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Introduction

The development of the mammalian skeleton takes place in distinct phases involving the initial migration of cells to the site of future bone, condensation of mesenchymal cells, and finally the differentiation of progenitors into chondrocytes and osteoblasts. During intramembranous bone formation, which gives rise to the flat bones of the skull, mesenchymal cells differentiate directly into bone-forming osteoblasts. By contrast, in endochondral bone formation, bones are formed through a 2-stage mechanism that begins with the formation of a chondrocyte anlage, onto which osteoblasts then differentiate and deposit bone. Endochondral bone formation occurs in close spatial and temporal association and proximity to capillary invasion, suggesting that angiogenesis and osteogenesis are coupled.

The initial signals for blood vessel invasion into bone are unknown, but tissue hypoxia is believed to be critical for commencement of the angiogenic cascade (1). Hypoxia triggers the changes in oxygen-regulated gene expression via the activation of the Per/Arnt/Sim (PAS) subfamily of basic helix-loop-helix (bHLH) transcription factors (2). The hypoxia-inducible factors (HIFs) activate genes encoding proteins that mediate adaptive responses (e.g., angiogenesis) to reduced oxygen availability (3). The HIF complex consists of 1 of 3 α subunits (HIF-1α, HIF-2α, or HIF-3α) bound to the aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIFβ. The level of HIF-1α and HIF-2α proteins is regulated by ongoing ubiquitination and proteasomal degradation following enzymatic prolyl hydroxylation on an oxygen-dependent degradation domain (ODD) (4). The E3 ligase von Hippel–Lindau protein (pVHL) binds directly to hydroxylated HIFα subunits and regulates their polyubiquitination and degradation by the proteasome (5). During hypoxia, prolyl hydroxylation is blocked, leading to HIFα stabilization, subsequent nuclear import, and dimerization with ARNT, which initiates the transcription of HIF-responsive genes (6). As indicated above, formation of endochondral bone coincides with capillary ingrowth and angiogenesis. Furthermore, disruption of normal afferent blood supply, which occurs following bone fracture, leads to hypoxia of adjacent tissue. Based on these observations, we reasoned that cells of mesenchymal origin, including osteoblasts, are ideally positioned in bone to sense and respond to fluctuations in oxygen and nutrient supply. Consistent with this concept, osteoblasts and osteocytes respond to hypoxia by elevating the level of HIFα, which in turn transactivates Vegf and other HIF target genes. We therefore hypothesize that osteoblasts use the HIFα pathway to sense reduced oxygen tension and transmit signals that impinge on angiogenic and osteogenic gene programs.
In this study, we used a genetic approach to determine the cellular and molecular effect of gain or loss of HIF function by conditional mutagenesis in osteoblasts during bone development. We show that constitutive activation of the HIFα pathway in mice promotes robust bone modeling and acquisition in long bones but not in the skull. This occurs through upregulation of Vegf and possibly other angiogenic factors primarily through cell- (osteoblast-) nonautonomous mechanisms. Conversely, loss of Hif1a in osteoblasts results in narrow, less vascularized bones. These results suggest that activation of the HIFα pathway in osteoblasts during bone development couples angiogenesis to osteogenesis.

Results

Primary osteoblasts express components of the HIFα pathway. Oxygen-sensitive cells use the HIFα pathway to sense and respond to changes in ambient oxygen. As a first step in studying the role of HIFs in osteoblasts, we determined the expression of components of this pathway in primary mouse osteoblasts. Osteoblasts expressed abundant pVHL and prolyl hydroxylase 1 and 3 (PHD1 and -3) (Figure 1A). Antibodies against mouse PHD2 were not available at the time of this study. As shown in Figure 1, B and C, exposure of osteoblasts to 2% O2 resulted in translocation of both HIF-1α and HIF-2α from the cytoplasm to the nucleus; nuclear translocation was more complete for HIF-1α than for HIF-2α. Exposure of osteoblasts to hypoxia was associated with upregulation of HIF target genes Vegf and type 1 glucose transporter (Glut1) mRNA expression (Figure 1, D and E). Therefore, osteoblasts possess the major components of the HIFα pathway.

Mice lacking Vhl in osteoblasts have upregulated HIFα. To investigate the function of HIFα in bone development in vivo, we created genetic mouse models engineered for manipulation of the levels of HIFα in osteoblasts. In the first model, we generated mice that overexpressed HIFα in osteoblasts by disrupting Vhl. Mice expressing the Cre recombinase driven by an osteocalcin (OC) promoter (OC-Cre) (7) were crossed with mice homozygous for the loxP-flanked (floxed) Vhl allele (hereafter designated as control) (8) to obtain mice lacking Vhl (hereafter designated ΔVhl) and therefore overexpressing HIFα in osteoblasts. To determine the specificity of the Vhl deletion in osteoblasts, we performed allele-specific PCR using DNA isolated from selected tissues. These results demonstrated that Cre-mediated recombination of the floxed Vhl allele (Δflo) occurred only in bone tissue (Figure 2A). Immunohistochemistry on fixed sections of distal femur showed upregulation of both HIF-1α and HIF-2α in osteoblasts lining bone surfaces of the ΔVhl mice (Figure 2B). Activation of the HIFα pathway in osteoblasts was also demonstrated in vitro following infection with adenoaviral Cre to delete the floxed Vhl allele. pVHL levels were eliminated in both the cytoplasm and nucleus, which was associated with increased expression of both HIF-1α and HIF-2α proteins in the nuclear fraction (Figure 2C).
were accompanied by increased expression of mRNA for the HIF target genes Vegf and Glut1 (Figure 2D). Expression of transcripts for all 3 Vegf isoforms (Vegf120, Vegf164, and Vegf188) was equally upregulated following Vhl deletion (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI31581DS1).

Loss of Vhl in osteoblasts increases long bone volume. ΔVhl mice were born at the expected mendelian ratios and were similar in size and weight to control littermates. μCT of long bones revealed striking and progressive increases in bone volume in mutant femurs compared with wild-type controls (Figure 3, A and B). To quantitatively assess bone turnover, static and dynamic histomorphometric analyses were performed on femurs from both ΔVhl mice and control littermates at 3 weeks of age, when bone modeling in the mouse is very active (7). Consistent with the μCT results, bone volume was significantly increased (P = 0.014; Figure 3C), whereas trabecular separation was greatly reduced (P = 0.008; Figure 3D) in ΔVhl mice. However, at this time, neither the number of osteoblasts (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI31581DS1) nor the rate of bone formation (Figure 3E) was significantly increased in the 7-day-old mice compared with controls (Figure 3F). In addition, osteoblast labeling showed an accelerated bone formation rate in the mutant from 7-day-old mice within the first week of life but appears to decline at latter stages of postnatal development.

Figure 2
Osteoblast-specific, Cre-mediated deletion of Vhl. (A) PCR analysis of Cre-mediated recombination in selected tissues from a ΔVhl mouse. The recombined allele (Δflox) was present exclusively in bone tissue. (B) Representative histological sections of distal femurs from 6-week-old control and ΔVhl mice after staining with antibodies against pVHL (left), HIF-1α (middle), or HIF-2α (right) as described in Methods. Sections were counterstained with hematoxylin. Red arrows indicate positive staining and black arrows negative staining in osteoblasts. Original magnification, ×400. (C and D) Confluent monolayers of Vhl floxed primary osteoblasts were infected with either Ad-GFP or Ad-CreM1 (100 MOI) for 48 hours. (C) Proteins in the cytoplasm and nucleus were extracted separately and analyzed by immunoblotting with antibodies against pVHL, HIF-1α, and HIF-2α. Immunoblots for TBP and α-tubulin were used as loading controls for nuclear and cytoplasmic proteins, respectively. TBP, TATA box–binding protein. (D) Total mRNA was extracted from confluent monolayers of osteoblasts 48 hours after adenoviral infection, and gene expression for Vhl, Vegf, and Glut1 was determined by quantitative real-time PCR. White bars represent Ad-GFP infection; black bars represent Ad-CreM1 infection. *P < 0.05; **P < 0.01.

The increased accumulation of bone seen in the mutant animals could theoretically be due to defective bone resorption, which would be expected to be associated with decreased number and activity of osteoclasts. However, the number of osteoclasts expressed either as number per bone surface or number per total tissue area was not significantly different from that of controls at 3 weeks (Supplemental Figure 3, A and B). Moreover, the serum level of osteoprotegerin (OPG), an osteoclast inhibitory cytokine, was similarly unaffected in the mutants (Supplemental Figure 3C). Therefore, these data suggest that the increased bone volume in the Vhl mutants resulted primarily from increased numbers and activity of osteoclasts at an early age. Indeed, serum levels of osteoclast-derived tartrate-resistant acid phosphatase form 5b (TRAP 5b) were modestly decreased in 6-week-old ΔVhl mice (Supplemental Figure 3D). These results suggest that bone turnover declines in
the mature ΔVhl mice, accounting at least in part for the progressive increase in postnatal bone acquisition.

Overexpression of HIFα in osteoblasts does not affect calvarial bone formation. As indicated above, the mechanisms responsible for formation of the flat bones of the skull (intramembranous ossification) are different than those that form the long bones (endochondral ossification). Surprisingly, mice lacking Vhl had no detectable changes in calvarial bone morphology. Gross μCT and histological analysis showed that cranial bone was similar in control and ΔVhl mice (Figure 4A). Quantitative histomorphometric analysis showed that bone volume (Figure 4B) and osteoblast numbers (Figure 4C) in the ΔVhl mice were not significantly different from those in controls. The lack of effect of the Vhl mutation on calvarial bone was not due to incomplete excision of the Vhl gene in this tissue (Figure 2A). Therefore, these results suggest that overexpression of HIFα in mouse osteoblasts appears to preferentially promote the acquisition of bone formed by endochondral processes.

Increased long bone volume in ΔVhl mice is associated with increased angiogenesis and Vegf production. At necropsy, we noted that the long bones from the Vhl mutants were more richly perfused with blood compared with control bones (Figure 5A), suggesting that deletion of Vhl may affect angiogenesis during bone development. To directly assess the impact of the Vhl mutation on bone vasculature we performed contrast-enhanced μCT imaging. μCT imaging of vasculature in Microfil-perfused bones revealed a striking increase in the density of the vasculature in ΔVhl as early as 7 days of age, with progressively increased vessel numbers with age (Figure 5B). Quantitative analysis showed that vessel surface and volume were both increased in ΔVhl relative to controls (Figure 5, C and D). These observations suggest that the loss of Vhl with consequent upregulation of HIFα in osteoblasts increases the production of angiogenic factors that promote formation of the bone vasculature. Consistent with this idea, the expression of 2 HIF target genes, Vegf(Figure 5E) and phosphoglycerate kinase (Pgk) (Supplemental Figure 4A), was upregulated in trabecular bone of ΔVhl femurs. The serum level of VEGF was not elevated in ΔVhl mice, as determined by ELISA (Supplemental Figure 4B), indicating that HIFα-mediated upregulation of VEGF only occurred locally in bone.

To further investigate the role of VEGF in promoting angiogenesis in the ΔVhl mice, we performed an angiogenesis assay using explants of E17.5 mouse metatarsals. Control metatarsals exhibited a small degree of endothelial sprouting (Figure 5F), which was greatly enhanced by treatment with a recombinant VEGF (Figure 5G). By contrast, endothelial sprouting from ΔVhl metatarsals was...
much greater than that from controls and was virtually abolished by preincubation with a VEGF-neutralizing antibody (Figure 5I), but not with a control IgG (Figure 5H). This finding suggests that VEGF is upregulated in the long bones of the ΔVhl mice and contributes to increased angiogenesis in bone tissue.

Upregulation of HIFα in osteoblasts independent of angiogenesis does not alter osteoblast proliferation and apoptosis. The results described above suggested that increased long bone volume in ΔVhl mice occurred in part through cell-autonomous effects on angiogenesis. To investigate possible cell- (osteoblast-) autonomous effects of HIFα overexpression on the osteoblast performance independent of angiogenesis, we determined the effect of HIFα upregulation on osteoblast proliferation, survival, and differentiation following disruption of Vhl with adenovirally driven Cre. Deletion of Vhl in this manner resulted in the desired upregulation of HIF-1α and HIF-2α (Figure 2C) but had no significant effect on proliferation and apoptosis as assessed using flow cytometry of BrdU- (Figure 6, A and B) and annexin V–stained cells (Figure 6, C and D). We next determined the effect of HIFα upregulation on osteoblast differentiation by growing cells in ascorbate-containing medium and staining for alkaline phosphatase (ALP) and von Kossa to assess bone mineral deposition. Disruption of Vhl had no appreciable effect on ALP expression and only slightly increased calcified nodule formation relative to that of control cells infected with a GFP virus (Figure 6, E–G). Moreover, the expression of runt-related transcription factor 2 (Runx2) and OC, markers for early and late osteoblast differentiation, respectively, was not significantly altered in the Vhl-deficient cells (Figure 6H). These results suggest that loss of Vhl and upregulation of HIFα in osteoblasts have minimal effects on in vitro osteoblast performance and further suggest that overexpression of HIFα in osteoblasts in vivo promotes bone formation primarily by cell-autonomous effects on angiogenesis.

Inactivation of Hif1α in osteoblasts decreases bone volume and vascularity. To definitively establish the role of HIFs in osteoblasts, we created a second mouse model lacking Hif1α in osteoblasts (ΔHif1α) by crossing the OC-Cre mouse (7) with a mouse carrying a conditional, floxed Hif1α allele (control) (9). Mice lacking Hif1α in osteoblasts were viable and developed normally. Allele-specific PCR analysis on tissues from these animals showed that recombination of the floxed Hif1α allele (Δfloxed) occurred exclusively in bone tissue (Figure 7A). Immunohistochemistry confirmed the loss of HIF-1α protein and increased HIF-2α expression in osteoblasts from ΔHif1α mice (Figure 7B). μCT images at the mid-shaft of the femur showed that the diameter of the mutant bones was reduced relative to that of controls (Figure 7C). Histomorphometric analysis at this site revealed a significant reduction in the osteoid volume along with the reduced number of osteoblasts in the mutant bones (Supplemental Figure 5, A and B). μCT analysis of Microfil-perfused bones from 3-week-old mice showed a reduction in vascular density in ΔHif1α mice compared with controls (Figure 7D). Thus, loss of Hif1α in osteoblasts appears to result in thinner, less vascularized long bones and produces a reverse bone phenotype to that of the ΔVhl mice. Even so, the reduction in bone and blood vessel volume in these animals was less pronounced than we anticipated and suggested that other factors may function redundantly with HIF-1α in bone tissue. Since HIF-2α and HIF-1α are known to exert overlapping functions in a number of tissues, we investigated the effect of deletion of Hif1α on HIF-2α expression using primary mouse osteoblasts in vitro. Introduction of Cre-expressing adenovirus into Hif1α floxed primary osteoblasts knocked down the levels of HIF-1α and increased HIF-2α protein levels (Figure 7E). These changes were associated with upregulation of Hif2α and Vegf mRNA levels (Figure 7F). Based on these results, we conclude that deletion of Hif1α in osteoblasts leads to compensatory increases in expression of HIF-2α, which can partially substitute for the loss of HIF-1α function.

To further investigate the role of HIF-1α in postnatal bone formation, we created a third mouse model lacking both Vhl and Hif1α in osteoblasts (ΔVhl/ΔHif1α) by crossbreeding the AVhl and ΔHif1α mice. If HIF-1α functions independently of HIF-2α or other factors to promote angiogenesis and osteogenesis, then mice lacking both Vhl and Hif1α should exhibit a bone phenotype similar to that of the ΔHif1α mice. However, 6-week-old double-mutant mice had significantly increased cortical (Figure 8A) and trabecular bone volume (data not shown) relative to control littermates. The bone volume in the double mutants remained lower than that seen in the ΔVhl single knockout mice (compare Figure 8A with Figure 3B). By contrast, vascular densities were greatly increased in the double-mutant bones and were similar to those seen in the ΔVhl mice (Figure 8B). This result suggested that the lack of both Vhl and Hif1α caused upregulation of HIF-2α abundance and function. In agreement with this idea, osteoblasts lining trabecular surfaces of the double mutants had increased HIF-2α immunoreactivity compared with controls (Figure 8C) and...
elevated Vegf mRNA expression (Figure 8D). Taken together, these results suggest that in the absence of HIF-1α, HIF-2α functions like HIF-1α to enhance VEGF production and drive angiogenesis.

Discussion
In this article, we present genetic evidence that the HIFα pathway is critical for coupling angiogenesis to osteogenesis during long bone formation. Activation of HIFα in osteoblasts through disruption of its degradation pathway produced robust bone modeling early in development. We attribute the increased bone formation to the increased angiogenesis, which appears to be mediated primarily by VEGF produced by the HIFα-overexpressing osteoblasts. Conversely, disruption of Hif1α in the osteoblast produced the reverse phenotype, i.e., thinner and less vascularized bones, clearly demonstrating the importance of the HIFα pathway in osteoblast-driven bone formation.

The development of the skeletal elements occurs in close association with and proximity to capillary ingrowth. The importance of a vascular supply in skeletogenesis was first demonstrated experimentally by Trueta and colleagues (10, 11), who demonstrated that bone mineralization and the expansion of the hypertrophic zone in the growth plate were decreased following the interruption of the blood supply to the growth plate. More contemporary studies by Gerber and Ferrara showed that impairment of angiogenesis decreased trabecular bone formation as well as the expansion of the hypertrophic zone into the growth plate (12). Moreover, it is known that the rate of bone formation and blood flow in the

Figure 5
Increased angiogenesis in long bones of ΔVhl mice. (A) Photograph of hind limbs from ΔVhl and control mice. (B) Representative μCT images of vasculature in Microfil-perfused femurs from 7-day-old and 3-week-old ΔVhl and control mice. Scale bar: 1.0 mm. (C and D) Morphological analysis of vessel surface and volume within femurs from Microfil-perfused ΔVhl (black bars; n = 3) and control (white bars; n = 3) mice. Data represent mean ± SEM. **P < 0.01. (E) In situ hybridization analysis with Vegf mRNA on histological sections from 3-day-old control and Vhl mutant femurs. Original magnification, ×40. (F–I) In vitro angiogenesis assay. Metatarsals were dissected from control and ΔVhl E17.5 fetuses and cultured in α-MEM for 14 days. The assay was performed using anti-CD31 antibody as described in Methods. Representative images are shown. Original magnification, ×25. (F) Little detectable endothelial sprouting from control metatarsal. (G) Massive endothelial sprouting from ΔVhl metatarsal remained intact after treatment with a mouse control IgG (100 ng/ml). (H) Extensive endothelial sprouting from ΔVhl metatarsal remained intact after treatment with a mouse control IgG (100 ng/ml). (I) Specific inhibition of endothelial sprouting in ΔVhl metatarsal using a VEGF-neutralizing antibody (100 ng/ml). Data are representative of 3 independent experiments.
The mature animal are tightly coupled (13). In this regard, the circulation is believed to be the major source for osteoclast precursors and the delivery of growth factors to individual remodeling units. Hauge et al. have suggested the existence of a discrete anatomical structure that they termed the bone remodeling compartment, which would serve to regulate bone remodeling (14, 15). Cells lining this structure express mediators of osteoclastogenesis including OPG and receptor activator of NF-κB ligand (RANKL), consistent with a role in coupling bone formation and resorption (15). Together, these observations support the existence of anatomical and molecular mechanisms (e.g., HIFα) that coordinate angiogenesis and osteogenesis.

The results presented herein demonstrate that activation of the HIFα pathway preferentially influenced modeling of the endochondral bones. As reviewed above, endochondral bones form through a process whereby cells from the paraxial mesoderm and lateral plate mesoderm differentiate into chondrocytes to form an avascular cartilage anlage. This template is subsequently vascularized and then converted into bone. During this time, angiogenesis is essential for the normal differentiation of chondrocytes and appropriate alignment of the chondrocytes in the growth plate. Indeed, previous studies by Schipani et al. have shown that the HIFα pathway is required for the normal endochondral growth plate development. In their model, chondrocytes lacking Hif1a in the center of both the proliferative and the upper hypertrophic zones underwent programmed cell death, which caused disorganization of chondrocyte palisades and led to the misshaped growth plates (9). However, in contrast to the findings of the present study, constitutive activation of HIF-1α in chondrocytes through disruption of Vhl had no apparent effect on angiogenesis but instead produced cell-autonomous effects that impacted survival and function of chondrocytes (16).

A striking observation in the current studies was that manipulation of the HIFα levels in osteoblasts did not influence the formation of the flat bones of the skull. These bones and those that compose the clavicles are formed though an intramembranous process involving condensing mesenchymal stem cells that derive from the neural crest and are already resident in the developing skull. These precursor cells differentiate directly into osteoblasts apparently without a requirement for angiogenesis (17). This distinct difference in the development programs and embryological origin of the bone precursor cells responsible for endochondral and intramembranous bone is the most likely explanation for the site-specific skeletal phenotype of the Vhl-deficient mice.
Our studies provide additional insight into the functional relationship between HIF-1α and HIF-2α. Osteoblast-specific deletion of *Hif1a* produced undervascularized, thinner bones, whereas overexpression of both HIF-1α and HIF-2α by loss of *Vhl* yielded dramatically thicker, heavily vascularized bones. However, overexpressing only HIF-2α in the osteoblasts by deleting both *Hif1a* and *Vhl* phenocopied the vascular effects seen in the *Vhl* mutants but did not produce the same amount of bone. This suggests some degree of specificity for different HIFα proteins in these 2 tissues; i.e., it is possible that HIF-1α and HIF-2α act redundantly to promote angiogenesis, but they act differently with respect to inducing osteogenesis per se. Though HIF-1α and HIF-2α are homologous and both contain the conserved ODD domain (18), several previous studies suggest that HIF-1α and HIF-2α are not entirely redundant. For example, there are distinct differences in the expression levels of HIF-1α and HIF-2α in different cell types (19, 20). In addition, mice globally deficient in either *Hif1a* or *Hif2a* die at different times during development, further suggesting that HIF-1α and HIF-2α have distinct functions in development (21–23). Moreover, the sets of genes regulated by these transcription factors, while overlapping, are not identical. For example, HIF-1α appears to be more important than HIF-2α with regard to regulation of hypoxia-inducible glycolytic genes such as *Pgk1*, whereas HIF-2α is a predominant regulator of hypoxic *Vegf* and erythropoietin (*Epo*) (24–26). Taken together, the data suggest that HIF-1α and HIF-2α have evolved to perform distinct functions in selected tissues but in certain contexts can substitute for one another.
of hypoxia-inducible genes in vivo (16, 27–30). Furthermore, a recent study using conditional expression of HIF-1α and HIF-2α variants that cannot be modified by prolyl hydroxylation showed that simultaneous expression of these stable HIFα molecules in liver and skin caused pathological changes that were indistinguishable from those seen when Vhl was deleted. In addition, activation of HIF-2α (with or without HIF-1α activation) induced expression of a set of genes that were also upregulated upon loss of Vhl, suggesting that dysregulation of HIF target genes is primarily responsible for the pathological changes seen after Vhl inactivation in skin and liver (31). Therefore, even though pVHL is capable of binding other cellular proteins and possibly exerts functions separate from its role as an HIFα E3 ligase (32, 33), it appears that its primary role is to control cellular levels of HIFα. Consequently, we attribute the skeletal effects seen in the Vhl mutant mice described in this article to increased HIFα activity rather than to HIF-independent phenomena.

Finally, our results enable the construction of a model for the role of the HIFα pathway in bone formation. In this model, osteoblasts residing on the nascent bone surface sense reduced oxygen or nutrient levels and upregulate HIFα subunits. Elevated HIFα transactivates target genes such as Vegf, which then stimulate new blood vessel formation and invasion into bone. This process is exponential, with ever-increasing numbers of new blood vessels introducing more osteoblast progenitors, which then mature and function to form more individual bone formation units. We speculate that the initial wave of osteogenesis results from signals emanating from the circulation, although it is also possible that osteoblast progenitors could themselves arrive from circulation (34, 35).

In this regard, a number of osteoblast-lineage cells that express bone-specific proteins are known to be present in the human circulation, especially during the adolescent growth spurt (36). On the other hand, it is also possible that VEGF might act in an autocrine mode to indirectly stimulate osteoblast differentiation, thereby accelerating bone mineralization (37–39). Regardless of the precise cellular mechanisms, our study clearly shows that HIF-dependent angiogenesis is a primary stimulus for osteoblast-mediated osteogenesis early during endochondral bone formation.

**Figure 8**
Bone and vascular phenotype in Vhl and Hif1a double-knockout mice. (A) Representative μCT images of femoral cross sections from 6-week-old ΔVhl/ΔHif1a double mutant mice and wild-type controls. Scale bars: 1.0 mm. (B) Representative images of Microfil-perfused femurs from 3-week-old ΔVhl/ΔHif1a double-mutant mice and control litters. Scale bar: 1.0 mm. (C) Immunohistochemical analysis of HIF-2α level in femoral sections from 6-week-old ΔVhl/ΔHif1a double-mutant mice and control litters. Sections were counterstained with hematoxylin. Red arrows indicate positive and black arrows negative staining in osteoblasts. Original magnification, ×400. (D) In situ hybridization analysis with Vegf mRNA on histological sections from 6-week-old control and double-mutant femurs. Original magnification, ×100.
inferior vena cava was severed. The vasculature was flushed with 0.9% normal saline containing heparin sodium (100 U/ml) at a pressure of approximately 100 mmHg via a needle inserted into the left ventricle. The specimens were then pressure fixed with 10% neutral buffered formalin. Formalin was flushed from the vessels using heparinized saline, and the vasculature was injected with a radiopaque silicone rubber compound containing lead chromate (Microfil MV-122; Flow Tech). Samples were stored at 4°C overnight for contrast-agent polymerization. Mouse femurs were dissected from the specimens and soaked for 4 days in 10% neutral buffered formalin to ensure complete tissue fixation. Tissues were subsequently treated for 48 hours in a formic acid–based solution, Ca-Ex II (Fischer Scientific), to decalcify the bone and facilitate image thresholding of the femoral vasculature from the surrounding tissues. Images were obtained using a high-resolution (16-μm isotropic voxel size) μCT imaging system (μCT40; SCANCO Medical). A threshold of 306 was initially chosen based on visual interpretation of thresholded 2D tomograms. Histomorphometric parameters including vessel volume, connectivity, number, thickness, separation, and degree of anisotropy were evaluated.

**Fetal mouse metatarsal angiogenesis assay.** This assay was performed following the established method as discussed previously (41). Briefly, E17.5 embryos were removed from timed-pregnant mice, and metatarsals were dissected. The isolated metatarsals were cultured in 24-well tissue culture plates in 150 μl of α-MEM supplemented with 10% heat-inactivated FBS and 1% of penicillin/streptomycin for 72 hours. Two hundred and fifty microliters of fresh medium were then replaced, and metatarsals were cultured for 14 days, with replacement of medium every 3 days. Explants were then fixed in zinc formalin for 15 minutes at room temperature and subsequently stained for CD31 using a rat polyclonal antiseraum against mouse CD31 (BD Biosciences — Pharmingen). Cultures were performed in sextuplicate, and each complete experiment was repeated at least twice.

**Primary osteoblast isolation and culture, hypoxic exposure, and adenosine infection.** Osteoblasts were isolated from calvaria of newborn mice by serial digestion in 1.8 mg/ml of collagenase type I (Worthington Biochemical Corp.) solution. Calvaria were digested in 10 ml of digestion solution for 15 minutes at 37°C under constant agitation. The digestion solution was then collected, and the digestion was repeated an additional 4 times. Digestion solutions 3–5, which contained the osteoblasts, were pooled together. After centrifugation, osteoblasts were obtained and cultured in α-MEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator supplied with 5% CO2. To examine the effect of experimental hypoxia, cells were cultured under hypoxic conditions (2% oxygen) at different times during culture or maintained under normoxic conditions (21% oxygen). All endpoints measured in hypoxic cells were compared with those in cells kept under normoxic conditions. Monolayer osteoblasts were infected with control adenovirus (Ad-GFP) or Cre recombinase virus M1 (Ad-CreM1) (Vector Laboratories) at an MOI of 100 for most experiments. Osteoblasts were harvested after 48 hours. Total mRNA was extracted from infected osteoblasts for deletion validation by real-time PCR, and proteins were extracted for immunoblotting. The remaining cells were replated on 100-mm tissue culture plates and subjected to proliferation and apoptosis assays or on 6-well plates for the mineralization assay.

**Osteoblast proliferation and apoptosis in vitro.** Mouse primary osteoblasts were plated on 100-mm plates with the density of 105 cells/plate, and cultured in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin for 24 hours. Cells were then starved in α-MEM with 1% FBS for 12 hours, followed by the replacement with 10% FBS in α-MEM and cultured for an additional 12 hours. For the proliferation assay, BrdU (10 μM) was added to the culture medium 2 hours before harvesting. Cells were stained with fluorescent anti-BrdU antibody and 7-amino-actinomycin D following the manufacturer’s instructions (BD Biosciences). For the apoptosis assay, annexin V–PE (BD Biosciences) and 7-AAD were added for staining. Osteoblasts were analyzed by FACScalibur (BD) and 20,000 events were collected. ALP and von Kossa staining. Osteoblasts were plated on 6-well plates with a density of 104 cells/well and cultured in α-MEM until they were confluent. Medium was then changed to osteogenic medium with the addition of β-glycerophosphate (10 mM) and ascorbic acid (50 μg/ml) to α-MEM. Osteoblasts were then cultured for 14 days, with the medium changed every 3 days. Histochemo staining for ALP activity in the cells was determined using naphthol AS-MX phosphate (Sigma-Aldrich) as a substrate and fast red TR salt as a coupler. Von Kossa staining was carried out by adding 3% silver nitrate solution to formalin-fixed cells and exposing cells to UV light. The deposits of calcium were demonstrated by the formation of black nodules. Densitometric analysis of ALP and von Kossa staining was performed using NIH ImageJ 1.36b (http://rsb.info.nih.gov/ij/).

**Quantitative real-time PCR.** Total RNA was extracted from osteoblasts using the TRIzol method, as recommended by the manufacturer (Invitrogen). The yield and purity of RNA was estimated spectrophotometrically using the A260/A280 ratio. Three micrograms of RNA were reverse transcribed into cDNA using SuperScript first-strand synthesis system (Invitrogen). One microliter of cDNA was subjected to PCR amplification using SYBR GREEN PCR Master Mix (Applied Biosystems) and sequence-specific primers (VH1, 5′-GCTATTTTGCGCACCATA-CACA-3′ and 5′-CATCTTCTCTATGTGCTGGCTTT-3′; Hif1α, 5′-CAAGATCTC- GGCGAAGCAA-3′ and 5′-GGTGAAGCTCATAACAGAAAGCTTT-3′; Hif2α, 5′-CAACCTGACGCTAGTGATC-3′ and 5′-CACCACGTCC- GTTCTTCTCGGAT-3′; Vegf120, 5′-GGCGGATCAACACT- CACAAA-3′ and 5′-CAGGCTGCTTGTCACATTTCCTTTCTT-3′; Vegf164, 5′-AAAGGACAAAAAGCCAGAAACAC-3′ and 5′-GTGTTAATCATAGCTGCCGTCGCTC-3′; Vegf188, 5′-GCGGATCAACACTCACA AAA-3′ and 5′-GAAAGGTCCTAGTGAGGACG-3′; Glut1, 5′-GAGGGCCTCGTGTCCATGAT-3′ and 5′-ACGAGCAAGCCAGAAGAGCC-3′ and 5′-CATCTGCTCCTCGTCACAG-3′; Oc, 5′-TCAGGCTTCTCTGCTGAC-3′ and 5′-GGACGCTGCTGACATCAGGCC-3′; β-actin, 5′-CCCCAGCCAAGAGAGG-3′ and 5′-GTCGAGACCAGGAGT-3′). PCR reactions were performed in triplicate for each cDNA, averaged, and normalized to endogenous β-actin reference transcripts.

**Immunoblotting analysis.** Whole cell lysate was obtained by homogenizing cell monolayers with cell lysis buffer in the presence of a protease inhibitor cocktail (Roche). Proteins in the cytoplasm and nucleus were separated using NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer’s instructions (Pierce). Thirty micrograms of protein extracts were loaded onto an SDS mini-PAGE system after concentrations were determined by the Bradford method. After electrophoresis, proteins were transferred to a PVDF membrane using a Bio-Rad semi-dry transfer system. Protein transfer efficiency and size determination were verified using prestained protein standards. Immunblots were incubated overnight at 4°C with anti–HIF-1α (NB100-105; Novus Biologicals), anti–HIF-2α (NB100-122; Novus Biologicals), or anti-tubulin (Santa Cruz Biotechnology Inc.) primary antibodies, and washed with 0.1% Tween-20 for 1 hour at room temperature and subsequently incubated overnight with primary antibodies at 4°C. Signals were detected using an HRP-conjugated secondary antibody and the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Antibodies used were anti-pvHL (BD Biosciences — Pharmingen), anti–HIF-1α (NB100-105; Novus Biologicals), anti–HIF-2α (NB100-122; Novus Biologicals), anti–TATA box–binding protein (anti-TBP) (Abcam), and anti-tubulin (Santa Cruz Biotechnology Inc.).

**Immunofluorescence and confocal microscopy.** Immunofluorescence assays were performed as previously described (42). Briefly, primary osteoblasts were plated on lysine-treated glass coverslips placed in 6-well plates. After culturing for 24 hours under either normoxic or hypoxic conditions, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.2% Triton X-100 for 2 minutes, and incubated overnight at 4°C with anti–HIF-1α (NB100-105; Novus Biologicals) or anti–HIF-2α (NB100-122; Novus Biologicals) antibodies at a dilution of 1:100.
After washing, coverslips were incubated with Alexa Fluor 488-labeled goat anti-mouse or anti-rabbit IgG (H+L) secondary antibody (Molecular Probes; Invitrogen) for 1 hour and mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories). Fluorescence localization was detected by confocal microscopy with a laser scanning microscope (LSM 510; Zeiss). Images were obtained with the same confocal settings for each set of experiments and no autofluorescence was detected at these settings. Controls in which primary or secondary antibodies were omitted showed no staining. Images were processed using Adobe Photoshop 7.0 software.

ELISA. VEGF, OC, OPG, and TRAP 5b in mouse sera were assayed using an ELISA according to manufacturer’s recommendations. ELISA kits used were mouse VEGF Quantikine ELISA kit (R&D Systems), mouse OC ELISA kit (Biomedical Technologies Inc.), mouse OPG assay kit (Biomedica), and mouse TRAP assay kit (IDS). Optical density was determined using a plate reader (BioTek).

Statistics. For statistical analysis and comparison of homotypometric parameters in control and mutant mice, Mann-Whitney U test was performed. For statistical analysis of gene expression data, ELISA data, and data collected by flow cytometry, pairwise 2-tailed Student’s t test was performed. Error bars represent SEM. Statistical significance was defined as P < 0.05.

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