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Inactivation of *Vhl* in Osteochondral Progenitor Cells Causes High Bone Mass Phenotype and Protects Against Age-Related Bone Loss in Adult Mice

Tujun Weng¹, Yangli Xie¹, Junlan Huang¹, Fengtao Luo¹, Lingxian Yi¹, Qifen He¹, Di Chen², and Lin Chen¹

¹Center of Bone Metabolism and Repair, State Key Laboratory of Trauma, Burns and Combined Injury, Trauma Center, Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing, China

²Department of Biochemistry, Rush University Medical Center, Chicago, IL, USA

Abstract

Previous studies have shown that disruption of von Hippel–Lindau gene (*Vhl*) coincides with activation of hypoxia-inducible factor α (HIF α) signaling in bone cells and plays an important role in bone development, homeostasis, and regeneration. It is known that activation of HIF1 α signaling in mature osteoblasts is central to the coupling between angiogenesis and bone formation. However, the precise mechanisms responsible for the coupling between skeletal angiogenesis and osteogenesis during bone remodeling are only partially elucidated. To evaluate the role of *Vhl* in bone homeostasis and the coupling between vascular physiology and bone, we generated mice lacking *Vhl* in osteochondral progenitor cells (referred to as *Vhl* cKO mice) at postnatal and adult stages in a tamoxifen-inducible manner and changes in skeletal morphology were assessed by micro-computed tomography (μ CT), histology, and bone histomorphometry. We found that mice with inactivation of *Vhl* in osteochondral progenitor cells at the postnatal stage largely phenocopied that of mice lacking *Vhl* in mature osteoblasts, developing striking and progressive accumulation of cancellous bone with increased microvascular density and bone formation. These were accompanied with a significant increase in osteoblast proliferation, upregulation of differentiation marker Runx2 and osteocalcin, and elevated expression of vascular endothelial growth factor (VEGF) and phosphorylation of Smad1/5/8. In addition, we found that *Vhl* deletion in osteochondral progenitor cells in adult bone protects mice from aging-induced bone loss. Our data suggest that the VHL-mediated signaling in osteochondral progenitor cells

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Address correspondence to: Lin Chen, PhD, MD, Center of Bone Metabolism and Repair, State Key Laboratory of Trauma, Burns and Combined Injury, Trauma Center, Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing 400042, China. linchen70@163.com.

Additional Supporting Information may be found in the online version of this article.

Disclosures

All authors state that they have no conflicts of interest.

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plays a critical role in bone remodeling at postnatal/adult stages through coupling osteogenesis and angiogenesis. © 2014 American Society for Bone and Mineral Research.

Keywords

VON HIPPEL–LINDAU GENE (VHL); HYPOXIA–INDUCIBLE FACTOR α (HIF α); BONE MASS; MUTANT MICE; AGING OSTEOPOROSIS

Introduction

Skeleton is continuously remodeled throughout entire life. During bone remodeling, osteoblast-mediated bone formation is tightly coupled with osteoclast-induced bone resorption, replacing damaged bone with new bone.⁽¹⁾ With increasing age, osteoblast and osteoclast activities are dysregulated and, consequently, a negative bone balance leads to osteoporosis.⁽²⁾ Osteoporosis is a skeletal disease affecting a large population of elderly women, with low bone mass and high susceptibility to fragility fracture.⁽³⁾ A better understanding of bone remodeling and osteogenesis will help us develop more effective strategies to manage patients with dysregulated bone remodeling and osteoporosis.

Skeleton is a highly vascularized tissue, where bone remodeling is tightly coupled with angiogenesis. Increased numbers of blood vessel brings more mesenchymal progenitor cells to the surface of trabecular bone, where they differentiate into mature osteoblasts and lay down new bone.⁽¹⁾ A recent study has shown reduced local blood supply to the tibia metaphysis in the ovariectomy-induced osteoporosis rat model, highlighting an association between bone vasculature and adult bone homeostasis.⁽⁵⁾ Mice lacking the von Hippel-Lindau gene (*Vhl*) in mature osteoblasts had increased angiogenesis in bone tissue and reduced estrogen deficiency-induced bone loss, suggesting that increased angiogenesis may potentially antagonize estrogen deprivation-induced osteoporosis through coupling of osteogenesis.⁽⁶⁾

The precise mechanism responsible for the coupling between angiogenic and osteogenic processes during bone remodeling remains to be defined. Under normoxic conditions VHL is critical for proteolysis of hypoxia-inducible factor α (HIF α).⁽⁷⁾ Wang and colleagues⁽⁸⁾ demonstrated that mice with overexpression of HIF α signaling through deletion of *Vhl* in mature osteoblasts developed extremely dense, heavily vascularized long bones. It is hypothesized that high expression of VEGF contributes to the coupling between skeletal angiogenesis and increased bone mass in osteoblast-specific *Vhl* knockout (KO) mice.⁽⁸⁾ This is the first observation showing that upregulation of HIF α and VEGF in osteoblasts promotes bone formation secondary to angiogenesis. Overexpression of VEGF in osteochondral progenitor cells, a downstream target of HIF α signaling, also led to aberrant skeletal vascularization and ossification in mice.⁽⁹⁾ It is likely that there is crosstalk between mesenchymal progenitor cells and vascular endothelial cells, but direct genetic evidence and the underlying mechanisms responsible for this coupling remain to be determined.

Conventional *Vhl*-deficient mice are embryonic lethal as a result of abnormalities of placental vasculogenesis.⁽¹⁰⁾ Mice lacking *Vhl* in chondrocytes developed a severe

dwarfism, increased extracellular matrix deposition, and reduced chondrocyte proliferation during growth plate development; however, no apparent change in angiogenesis was observed.⁽¹¹⁾ In contrast, targeted deletion of *Vhl* in osteoblasts, coinciding with activation of HIF α , significantly increased bone volume with a striking increase in bone vascularity.⁽⁸⁾ In addition, it has been reported that activation of the HIF α pathway by deletion of *Vhl* in osteoblasts or treatment with a small molecular inhibitor of HIF prolyl hydroxylation (desferrioxamine [DFO]) markedly increased vascularity and accelerated bone regeneration in response to distraction osteogenesis.⁽¹²⁾ On the contrary, mice with partial HIF-1 α deficiency displayed enhanced bone regeneration subsequent to fracture as a result of decreased chondrocytic and osteoblastic apoptosis.⁽¹³⁾ These findings suggest that the exact roles of VHL-regulated HIF α pathway may be different in specific cell types and developmental stages during the process of bone remodeling and regeneration.

In the current study, to evaluate the role of VHL in mesenchymal progenitor cells and in bone homeostasis in postnatal mice, we decided to use *Col2-CreER^{T2}* inducible transgenic mice,⁽¹⁷⁾ in which the *Col2a1* promoter drives Cre recombinase expression in a subset of mesenchymal osteochondral progenitor cells that give rise to chondrocytes and osteoblasts,^(14–16) to generate mice lacking *Vhl* in osteochondral progenitor cells at postnatal stage in a tamoxifen (TM)-inducible manner. We found that deletion of the *Vhl* gene in osteochondral progenitor cells induced by TM in the adult skeleton causes accumulation of excessively ossified bone accompanied with enhanced angiogenesis and protects mice from age-related bone loss.

Subjects and Methods

Animals

Vhl^{fl/fl} mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and *Col2-CreER^{T2}* mice were generated in author DC's laboratory.⁽¹⁷⁾ These two mouse lines were maintained in C57BL/6J background. The *Col2-CreER^{T2};R26R* mice were generated by breeding *Col2-CreER^{T2}* mice with Rosa26 transgenic reporter mice. To generate *Col2-CreER^{T2};Vhl^{fl/fl}* (hereafter referred to as *Vhl* cKO) mice, *Vhl^{fl/fl}* mice were crossed with *Col2-CreER^{T2}* transgenic mice. The *Col2-CreER^{T2};Vhl^{fl/fl}* mice and their Cre-negative control littermates (*Vhl-flox*) were intraperitoneally injected with TM at a specific time point (1 mg/10g body weight, daily for 5 days). Genotypings for *Vhl* and the Cre transgene were determined by PCR analysis as described⁽⁸⁾ and primer sequences will be provided upon request. All animal experiments were performed according to protocols approved by the Institutional Animals Care and Use Committee of Daping Hospital (Chongqing, China).

μ CT scanning and analysis

Bone tissues from *Vhl* cKO and Cre-negative control mice were scanned with a Scanco vivaCT 40 instrument (Scanco, Brüttisellen, Switzerland) and analyzed for bone structure. Briefly, serial 10.5- μ m tomographic images were acquired at 70kV and 113 mA. To segment bone from bone marrow, the constant threshold for trabecular and cortical bone was 180 and 220, respectively. The region of interest (ROI) in trabecular bone was located at 10 slices (105 μ m) below the lowest point of growth plate and the length was extended for 100

slices (1050 μm). Trabecular morphometry parameters analyzed included the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp). Cortical ROI was selected from the femoral midshaft and the thickness was extended for 50 slices (525 μm). Cortical thickness (Ct.Th) and cortical bone fraction (Ct.Ar/Tt.Ar) were used to analyze cortical morphometry. 2D and 3D images were also obtained.

Bone histology and histomorphometry

Bone tissues were fixed in 4% paraformaldehyde in 0.1M phosphate buffer and decalcified in 15% EDTA-PBS for 2 weeks and embedded with paraffin. 6- μm -thick sections were stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP; Sigma, St. Louis, MO, USA) according to standard methods. Reticulin staining was performed according to provided instruction (GenMed Scientifics Inc., Arlington, MA, USA). TRAP-stained slides were used for histomorphometric analysis to evaluate the osteoclast activity and bone resorption.

For in vivo fluorescent labeling, calcein (20 mg/kg body weight; Sigma) was intraperitoneally injected twice, at day 10 and day 3 before tissue collection at 4 months of age. After dehydration, the undecalcified tibias were embedded in methylmethacrylate, and then 10- μm -thick and 5- μm -thick sections were used for fluorescent analysis and von Kossa/toluidine blue staining, respectively. Histomorphometric analyses were performed with the OsteoMeasure (OsteoMetrics, Inc., Decatur, GA, USA) bone analysis system. The ROIs for trabecular bone data collection were measured in an area 1.5mm in length from 0.5mm below the growth plate of the proximal tibias. All histomorphometric parameters are reported in accordance with standard criteria.⁽¹⁸⁾

Immunohistochemistry

For immunohistochemistry (IHC) analysis, decalcified tibia or femur sections were deparaffinized by xylene, deprived of endogenous peroxidase activity with 3% H_2O_2 for 30 minutes, and treated with trypsin for 10 minutes to retrieve antigen, then blocked with normal goat serum for 30 minutes. Sections were incubated at 4°C overnight with primary antibody followed by biotinylated secondary antibodies and a horseradish peroxidase (HRP)-conjugated streptavidin-biotin staining technique (DAB kit; ZSGB-BIO, Beijing, China). The primary antibodies used in this study were as follows: rabbit anti-Hif1 α polyclonal antibody (1:100 dilution; Boster, Wuhan, China), rabbit anti-Hif2 α polyclonal antibody (1:100 dilution; Abcam, Cambridge, MA, USA), mouse anti-BrdU monoclonal antibody (1:500 dilution; Sigma), goat polyclonal antibody anti-Runx2 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal antibody anti-Osteocalcin (1:50 dilution; Santa Cruz Biotechnology), mouse anti-VEGF monoclonal antibody (1:100 dilution; Abcam), rat anti-CD31 monoclonal antibody (1:100 dilution; Abcam), rabbit anti-p-Smad1/5/8 polyclonal antibody (1:100 dilution; Cell Signaling Technology, Danvers, MA, USA).

Real-time PCR

RNA was extracted from bone tissues and primary chondrocytes using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Isolation and culture of chondrocytes has been described⁽¹⁹⁾; primary cultures were treated with 4-hydroxytamoxifen (4-OH-TM) (1 μ M) or vehicle for 48 hours to targeted *Vhl* deletion. Total RNA was reverse-transcribed to cDNA using a PrimerScript RT reagent kit (TaKaRa, Dalian, China). Real-time PCR was performed to measure the relative mRNA levels using the MxPro system (Stratagene, Agilent Technologies, Santa Clara, CA, USA) with SYBR Green Mix (TaKaRa). All samples were measured in triplicate and normalized to internal control Cyclophilin A. The annealing temperature was 57°C. Primer sequences have been described.⁽²⁰⁾

ELISA assay

Sera were collected from 12-month-old *Vhl* cKO and Cre negative control mice and osteocalcin levels were determined using the Mouse Osteocalcin ELISA kit (Immunotopics, Inc., San Clemente, CA, USA).

Statistical analysis

Results are reported as the mean $\pm$ SD. Unpaired Student's *t* tests were used to determine statistical difference between groups. Values of *p* < 0.05 were considered significant.

Results

Inactivation of *Vhl* in osteochondral progenitor cells of adult mice leads to dramatically increased cancellous bone

It has been reported that the *Col2a1* promoter drives Cre recombinase expression in osteochondral progenitor cells which give rise to chondrocytes and osteoblasts.^(14,15) We first performed LacZ staining to examine the Cre-mediated recombination in adult bone. The *Col2-CreER^{T2};Rosa26R* mice and their Cre-negative control littermates were intraperitoneally injected with TM at the age of 2 months and 1 month later femurs and tibias were dissected for LacZ staining. Histologically, LacZ-positive staining was observed in growth plates, articular chondrocytes, and osteoblast lineage in trabecular bone surface (Supplemental Fig. S1). Thus, the *Col2a1-CreER^{T2}* mice used in our study was a valuable tool for gene deletion in osteochondral progenitor cells by TM-induced temporally controlled manner.

To investigate the effects of HIF α overexpression on bone homeostasis at adult stage, *Vhl* was deleted in osteochondral progenitor cells of adult bone using TM-inducible system. TM injection into adult *Vhl* cKO mice and Cre-negative control mice for 5 continuous days dramatically changed the bone micro-structure in *Vhl* cKO mice at the age of 2 months and 4 months (Fig. 1A – C). One month after TM administration, μ CT images of tibias showed a striking increase in trabecular bone volume in *Vhl* cKO mice compared to that in Cre-negative control mice (Fig. 1A). X-ray and 2D μ CT analyses revealed that *Vhl* cKO mice exhibited higher amounts of trabecular bone in vertebrae than their wild-type littermates over 2 months after TM induction (Fig. 1B). Consistently, the results of H&E staining also

revealed increased trabecular bone volume in tibias and vertebrae from *Vhl* cKO mice at 4-months-old after TM injection (Fig. 1C). Quantification showed that trabecular bone volume (BV/TV) and the number of trabecular bone (Tb.N) in vertebrae from *Vhl* cKO mice were largely greater than that in Cre-negative control mice (Fig. 1D). It has been reported that overexpression of VEGF in osteochondral progenitor cells induced large bone mass associated with bone marrow fibrosis⁽⁹⁾. To examine whether the increased osteogenesis was accompanied with abnormal fibrosis, we performed reticulin staining to detect collagen IV-positive fibers and no significant fibrosis was found in the bone marrow (Supplemental Fig. S2). These data suggest that inactivation of *Vhl* in osteochondral progenitor cells in adult mice caused formation of abundant cancellous bone without fibrosis.

Although increased bone mass was observed in long bones from *Vhl* cKO mice, no significant changes in calvarial bone morphology and thickness as shown by μ CT analysis were observed (Fig. 2A). In addition, we also compared cortical bone at mid-shaft of femurs between Cre-negative control and *Vhl* cKO mice (Fig. 2B). No significant changes of cortical bone thickness and cortical bone fraction (Ct.Ar/Tt.Ar) in the mid-shaft of femurs were observed in *Vhl* cKO mice 2 months after TM induction as assessed by μ CT (Fig. 2C, D).

High bone mass phenotype in mice with inactivation of *Vhl* is characterized with enhanced bone formation and bone resorption

To determine the cellular basis responsible for the bone phenotype in *Vhl* cKO mice, static and dynamic histomorphometric assess were performed on proximal tibias from male *Vhl* cKO and Cre-negative control mice at 4 months old after 2 months' TM induction. Von Kossa/toluidine blue staining revealed a significantly increased trabecular bone and osteoblast lineage cells surrounding cancellous bone in *Vhl* cKO mice (Fig. 3A). Double calcein incorporation examination showed aberrant and disorganized labeling in the tibias from 4-monthold *Vhl* cKO mice, suggesting that inactivation of *Vhl* in osteochondral progenitor cells results in increased number of osteoblasts as well as increased osteogenesis, leading to the formation of large amount of woven bone (Fig. 3B). Consistent with the μ CT results, bone volume (BV/TV) and trabecular number (Tb.N) was significantly increased ($p < 0.001$, Fig. 3C), whereas trabecular separation (Tb.Sp) was greatly reduced ($p < 0.001$, Fig. 3C) in *Vhl* cKO mice. Histomorphometric assay also showed that the number of osteoblasts per tissue area (N. Ob/T.Ar) and number of osteoblasts/bone perimeter (N.Ob/B. Pm) (data not shown) were significantly increased in *Vhl* cKO mice (Fig. 3D). Next we examined the potential contribution of osteoclasts to the high bone mass phenotypes. We found that *Vhl* cKO mice had more TRAP-positive osteoclasts in tibia sections (Fig. 3C). The number of mature osteoclasts per tissue area (N.Oc/T.Ar) was significantly increased in *Vhl* cKO mice compared to that in Cre-negative control mice (Fig. 3D). Supportively, real-time PCR analyses displayed that the expressions of *Trap* and matrix metalloproteinase 9 (*Mmp9*) were markedly increased in long bones of *Vhl* cKO mice (Fig. 3E). In addition, serum osteocalcin, a marker of bone turnover, was significantly increased in *Vhl* cKO mice compared to that in Cre-negative control mice (Supplemental Fig. S3). Our results suggested that the osteosclerosis phenotype in *Vhl* cKO mice was characterized with high bone turnover; ie, enhanced bone formation and bone resorption.

Inactivation of *Vhl* in osteochondral progenitor cells in adulthood protects mice from aging-induced bone loss

Aging is one of the main contributors leading to osteoporosis. To determine the role of *Vhl* in osteochondral progenitor cells in the pathogenesis of aging-related bone loss, we compared the trabecular bone microarchitecture in distal femora between young (4-month-old) and older mice (12-month-old) in male *Vhl* cKO and their control littermates by μ CT analysis. μ CT images showed larger amount of ossified bone in the femora of *Vhl* cKO mice from both young and older mice (Fig. 4A). In Cre-negative control mice, bone volume (BV/TV) at the distal femur was significantly decreased in the 12-month-old group compared to the 4-month-old group ($21.8\% \pm 3.1\%$ and $8.1\% \pm 3.2\%$, respectively, $p < 0.001$) (Fig. 4B). In addition, a significant increase in trabecular separation (Tb.Sp, $+70.6\%$, $p < 0.001$) and decrease in trabecular number (Tb.N, -40.5% , $p < 0.001$) were observed in the Cre-negative control femurs during the aging process (Fig. 4B). However, in contrast to Cre-negative control mice, *Vhl* cKO mice showed no trabecular bone loss and conversely, increased bone volume (BV/TV) in *Vhl* cKO mice was observed in the 12-month-old group compared to that in the 4-month-old group ($87.9\% \pm 7.2\%$ and $98.4\% \pm 0.7\%$, respectively, $p < 0.05$) (Fig. 4B). Correspondingly, a significant increase in trabecular thickness (Tb.Th, $\pm 187.5\%$, $p < 0.001$) was observed in the *Vhl* cKO femurs during the aging process (Fig. 4B). These results indicate that inactivation of *Vhl* in osteochondral progenitor cells may prevent aging-induced bone loss.

Deletion of *Vhl* in osteochondral progenitor cells promotes osteoblast proliferation, differentiation and upregulates VEGF expression and bone morphogenic protein signaling

To determine the mechanism responsible for the increased bone mass, we first detected HIF α expression in osteoblast lineage cells. As expected, IHC data showed that expression of HIF-1 α and HIF-2 α in osteoblast lineage cells was higher in *Vhl* cKO mice than those in Cre-negative control mice (Fig. 5A). Then we investigated proliferation and differentiation of osteoblast lineage cells in adult bone from *Vhl* cKO mice. Results of bromodeoxyuridine (BrdU) incorporation assay on adult *Vhl* cKO bones displayed markedly increased proliferating osteoprogenitor cells and/or osteoblasts 7 days after TM induction (Fig. 5B). Quantification results showed that an over twofold increase in BrdU-positive cells at the tibia was observed in *Vhl* cKO mice compared with the Cre-negative control mice (Fig. 5B). Increased osteoblast proliferation may greatly contribute to the increased bone formation in *Vhl* cKO mice. Osteoblast differentiation was determined by IHC assay. Runx2 is a key transcription factor for osteoblast differentiation and osteocalcin is a mature osteoblast marker. Remarkable increases in Runx2-positive and osteocalcin-positive staining osteoblasts were observed on trabecular bone surface in the *Vhl* cKO mice at 12 months of age (Fig. 5C). H&E staining also displayed abundant osteoblast lineage cells at the trabecular bone surface in *Vhl* cKO mice (Fig. 5D).

VEGF is required for effective coupling between angiogenesis and osteogenesis. IHC for VEGF showed excessive staining in adult *Vhl* cKO bone at 4 months of age (Fig. 6A). Real-time PCR showed that loss of *Vhl* led to increased *Vegf* expression in chondrocytes (Fig. 6D). We also checked the expression of CD31, a marker for vascular endothelium, and found strongly increased microvascular density in the metaphyseal from *Vhl* cKO mice (Fig.

6B). Bone morphogenic protein (BMP) signaling plays important roles in osteoblast differentiation and bone formation and it has been reported that hypoxia enhances BMP-2 expression in osteoblasts.^(21,22) We have examined whether BMP signaling is involved in the *Vhl* deletion-induced bone phenotype. Increased expression of *Bmp-4* and *Bmp-7* were found in chondrocytes from *Vhl* cKO mice (Fig. 6D). Immunohistologically, p-Smad1/5/8, the canonical downstream mediators of BMP signaling, were also significantly increased in the osteoblast lineage cells in *Vhl* cKO mice 7 days after TM injection (Fig. 6C).

Discussion

In the current study, we provide genetic evidence for the first time that deletion of the *Vhl* gene in osteochondral progenitor cells at the adult stage dramatically increases cancellous bone characterized with enhanced angiogenesis and osteogenesis, and protects mice against aging-induced bone loss.

The phenotypes observed in our *Vhl* cKO mice with targeted deletion of *Vhl* in osteochondral progenitor cells at adult bone largely phenocopy mice lacking *Vhl* in mature osteoblasts, but are quite different from the severe dwarfism phenotype observed in mice lacking *Vhl* caused by the *Col2a1* promoter-driven Cre recombinase.^(8,11)

Surprisingly, inactivation of *Vhl* in osteochondral progenitor cells does not significantly affect calvarial bone morphology and the thickness of cortical bone at femoral mid-shaft. The reasons for the site-specific effect of *Vhl* cKO mice are not presently clear. We speculate that the Cre-expression pattern may be related to the negative phenotype of cortical bone in our *Vhl* cKO mice. Consistently, it has been reported that activation of the HIF α pathway in osteoblasts preferentially influenced modeling of the endochondral bones.⁽⁸⁾ In addition, the phenotype differences observed in *Col2-Cre; Vhl^{fl/fl}* and our *Vhl* cKO mice (*Col2-CreER^{T2}; Vhl^{fl/fl}*) could also be related to the fact that the *Vhl* gene was deleted at different stages; ie, developmental and adult stage. These genetic evidences suggest that the roles of *Vhl* in skeleton depend on specific cell types and developmental stages.

Hypoxia signaling stimulates VEGF production in bone, which then increases blood vessel growth into bone, bringing osteoprogenitor cells to the trabecular surfaces to form bone following proliferation, differentiation, and matrix synthesis.⁽²³⁾ As reported previously, osteoblast HIF α signaling, by using paracrine VEGF angiogenic signals, plays a central role in coupling angiogenic and osteogenic process during long-bone development.⁽⁸⁾ Increased expression of VEGF and CD31⁺ endothelial cells were also observed in our *Vhl* cKO mice at 12 months of age, which is 10 months after TM induction. We postulate that VEGF produced from osteochondral progenitor cells and their progeny, especially osteoblast lineage cells, plays a vital role in coupling of bone formation and skeletal angiogenesis in adult bone. Consistent with these findings, mice with VEGF overexpression in skeletal progenitors displayed extremely dense, heavily vascularized long bones.⁽⁹⁾ However, comparing to the aberrant bone marrow fibrosis in VEGF overexpression mice, no fibrosis was found in our *Vhl* cKO mice determined by reticulin staining, suggesting that some functions of *Vhl* in skeleton are independent from the VEGF signaling.

In contrast to the mice lacking *Vhl* in mature osteoblasts, we further found that induction of *Vhl* deletion in osteochondral progenitor cells led to increased proliferation and differentiation of osteoblast lineage cells in vivo. BrdU incorporation assay showed that increased proliferative response of osteoprogenitors and/or osteoblasts potentially contributed to the high bone mass phenotype observed in *Vhl* cKO mice. Higher expression of Runx2 and osteocalcin, a mature osteoblast marker gene, was observed in *Vhl* cKO mice even at 12 months, suggesting that osteoblast differentiation process was accelerated in the adult *Vhl* cKO bone. Our results suggest that, in addition to increased skeletal angiogenesis, enhanced osteoblast proliferation and differentiation also contribute to the increase in cancellous bone.

The mechanisms for the changes in osteoblasts are not presently clarified. The role of activation of BMP signaling in osteoblast differentiation and new bone formation is well recognized.⁽²²⁾ Phosphorylation of Smad1/5/8 was found to be increased in the metaphysis of *Vhl* cKO mice 1 week after TM induction, suggesting BMP signaling was activated after *Vhl* inactivation in osteoprogenitors. It has been reported that hypoxia induces BMP-2 expression in osteoblasts and hypoxia and VEGF upregulate BMP-2 production in microvascular endothelial cells.^(21,24) However, whether activation of BMP signaling in our *Vhl* cKO mice is the result of activation of HIF α /VEGF signaling needs further investigation. In addition, the activation of BMP signaling in stromal cells/osteoblasts may also contribute to the coupling of angiogenic and osteogenic processes. Ma and colleagues⁽²⁵⁾ reported that nicotine exposure suppressed bone healing with enhanced angiogenesis and inhibited BMP-2 expression, suggesting that reduced BMP signaling is associated with uncoupled angiogenesis and osteogenesis in nicotine-compromised bone healing. Thus, we speculate that activation of BMP signaling in osteoprogenitors observed in our *Vhl* cKO mice may also contribute to the osteosclerosis bone phenotype.

In this study we also found that inactivation of *Vhl* in osteochondral progenitors at the adult stage led to progressively increased trabecular bone volume in older mice, which for the first time indicates that loss of VHL in osteochondral progenitor cells protects mice from bone loss secondary to aging. Comparing to extensively studied estrogen deprivation-associated osteoporosis, mechanisms responsible for age-related osteoporosis are still poorly understood.⁽²⁶⁾ Previous studies suggest that there is a significant reduction in bone formation and bone mass with aging.⁽²⁷⁾ The transition from osteoblastogenesis to predominant adipogenesis in the bone marrow was considered as main pathogenesis for aging-related osteoporosis.⁽²⁸⁾ Recent study suggests that intracellular VEGF regulates the balance between osteogenic and adipogenic differentiation in mesenchymal stem cells and mice lacking *Vegf* in mesenchymal progenitor cells exhibit an osteoporosis-like phenotype characterized by reduced bone mass and increased bone marrow fat content.⁽²⁹⁾ In addition, decreased proliferative capacity of osteoprogenitor cells also contribute to age-related decrease in bone formation.⁽³⁰⁾ Moreover, a significant change in blood vessels was observed during the development of osteoporosis and reduced local blood supply to bone was also found in ovariectomy (OVX)-induced osteoporosis, highlighting a possible role of a microvascular defect in the pathogenesis of osteoporosis.^(5,31) In the current study, mice lacking *Vhl* in osteochondral progenitor cells display upregulated VEGF expression and

higher microvascular density, which may play a great role in preventing aging-induced bone loss. Together, we speculate that upregulated VEGF and BMP activity may coordinately contribute to the protective effect of *Vhl* cKO mice against aging-related bone loss.

In conclusion, by using an inducible deletion approach to avoid the potentially confounding effect owing to the developmental window, we here show that mice with targeted deletion of *Vhl* in osteochondral progenitor cells at the adult stage develop extremely dense, heavily vascularized long bones. More importantly, mice lacking *Vhl* in osteochondral progenitor cells at adult bone did not develop aging-related bone loss. Our results suggest that VHL/HIF in osteochondral progenitors plays an important role in the bone homeostasis at the adult stage by coupling angiogenesis and osteogenesis. VHL and HIF α may serve as novel therapeutic targets for drug development for the treatment of osteoporosis and other bone loss-associated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Inactivation of *Vhl* in osteochondroprogenitors in adult mice causes large amounts of cancellous bone in tibias and spine. (A) Representative 3D μ CT images of tibias from Cre-negative controls (*Vhl*-floxed mice) and *Vhl* cKO at 2 months old. Cre-negative control and *Vhl* cKO mice were treated with same amounts of TM. Mice were injected with TM daily for 5 continuous days at the age of 1 month and analyzed 1 month later. (B) Representative image of spine from 4-month-old Cre-negative control and *Vhl* cKO mice. Mice at the age of 2 months were injected with TM and analyzed 2 months later. X-ray (top) and μ CT (bottom) images showed increased bone mass in *Vhl* cKO mice. (C) Representative images stained with H&E in tibias and spine showed excessive trabecular bone in *Vhl* cKO mice 2 months after TM treatment. Original magnification: (top) $\times 40$; (bottom) $\times 20$. (D) Quantification of BV/TV and Tb.N in spine was determined by bone histomorphometry analysis. Boxed areas showed in C were analyzed with OsteoMeasure software. ** $p < 0.001$. BV/TV = trabecular bone volume; Tb.N = trabecular number; TM = tamoxifen.

Fig. 2.

Induced disruption of *Vhl* in osteochondral progenitor cells does not alter calvarial bone and cortical bone in femurs. Mice were injected daily with TM for 5 days at the age of 2 months and analyzed 2 months later. (A) Representative μ CT images of calvaria from 4-month-old Cre-negative control and *Vhl* cKO mice. The top is 3D reconstructed μ CT images of calvaria and the bottom is 2D μ CT images of calvaria. (B) 2D cortical bone images at the femoral mid-shaft from 4-month-old Cre-negative control and *Vhl* cKO mice. (C) Comparable cortical thickness in mid-femoral between Cre-negative control and *Vhl* cKO mice quantified by μ CT. (D) No changes in Ct.Ar/Tt.Ar in the mid-shaft of femurs from Cre-negative control and *Vhl* cKO mice were observed assessed by μ CT. Ct.Ar/Tt.Ar=cortical bone fraction; TM=tamoxifen.

Fig. 3.

Histological and histomorphometric analysis showed that enhanced bone formation and bone resorption in Cre-negative control and *Vhl* cKO mice at age of 4 months. Mice were treated with TM for 5 days at 2 months of age. (A) Von Kossa/toluidine blue-stained undecalcified sections in the distal femurs from Cre-negative control and *Vhl* cKO mice. Calcified bone was stained in black, and blue osteoblasts was surrounding the black trabeculae. Original magnification: (top) $\times 40$; (bottom) $\times 200$. (B) Disorganized double-calcein labeling was observed in the tibias from 4-month-old *Vhl* cKO mice. Original magnification: $\times 400$. (C) Representative images of TRAP staining in the proximal tibia from Cre-negative control and *Vhl* cKO mice. Original magnification: $\times 400$. (D) Quantification of structural and cellular parameters in the proximal tibias in Cre-negative control and *Vhl* cKO mice was determined by OsteoMeasure image-analysis software. (E) Real-time RT-PCR analysis showed that expression of osteoclast-related genes *Trap* and *Mmp-9* in long bones from *Vhl* cKO mice was higher than that in Cre-negative control mice at age of 4 months. BV/TV = bone volume/tissue volume; Tb.Sp = trabecular separation; Tb.N = trabecular number; Tb.Th = trabecular thickness; N.Ob/T.Ar = osteoblast number per tissue volume; N.Oc/T.Ar = osteoclast number per tissue volume; TM = tamoxifen.

Fig. 4.

Mice with *Vhl* deletion in osteochondroprogenitors in adult protected from aging-induced bone loss. (A) Representative μ CT 3D images in distal femoral trabeculae from Cre-negative control and *Vhl* cKO mice at age of 4 months and 12 months. Mice were injected with TM for 5 days at 2 months old. (B) Changes of trabecular morphology in the distal femora from Cre-negative control and *Vhl* cKO mice measured by μ CT at the age of 4 months and 12 months. * $p < 0.05$; ** $p < 0.001$. BV/TV = bone volume/tissue volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number; TM = tamoxifen.

Fig. 5.

Inactivation of *Vhl* in osteochondroprogenitors accelerated proliferation and differentiation on osteoblast lineage. (A) IHC for HIF-1α (left) and HIF-2α (right) in the distal femora from 4-month-old Cre-negative control and *Vhl* cKO mice 2 months after TM treatment. Original magnification, ×200. (B) IHC detecting incorporated BrdU in tibiae from Cre-negative control and *Vhl* cKO mice, which was at age of 1 month and after TM injection for 7 days. Original magnification, ×200. Quantification of BrdU showed higher proliferation of osteoblast lineage in the *Vhl* cKO mice compared with that in Cre-negative controls. * $p < 0.05$. (C) IHC for osteoblast differentiation markers Runx2 (left) and osteocalcin (right) in the tibiae from Cre-negative control and *Vhl* cKO mice at age of 12 months. Original magnification, ×400. (D) H&E staining exhibited increased osteoblast lineage cell around trabecular bone in *Vhl* cKO mice at 4 months of age after deletion of *Vhl* for 2 months. IHC = immunohistochemistry; TM = tamoxifen.

Fig. 6. Deletion of VHL in osteochondral progenitor cells enhances VEGF expression and activates BMP signaling. (A) Immunohistochemical analysis of VEGF level in tibial sections from 4-month-old *Vhl* cKO mice and Cre-negative controls 8 weeks after TM injection. Original magnification: $\times 400$. (B) IHC for CD31-positive endothelium in tibial sections from Cre-negative control and *Vhl* cKO mice at 4 months old. Original magnification: $\times 200$. (C) IHC analysis showed increased expression of p-smad1/5/8 in tibiae of *Vhl* cKO mice compared with that in Cre-negative control mice 7 days after TM induction at 1 month old. Magnification: (left) $\times 40$ and (right) $\times 400$. (D) Real-time RT-PCR mRNA expression analyses were performed for evaluating expression of *Vhl*, *Vegf*, *Bmp-2*, *Bmp-4*, and *Bmp-7* in chondrocytes isolated from knee joint of Cre-negative control and *Vhl* cKO mice at 5 days old after 4-OH-TM treatment for 48 hours and results were expressed as fold changes compared to Cre-negative controls. The real-time RT-PCR analysis was repeated three times. * $p < 0.05$. TM = tamoxifen; VEGF = vascular endothelial growth factor; BMP = bone morphogenic protein; IHC = immunohistochemistry.