, and b4+ are labeled). CID, collision induced dissociation. (B) Mut-Reg1cp retrieves KLF3, as detected by immunoblotting. (C) Reg1cp expression level in HMECs transfect with Reg1cp-mut or Reg1cp-wt plasmid. (D) KLF3 retrieves Reg1cp RNA specifically, as detected by qRT-PCR. (E) Sanger sequencing results of transcripts pulled down by an anti-KLF3 antibody. (F and G) Western blotting analysis (F) and quantitation (G) of the relative levels of KLF3 protein expression in Hek293T cells transfected with Reg1cp-mut or Reg1cp-wt plasmids. (H) EMSA of the binding of KLF3 with JunB CACCC probe in vitro. Shift band stands for specific KLF3/probe complexes. Ab,
endothelium-specific knockout of Klf3cdh5 in ECs increased the amount of CD31hiEMCNhi endothelium, the bone volume, the number of osteoblasts, and bone marrow COL 1+ area in the aged mice without disturbing the number of TRAP+ osteoclasts (Fig. 8, A and B). However, endothelium-specific knockout of Klf3 did not affect the number of osteoclasts and their function, as indicated by tartrate-resistant acid phosphatase (TRAP) staining (Fig. 6, M and N) and serum CTX levels (Fig. 6 F). Taken together, these results suggested that the Klf3cdh5−/− mice had increased numbers CD31hiEMCNhi vessels and increased bone formation.

Molecular docking identified a natural compound as a KLF3 inhibitor
To identify novel small molecule inhibitors of KLF3, we performed virtual screening based on compounds docking to the binding pocket of KLF3 protein and obtained a small number of diverse compounds (Table S6). The top four compounds, Calenduloside E, Theaflavin 3’-O-gallate, Ophiopogonin D, and Picefeltarrenin IB, were selected for further experimentation. We treated HMECs with the four compounds for 48 h, and then detected the expression levels of CD31hiEMCNhi, JunB, and VEGFA. Ophiopogonin D treatment significantly increased the CD31hiEMCNhi ECs, bone volume, serum OCN levels, numbers of osteoblasts and ALP+ osteoprogenitors on the bone surfaces, and the COL 1+ area in the bone marrow (Fig. 9, A–H, and J–O), without changing the number of TRAP+ osteoclasts (Fig. 9, I, P, and Q). Taken together, these results suggested that inhibition of KLF3 in ECs by intraperitoneal administration of Ophiopogonin D promoted CD31hiEMCNhi vessel formation and stimulated new bone formation in both aged and OVX osteoporosis mouse models.

Discussion
Angiogenesis and osteogenesis show a close spatial-temporal association during skeletal development (Maes and Clemens,

The KLF3 inhibitor promotes CD31hiEMCNhi vessels and bone formation in aged mice and in an OVX model of osteoporosis
To investigate the therapeutic effects of Ophiopogonin D on osteoporosis, 2-mo-old C57/B6 mice after OVX surgery and 12-mo-old C57/B6 mice were treated intraperitoneally with Ophiopogonin D at 20 mg/kg every other day for 3 mo. Ophiopogonin D treatment increased the expression of JunB and VEGFA in HMECs treated with Ophiopogonin D (Fig. 6, J and K). These data indicated that Ophiopogonin D acts as a KLF3 inhibitor and has a positive effect on the formation of the CD31hiEMCNhi endothelium.
Figure 4. Mutant Reg1cp abolishes the role of KLF3 in CD31highEMCNhigh vessel formation. (A) ChIP-PCR assays with anti-KLF3 antibodies or anti-IgG antibodies using specific primers targeting the promoter regions of JUNB. (B) qRT-PCR analysis of the JUNB expression level after anti-KLF3 or anti-IgG ChIP. (C) HMECs were transfected with luciferase reporter carrying WT-pGL3-JunB or MUT-pGL3-JunB, respectively, and cotransfected with the Klf3 plasmid or vector. Firefly luciferase values, normalized for renilla luciferase, are presented. (D and E) Western blotting analysis (D) and quantitation (E) of the relative levels of KLF3, JUNB, and VEGFA protein expression. (F) ChIP-PCR assays with anti-KLF3 antibodies or anti-IgG antibodies in HMECs transfected with Reg1cp-mut or Reg1cp-wt plasmids. (G) JUNB expression level of anti-KLF3 or anti-IgG ChIP. (H and I) Western blotting analysis (H) and quantitation (I) of the relative levels of KLF3, JUNB, and VEGFA protein expression. (J) ChIP-PCR assays with anti-KLF3 antibodies or anti-IgG antibodies in HMECs with different Reg1cp genotypes. Het, heterozygous mutation; Hom, homozygous mutation. (K) JUNB expression level of anti-KLF3 or anti-IgG ChIP. (L and M) Western blotting analysis (L) and quantitation (M) of the relative levels of JUNB and VEGFA protein expression. (N and O) Representative images (N) and relative quantification (O) of a transwell migration assay. Scale bar, 150 µm. (P and Q) Representative images (P) and relative quantification (Q) of tube branch numbers of a Matrigel tube formation
Specialized CD31^{hi}EMCN^{hi} bone capillaries identified in the skeletal system have a close relationship with perivascular osteoprogenitors and mediate their differentiation (Kusumbe et al., 2014). Xu et al. (2018) demonstrated that osteoblast-derived SLIT3 could increase the formation of the CD31^{hi}EMCN^{hi} endothelium. Our previous studies revealed that PDGF-BB secreted by preosteoclasts could induce the CD31^{hi}EMCN^{hi} vessel subtype, subsequently preventing bone loss in osteoporosis (Xie et al., 2014). The microRNA miR-497~195 cluster contributes to the increase in CD31^{hi}EMCN^{hi} vessels and bone formation in aged mice (Yang et al., 2017). The decreased abundance of CD31^{hi}EMCN^{hi} vessels is an early marker of bone loss in humans (Wang et al., 2017). Therefore, it is a rational therapeutic strategy to target osteoporosis via the induction of CD31^{hi}EMCN^{hi} vessels. In the present study, we identified a specific point mutation in lncRNA Reg1cp that is involved in HBM pathogenesis. Mutant Reg1cp regulates the coupling of angiogenesis with osteogenesis via promoting CD31^{hi}EMCN^{hi} vessel formation in bone marrow. Furthermore, we provided a practical treatment option for age-related bone loss based on the mechanism of Mut-Reg1cp in regulating bone metabolism.

The major hormonal regulators of bone remodeling are hCT, PTH, 25-OHVD, and estrogen. Growth factors such as insulin-like growth factors, transforming growth factor-β (TGF-β), fibroblast growth factors, epidermal growth factor, WNTs, and bone morphogenetic proteins also play crucial roles in the regulation of bone remodeling (Khosla et al., 2012; Baron and Kneissel, 2013; Siddiqui and Partridge, 2016; Karner and Long, 2017; Lim et al., 2017). Our research began with the clinical study, we identified a specific point mutation in lncRNA Reg1cp that resulted in human high bone mass.
Figure 5. Endothelial-specific Klf3 knockout mice show increased CD31hiEMCNhi vessels and bone formation. (A) Expression level of Klf3 in ECs. (B) qRT-PCR analysis of JunB and Vegfa levels in ECs. (C and D) Representative images (C) and quantitation (D) of CD31 (green) and EMCN (red) immunostaining in femora from endothelial-specific Klf3 knockout mice (Klf3cdh5−/−) and their littermate controls (Klf3flox/flox). Scale bars, 100 µm. (E and F) Quantitation (E) and FACS analysis dot plot (F) of CD31hiEMCNhi ECs (Type H ECs) from long bones of 1-, 3-, and 12-mo-old Klf3cdh5−/− mice and their littermate controls. (G–K) Representative μCT images (G) and quantitative μCT analysis (H–K) of trabecular bone microarchitecture in femora. (L and M) Immunohistochemical...
whether Mut-Reg1cp is involved in other signaling pathways; however, this should be addressed in a future study.

With the progressive aging of the general population, the medical and socioeconomic burden of osteoporosis will increase further. One of the major requirements for novel treatments of bone loss is to develop anabolic agents that can stimulate bone formation. Ophiopogonin D is a natural compound isolated from the traditional Chinese herbal agent Radix Ophiopogon japonicus. It was reported to have anti-osteoporotic effects in a murine OVX model (Huang et al., 2015). In the present study, we identified that Ophiopogonin D functions as a mimic of mutant Reg1cp. Ophiopogonin D could bind to KLF3 and suppress its function, thus further promoting angiogenesis. Aged and OVX osteoporosis mice treated with Ophiopogonin D showed increased numbers of CD31hiEMCNhi vessels and new bone formation.

In summary, we revealed a specific mutation in the IncRNA Reg1cp that is involved in HBM pathogenesis by promoting angiogenesis during coupling with osteogenesis. We identified a natural compound that could act as a mimic of mutant Reg1cp, which increased the number of CD31hiEMCNhi vessels and promoted bone formation in aged and OVX osteoporosis mice. Our findings provide a potentially novel strategy to treat osteoporosis.

Materials and methods

Whole exome sequencing

Genomic DNA was collected and extracted from peripheral whole blood samples using a FlexiGene DNA kit (51206; Qiagen). Whole exome sequencing was conducted by Sangon Biotech by using the Agilent SureSelect Human All Exon V5+UTR and HiSeq X Ten Reagent Kit v2.5 for capture and Illumina sequencing platforms for data generation (paired end, 300 cycles). Sequencing was performed with 150-bp paired-end reads on the Illumina HiSeq XTen sequencing platform. The whole exome sequencing data were deposited on the Sequence Read Archive under accession no. PRJNA542422.

Bone density measurements

We measured BMD in the lumbar spine, femoral neck, and hip by dual-energy x-ray absorptiometry using a Hologic device (Hologic Discovery). The results are expressed as BMD and Z-scores (the number of SDs from the mean value for persons in the general population matched for age, sex, and race).

Massive sequencing and biochemistry

Human peripheral whole blood samples were obtained from 1,465 healthy subjects (616 males and 849 females) with ages ranging 20–80 yr. These volunteers were all residents of Changsha and surrounding areas. (The whole blood samples collection was conducted by the Physical Examination Center, Endocrinology Department of Xiangya Hospital of Central South University, Changsha, China and Department of Metabolic Endocrinology of the Second People’s Hospital of Xiangyang, Xiangxi China.) All subjects were screened using a detailed questionnaire, disease history, and physical examination. Subjects were excluded from the study if they had conditions affecting bone metabolism, including diseases of the kidney, liver, parathyroid, or thyroid, hyperprolactinemia, oophorectomy, rheumatoid arthritis, ankylosing spondylitis, malabsorption syndromes, malignant tumors, hematologic diseases, menopause before age 40 yr, or previous pathological fractures within 1 yr. If the subjects had received treatment with glucocorticoids, estrogens, thyroid hormone, FTH, fluoride, bisphosphonate, calcitonin, thiazide diuretics, barbiturates and anti-seizure medication, they were also excluded.

Genomic DNA was collected and extracted from peripheral whole blood samples using a FlexiGene DNA kit (51206; Qiagen). The genomic DNA met the sequencing requirements of the purity (OD 260/280 > 1.8) and concentration (50 ng/ml) of each sample. Reg1cp gene was amplified in 1,465 subjects by using DNA polymerase (MIX [GREEN]), and the primers of forward: 5'-GGAGCGCTTGTGTTAGAGAAACTG-3'; reverse: 5' -CTCTCTCCTACACATCTGACATAAAC-3'. Forward and reverse sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems), and the products were analyzed on an ABI 3730XL automated sequencer (PE Applied Biosystems). The clinical study was approved by the Ethics Committee of Xiangya Hospital of Central South University, and written informed consent was obtained from all participants before whole blood collection. Serum PINP and β-CTX were measured with the use of commercial kits (FC-007; Human PINP ELISA Kits, Phicon; FC-008; Human β-CTX ELISA Kits, Phicon).

Human samples

Human trabecular bone and bone marrow samples were obtained from 23 patients (13 male and 10 female) based on the inclusion and exclusion criteria. 20 patients with aseptic necrosis of femoral head or fracture undergoing hip arthroplasty replacement or open reduction internal fixation (ORIF), with ages ranging from 34 to 79 yr; one patient with ulna fracture, female, 27 yr old; and two patients with tibia fracture undergoing ORIF, female, 43 and 52 yr old respectively. We also performed Reg1cp sequencing of genomic DNA collected and extracted from bone marrow cells from those surgery patients. Among them, one male patient, 37 yr old, with femur shaft fracture undergoing ORIF was detected with Reg1cp heterozygous mutation (human trabecular bone and bone marrow collection were conducted by the Department of Orthopedics, Xiangya Hospital of Central South University, Changsha, China). All subjects were screened using a detailed questionnaire, disease history, and physical examination. Subjects were excluded...
Figure 6. Endothelial-specific Klf3 knockout in OVX mice show increased CD31hiEMCNhi vessels and bone formation. (A and B) FACS analysis dot plot (A) and quantification (B) of CD31hiEMCNhi ECs (Type H ECs). (C and D) Representative μCT images (C) and quantitative μCT analysis (D) of trabecular bone microarchitecture in femora. (E and F) Serum levels of OCN (E) and CTX (F) at the time of harvest. (G and H) Representative images (G) and quantification (H) of ALP (green) immunostaining in femora. Scale bar, 200 µm. (I and J) Immunohistochemical staining (I) and quantification (J) of OCN+ cells (brown) in femora. Scale bar, 50 µm. (K and L) Immunohistochemical staining (K) and quantification (L) of COL 1 (green) in femora. Scale bar, 200 µm. (M) Representative images of TRAP staining of femora. Scale bar, 50 µm. (N) Quantification data of TRAP+ cells in trabecular bone surface. Number of TRAP+ cells per bone perimeter (Tb.N.Trap+/B.Pm) was measured. n = 6 mice in each group from three independent experiments. Data are shown as the mean ± SD. **, P < 0.01; ANOVA.
Figure 7. A natural compound is identified as a KLF3 inhibitor by molecular docking. (A–D) qRT-PCR analysis of the relative levels of CD31 (A), EMCN (B), JUNB (C), and VEGFA (D) expression in HMECs treated with four different compounds. n = 3 in each group from three independent experiments. (E) The structure of Ophiopogonin D selected by molecular docking. (F) Crystal structure of Ophiopogonin D bound to Klf3. (G) HPLC-MS chromatograms of Ophiopogonin D reference substance (upper panel) and KLF3 recruit ligand (lower panel). Representative of two independent experiments. (H) ChIP-PCR assays with anti-Klf3 antibodies or anti-IgG antibodies in HMECs treated with Ophiopogonin D and control groups. Representative of three independent experiments. (I) qRT-PCR analysis of JUNB expression after anti-KLF3 or anti-IgG ChIP. n = 3 in each group from three independent experiments. (J and K) Western blotting analysis (J) and the quantification (K) of the levels of JUNB and VEGFA in HMECs treated with vehicle or different doses of Ophiopogonin D. Representative of three independent experiments. Data are shown as the mean ± SD. *, P < 0.05; **, P < 0.01; Student’s t test (A–D and I) and ANOVA (K).
Figure 8. Ophiopogonin D treatment promotes CD31\(^{hi}\)EMCN\(^{hi}\) vessels and bone formation in aged mice. 12-mo-old C57/B6 mice were intraperitoneally treated with Ophiopogonin D at 20 mg/kg every other day for 3 mo. (A) qRT-PCR analysis of JunB level in ECs. (B) qRT-PCR analysis of Vegfa level in ECs. (C and D) FACS analysis dot plot (C) and quantification (D) of CD31\(^{hi}\)EMCN\(^{hi}\) ECs (Type H ECs). (E and F) Representative images (E) and quantification (F) of CD31 (green) and EMCN (red) immunostaining in femora from Ophiopogonin D–treated mice and the vehicle control group. G, growth plate. B, bone. Scale bar, 100 \(\mu\)m. (G–K) Representative \(\mu\)CT images (G) and quantitative \(\mu\)CT analysis (H–K) of trabecular bone microarchitecture in femora. (L and M) Representative
from the study if they had conditions affecting bone metabolism, including diseases of the kidney, liver, parathyroid, or thyroid, hyperprolactinemia, oophorectomy, rheumatoid arthritis, ankylosing spondylitis, malabsorption syndromes, malignant tumors, hematologic diseases, or menopause before age 40 yr. If the subjects had received treatment with glucocorticoids, estrogens, thyroid hormone, PTH, fluoride, bisphosphonate, calcitonin, thiazide diuretics, barbiturates, or anti-seizure medication, they were also excluded. These participants had trabecular bone and bone marrow collection performed during bone fracture surgery or hip arthroplasty replacement. The clinical study was approved by the Ethics Committee of Xiangya Hospital of Central South University, and written informed consent was obtained from all participants before bone marrow collection.

Mice
To generate endothelium-specific Klf3 knockout mice, mice carrying loxP-flanked Klf3 alleles (Klf3flo/flo) and Cdhs-Cre transgenics were interbred. The Klf3flo/flo littermates were used as controls. The Cdhs-Cre transgenic mice (017968) were purchased from Jackson Laboratory; loxP-flanked Klf3 mice were purchased from Cyagen Biosciences. For endothelium-specific Klf3 knockout experiments, six male mice were used for each group at each observed time point (1, 3, and 12 mo) for each independent experiment. All mice we used were on a C57/B6 background. 2-mo-old female mice were used to perform OVX surgery and were sacrificed at 5 mo old. For Ophiopogonin D treatment experiments, 12-mo-old C57/B6 mice were intraperitoneally treated with Ophiopogonin D at a dosage of 20 mg/kg every other day for 3 mo. 2-mo-old C57/B6 mice after undergoing OVX surgery were intraperitoneally treated with Ophiopogonin D at dosage of 20 mg/kg 1 wk after surgery every other day for 3 mo. Six mice were used for each group for each independent experiment. Mice treated with vehicle treatment were used as controls. All animal care protocols and experiments were reviewed and approved by the Animal Care and Use Committees of the Laboratory Animal Research Center at Xiangya Medical School of Central South University. All mice were maintained in the specific pathogen-free facility of the Department of Laboratory Animals, Central South University.

Primary cell isolation and cell culture
For mouse bone marrow EC isolation, we collected bone marrow from tibiae and femurs of mice in sterile Ca²⁺- and Mg²⁺-free PBS. For human bone marrow EC isolation, we collected bone marrow from the patients with aseptic necrosis of femoral head or fracture undergoing hip arthroplasty replacement or ORIF. After the bone was crushed, the mixture was digested with type IA collagenase (Sigma-Aldrich) to obtain a single-cell suspension. Mice ECs were then sorted using EMCN antibody (SC-65495) and Dynabeads sheep anti-Rat IgG (Invitrogen). Human ECs were sorted using EMCN antibody (ab45772; Abcam). Sorted ECs were then plated on dishes coated with fibronectin and cultured in EC growth medium (EBM-2; Clonetics; Lonza) supplemented with EGM-2 Single Quots (CC-4176; Clonetics; Lonza). At first passage, cells were again magnetic cell separation sorted with EMCN antibody and plated for culture. Cells were fed every other day and passed upon confluency. Cultures were maintained at 37°C with 5% CO₂ in a humidified atmosphere.

For human endothelial progenitor cell isolation, peripheral whole blood samples were collected from Reg1cp/+mat and age-matched Reg1cp/+ subjects recruited by the Endocrinology Department of Xiangya Hospital of Central South University, as described earlier in Human samples and Massive sequencing and biochemistry. Total mononuclear cells were isolated from blood by density gradient centrifugation with Histopaque-1077 (10771; Sigma-Aldrich). Mononuclear cells were plated in 0.5 ml EC growth medium (EBM-2; Clonetics; Lonza) supplemented with EGM-2 Single Quots (CC-4176; Clonetics; Lonza) on fibronectin/gelatin–coated 24-well plates. After 7 d of culture, the attached endothelia cells were collected for RNA extraction.

For human BMSCs isolation, bone marrow cells were collected and incubated with FITC-, APC-, and PE-conjugated antibodies that recognized human Stro-1 (340106; BioLegend), CD45 (304012; BioLegend), and CD146 (361008; BioLegend) at 4°C for 30 min. The acquisition was performed on a FACS Aria model (BD Biosciences), and the analysis was performed using FACS DIVA software version 6.1.3 (BD Biosciences). The sorted human CD146⁺Stro-1⁺CD45⁻ BMSCs were cultured with α-MEM (Gibco-BRL) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

For human osteoblasts isolation, the bone samples were collected from patients with aseptic necrosis of femoral head or fracture undergoing hip arthroplasty replacement or ORIF and were cut into 1- to 2-mm lengths. Then we used warmed collagenase solution (4 mg/ml type IA collagenase in α-MEM) to incubate bone pieces three times at 25°C for 25 min. After that the bone samples were incubated with warmed collagenase solution (4 mg/ml type IA collagenase in α-MEM) and EDTA solution (5 mM EDTA solution PBS with 1% BSA) to incubate bone pieces at 25°C alternately; we aspirated the solution and retained it for cell plating. The isolated primary osteoblasts were cultured with α-MEM (Gibco-BRL) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

For preosteoclasts, we harvested monocytes and macrophages from bone marrow samples of patients with aseptic necrosis of femoral head or fracture undergoing hip arthroplasty.
replacement or ORIF. Bone marrow cells were cultured overnight on Petri dishes in α-MEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. After discarding the adherent cells, we incubated floating cells with M-CSF (30 ng/ml; R&D Systems) to obtain pure monocytes and macrophages. Upon incubation of monocytes and macrophages with 30 ng/ml M-CSF and 60 ng/ml receptor activator of nuclear factor-κ B ligand (PeproTech), all cells became preosteoclasts after a 3-d culture.

**Osteogenic differentiation assay**
Isolated BMSCs were cultured in 6-well plates at 2 × 10^6 cells/well with α-MEM (Gibco-BRL) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM dexamethasone,
10 mM β-glycerol phosphate, and 50 mM ascorbate-2-phosphate for 21 d. Culture medium was changed every 3 d. Cells were collected for RNA extraction or stained with 2% Alizarin Red S (Sigma-Aldrich) at pH 4.2 to evaluate the cell matrix mineralization.

EC culture and functional assays
HMECs were cultured in MCDB131 medium (Gibco) containing 10% FBS (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were fed every other day and passaged upon confluency. Cells were maintained at 37°C with 5% CO2 in a humidified atmosphere. EC migration assay was set up in transwell 24-well plates with 8-μm pore filters. In brief, we seeded 10^5 cells per well in the upper chamber after 1 h serum starvation. After 12 h incubation, we removed the cells in the upper surface of each filter using cotton swabs. The cells that migrated into the lower surface were fixed with 4% paraformaldehyde for 30 min and then stained with crystal violet. We counted the cell number in four random microscope visual fields in each well (three wells for each condition). EC tube formation assay was conducted in 48-well plates precoated with Matrigel (BD Biosciences). In brief, we seeded 10^5 cells per well after 1 h serum starvation. After 12 h incubation at 37°C, we observed the tube formation of HMECs and quantified the number of tube branches by counting four random microscope visual fields in each well (three wells for each condition).

Construction of mutant Reg1cp HMEC cell line
The mutation Reg1cp HMEC cell line was constructed using a LentriCRISPRv2 system. At first, the editing efficiency of predicted small guide RNAs was detected in Hek293T cells. The one that had the highest efficiency was chosen to construct lentiviruses. The sequence of small guide RNA used in this study is 5′-GGCCCATGCTGAGTTGCCCCA-3′. Then, the HMEC cells were infected with lentiviruses. 2 d after infection, 5 μg/ml puromycin was added to the culture medium to kill off cells not transduced by virus until all cells in the no virus infection control group died out. Afterwards, the lentivirus-infected HMEC cells were transfected with Mut-Reg1cp expressing plasmids or empty plasmids as the editing template. The single subclone of transfected HMECs was selected and validated for editing efficiency. The genomic DNA was isolated with a Flexigene DNA kit (Qiagen) according to the protocol of the manufacturer. The genomic DNA was analyzed by further Sanger sequencing. The single subclones with heterozygous or homozygous mutation on the identified site of Reg1cp were used for further experiments.

RNA immunoprecipitation and RNA pulldown
RNA immunoprecipitation was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17–700; Millipore) according to the manufacturer’s instructions. RNA for in vitro experiments was transcribed using TranscriptAid T7 High Yield Transcription Kit (K0441; Thermo Fisher Scientific) according to the manufacturer’s instructions. 5 pmol of 30-biotinylated RNA was used in each pulldown assay. The RNA pulldown experiment was performed using a Ribocycler profiler RiboTrap Kit (RN1011/RN1012; MBL) according to the manufacturer’s instructions.

Luciferase reporter assay
For functional analysis of klf3, the segments of the human JUNB promoter, including the predicted klf3 binding site, were PCR-amplified. The PCR products were purified and inserted into the XbaI-FseI site immediately downstream of the stop codon in the pGL3 control luciferase reporter vector (Promega), resulting in human WT-pGL3-JunB. The JUNB mutants for the klf3 seed regions were prepared using the Quik Change Site-Directed Mutagenesis Kit (Strategene) to get human MUT-pGL3-JunB. Human HMECs were transfected with either WT or mutant pGL3 construct, the pRL-TK renilla luciferase plasmid (Promega), and klf3 plasmid or vector for 48 h using Lipofectamine 2000 (Invitrogen). The dual luciferase reporter assay system (Promega) was used to quantify luminescent signal using a luminometer (Glomax; Promega).

RNA extraction and qRT-PCR
Total RNA from cells was extracted using Trizol reagent (Invitrogen). RT was performed using 1 μg total RNA by using the PrimeScript RT reagent Kit (Takara). Amplification reactions were set up in 25-μl reaction volumes containing SYBR Green PCR Master Mix (PE Applied Biosystems), 1 μl volume of cDNA, and amplification primers. Relative quantification was calculated by normalizing the test crossing thresholds (Ct) with the β-actin amplified control Ct. The results were normalized to β-actin. Primer sequences are listed in Table S7.

ChIP assay
ChIP assay was performed using SimpleChip Kit (9003; Magnetic Beads; Cell Signaling Technology) according to manufacturer’s instructions. Briefly, chromatin was cross-linked with 1% formaldehyde and sheared to 100- to 500-bp fragments by sonication (Sonics VCX130; 30% amplitude, 15 s on and 45 s off for four cycles). Klf3 antibody (1:100; PA5-18030; Invitrogen Antibodies) and normal rabbit IgG (2729; belongs to SimpleChip Kit) were used to immunoprecipitated the relevant protein–DNA complex. The cross-linked DNA and protein were reversed by addition of 5 M NaCl; DNA was extracted by phenol/chloroform and precipitated with ethanol and glycogen. Purified DNA was used to perform normal PCR or was quantified using real-time PCR. The primer sequences are listed in Table S8.

EMSA

The EMSA was operated using a LightShift chemiluminescent EMSA kit (GS009; Beyotime Biotechnology) according to the manufacturer’s instructions. 3 μl nuclear extract was used in each binding reaction in a total volume of 20 μl. Unlabeled WT and mutant probes were added to the binding reaction as competition experiments. 2 μg of anti-Klf3 antibodies (PA5-18030; Invitrogen Antibodies) was added to the binding reaction as supershift experiments. 20 min later, biotin-labeled WT probe was added. The probes used for EMSA are listed in Table S9.
Co-immunoprecipitation assays
HMECs were transfected with Reglc-pmut plasmid, His-Klf3, Myc-CTBP1, and HA-CTBP2. Following transfection, cells were lysed on ice in 1 ml of cell lysis buffer for 30 min. Supernatants were collected by centrifugation; two 50-μl aliquots were kept for input, whereas the remaining whole cell extracts were used for immunoprecipitation. Extracts were incubated with 5 μg of antibody (αβ9110; anti-HA tag antibody; Abcam; αβ9106; anti-Myc tag antibody; Abcam; αβ9108; anti-His tag antibody; Abcam) overnight at 4°C, and the products were collected on Dynabeads Protein G.

Western blot
Total cell lysates were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% milk (170–6404; Bio-Rad) and incubated with specific antibodies to klf3 (1:1,000; PA5-18030; Invitrogen Antibodies), JunB (1:1,000; 3753; Cell Signaling Technology), VEGFa (1:1,000; 19003–1-AP; Proteintechn, α-Tubulin (1:2,000; 11224–1-AP; Proteintechn), VEGFR2 (1:1,000; 2479; Cell Signaling Technology), and pVEGFR2 (1:1,000; 2478; Cell Signaling Technology), then reprobed with appropriate HRP-conjugated secondary antibodies. Blots were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (SD251210; Thermo Fisher Scientific). Phosphorylation of VEGFR2 was performed as previously described (Lee et al., 2007). Phosphorylation of VEGFR2 under hypoxia was measured after exposure to CoCl2 (100 mM) for 24 h.

ELISA analysis
We performed OCN or Ctx ELISA analysis of serum using a mouse OCN Enzyme Immunoassay kit (Biomedical Technologies Inc.) or a RatLAPS Enzyme Immunoassay kit (Immunodiagnostic Systems). We did all ELISA assays according to the manufacturers’ instructions.

Molecular docking
The first run of virtual screening was performed using Autodock Vina. First, receptor structure was processed in MGLTools 1.5.6 by addition of hydrogen atoms and Gasteiger charges. Openbabel was then employed to convert compounds into PDBQT files. The first run of virtual screening was performed using Autodock Vina. Only one pose was output for each compound. Grid score = −50 kcal/mol was used as the threshold in filtration. Results for all sites were combined, and duplicates were removed. Finally, clustering analysis was performed to obtain a small number of diverse compounds.

HPLC-MS assays
To demonstrate the direct binding of Ophiopogonin D to KLF3, 2 μg of Ophiopogonin D was incubated with 10 μg of human His-tag KLF3 protein (NB2P-23175; Novus Biologicals) in 150 μl of a buffer (pH 7.5) consisting of 100 mM Tris, 10% glycerol, 50 mM KCl, and 1 mM EDTA at room temperature for 2 h. Ni-NTA Agarose (30210; Qiagen) was used to separate the protein-ligand complexes, and the ligands were dissociated from KLF3 protein using 400 μl of methanol. Finally, we analyzed the recruited ligands by HPLC-MS assays.

Flow cytometry
For the analysis or sorting of mice CD31hiEMCNhi cells, femora and tibiae were dissected from mice after euthanization. Then the epi- physis, muscles, and periosteum were removed. The metaphyseal and diaphyseal regions of the bone were crushed in ice-cold PBS to get the bone marrow. For the analysis of human CD31hiEMCNhi cells in bone, human trabecular bone and bone marrow samples were obtained from people undergoing bone fracture surgery or hip arthroplasty replacement. Bone pieces and whole bone marrow samples were digested using 1 mg/ml type IA collagenase at 37°C for 20 min to obtain single-cell suspensions. For mice samples, after filtration and washing, the cells were counted and incubated for 45 min at 4°C with EMCN antibody (1:100; SC-65495; Santa Cruz Biotechnology), then washed and further incubated with APC-conjugated CD31 antibody (1:100; FAB3628A; R&D Systems) for 45 min at 4°C. For human samples, the cells were incubated with EMCN antibody (1:100; ab45772; Abcam), then washed and further incubated with CD31 antibody (1:100; 561654; BD Biosciences). We performed acquisition on a FACScan cytometer (BD Immunocytochemistry Systems). For demarcating and sorting CD31hiEMCNhi cells, first standard quadrant gates were set. Subsequently, CD31hiEMCNhi cells were distinguished from the total double-positive cells in quadrant 2, and the gates of quadrant 2 were set at >10^3 log Fl-4 (CD31-APC) fluorescence and >10^3 log Fl-2 (EMCN-PE) fluorescence.

μCT
Femora were dissected from mice, carefully removing the attached muscle, and fixed overnight with 10% formalin at 4°C. The μCT analyses were performed using high-resolution μCT (Skyscan 1172; Bruker microCT). The scanner was set at a voltage of 65 kV, a current of 153 μA, and a resolution of 15 μm per pixel. The image reconstruction software (NRecon, version 1.6; Bioz), data analysis software (CT Analyser, version 1.9; Bruker microCT) and three-dimensional model visualization software (μCT Volume, version 2.0; Bruker microCT) were used to analyze the parameters of the distal femoral metaphyseal trabecular bone. The region of interest for analysis was 5% of femoral length below the growth plate. Trabecular bone volume per...
tissue volume, trabecular number, trabecular separation, and trabecular thickness were measured.

**Histochemistry and histomorphometry**

Femora were dissected from mice, carefully removing the attached muscle, and fixed overnight with 10% formalin at 4°C. After washing three times with ice-cold PBS, the samples were decalcified at 4°C using 10% EDTA (pH 7.4) for 21 d and then embedded in paraffin. 4-μm-thick sagittal-oriented sections of the knee joint medial compartment were used for staining. The slides were processed for TRAP staining, which was performed using a standard protocol (Sigma-Aldrich). Histomorphometric analysis of two-dimensional parameters of the trabecular bones was performed using OsteoMeasureXP Software (OsteoMetrics). Osteoclast surface per bone surface and osteoclast number per bone perimeter parameters were used to measure the bone resorption.

**Immunocytochemistry and histomorphometry**

Femora were dissected from mice, carefully removing the attached muscle, and fixed overnight with 10% formalin at 4°C. After washing three times with ice-cold PBS, the samples were decalcified at 4°C using 10% EDTA (pH 7.4) for 21 d and then embedded in paraffin. 4-μm-thick longitudinally oriented bone sections of femora samples were used for staining. The sections were stained with individual primary antibodies to OCN (1:100; M137; Takara Bio) at 4°C overnight. We used the HRP-streptavidin detection system (Dako) to detect immunoactivity. Then we counterstained the sections with hematoxylin (Sigma-Aldrich). Histomorphometric analysis of two-dimensional parameters of the trabecular bones was performed using OsteoMeasureXP Software (OsteoMetrics). Osteoblast number per bone perimeter parameters were used to measure the bone formation.

**Immunofluorescence and histomorphometry**

For immunofluorescence staining, freshly dissected femora collected from transgenic mice and their control littermates were first fixed in ice-cold 4% paraformaldehyde solution for 4 h, then decalcified in 0.5 M EDTA (pH 7.4) at 4°C for 24 h (1- and 3-mo-old mice) or for 48 h (12- and 15-mo-old mice). The bone samples were then incubated in 20% sucrose and 2% polyvinylpyrrolidone solution overnight, as described previously (Kusumbe et al., 2013). For type H vessel staining, we embedded the tissues in 8% gelatin (porcine) in the presence of 20% sucrose and 2% polyvinylpyrrolidone. 40-μm-thick longitudinally oriented bone sections were stained with primary antibodies to mouse CD31 (1:50; ab28364; Abcam) and EMCN (1:50; V.7C7; Santa Cruz Biotechnology) overnight at 4°C. For other immunofluorescence staining, we embedded the tissues in optimal cutting temperature compound. 4-μm-thick longitudinally oriented bone sections were stained with individual primary antibodies to mouse COL 1 (1:50; AB765P; Millipore), ALP (1:200; ab108337; Abcam), Aggrecan (1:50; AB1031; Millipore), Sox9 (1:200; ab185230; Abcam), and Klf3 (1:100; ab49221; Abcam) overnight at 4°C. Subsequently, we used secondary antibodies conjugated with fluorescence at room temperature for 1 h while avoiding light. We used isotype-matched controls, such as polyclonal rabbit IgG (AB-105-C; R&D Systems), polyclonal goat IgG (AB-108-C; R&D Systems), and monoclonal rat IgG2A (54447; R&D Systems) under the same concentrations and conditions as negative controls. We counted the numbers of positively stained cells in four random visual fields in the distal metaphysis of the femur in five sequential sections per mouse in each group.

**Calcein double-labeling**

To examine dynamic bone formation, we injected mice intraperitoneally with 0.08% calcein (20 mg/kg, body weight; Sigma-Aldrich) 8 d and 2 d before euthanasia. Calcein double-labeling in undecalcified bone slices was observed under a fluorescence microscope. Four randomly selected visual fields in the distal metaphysis of the femur were measured to test trabecular bone formation in femora.

**Statistical analyses**

The data are presented as mean ± SD. For comparisons of two groups, the two-tailed Student’s t test was used. For comparisons of multiple groups, one-way ANOVA was used. Differences were considered significant at P < 0.05. No randomization or blinding was used, and no animals were excluded from analysis. Sample sizes were selected on the basis of previous experiments.

**Study approval**

The clinical study was approved by the Ethics Committee of Xiangya Hospital of Central South University, and written informed consent was obtained from all participants. All animal care protocols and experiments were reviewed and approved by the Animal Care and Use Committees of the Laboratory Animal Research Center at Xiangya Medical School of Central South University.

**Online supplemental material**

Fig. S1 shows the family trees of affected subjects. Fig. S2 shows the secondary structure prediction of Reg1cp and mutated Reg1cp. Fig. S3 shows that mutant Reg1cp does not retrieve KLF8 and KLF12 and does not affect the recruitment of corepressors CTBP1 and CTBP2 and KLF3. Fig. S4 demonstrates that endothelial-specific Klf3 knockout mice show no change in osteoclast number, Wnt signaling, Tphl expression, and growth plate morphology. Fig. S5 shows that Ophiopogon D treatment does not affect osteoclast number and growth plate morphology in aged mice. Table S1 shows clinical information of the 67 Reg1cp+/+ individuals. Table S2 shows the index of bone mass in 26 male Reg1cp+/+ individuals and 590 male Reg1cp+/− individuals. Table S3 shows the index of bone mass in 41 female Reg1cp+/+ individuals and 808 female Reg1cp+/− individuals. Table S4 shows a biochemical survey for bone remodeling parameters in 26 male Reg1cp+/+ individuals and 226 male Reg1cp+/−. Table S5 shows a biochemical survey for bone remodeling parameters in 41 female Reg1cp+/+ individuals and 213 female Reg1cp+/− individuals. Table S6 shows the top 21 selected small molecules. Table S7 shows nucleotide sequences of primers used for quantitative RT-PCR detection. Table S8 shows nucleotide sequences of primers used for ChIP-PCR. Table S9 shows information from the biotin-labeled probe used for EMSA.
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