THE ROLE OF THE HYPOXIA-INDUCIBLE FACTOR PATHWAY IN BONE DEVELOPMENT AND REPAIR

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA

2007
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ABSTRACT

Osteogenesis and angiogenesis are tightly coupled during bone formation and repair. Blood vessels not only carry oxygen and nutrients to the developing bone, but also play an active role in bone formation and remodeling by mediating the interaction between osteoblasts, osteocytes, osteoclasts and vascular cells at a variety of levels. Tissue hypoxia is believed to be a major stimulus for angiogenesis. Hypoxia activates the hypoxia-inducible factor (HIF) pathway and triggers hypoxia-responsive gene expression such as vascular endothelial growth factor (VEGF), which plays a critical role in angiogenesis, endochondral bone formation and bone repair following fracture.

In this dissertation, I investigated the mechanisms through which osteoblasts use the HIF pathway to sense reduced oxygen tension and transmit signals that impinge on angiogenic and osteogenic gene programs during bone formation. Using a genetic approach, I have demonstrated that overexpression of HIF in mouse osteoblasts through disruption of the von Hippel-Hindau (pVHL)-mediated degradation pathway results in profound increases in angiogenesis and osteogenesis which appear to be mediated by cell nonautonomous mechanisms involving VEGF. Based on these results, the role of the HIF pathway during bone repair was also investigated by using a murine model of distraction osteogenesis in which repair is accomplished through osteoblast-mediated new bone formation. Mice with overexpressed HIF, through either deletion of Vhl gene or treatment of prolyl hydroxylase inhibitors, showed a markedly increased vascularity and
accelerated bone regeneration in response to distraction osteogenesis. The increased bone regeneration in \textit{Vhl} mutant mice was eliminated by concomitant infusion of VEGF receptor antibodies, suggesting that VEGF is required for bone regeneration. These findings suggest that the HIF/VEGF pathway also plays an important role in coupling angiogenesis and bone formation during repair. The precise mechanism for angiogenic-osteogenic coupling during skeletal development, as to what extent VEGF functions independent of endothelial cells, is currently under investigation.
ACKNOWLEDGEMENTS

Graduation is a time to thank the people who helped me through the last four years. I would like to express my most sincere gratitude to my mentor, Dr. Thomas Clemens, for his agreeing to supervise me, continuous guidance and encouragement. Our discussions provided an ocean of ideas that continually found me in the depths of bone biology, sink or swim. I have learned various things from him, such as the way of critical thinking, proceeding research, presenting in national meetings, and having a sense of humor. Thanks for believing in me, Dr. Clemens.

I wish to thank the members of my thesis committee, Dr. Stephen Barnes, Dr. Xu Cao, Dr. Stuart Frank and Dr. Rosa Serra, for their guidance and help throughout my thesis work. They instilled in me not only the knowledge, but the essence of being a scientist: seriousness, honesty, creativity and self-confidence. These are treasures that will remain intact my whole life. I am also grateful to Dr. Stephen Barnes, for chairing my committee meeting and dissertation defense.

The help from people in Dr. Clemens laboratory contributed greatly to my research. They saw me through all the joys and frustrations of this research, while helping me stay focused on the present. I cannot thank them more. Thank you, Xuemei, Chao, Judy, Doug, Keertik, and Mahendra, for your excellent work, wonderful suggestion and friendship.

My graduate studies were also accelerated by the help of Pharmacology and Toxicology staff, and several Pathology faculty and staff. Thank you for your support.
I dedicate this thesis to my family — my parents, my husband Lidong and my lovely daughter Rachel. In the last four years I have experienced a tremendous change in my life. Their endless love and unwavering support encouraged me to face challenges bravely, to overcome obstacles successfully, to believe in myself, and to always try my best. With them, my dream has come true.
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<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>Ad-GFP</td>
<td>adnoviral green fluorescent protein</td>
</tr>
<tr>
<td>Ad-CreM1</td>
<td>adnoviral CreM1</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DFO</td>
<td>desferrioxamine</td>
</tr>
<tr>
<td>DO</td>
<td>distraction osteogenesis</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>epo</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Glut-1</td>
<td>type 1 glucose transporter</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia-responsive element</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>L-mim</td>
<td>L-mimosine</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum Essential Medium alpha</td>
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</table>
MSCs  mesenchymal stromal cells

O₂  oxygen

OC  osteocalcin

ODD  oxygen-dependent degradation domain

OPG  osteoprotegerin

PAS  Per/Arnt/Sim

PCR  polymerase chain reaction

Pgk  phosphoglycerate kinase

PHD  prolyl hydroxylase

pVHL  von Hippel-Lindau protein

RANKL  receptor activator of NF-κB ligand

rhVEGF  recombinant human vascular endothelial growth factor

Runx2  runt-related transcription factor 2

TBP  TATA box-binding protein

TRAP 5b  tartrate-resistant acid phosphatase form 5b

VEGF  vascular endothelial growth factor

VEGFR1  vascular endothelial growth factor receptor 1

Vhl  von Hippel-Lindau

ΔHif1α mice  mice with conditional deletion of Hif1α in osteoblasts

ΔVhl mice  mice with conditional deletion of Vhl in osteoblasts

ΔVhl/ΔHif1α mice  mice with conditional deletion of Vhl and Hif1α in osteoblasts
INTRODUCTION

Osteogenesis and angiogenesis are tightly coupled during skeletal development and repair. Blood vessels not only carry oxygen and nutrients to the developing bone, but also play an active role in bone formation and remodeling by mediating the interaction between osteoblasts, osteocytes, osteoclasts and vascular cells at a variety of levels. Tissue hypoxia is believed to be a major stimulus for angiogenesis. Hypoxia activates the hypoxia-inducible factor (HIF) pathway, which is a central regulator of hypoxia-adaptation in vertebrates. HIF remains inactive under normoxic conditions through the von Hippel-Hindau protein (pVHL)-mediated polyubiquitination and proteasomal degradation. Activation of the HIF pathway by hypoxia triggers hypoxia-responsive gene expression such as vascular endothelial growth factor (Vegf), which plays a critical role in angiogenesis, as well as endochondral bone formation and bone repair following fracture. To date, however, the role of the HIF pathway in osteoblast-mediated osteogenesis is poorly understood.

In this dissertation research, we tested the hypothesis that osteoblasts use the HIF pathway to sense reduced oxygen tension and transmit signals that affect angiogenic and osteogenic gene expression. In the first part of my thesis work, which is presented following this introduction, we investigated the role of the HIF pathway in bone formation. Using a genetic approach, we have demonstrated that overexpression of HIFs in mouse osteoblasts through disruption of Vhl gene results in profound increases in angiogenesis and osteogenesis which appear to be mediated by cell non-autonomous mechanisms in-
volving VEGF. These findings provide the theoretical basis for the second part of my dissertation research, in which the importance of the HIF pathway in bone regeneration following distraction was defined. Using genetic and pharmacological approaches, we upregulated HIFα levels either by disrupting Vhl in osteoblasts or blocking prolyl hydroxylase activity, and then examined the effect of upregulated HIFα in bone regeneration following tibial distraction. These results suggest that activation of the HIF pathway accelerates bone healing by stimulating both angiogenesis and osteogenesis, and also demonstrate the feasibility of developing HIFα activating agents to facilitate bone regeneration. In the following pages, I will briefly introduce bone biology, angiogenesis in bone formation and following bone repair, as well as the HIF pathway, its function and study approach.

Importance of Oxygen Delivery and Vasculature in Bone Development

Mechanisms of Bone Development

The development of the mammalian skeleton takes place in distinct phases. Mesenchymal cells initially migrate into the site of future bone. This is followed by the condensation of mesenchymal cells and finally the differentiation of progenitors into chondrocytes and osteoblasts to form future cartilage and bone, respectively. The vertebrate skeleton is derived from three distinct embryonic lineages. Cranial neural crest cells give rise to the craniofacial skeleton, paraxial mesoderm (somites) produces the axial skeleton, and lateral plate mesodermal cells form the limb skeleton (1).

Two different mechanisms, intramembranous and endochondral ossification, are responsible for formation of the vertebrate skeleton. Intramembranous ossification forms
the flat bones of the skull and clavicle. In this process, capillaries invade into the mesenchymal zone, and the mesenchymal cells differentiate directly into osteoblasts. Endochondral ossification accounts for the development of most other bones, including the long bones. In this process, cartilage, an avascular tissue, provides a template for bone formation. In vertebrate long bones, endochondral ossification occurs in the primary site (the mid-diaphysis) and the secondary site (epiphyseal plate). Mesenchymal cells first become condensed, and further differentiate into chondrocytes. Chondrocytes then undergo stepwise differentiation. First, a region of resting chondrocytes differentiates into a zone of proliferating chondrocytes, thereby forming two growth plates between the diaphysis (shaft of a long bone) and the epiphysis (head/end of a long bone) by the segregation of chondrocytes at different stages of differentiation (2). Growth plates are responsible for the elongation of long bones. As development progresses, chondrocytes in the center of the cartilage stop proliferating and differentiate into hypertrophic chondrocytes. Hypertrophic chondrocytes express high levels of alkaline phosphatase and collagen type X, and lose expression of cartilage markers such as collagen II and IX (3). During this phase, the surrounding extracellular matrix (ECM) is remodeled and calcifies. Terminally differentiated chondrocytes subsequently undergo apoptotic cell death. In addition, chondroclasts and preosteoclasts erode the cartilage, creating cavities within the bone and allow blood vessel invasion from perichondrium, the membrane of fibrous connective tissue surrounding cartilage template. Following vascular invasion, osteogenic pregenitors are recruited to this area, and use the calcified ECM as a scaffold to secret osteoid, where they subsequently deposit trabecular bones. At the same time, osteoblasts differentiate on the perichondrium surface to form bone collar, which serves as a support
for the new bone. Thus, an avascular tissue (cartilage) is gradually converted into one of the most highly vascularized tissues in vertebrate body (bone).

**Oxygen Delivery and Vasculature in Skeletal Development**

During skeletal development, both endochondral and intramembranous ossification are associated with vessel in-growth. Blood vessel invasion from the metaphyseal region into the avascular cartilage coincides with the formation of bone on the cartilaginous template. Mature long bones are vascularized by a nutrient artery and an intricate vascular network of vessels, capillaries, and blood sinusoids (4). The nutrient artery arises from the systemic circulation, enters the diaphysis, and then branches into ascending and descending medullary arteries within the marrow cavity. These vessels are then further subdivided into arterioles, which penetrate the endosteal surface to form the primary supply of the diaphyseal cortex (5).

The importance of angiogenesis in skeletogenesis was first recognized over 50 years ago. Coolbaugh (6) examined the physical properties of bone including bone density, tensile strength and modulus of elasticity, following surgical disruption of blood supply to bone. An initial decrease in the density of the compacta was observed following interruption of the blood supply to bone. Subsequent studies by Trueta (7;8) demonstrated that bone mineralization and the expansion of the hypertrophic zone in the growth plate were decreased following the interruption of blood supply to the growth plate. More recently, Gerber and Ferrara (9) provided more direct evidence that vascular invasion into growth plate cartilage is necessary for long bone formation during endochondral ossification. In these studies, experimental blockade of angiogenesis with a soluble
VEGF receptor 1-immunoadhesin, mFlt(1-3)-IgG, decreased trabecular bone formation and resulted in the expansion of the hypertrophic zone of the growth plate. Taken together, these studies indicate that blood vessels not only carry oxygen and nutrition to the developing bone, but also play an active role in bone formation.

The close spatial relationship between vascular cells, chondrocytes, osteoblasts, and osteoclasts during bone development, modeling, and remodeling suggests that these cell populations interact at a variety of levels (10). In a simple analogy, the circulatory system serves as a highway upon which oxygen, nutrients, and endocrine factors are transported to cells within the skeleton, and carbon dioxide (CO₂) and metabolic end products are cleared. Rather than passively observing this exchange, an increasing body of evidence indicates that osteoblasts and adjacent endothelial and smooth muscle cells actively mediate physiologic processes required for oxygen and nutrient exchange. As will be discussed in detail below, osteoblasts exhibit properties of other well-established oxygen-sensing cells including the molecular mechanisms for oxygen and nutrient sensing. Moreover, the circulation is believed to be the major source for osteoclast precursors and provides a conduit for growth factors to individual bone remodeling units. Hauge and Eriksen (11;12) suggested the existence of a discrete structure termed the bone remodeling compartment, which serves to coordinate bone remodeling. This self-contained unit is comprised of a blood vessel, osteoclasts and osteoblasts and is sealed off from the adjacent bone. Cells lining this compartment express mediators of osteoclastogenesis including osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL), required for coupling bone formation and resorption (12). Thus, compartmentalization of
vasculature with bone cells would provide an anatomical mechanism to functionally link
the vasculature to bone formation and remodeling.

Hypoxia is believed to be the major stimulus for initiation of the angiogenic cas-
cade during development and following bone injury. During early organogenesis, the or-
derly programs of differentiation and migration involve hypoxia-driven diffusion of oxy-
gen in the embryo. In turn, molecular responses to oxygen gradients mediate the proper
differentiation and maintenance of the developing vasculature (13). Multiple cell types
are involved in this process, including endothelial cells, pericytes, smooth muscle cells,
and leukocytes. In addition, an expanding list of factors have emerged that coordinate the
angiogenic response (14;15). Among these factors, VEGF is a well-characterized pro-
angiogenic factor that is activated by hypoxia and plays a critical role in angiogenesis.

The Role of VEGF in Angiogenesis, Osteogenesis and Bone Regeneration

During endochondral bone formation, VEGF is abundantly released by hypertro-
phic chondrocytes and functions in this microenvironment to initiate blood vessel inva-
sion into cartilage (9;16;17). Gerber et al. (9) found that administration of mFlt (1-3)-IgG
completely blocked neoangiogenesis in the growth plates of 24-day-old mice, suggesting
that growth plate vascularization is VEGF-dependent. Studies by Zelzer and Olsen (18)
in the developing mouse tibia provided evidence that blood vessels were first recruited to
the perichondrium as early as embryonic 13.5-14.5 (E13.5-14.5) through the actions of
VEGF produced in perichondrial cells. This was followed by vessel invasion into the
hypertrophic cartilage from the perichondrium at E14.5. Consistent with these observa-
tions, mice expressing only the soluble isoform of VEGF, VEGF$_{120}$, but lacking VEGF$_{164}$
and VEGF$_{188}$ isoforms, exhibited delayed blood vessel penetration into the perichondrium (17). This finding also suggests that VEGF$_{164}$ and VEGF$_{188}$ might specifically function to coordinate perichondrial angiogenesis.

VEGF-dependent angiogenesis is also critical for the cellular responses necessary for bone repair following fracture. Fracture healing recapitulates many stages of the developmental endochondral bone formation program. Thus, VEGF produced in the newly formed cartilaginous callus stimulates neoangiogenesis of the cartilage. Hypertrophic chondrocytes undergo apoptosis which is followed by callus remodeling by osteoclasts and replacement with woven bone which subsequently mineralizes. During this time, the pattern and timing of VEGF expression in the callus resemble what are seen during long bone development (19-21). Moreover, Street et al. (22) demonstrated that inhibition of VEGF using Flt-IgG in a mouse femur fracture model dramatically reduced angiogenesis, bone formation, and callus mineralization. By contrast, exogenous VEGF enhanced bone formation in both fractured mice and rabbits with segmental radius defects.

There is some experimental support for the concept that VEGF functions independently of angiogenesis on chondrocyte differentiation, osteoclast recruitment, and osteoblast differentiation (18). VEGF has been shown to act as a potent chemoattractant to induce migration of cultured osteoblasts (23), as well as to promote human osteoblast proliferation (24). VEGF and its receptors (VEGFR-1/ Flt-1 and VEGFR-2/ Flk-1) are expressed in hypertrophic chondrocytes and osteoblasts. Deckers et al. (25) have suggested that exogenous VEGF enhances osteoblast differentiation and VEGF expression is increased as osteoblasts mature. These studies suggest that VEGF stimulates mature osteoblast differentiation by acting as an autocrine and/or paracrine factor. However, direct
effects of VEGF on differentiation of osteoblastic precursors have been questioned. Vil-
lars et al. (26) concluded that VEGF did not directly affect the differentiation of human
bone marrow stromal cells (hMSCs), consistent with the result that VEGFR-1 and -2
were not detected in hMSCs at mRNA levels (27;28). Alternatively, using recombinant
hMSCs, Mayer et al. demonstrated that high expression of VEGF accelerated, while high
expression of soluble Flt-1 reduced, late stages of osteoblast differentiation and minerali-
ization (28). Using an ex vivo muscle-derived stem cell-based gene therapy method, Peng
et al. (29) demonstrated that VEGF alone did not improve bone regeneration, but instead
acted synergistically with bone morphogenetic protein 4 (BMP4) to increase recruitment
of MSCs, to enhance cell survival, and to augment cartilage formation in the early stages
of endochondrial bone formation. Thus, while it is clear that VEGF exerts many of its
actions on bone indirectly by stimulation of angiogenesis, whether or to what extent this
factor functions independently of endothelial cells remains to be determined.

VEGF production in bone cells is regulated by a variety of signaling pathways in-
cluding prostaglandins E1 and E2, transforming growth factor β (TGFβ), bone morpho-
genetic proteins (BMPs), fibroblast growth factor 2 (FGF-2), insulin-like growth factor 1
(IGF-1), endothelin-1, and vitamin D3 (18). A proximate control point in all of these
pathways involves the HIF pathway.

Regulation of the HIF Pathway and Function in Bone Formation and Repair

*The Hypoxia-Inducible Factors (HIFs)*

HIF is an αβ heterodimeric transcription factor that mediates the adaptation of
many multicellular organisms to molecular oxygen, and is essential for the maintenance
of cellular oxygen homeostasis. The HIF family comprises three α subunits: HIF-1α, HIF-2α and HIF-3α (Figure 1). The expression of the α subunits is elevated during hypoxia and is maintained at low levels in most cells under normoxic conditions. Unlike the α subunit, the β subunit (also known as the aryl hydrocarbon receptor nuclear translocator, ARNT) is constitutively expressed in the nucleus in an oxygen-independent manner (30). HIFs are members of the basic helix-loop-helix (bHLH) transcription factor family, with each containing two Per/Arnt/Sim (PAS) domains (31) along with a bHLH domain (32). The bHLH and PAS domains are required for heterodimer formation between the α and β subunits and for the binding of DNA (33). HIF-1α and HIF-2α contain two transactivation domains in the C-terminal known as the N-terminal (N-TAD) and C-terminal (C-TAD) transactivation domains. The N-TAD overlaps with the oxygen-
dependent degradation (ODD) domain, which contains a number of prolyl residues that are recognized and hydroxylated by specific prolyl hydroxylase domain (PHD) enzymes (34). The C-TAD interacts with coactivators such as p300/CBP, which is required for full HIF activity.

Under normoxia, HIFα is inactivated by two different hydroxylases (Figure 2). Prolyl hydroxylation at residues 402 and 564 within the ODD domain mediates the binding of the E3 ubiquitin ligase pVHL, a component of the complex that targets HIFα for proteasomal degradation, while asparaginyl hydroxylation disrupts coactivator associa-
tion (35). Hydroxylation of HIFα requires molecular oxygen and iron, and is inhibited by hypoxia. Consequently, the HIFα subunit accumulates in the cytoplasm, and then translocates to the nucleus where it dimerizes with the HIF-1β subunit. This dimer then binds to hypoxia-response elements (HRE) within promoters of hypoxia-responsive genes (36). Genes containing functional HREs include those involved in angiogenesis (Vegf, endothelin-1), maturation of red blood cells (erythropoietin, transferrin), energy metabolism (glucose transporter 1 and 3), and cell proliferation and viability (insulin-like growth factor 2, p21) (37).

A second HIFα isoform, HIF-2α has limited (48%) identity with HIF-1α, but shares the conserved pVHL-binding domain, which renders HIF-2α a substrate of pVHL. Under hypoxic conditions, HIF-2α appears to be regulated by hypoxia in the same way as HIF-1α (38). Therefore, it is not surprising that HIF-1α and HIF-2α can function redundantly to promote the expression of the same set of target genes (39). However, there are differences in expression levels of these two HIFα isoforms among different cell types. The HIF-1α isoform is the predominant protein expressed in epithelial cells, whereas HIF-2α is mainly expressed in endothelial cells and fibroblasts (32). In addition, mice globally deficient in either Hif1α or Hif2α die at different times during development, further suggesting that HIF-1α and HIF-2α exert distinct developmental functions (40-42). Moreover, the sets of genes regulated by these transcription factors, while overlapping, are not identical (43-45). Taken together, it appears that HIF-1α and HIF-2α have evolved to perform distinct functions in selected tissues, but in certain contexts can substitute for one another.
Role of HIF-1α in Skeletal Development and Bone Injury

Recent studies from Schipani and colleagues (46) suggest that HIF-1α is required for endochondral bone development. They created a mouse model wherein Hif1α was deleted in the cartilaginous growth plate of developing bone. These mutant animals developed shorter and deformed limbs compared to their control littermates. This phenotype was associated with misshaped growth plates and disorganized chondrocytes within the growth plate. They revealed that within the hypoxic environment of developing growth plate, chondrocytes lacking Hif1α underwent massive cell death, suggesting that HIF-1α is required for chondrocyte survival and adaptation to hypoxia. An alternative mouse model with overexpressed HIF-1α in chondrocytes via conditional deletion of Vhl displayed a unique cartilage phenotype that was characterized by hypocellularity, a dramatic decrease of chondrocyte proliferation, and an increased extracellular matrix deposition between cells (47). These results suggested that the altered endochondral ossification process in Vhl mutant mice could result from the accumulation of HIF-1α in growth plate chondrocytes, and, consequently, from de-regulation of HIF-1α target gene expression (47).

Little is known regarding the role of HIF-1α in osteoblasts. It is known that the blood vessel invasion during endochondral bone development is associated with the appearance of osteoblasts that form trabecular bone. Therefore, it is possible that osteogenesis is directly dependant on angiogenesis during skeletal development. In this scenario, the condensing mesenchymal cells that ultimately differentiate into osteoblasts may become hypoxic or nutrient deprived due to the lack of sufficient blood supply. These cells would then upregulate HIF-1α, triggering the transcriptional activation of
pro-angiogenic genes such as \textit{Vegf}. The expression of this pathway has been demonstrated both in primary osteoblast cultures and osteoblast-like cell lines, in which the VEGF secretion and gene expression were elevated following exposure to hypoxia or hypoxia-mimicking metals (cobalt and nickel) (48;49). In addition, in developing long bones, osteoblasts on the periosteal surface may recruit more of these pro-angiogenic molecules to support the growth of the central developing diaphysis. During osteoblastic bone formation, HIF-1\textalpha would be expected to function through transactivation of the pro-angiogenic gene, \textit{Vegf}. However, the possibility of HIF-1\textalpha promoting osteogenesis independent of vascular supply cannot be ignored. HIF-1\textalpha may promote the differentiation of pre-osteoblasts into mineralizing osteoblasts and ultimately lead to bone formation by either enhancing cell proliferation, inhibiting apoptosis, or both. The experiments designed in this research will investigate these mechanisms.

The involvement of HIF-1\textalpha as a signaling molecule for bone repair is, on the surface, somewhat more intuitive. Thus, the abrupt interruption of blood and nutrient supply following distraction leads to neovascularization of the injured tissue and de novo osteoblast-mediated bone formation. Faced with a hostile metabolic insult, cells with the capability of osteoblastic potential upregulate HIF-1\textalpha and send out angiogenic or osteogenic signals to bring in the “repair” molecules. The second part of this dissertation research will attempt to define the effect of HIF-1\textalpha activation in response to bone injury.

\textit{Genetically Altered Mouse Models for Study of HIF-1\textalpha and Bone Formation}

Recent advances in methodologies to manipulate the mouse genome provide powerful approaches to investigate functions of certain gene products. The commonly
used method is genetic overexpression or knockout of a particular gene during embryonic development. However, this approach may result in death at an early stage of development if the target gene is critical to the animal survival. For example, mice that lack $Hif1\alpha$ globally ($Hif1\alpha^{-/-}$) die around day 9 of gestation. $Hif1\alpha^{-/-}$ embryos exhibit neural tube defects, cardiovascular malformations, and marked cell death within the cephalic mesenchyme (40). Conditional knockout or overexpression models are theoretically ideal for examination of gene product function in specific tissues. To accomplish this, the most commonly used strategy to date has been the Cre/LoxP system. The Cre recombinase of the P1 bacteriophage is a 38kD protein that belongs to the integrase family of site-specific recombinase. It recognizes two LoxP sites, each of which contains a 34bp consensus sequence consisting of a core spacer sequence of 8bp and two 13bp palindromic flanking sequences. A single Cre recombinase molecule binds to each palindromic half of a LoxP site, followed by the formation of a tetramer, thus bringing two LoxP sites together (50). Since the recombination occurs within the spacer area of the LoxP sites, the target locus could be excised once it is flanked by LoxP sites and inserted into the spacer area. Accordingly, conditional mutagenesis can be achieved by choosing a tissue-specific promoter to drive the Cre transgene expression. Based on this principle, we have developed mouse models with conditional deletion of either $Vhl$, $Hif1\alpha$, or both in osteoblasts. In the first part of this thesis, we characterized the skeletal phenotype of these mutant mice to determine the role of HIFs during bone development.
Validation of a Mouse Model for Distraction Osteogenesis

As discussed above, angiogenic processes also appear to operate during fracture repair to coordinate the concurrent processes of endochondral and intramembranous bone formation. However, understanding the importance of angiogenesis to osteogenesis during fracture repair is complicated by the contributory role of chondrocytes during formation of the callus. By contrast, neoosteogenesis following distraction of the long bone occurs mainly in the absence of an endochondral platform. In this surgical procedure, intramembranous bone formation is locally induced by the application of an external fixation device that applies gradual mechanical distraction across an osteotomy (51). The precise sequence of events that accompanies healing of a distracted bone is still poorly understood. However, the rapid pace at which new bone is formed during distraction and the subsequent consolidation periods places a significant demand on blood supply and therefore constitutes a powerful model for understanding the interplay between angiogenic and osteogenic processes, and for examining the role of hypoxia in the regulation of these processes.

Distraction osteogenesis (DO) has been performed in the clinic to lengthen limbs, and this procedure usually takes years to be completed. Drs. Gerstenfeld and Einhorn (52) recently adapted this approach to a mouse model, in which tibial DO was developed using a dental device. This model enables us to study bone regeneration following distraction in a short period of time. As illustrated in Figure 3, DO is characterized by three separate phases: 1) the latency phase; immediately following osteotomy before any distraction is performed; 2) the active distraction phase; a period in which there is active distraction of the bony segments. In this phase, a migrating zone of proliferating, fibroblast-
like cells appear and align themselves parallel to the vector of elongation and deposit collagen in bundles. Capillaries then form between these bundles and osteoblasts emerge and arrange themselves along the collagen fibers; and 3) the consolidation phase; when the active distraction has ended and the healing of the callus begins, leading to the formation of woven bone and subsequent remodeling into lamellar bone.

Figure 3. Time course of distraction osteogenesis in the mouse. Immediately following osteotomy is the 7-day period of latency phase (LT). Distraction (DO) starts at day 7 and lasts for 10 days. At the end of distraction phase is the beginning of consolidation phase (CD) which lasts for 21 days.

Figure 4 (top panel) depicts representative X-rays at the different stages of the distraction protocol. Representative histological sections are presented corresponding to each of the distraction phases (Figure 4, lower panel). Immediately following osteotomy (day 0), a slight swelling in the subperiosteal areas of the adjacent bone is observed, together with the development of a hematoma (not shown). At the end of the latency phase (LT) (day 7) very little new bone can be seen. At day 3 of distraction (DO3), abundant mesenchymal cells are apparent in the osteotomy gap, but little well-formed new bone is observed at this time. At the end of the distraction period (17 days after osteotomy), new trabecular bone is seen within the distraction gap. Large vascular sinusoids as well as smaller well-formed blood vessels are seen within the newly formed bone adjacent to the
marrow space of the bone. During the consolidation (CD) period, the osteotomy gap fills with dense woven bone and begins to remodel. The marrow space then expands in the spaces that were originally occupied by the initial penetrating vessels that grew into the repair tissue. During consolidation, repaired tissues show an increase in vascular elements, which is aligned parallel to the direction of the distraction force. In the second part of this dissertation, we determined the role of HIF pathway in angiogenesis and osteogenesis following distraction of mouse bone using this procedure.

Figure 4. Adaptation of an oral aveolar bone distractor for distraction of murine long bones. Top panels show post operative X-ray analysis at the end of the latency phase (LT), at day 3 of the distraction phase (DO3), at the end of the distraction period 8 hours after the last turn of the appliance (DO), and at 14 days of the consolidation phase (CD). Note the alignment of the bony segments throughout the course of distraction. Bottom panels show H&E stained histological sections of the distraction gap in the mouse tibia. Each section corresponds to the time points shown in the top panel.

Specific Aims

Aim 1: Investigate the Role of the HIF Pathway in Bone Development

As discussed previously, under normoxia, HIFα is degraded by the pVHL E3 ubiquitin ligase complex following prolyl hydroxylation. To overexpress HIFα in osteoblasts, we created mice lacking the Vhl gene in osteoblasts (ΔVhl) using a Cre/LoxP system. The same strategy was used to generate mice lacking Hif1α (ΔHif1α) or both Vhl and Hif1α (ΔVhl/ΔHif1α) in osteoblasts. Bone phenotypes of these mutant mice were compared with their wild type littermates at specific time points during embryonic and postnatal development. Primary osteoblasts obtained from the calvaria of these mice were examined the effect of HIFα overexpression or deletion on osteoblast proliferation, differentiation, and mineralization.

Aim 2: Define the Importance of the HIF Pathway in Bone Regeneration Following Distraction Osteogenesis

Using the Vhl deficient mouse model developed in Aim 1, we determined the importance of HIF-1α in neovascularization and osteogenesis following distraction of the mouse tibia. Additionally, bone regeneration was also examined following pharmacological upregulation of the HIF pathway using prolyl hydroxylase inhibitors.
THE HYPOXIA-INDUCIBLE FACTOR α PATHWAY COUPLES ANGIOGENESIS TO OSTEOGENESIS DURING SKELETAL DEVELOPMENT

by

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Format adapted and errata corrected for dissertation
Abstract

Skeletal development and turnover occur in close spatial and temporal association with angiogenesis. Osteoblasts are ideally situated in bone to sense oxygen tension and respond to hypoxia by activating the hypoxia inducible factor α (HIFα) pathway. Here we provide evidence that HIFα promotes angiogenesis and osteogenesis by elevating VEGF levels in osteoblasts. Mice overexpressing HIFα in osteoblasts through selective deletion of the von Hippel-Lindau (Vhl) gene expressed high levels of Vegf and developed extremely dense, heavily vascularized long bones. By contrast, mice lacking Hif1α in osteoblasts had the reverse skeletal phenotype of that of the Vhl mutants: long bones were significantly thinner and less vascularized than those of controls. Loss of Vhl in osteoblasts increased endothelial sprouting from the embryonic metatarsals in vitro but had little effect on osteoblast function in the absence of blood vessels. Mice lacking both Vhl and Hif1α had a bone phenotype intermediate between those of the single mutants, suggesting overlapping functions of HIFs in bone. These studies suggest that activation of the HIFα pathway in developing bone increases bone modeling events through cell-nonautonomous mechanisms to coordinate the timing, direction, and degree of new blood vessel formation in bone.
Introduction

The development of the mammalian skeleton takes place in distinct phases which involve 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 which 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 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 destruction 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, were 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 Hif1α 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 up-regulation of HIF target genes Vegf and type-1 glucose transporter (Glut-1) 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, allele-specific PCR was performed using DNA isolated
from selected tissues. These results demonstrated that Cre-mediated recombination of the floxed Vhl allele (Δflox) 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 adenoviral Cre to delete the floxed Vhl allele. pVHL levels were eliminated both in the cytoplasm and nucleus, which was associated with increased expression of both HIF-1α and HIF-2α proteins in the nuclear fraction (Figure 2C). These changes were accompanied by increased expression of mRNA for the HIF target genes Vegf and Glut-1 (Figure 2D). Expression of transcripts for all 3 Vegf isoforms (Vegf120, Vegf164 and Vegf188) was equally upregulated following Vhl deletion (Supplemental Figure 1).

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 2A) nor the rate of bone formation (Figure 3E) was elevated relative to controls, suggesting that the in-
creased bone volume in ∆Vhl mice might have occurred earlier. To investigate this possibility, histomorphometric analysis was performed on femurs from 7-day-old ∆Vhl and control mice. At this time, double calcein labeling showed an accelerated bone formation rate in the mutant mice compared with that of controls (Fig 3F). In addition, osteoblast number was significantly increased in the 7-day-old ∆Vhl mice compared with controls ($P = 0.039$; Figure 3G). Thus, these results demonstrate that bone modeling is increased in ∆Vhl mice as early as 7 days of age. To exclude the possibility that the greatly increased trabecular bone volume was masking an absolute increased osteoblast number in ∆Vhl mice, we measured osteoblast numbers per tissue area in ∆Vhl and control mice at 3 (Supplemental Figure 2B) and 6 weeks of age (not shown). Osteoblast numbers calculated per tissue area were not significantly altered in ∆Vhl bone. As an additional measure of osteoblast activity, we assayed the level of serum OC in ∆Vhl and control mice. These results show a slight and insignificant decrease in OC in the Vhl mutants (Supplemental Figure 2C). Taken together, these data demonstrate that bone modeling is increased in ∆Vhl mice within the first week of life but appears to decline at latter stages of post-natal development.

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 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 osteoblasts 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 ∆Vhl mice (Supplemental Figure 3D). These results suggest that bone turnover declines in the mature ∆Vhl 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 (intramembraneous 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 ∆Vhl mice (Figure 4A). Quantitative histomorphometric analysis showed that bone volume (Figure 4B) and osteoblast numbers (Figure 4C) in the ∆Vhl mice were not significantly different from those in controls. The lack of effect of the Vhl mutation on calvarial bone is 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 mice 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 which 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 ∆Vhl 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 ∆Vhl mice occurred in part through cell nonautonomous 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 overexpression 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, F and 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 ef-
ffects on in vitro osteoblast performance and further suggest that overexpression of HIFα in osteoblasts in vivo promotes bone formation primarily by cell-nonautonomous 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 (Δflox) 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 tis-
sues, 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 lacking both *Vhl* and *Hif1α* in osteoblasts (*∆Vhl/∆Hif1α*) by cross breeding the *∆Vhl* and *∆Hif1α* 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 the *∆Hif1α* 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 below that seen in the *∆Vhl* 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 *∆Vhl* 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 paper, 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 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 which 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 hypothesis that blood vessel invasion is critical for bone formation and turnover and 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. (9) have shown that the HIFα pathway is required for the normal endochondral growth plate development. In their model, chondrocytes lacking Hif1α 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. However, in contrast to the current studies, constitutive activation of HIF-1α in chondrocytes though 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 which 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 Hif1α 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 Hif1α 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 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 Hif1α or Hif2α 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 Pgkl, whereas HIF-2α is a predominant regulator of hypoxic Vegf and erythropoietin (Epo) (24-26). Taken to-
gether, 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.

Although a number of functions have been ascribed for the pVHL protein ligase, several lines of evidence including the studies described here suggest that its primary role is to regulate the destruction of HIFα subunits. Mice lacking Vhl in liver, growth plate, thymus, colon, and myeloid cells all demonstrate increased HIFα levels, increased expression of Vegf and increased angiogenesis underscoring the importance of pVHL as a negative regulator 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 prolyl hydroxylated 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 of HIF-1α activation) induced 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 a 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 paper 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 the endochondral bone formation.
Methods

Generation of Conditional Vhl, Hif-1α, and Double Knockout Mice

OC-Cre transgenic mice were created by microinjection of a DNA construct with human OC promoter driven Cre recombinase into fertilized eggs of the F-VBN mouse strain (7). Mice with osteoblast-specific inactivation of Vhl or Hif-1α were obtained by intercrossing OC-Cre mice with mice homozygous for a floxed Vhl allele (27), or homozygous for a floxed Hif-1α allele (9). To generate mice lacking both Vhl and Hif1α in osteoblasts, we first crossed ΔHif1α mice with mice homozygous for a floxed Vhl allele and then interbred the offspring for 3 generations. Genotyping was performed as previously described (7;9;27). All procedures involving mice were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Skeletal Phenotyping, Histological Analysis and in situ Hybridization

The intact right femur was scanned using μCT (μCT20; Scanco Medical) to assess bone mass, density, geometry, and trabecular microarchitecture. Parameters computed from these data include trabecular thickness, number, separation and connectivity at the distal femoral metaphysis and cortical thickness and cross-sectional area at the mid-diaphysis. Dynamic bone formation rate was measured by injection of 2 sequential doses of calcein (8 mg/10 ml sterile saline) delivered in a total of 0.25 ml 2 and 6 days prior to sacrifice. Mouse femur and calvaria were dissected, fixed in 10% buffered formalin, decalcified in 8% Na2EDTA, paraffin embedded, and stained with hematoxylin and eosin by standard methods. For immunohistochemistry, antigen retrieval was performed by boiling in 10 mM sodium citrate (pH 6.0) for 5 minutes. Sections were incu-
bated with antibodies to HIF-1α (C-19, Santa Cruz Biotechnology Inc.), HIF-2α (Novus Biologicals), and pVHL (BD Biosciences — Pharmingen). In situ hybridization was performed using complementary $^{35}$S-labeled riboprobes as described previously (9).

Imaging of Blood Vessels

Blood vessels in bone were imaged using μCT. Specimens were prepared in accordance with previously described methods (40). Briefly, after animals were euthanized, the thoracic cavity was opened and the 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, Cal-Ex II (Fisher 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 (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 fetal bovine serum and 1% of penicillin/streptomycin for 72 hours. Two hundred and fifty micro-liters 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 min at room temperature and subsequently stained for CD31 using a rat polyclonal antiserum 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 Experiment and Adenovirus 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 contain the osteoblasts, were pooled together. After centrifugation, osteoblasts were obtained and cultured in α-MEM containing 10% fetal bovine serum 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 Biolabs) at 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 $10^6$ cells/plate, and cultured in $\alpha$-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin for 24 hours. Cells were then starved in $\alpha$-MEM with 1% fetal bovine serum for 12 hours, followed by the replacement with 10% fetal bovine serum in $\alpha$-MEM and cultured for an additional 12 hours. For the proliferation assay, BrdU (10 $\mu$M) was added to the culture medium 2 hours before harvesting. Cells were stained with fluorescent anti-BrdU antibody and 7-amino-actinomycin D following 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 FACS Calibur (BD) and 20,000 events were collected.

ALP and von Kossa Staining

Osteoblasts were plated on 6-well plates with a density of $10^5$ cells/well and cultured in $\alpha$-MEM until they were confluent. Medium was then changed to osteogenic me-
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. Histochemical staining for ALP activity in the cells was determined using naphthol AS-MX phosphate (Sigma) 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 \( Vhl \), 5’-GCCTATT TT-TGCCAACATCACA-3’ and 5’-TCATTCTCTCTATGTGCTGGCTTT-3’; \( Hif1\alpha \), 5’-CAAGATCTCGGCGAAGCAA-3’ and 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Hif2\alpha \), 5’-CAACCTGCAGCCTCAGTGTATC-3’ and 5’-CACCACGTCGTTCTTCTCTC- GAT-3’; \( Vegf \), 5’-CCACGTCGACAGACATCA-3’ and 5’-TCATTCTCTATGTGCTGGCTTT-3’; \( Vegf_{120} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{164} \), 5’-ACAGGACAAAGCCAGAAAAACAC-3’ and 5’-GT TAAACTCAAGCTCGCCCTC-3’; \( Vegf_{188} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{200} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{220} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{240} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{260} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{280} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{300} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{320} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{340} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{360} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{380} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{400} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{420} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{440} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{460} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{480} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{500} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{520} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{540} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{560} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{580} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{600} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{620} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{640} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{660} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{680} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{700} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{720} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{740} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{760} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{780} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{800} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{820} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{840} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{860} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{880} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{900} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{920} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{940} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{960} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{980} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1000} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1020} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1040} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1060} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1080} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1100} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1120} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1140} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1160} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1180} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1200} \), 5’-GGTGAGCCTCATAACAGAAGCTTT-3’; \( Vegf_{1220} \), 5’-GGTGAGCCTCATAA
3’ and 5’-GAACAAGGCTCACAGTGAACGC-3’; Glut1, 5’-GGGCATGTGCTTCA- 
GTATGT-3’ and 5’-ACGAGGAGCACCCTGAAGAT-3’; Runx2, 5’-ATGCTTCATTCE- 
GCCTCAC-3’ and 5’-CTCACGTCGCTCATCTTTG-3’; OC, 5’-TCTGCTCACTCTGCT- 
GAC-3’ and 5’-GGAGCTGCTGTGACATCC-3’; β-actin, 5’-CCCAGAGCAAGAGAG- 
G-3’ and 5’-GTCCAGACGCAGGATG-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 ex-
tracts 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 pre-stained protein markers. Membranes were then 
blocked with 5% dry milk in Tris-buffered saline Tween-20 for 1 hour at room tempera-
ture and subsequently incubated overnight with primary antibodies at 4°C. Signals were 
detected using a HRP-conjugated secondary antibody and the Supersignal west femto 
maximum sensitivity substrate (Pierce). Antibodies used were anti-pVHL (BD Biosci-
ences — 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 was 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 normoxia or hypoxia 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 anit-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 manufacturer’s recommendations. ELISA kits used were mouse VEGF Quantikine ELISA kit (R&D systems), mouse OC EIA kit (Biomedical Technologies Inc.), mouse OPG assay kit (Biomedica), and mouse TRAP assay kit (IDS). Optical density was determined using a plate reader (BioTek).
**Statistical Analysis**

For statistical analysis and comparison of histomorphometric 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 $P < 0.05$. 
Acknowledgements

The authors thank Drs. Buer Song and Rosa Serra for assistance with the in vitro angiogenesis assays. This work was supported by NIH grant R01 AR049410.
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icrocomputed tomography analysis of collateral vessel development after ischemic 

Figure 1. Primary mouse osteoblasts express components of the HIFα pathway. (A) Calvarial primary osteoblasts were obtained from wild type mice and cultured in α-MEM until confluent. Whole cell lysate was analyzed by immunoblotting using antibodies against pVHL (top), PHD1 (middle), and PHD3 (bottom) in experiments performed in duplicate. OB, osteoblast. (B and C) Confluent cell monolayers were exposed to 21% (normoxia) or 2% (hypoxia) O2 for 24 hours. (B) Proteins from cytoplasm (C) and nucleus (N) were extracted separately and analyzed by immunoblotting with antibodies against HIF-1α (top row) and HIF-2α (bottom row). (C) Nuclear translocation of HIF-1α (top row) and HIF-2α (bottom row) was assessed by confocal microscopy as described in Methods. DAPI was used to stain the nuclei. Original magnification, × 100. (D and E) Quantitative real-time PCR analysis was performed to determine Vegf and Glut-1 mRNA expression after cell monolayers were exposed to normoxia (white bars) or hypoxia (black bars) for the indicated times. *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 2. Osteoblast-specific, Cre-mediated deletion of Vhl. (A) PCR analysis of Cre-mediated recombination in selected tissues from a ∆Vhl mouse. The recombined allele (∆flox) was present exclusively in bone tissue. (B) Representative histological sections of distal femurs from 6-week-old control and ∆Vhl 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 Glut-1 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.
Figure 3. Disruption of Vhl in osteoblasts increases long bone volume. (A) Representative micro CT images of the femurs from ∆Vhl and control mice at the age of 3, 6 and 12 weeks. Scale bars, 5.0 mm. (B) Representative femoral cross sections from 6-week-old ∆Vhl and control mice. Scale bars, 1.0 mm. (C–E) Histomorphometric analyses were performed on femoral sections from ∆Vhl mice and controls at 3 weeks of age as described in Methods. Comparisons of trabecular bone volume (C), trabecular separation (D) and bone formation rate per osteoblast (E) between control (white bars; n = 6) and ∆Vhl mice (black bars; n = 7) are shown. Data represent mean ± SEM. *P < 0.05; **P < 0.01. (F) Seven-day-old mice were labeled with sequential doses of calcein before sacrifice. Representative calcein-labeled sections of distal femur from control and ∆Vhl mice are shown. Original magnification, × 400. (G) Quantitative histomorphometric measurement of osteoblast number was performed at the distal femur from 7-day-old ∆Vhl (black bars; n = 3) and control mice (white bars; n = 3). Data represent mean ± SEM. *P < 0.05.
Figure 4. Loss of Vhl in osteoblasts does not alter calvarial bone. (A) Representative µCT images of calvaria from 12-week-old ∆Vhl and control mice (top). Scale bar, 2.0 mm. The bottom panels show H&E stained sections of calvaria from 6-week-old control and ∆Vhl mice. Original magnification, × 100. (B and C) Quantitative histomorphometric measurement of bone volume and osteoblast number was performed on calvarial sections from 6-week-old ∆Vhl (black bars; n = 3) and control mice (white bars; n = 3). Data represent mean ± SEM.
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 in control metatarsal treated with recombinant VEGF (10 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.
Figure 6. Deletion of \textit{Vhl} in primary osteoblasts in vitro does not affect osteoblast proliferation and apoptosis. Confluent \textit{Vhl} floxed primary osteoblast monolayers were infected with either Ad-GFP or Ad-CreM1 (100 MOI). \textit{Vhl} mRNA expression in infected osteoblasts was determined by real-time PCR 48 hours after infection to assess deletion efficiency. Cell proliferation, apoptosis, and differentiation assays were performed as described in Methods. (A and B) Cell proliferation was assessed by flow cytometry using BrdU incorporation. (C and D) Cell apoptosis was assessed by flow cytometry using Annexin V-PE staining. (E) Mineralized nodule formation was determined by ALP (left) and von Kossa staining (right) 7 and 14 days after cells were cultured in osteogenic medium. (F and G) Densitometric analysis of ALP and von Kossa staining observed in E using NIH ImageJ 1.36b. Data represent mean ± SEM. (H) Measurement of \textit{Vhl}, \textit{Hif-1}α, runt-related transcription factor 2 (\textit{Runx2}) and \textit{OC} mRNA expression by quantitative real-time PCR at day 14 of osteogenic induction. **P < 0.01; ***P < 0.001.
Figure 7. Mice lacking $Hif1\alpha$ in osteoblasts have narrow, poorly vascularized long bones. (A) PCR analysis of Cre-mediated recombination in selected tissues from a $\Delta Hif1\alpha$ mouse. The recombined allele ($\Delta flosk$) was present exclusively in bone tissue. (B) Representative histological sections of distal femurs from 6-week-old control and $\Delta Hif1\alpha$ mice after staining with antibodies against HIF-1$\alpha$ (left) or HIF-2$\alpha$ (right) as described in Methods. Sections were counterstained with hematoxylin. Red arrows indicate positive and black arrows negative staining in osteoblasts. Original magnification, ×400. (C) Representative images of femoral cross sections from control and $\Delta Hif1\alpha$ mice. Scale bars, 1.0 mm. (D) Representative µCT images of vasculature in Microfil-perfused femurs from 3-week-old $\Delta Hif1\alpha$ and control mice. Scale bar, 1.0 mm. (E and F) Confluent monolayers of $Hif1\alpha$ floxed primary osteoblasts were infected with either Ad-GFP or Ad-CreM1 (100 MOI). (E) Proteins in the cytoplasm and nucleus were extracted separately 48 hours after infection. Immunoblotting analysis was performed with antibodies against HIF-1$\alpha$ and HIF-2$\alpha$. Immunoblots for TBP and $\alpha$-tubulin were used as loading controls for nuclear and cytoplasmic proteins, respectively. TBP, TATA box- binding protein. (F) Total mRNA was extracted from confluent monolayers of osteoblasts 48 hours after infection. $Hif-1\alpha$, $Hif-2\alpha$ and $Vegf$ mRNA expression was determined by quantitative real-time PCR. **$P < 0.01$. 
Figure 8. Bone and vascular phenotype in Vhl and Hif1α double-knockout mice. (A) Representative µCT images of femoral cross sections from 6-week-old ΔVhl/ΔHif1α double mutant mice and wild type controls. Scale bars, 1.0 mm. (B) Representative images of Microfil-perfused femurs from 3-week-old ΔVhl/ΔHif1α double-mutant mice and control littermates. Scale bar, 1.0 mm. (C) Immunohistochemistry analysis of HIF-2α level in femoral sections from 6-week-old ΔVhl/ΔHif1α double-mutant mice and control littermates. 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.
Supplemental Figure 1. *Vhl* deletion in mouse osteoblasts is associated with increased *Vegf* mRNA. Confluent monolayers of *Vhl* floxed primary osteoblasts were infected with either Ad-GFP or Ad-CreM1 (100 MOI) for 48 hours. Total mRNA was extracted from confluent monolayers and gene expression of *Vegf*\(_{all}\), *Vegf*\(_{120}\), *Vegf*\(_{164}\), and *Vegf*\(_{188}\) was determined by quantitative real-time PCR using sequence specific primers. White bars represent Ad-GFP infection; black bars represent Ad-CreM1 infection. *P* < 0.05; **P* < 0.01; ***P* < 0.001.
Supplemental Figure 2. Osteoblast numbers and serum osteocalcin in ΔVhl and control mice. (A and B) Histomorphometric analyses for osteoblast numbers were performed on femoral sections from ΔVhl mice (black bars; n = 7) and controls (white bars; n = 6) at 3 weeks of age as described in Methods. (A) Osteoblast numbers per trabecular bone surface. (B) Osteoblast numbers per tissue area. Data represent mean ± SEM. *P < 0.05. (C) Serum levels of OC were measured in control and ΔVhl mice at 6 weeks of age by ELISA, as described in Methods. Data represent mean ± SEM. n = 3.
Supplemental Figure 3. Osteoclast numbers and serum TRAP5b in △Vhl and control mice. (A and B) Histomorphometric analyses for osteoclast numbers on femoral sections from △Vhl mice (black bars; n = 7) and controls (white bars; n = 6) at 3 weeks of age as described in Methods. (A) Osteoclast numbers per trabecular bone surface. (B) Osteoclast numbers per tissue area. Data represent mean ± SEM. (C and D) Serum levels of OPG and TRAP 5b were measured in control and △Vhl mice at 6 weeks of age by ELISA, as described in Methods. Data represent mean ± SEM. n = 3.
Supplemental Figure 4. Elevated Pgk mRNA expression and unchanged circulating VEGF level in ΔVhl mice. (A) In situ hybridization analysis with Pgk mRNA on histological sections from 3-day-old control and Vhl mutant femurs was performed as described in Methods. Original magnification, × 40. (B) Serum levels of VEGF were measured in control and ΔVhl mice at 12 weeks of age by ELISA, as described in Methods. Data represent mean ± SEM. n = 5.
Supplemental Figure 5. Decreased bone volume and osteoblast numbers in mice lacking Hif1α in osteoblasts. Histomorphometric analyses were performed on femoral sections from ΔHif1α mice and controls at 3 weeks of age as described in Methods. Comparison of osteoid volume (A) and osteoblast numbers (B) between control (white bars; n = 6) and ΔHif1α mice (black bars; n = 6) are shown. Data represent mean ± SEM. *P < 0.05.
ACTIVATION OF THE HYPOXIA-INDUCIBLE FACTOR-1α PATHWAY ACCELERATES BONE REGENERATION

by

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Submitted to Journal of Clinical Investigation

Format adapted for dissertation
Abstract

The hypoxia inducible factor-1α (HIF-1α) pathway is the central regulator of adaptive responses to low oxygen availability and is required for normal skeletal development. Here we demonstrate that the HIF-1α pathway is activated during bone repair and can be manipulated genetically and pharmacologically to improve skeletal healing. Mice lacking pVhl in osteoblasts with constitutive HIF-1α activation in osteoblasts had markedly increased vascularity and produced more bone in response to distraction osteogenesis, whereas mice lacking HIF-1α in osteoblasts had impaired angiogenesis and bone healing. The increased vascularity and bone regeneration in the pVHL mutants were VEGF dependent and eliminated by concomitant administration of VEGF receptor antibodies. Small molecule inhibitors of HIF prolyl hydroxylation stabilized HIF/VEGF production and increased angiogenesis in vitro. One of these molecules (DFO) administered in vivo into the distraction gap increased angiogenesis and markedly improved bone regeneration. These results identify the HIF-1α pathway as a critical mediator of neoangiogenesis required for skeletal regeneration and suggest the application of HIF activators as therapies to improve bone healing.
Introduction

Bone has a unique ability to regenerate and repair itself postnatally. However, of the 6 million fractures reported annually in the United States, as many as 10% have impaired healing. This results in enormous direct and indirect cost to the individual and to society (1). A hallmark of impaired healing in humans and animals is a reduction in vascular supply and nutrient availability at the site of injury, suggesting that impaired angiogenic response is a major contributor to the pathology.

Bone regeneration recapitulates processes that operate during skeletal development and require close temporal and spatial coordination of events involving resident bone cells, marrow stromal elements and associated vascular structures (2;3). Angiogenesis is critical for bone regeneration and is dependent of hypoxic stimuli and vascular endothelial growth factor (VEGF) production. The hypoxia inducible factor (HIF) pathway is the central pathway for sensing and responding to changes in local oxygen availability in a wide variety of organisms. HIF impinges on gene programs which influence angiogenesis (e.g., Vegf, angiopoietins) and cellular metabolism (Glut-1). In addition, HIF can recruit inflammatory and mesenchymal cells and influence cell differentiation (4-7). Consequently, the HIF pathway is ideally suited to coordinate tissue response to injury.

HIF-1α levels are controlled by regulated proteolysis through an oxygen sensitive mechanism. Under normoxic conditions, HIF-1α undergoes prolyl hydroxylation and is ligated by von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase (8-10) and then degraded by the proteosome. The prolyl hydroxylases require iron, 2-oxoglutarate, and oxygen as cofactors (11). Under hypoxia, HIF-1α prolyl hydroxylation is inhibited, and
HIF-1α accumulates in the nucleus where it forms a dimer with the HIF-1β subunit (also known as ARNT). The dimer then complexes with coactivator p300 and transactivates HIF responsive genes (12).

We have recently identified the importance of the HIF pathway in skeletal development (13). Mice lacking \( Hif1a \) in osteoblasts were found to have decreased bone volume, whereas targeted deletion of pVHL resulting in increased HIF activation in osteoblasts significantly increased bone volume with a striking increase in bone vascularity. We therefore hypothesized that activation of HIF would increase vascularity and, subsequently, improve bone healing. In this study, we used both genetic and pharmacologic approaches to activate HIF in the mouse distraction osteogenesis (DO) model of skeletal repair. Our results demonstrate the requirement of the HIF-1 pathway in mediating the angiogenesis and osteogenic phases of bone repair and suggest the feasibility of molecular targeting of the HIF pathway to enhance bone regeneration.
Results

Development of Hypoxia and Expression of HIF-1α Pathway Components during DO

Histological analysis was performed in tissues harvested from eight week old male C57BL/6 mice subjected to DO (14). Following osteotomy, no manipulation was performed for 7 days of latency. After subsequent distraction over 10 days, new bone formation was apparent in the distraction gap at day 17 and healing was complete by day 38 (Figure 1A). Pimonidazole adducts immunostaining was used to assess tissue hypoxia in the distraction gap after 1 day of distraction. Cells that exhibited morphological features consistent with osteoblasts in the central region of the distraction gap were intensely stained with the antibody to the protein-pimonidazole adducts (Figure 1B). HIF-1α and VEGF were also localized to these cells by immunohistochemistry and in situ hybridization (Figure 1C). Within this distraction region, new blood vessels were also evident as assessed by immunohistochemistry for the endothelial marker CD31 (Figure 1C). These results demonstrate the development of hypoxia in bone lining cells during distraction in association with neoangiogenesis and the appearance of HIF-1α and VEGF.

Genetic Activation of the HIF-1α Pathway Promotes Angiogenesis and Enhances Bone Regeneration

We developed two mutant mouse models to investigate the function of HIFs in bone regeneration during DO. In the first model we overexpressed HIFs in osteoblasts by disrupting the HIF E-3 ligase, pVHL (13). As expected, loss of pVHL (Figure 2A) was accompanied by increased HIF-1α protein, increased VEGF mRNA and protein and increased CD31 immunostaining. To quantify the subsequent angiogenic response, we per-
formed micro computed tomography (µCT) angiography using Microfil. Increased vascularity was noted in the mutant (∆Vhl) mice at day 17 (Figure 2B). Significant increases in both vessel volume per total volume (VV/TV) and vessel number were observed in the mutants compared to controls (Figure 2B). The ∆Vhl mice generated more dense woven bone in the distraction gap compared to controls (Figure 2C and Supplemental Figure 1). µCT measurements showed significantly increased bone volume per total volume (BV/TV) and decreased trabecular separation in the ∆Vhl mice compared with the controls (Figure 2C). Thus, the increased vascularity observed in the ∆Vhl mice at the conclusion of DO was followed by increased bone formation. Biomechanical testing of the bones by three point bending showed that peak load and stiffness were significantly increased in ∆Vhl mice compared with the controls (Figure 2D). Nanoindentation showed no significant difference in elastic modulus and hardness between the mutants and controls (Figure 2D). Thus, the increased bone formation in the ∆Vhl mice led to an increase in structural integrity by increased bone volume with no difference in the material properties of the newly formed bone. Collectively, these results show that genetic activation of the HIF pathway in the ∆Vhl mice increased angiogenesis and bone regeneration.

VEGF Receptor Antibodies Inhibit Angiogenesis during DO

To determine the importance of VEGF signaling in the angiogenic response during bone regeneration, we administered VEGFR1 and 2 antibodies or non-immune IgG intraperitoneally every 3 days post-surgery until day 17. µCT angiography showed that mice given VEGF receptor antibodies had significantly decreased VV/TV, vessel number, and vessel surface with significantly increased vessel separation (Figure 3, A and B).
Inactivation of HIF-1α Impairs Angiogenesis and Bone Regeneration

We next examined whether inhibiting HIF-1α or its downstream target, VEGF, would impair angiogenesis and bone healing. We developed a second mouse strain with a targeted deletion of HIF-1α in osteoblasts and subjected them to DO. μCT angiography at day 17 showed decreased vascularity in the ∆Hif1α mice with significant decreases in VV/TV and vessel number compared with controls (Figure 4A). μCT analysis demonstrated that subsequent bone regeneration was decreased in ∆Hif1α mice compared with the controls (Figure 4B). These results suggest that HIF-1α and VEGF are required for normal angiogenesis and bone healing.

Pharmacological Activation of the HIF-1α Pathway Stimulates Angiogenesis and Accelerates Bone Regeneration

A family of oxygen sensitive prolyl hydroxylases (PHD1, 2, 3) hydroxylate HIFs under normoxia which promotes their subsequent E-3 ligation and proteosomal destruction. To identify HIF activators we next tested several agents known to inhibit prolyl hydroxylases for their ability to activate a HIF responsive reporter gene stably expressed in an osteoblast-like osteosarcoma cell line. Desferrioxamine (DFO) and L-mimosine (L-mim) strongly activated the reporter gene and increased nuclear accumulation of HIF-1α (Figure 5, A and B). DFO treatment increased VEGF expression in primary mouse bone marrow mesenchymal stem cells (MSCs) maintained under normoxic conditions (Figure 5C). We next tested DFO and L-mim for angiogenic activity using a standard matrigel tube formation assay with human umbilical vein endothelial cells (HUVEC). Exposure to DFO and L-mim increased formation of tube-like structures (Figure 5, D and E). To
further evaluate the angiogenic activity of the PHD inhibitors, we performed an angiogenesis assay using explants of E17.5 mouse metatarsals. Control metatarsals exhibited a small degree of endothelial sprouting, which was greatly enhanced by treatment with rhVEGF in a time-dependent manner (Figure 5F and Supplemental Figure 2). Continuous (14 days) exposure to DFO or L-mim was associated with detachment of the bone rudiments from the tissue culture plate possibly due to the known effect of PHD inhibitors on collagen processing (15). However, exposure to DFO and to a lesser degree L-mim for a shorter period increased endothelial sprouting without obvious toxicity (Figure 5F). Furthermore, treatment of mouse MSCs with these agents had no detectable effect on the ability of these cells to differentiate into osteoblasts in vitro as measured by alkaline phosphatase (ALP) staining (Supplemental Figure 3).

Because of the greater angiogenic activity of DFO in vitro, we selected it for evaluation in vivo in the DO model. DFO was administered directly into the distraction gap every other day over the period of active distraction from days 7-17. Separate experiments using methylene blue injections documented local delivery of the vehicle into the gap (Figure 6A). DFO strongly increased vascularity in the distraction gap, as demonstrated by significantly increased vessel number and vessel connectivity (Figure 6, B and D). X-ray and μCT analysis showed that bone regeneration was increased following DFO treatment, BV and BV/TV were significantly increased compared with the controls (Figure 6, A, C and E).
Discussion

In this paper, we demonstrate that tissue hypoxia which develops following a standard surgical distraction procedure in normal mice is accompanied by upregulation of the HIF/VEGF pathway and results in neoangiogenesis. We provide genetic evidence that the HIF-1 transcription factor is required to stimulate VEGF production and to mount a normal angiogenic and osteogenic response in a mouse model of bone repair. In addition, we provide proof of concept that this pathway can be targeted pharmacologically to augment bone regeneration.

Recent work from our laboratory and others have shown that HIF-1 and its downstream target, VEGF are upregulated in chondrocytes and osteoblasts within the fracture callous (16). VEGF has also been found in human fracture hematoma and serum following fracture (2;17;18). Moreover, local application of VEGF has been used to enhance healing and angiogenesis in mouse, rat, and rabbit fracture and bone defect models (19-22). The present studies provide new insights into the cellular and molecular mechanisms responsible for bone formation and repair. Neoangiogenesis is known to be required for bone to regenerate (23;24), such that animal models with defective or delayed angiogenesis have impaired healing (25). The current results show that activation of HIF-1α, either by genetic or pharmacologic means, leads to increased bone deposition proportional to the prior increase in vascularity. Therefore, the osteogenic process appears to be driven by angiogenesis which is stimulated through HIF activation and VEGF production. Previous work has shown that VEGF is expressed by osteoblasts and undifferentiated cells within the distraction gap (26) and that HIF-1α, VEGF-A, angiopoietin 1 and neuropilin are induced with each episode of distraction during the DO process, sug-
suggesting the involvement of these pathways in the angiogenic response (14). Furthermore, mechanical intervention induced by axial shortening following distraction resulted in improved healing in a rabbit DO model in association with HIF-1α upregulation suggesting that the HIF pathway might also link mechanical stimuli to the regenerative response (27). However, although the VEGF pathway is important in mediating angiogenesis, VEGF alone was insufficient to improve healing in a rabbit DO model (28). This may be because additional factors such as angiopoietins and Tie1 and 2 are required to restabilize endothelial cells following recruitment (29). Because HIFs are upstream of the entire angiogenic cascade they would be expected to induce additional factors required for re-supplying an intact vascular network in the injured bone tissue. Consequently, the HIF pathway is ideally suited for induction of angiogenesis in tissue repair and regeneration.

The precise mechanisms that couple angiogenesis to bone formation are still not known but appear to require crosstalk between osteoprogenitor cells and vascular endothelial cells. For example, the ΔVhl mice which overexpress HIFs and VEGF develop heavily vascularized long bones, yet when these VEGF overexpressing osteoblasts are cultured independent from blood vessels in vitro they proliferate and differentiate similar to wild type osteoblasts (13). This suggests that VEGF produced by the osteoblast stimulates neoangiogenesis in a cell non autonomous fashion. Indeed capillaries are uniformly observed in bone modeling and remodeling compartments (30;31). We propose that the development of new blood vessels would serve to increase the number of active bone (re)modeling units and provide a conduit for supply of circulating bone precursor cells and/or delivery of vessel-derived factors required for bone cell differentiation. Current
studies in our lab are aimed at identifying the precise cellular events responsible for coupling angiogenesis to osteogenesis.

The apparent requirement for angiogenesis in bone formation suggested the possibility that agents that stimulate angiogenesis might promote bone regeneration. In this study, we provide proof of principle that small molecules with HIF activating activity can be delivered into regenerating bone to augment healing. Previous work has identified a number of inhibitors of prolyl hydroxylases which are required for ligation and proteosomal degradation of HIF (15;32-36). The two agents used in this study, DFO and L-mim, inhibit PHDs through different mechanisms. DFO chelates iron, a cofactor required for enzyme activity, whereas L-mim is a competitive inhibitor of another cofactor 2-oxoglutarate. Both agents increased angiogenesis in standard HUVEC tube formation and fetal metatarsal endothelial sprouting assays in vitro. DFO significantly increased angiogenesis and bone formation in the DO model in vivo. Thus, in agreement with the genetic models of HIF activation, pharmacologic stimulation of HIF activity also induced a robust angiogenic response that was coupled to a subsequent osteogenic response. However, in contrast to the genetic models in which HIF-1α was targeted to the osteoblast, direct delivery of DFO into the distraction gap would be expected to increase HIF activity in all cells including bone progenitors and endothelial cells. In this regard, the resultant new bone formation caused by DFO appeared to be even more robust than that seen in the genetic model, with evidence of new bone extending to all regions reached by the DFO solution. The overall efficacy and lack of overt toxicity of DFO is encouraging, given its potential to inhibit prolyl hydroxylation of collagen 1, and suggests some selectivity of this compound to target the HIF PHDs. Nonetheless, additional
PHD inhibitors, custom peptides containing the HIF oxygen degradation domain (ODD) (37) or transgenic constructs (38) are currently available and might represent useful alternatives to increase bone regeneration. The low cost and relative stability of PHD inhibitors are clearly advantageous and suggest promise as agents to improve blood supply to skeletal and other tissues which require angiogenesis to regenerate.
Methods

Generation of Conditional Knockout Mice

All procedures involving animals were in compliance with the guiding principles of the "Care and Use of Animals," and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Osteocalcin Cre (OC-Cre) expressing mice (39) were crossed with mice homozygous for a floxed Vhl allele (40) to obtain mice lacking Vhl, and therefore overexpressing HIFs in osteoblasts (ΔVhl). Hif1α conditional knockout mice (ΔHif1α) were created by crossing OC-Cre mice with mice homozygous for a floxed Hif1α allele (41). After appropriate breeding, ΔVhl and ΔHif1α mice were generated. These mice have been previously described (13). PCR of DNA from tail biopsies was used to confirm the genotype.

Distraction Osteogenesis Model

DO was performed in the left tibiae of eight week old mice as previously described (14). Briefly, a modified 6-mm track distractor (KLS Martin, Jacksonville, FL) was attached to the tibia with 0.010 in. ligature wire (3 M Unitek, Monrovia, CA) and an osteotomy was performed with a saw. Following 7 days of latency (no mechanical perturbation), distraction was performed at 0.15 mm/day for 10 days, for a total lengthening of 1.5 mm (days 7-17). Up to 21 days were then allowed for consolidation (days 18-38). Three pairs of mice were examined at the indicated timepoints for all DO experiments.
Radiographic Evaluation

Radiographs were performed using a Faxitron x-ray machine. If the device changed position from its original placement or lost proper alignment during the experimental period, the animal was not used for any analysis. Computed tomography was used to quantify bone healing using the µCT-40 system (Scanco Medical, Bassersdorf, Switzerland) and related analysis software. A volume of interest (VOI) was selected which contained the distraction osteogenesis area. A bone segmentation threshold setting of 204 was employed. Direct calculation of histomorphometric parameters was performed including bone volume (BV), total volume (TV), ratio of BV and TV (BV/TV), bone surface (BS), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N). To evaluate the vascularity, µCT angiography was performed as described by Duvall, et al (42). At the end of the active distraction phase (day 17), the animals were euthanized and perfused with a radiopaque silicone rubber compound containing lead chromate (Microfil MV-122, Flow Tech, Carver, MA). The distracted bones were removed, decalcified and imaged by µCT. The VOI was defined as the distraction zone. Histomorphometric values including vessel volume, connectivity, number, thickness, and separation, as well as degree of anisotropy were calculated.

Histology, Immunohistochemistry and in situ Hybridization Analysis

Tibae were also harvested, fixed, and decalcified, with specimens embedded in paraffin for hematoxylin and eosin (H&E) staining, immunohistochemistry and in situ hybridization. Immunostaining was performed per standard protocols after deparaffinization and rehydration. Sections were incubated with primary antibodies for pimonidazole
(Hypoxyprobe™-1) adducts (Millipore), HIF-1α (C-19, Santa Cruz Biotechnology Inc.), VEGF, CD31 and pVHL (BD Biosciences – PharMingen). An HRP-Streptavidin detection system was used (Vector Laboratories). In situ hybridization was performed as described previously (41) by using complementary ³⁵S-labeled riboprobes for mouse VEGF mRNA.

**Biomechanical Testing**

Mice were sacrificed at day 38, and tibiae collected and fresh frozen. Specimens were tested to failure by three-point bending on 858 MiniBionix Materials Testing System (MTS Systems, Eden Prairie, MN, USA). Stiffness, peak load and toughness were calculated from the force displacement data. Depth-control nanoindentation tests were performed on the same specimens using a Nanoindenter XP (MTS Systems, Oak Ridge, TN) with a Berkovich diamond indenter. Elastic modulus and hardness were calculated by the established method (43).

**In vitro Evaluation of Prolyl Hydroxylase Inhibitors**

A U2OS human osteosarcoma cell line stably expressing a luciferase reporter construct under the control of a hypoxia response element (U2OS-HRE-luc) (44) was used to quantify HIF activation in response to commercially available agents described in published literature. U2OS-HRE-luc cells were treated for 24 hours and luciferase activity was detected using the Bright-Glo luciferase reagent (Promega) and a luminometer. Cells cultured in hypoxia (1% O₂) served as positive controls.
**Immunoblotting Analysis**

For Western blotting, nuclear protein was extracted using NE-PER kit (Pierce). The extracts were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. After probing with primary antibodies, the membranes were incubated with horseradish peroxidase-linked secondary anti-rabbit antibodies (Cell Signaling Technologies, Inc.) and bound antibodies were visualized using the Supersignal West Femto Maximum Sensitivity Substrate (Pierce). Antibodies used were anti-HIF-1α (R&D Systems, Inc.) and anti-TATA box-binding protein (anti-TBP) (Abcam).

**Quantitative Real-Time PCR**

Further in vitro evaluation of PHD inhibitors was performed using bone marrow mesenchymal stem cells (MSCs) isolated from marrow flushes of the femora and tibiae of normal FVBn mice. Ficoll column purification was performed and the adherent cells were subcultured. Cells were exposed to DFO or L-mim for 24 hours. Total RNA was extracted by the TRIZOL protocol (Invitrogen). Real time PCR was performed at 57 °C for 30 cycles in the Opticon Continuous Fluorescent Detector using IQTM SYBR Green supermix (Bio-Rad, Hercules, CA, USA). Triplicates were performed and results were normalized to β-actin. We used the following primers: *VEGF-A*: F5′- CCACGTCAGAGCAACATCA -3′ and R5′- TCATTCTCTCTATGTGCTGGCTTT -3′.

**In vitro Angiogenesis Assays**

Effects on endothelial cells were evaluated using a matrigel tube formation assay (45). HUVEC were cultured on matrigel chambers with the addition of DFO or L-mim
with VEGF or VEGF antibody as positive and negative controls. Numbers of tube-like structures were counted after 12 hours incubation. Metatarsal explant cultures were performed as previously described (46). Metatarsals from 17.5 day embryos were dissected and cultured in α-MEM with 10% FBS. Experimental bones were exposed to DFO or L-mim for 24 hours and medium was changed every 3 days. After 7 days, cultures were fixed and immunostained for CD31.

**ALP Staining**

Mouse MSCs were plated on 6-well plates with a density of $10^5$ cells/well and cultured in α-MEM until they were confluent. Medium was then changed to osteogenic medium (supplemented with dexamethasone ($10^{-8}$ M), β-glycerophosphate (10 mM) and ascorbic acid (50 µg/ml)) for 14 days, with the medium changed every 3 days. Histochmical 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.

**Administration of VEGFR Antibodies in DO Model**

To evaluate whether effects seen in the ΔVhl mice were mediated by VEGF, mice were treated with monoclonal antibodies against mouse VEGF receptors (VEGFR-1 (clone mF-1) and VEGFR-2 (clone DC101) (ImClone Systems)). Intraperitoneal injections were performed every 3 days after surgery for a total of 5 injections. Non-immune IgG injection served as a negative control.
Administration of PHD Inhibitors in DO Model

For pharmacologic activation, C57BL/6 mice were injected with 20 µL of saline (control) or DFO 200 µM in the distraction gap every other day from days 7-17, for a total of 5 doses. To confirm correct placement of injection, methylene blue injections were also performed.

Statistical Analysis

Comparisons were made using student’s t-test or Mann-Whitney test. Results were expressed as mean ± SD. Significance level used was \( P < 0.05 \).
Acknowledgements

We thank Tim Nagy and Maria Johnson for support on μCT analysis; Shafiul Chowdhury for assistance with nanoindentation. This work was supported by a grant from the NIH AR49410 (TLC) and a pilot grant through the UAB Core Center for Basic Skeletal Research P30AR046031 (SRG).
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Figure 1. Development of hypoxia and expression of HIF-1α pathway components during DO. (A) 8-week-old C57BL/6 mice were subjected to DO. Bone tissues were harvested at days 17, 24 and 38 following surgery. Representative X-ray images of distracted tibiae and H&E stained sections of distraction site show the progressive new bone formation in the distraction gap (between dashed lines represents distraction gap). (B) Mice were injected with pimonidazole immediately prior to distraction, and tissues were harvested 1 day after distraction. Hypoxia in the distraction gap was determined by immunostaining with an anti-pimonidazole adducts antibody. Black arrows indicate positive-stained bone-lining cells at low (middle, ×200) and high (right, ×400) magnification. (C) Representative histological sections of the distraction site from tissues harvested at day 24 post-surgery. VEGF mRNA expression in bone-lining cells (white arrows) is shown by in situ hybridization analysis. Sections were stained with antibodies against HIF-1α, VEGF, and CD31. Black arrows indicate positively stained cells in newly formed bone.
Figure 2. Genetic activation of the HIF-1α pathway increases neoangiogenesis and promotes bone regeneration. (A) 8-week old ΔVhl mice and control littermates were subjected to DO. Tissues were harvested at day 31 post-surgery and histological sections of distraction gap were prepared. Representative sections from the ΔVhl mice and controls

B

C

D

Figure 2. Genetic activation of the HIF-1α pathway increases neoangiogenesis and promotes bone regeneration. (A) 8-week old ΔVhl mice and control littermates were subjected to DO. Tissues were harvested at day 31 post-surgery and histological sections of distraction gap were prepared. Representative sections from the ΔVhl mice and controls
are shown after staining with antibodies against pVHL and HIF-1α. VEGF mRNA expression in bone-lining cells is shown by in situ hybridization and immunostaining, CD31 immunostaining is also shown. Black arrows show positive cells. (B) Representative µCT images of vasculature in Microfil-perfused distraction area from control and ∆Vhl mice at day 17 post-surgery. Quantitative measurements of vessel volume per total volume (VV/TV) and vessel number are shown. Data represent mean ± SD. *P < 0.05. (C) Representative µCT images of distraction area from control and the ∆Vhl mice at day 38 post-surgery. Quantitative measurements of bone volume per total volume (BV/TV) and trabecular separation (Tb.Sp) are shown. Data represent mean ± SD. *P < 0.05. (D) Three point bending tests (peak load and stiffness) and nanoindentation (elastic modulus and hardness) were performed on tibiae from the ∆Vhl mice and controls at day 38 post-surgery. Data shown represent mean ± SD. *P < 0.05. Con, control.
Figure 3. VEGFR is required for neoangiogenesis during DO. 8-week old C57BL/6 mice were injected intraperitoneally with monoclonal antibodies against VEGFR-1 and -2 every 3 days after surgery for a total of 5 injections. Non-immune IgG injection served as a negative control. At day 17 post-surgery, mice were perfused with Microfil and analyzed for vessel formation in the distraction gap. (A) Representative µCT images of vasculature in Microfil-perfused distraction area were shown. (B) Quantitative measurements of vessel volume per total volume (VV/TV), vessel number, vessel separation, and vessel surface are shown. Data represent mean ± SD. *P < 0.05, **P < 0.01.
Figure 4. Disruption of Hif1α in osteoblasts impairs angiogenesis and bone regeneration during DO. (A) Representative µCT images of vasculature in Microfil-perfused distraction area from control and ΔHif1α mice at day 17 post-surgery. Quantitative measurements of vessel volume per total volume (V/V) and vessel number are shown. (B) Representative µCT images of distraction area from control and ΔHif1α mice at day 38 post-surgery. Quantitative measurements of bone volume per tissue volume (BV/TV) and BV are shown. Data represent mean ± SD. *P < 0.05. Con, control.
Figure 5. Pharmacological activation of the HIF-1α pathway increases angiogenesis in vitro. (A) U2OS cells expressing an HRE reporter gene were exposed to hypoxia (Hyp), or treated with cobalt chloride (CoCl₂, 125 µM), DFO (200 µM), ethyl 3,4-dihydroxybenzoate (DHB, 700 µM), or L-mim (700 µM) under normoxia. Cells were harvested 24 hours after treatment and analyzed for luciferase activity. (B) U2OS cells were treated with DFO or L-mim at different doses for 24 hours followed by nuclear protein extraction. HIF-1α level in the nucleus was determined by immunoblotting analysis using an anti-HIF-1α monoclonal antibody. Immunoblot for TBP (TATA box-binding protein) was used as loading control. (C) Mesenchymal stem cells were collected from bone marrow of FVB/N mice following standard method and cultured until confluent. Cells were untreated (control), treated with DFO (10 and 200 µM) under normoxia or ex-
posed to hypoxia (Hyp) as a positive control for 24 hours. Total RNA was extracted from cells and VEGF mRNA expression was determined using quantitative real-time PCR. (D) Matrigel tube formation assay. HUVEC were cultured on matrigel chambers with the addition of DFO (50 and 200 µM) or L-mim (300 and 500 µM) with VEGF (10 ng/ml) as positive control. Tube formation was photographed 12 hours after treatment. Magnification, ×100. (E) Quantification of tube formation assay by counting tube-like structure numbers. Data represent mean ± SD. *P < 0.05. (F) In vitro metatarsal endothelial sprouting assay. Metatarsals were dissected from C57BL/6 E17.5 fetuses and cultured for 3 days for attachment. The explants were then cultured for another 6 days and then treated with DFO (50 µM) or L-mim (300 µM) for 24 hours with rhVEGF (10 ng/ml) as positive control followed by the detection of endothelial sprouting by immunostaining with anti-CD31 monoclonal antibody. Representative images are shown. Magnification, ×25.
Figure 6. Pharmacological activation of the HIF-1α pathway by DFO increases angiogenesis and promotes bone regeneration. 8-week-old wild type (C57BL/6) mice were subjected to DO. Mice were injected with DFO (200 µM) or saline as control in the distraction gap every other day from days 7-17 post-surgery. (A) Validation of local injection approach using methylene blue following the same protocol with DFO treatment. X-ray images show bone regeneration in the distraction gap in DFO treated and control mice at day 38 post-surgery. (B) Representative µCT images of vasculature in Microfil-perfused distraction area from DFO treated and control mice at day 17 post-surgery. (C) Representative µCT images of distraction area from DFO treated and control mice at day 38 post-surgery. (D) Quantitative measurements of vessel number and connectivity are shown. Data represent mean ± SD. *P < 0.05. (E) Quantitative measurements of bone volume and bone volume per total volume are shown. Data represent mean ± SD. *P < 0.05.
Supplemental Figure 1. Histological evidence of enhanced bone regeneration in the ΔVhl mice. H&E staining was performed on histological sections of distraction gap from control and the ΔVhl mice at days 24, 31 and 38 post-surgery. More dense woven bone was generated in the DO gap of the ΔVhl mice during consolidation phase (between dashed lines represent distraction gap; b, bone; m, marrow). Original magnification, ×40.
Supplemental Figure 2. rhVEGF stimulates metatarsal endothelial sprouting in a time-dependent manner. Metatarsals were dissected from C57BL/6 E17.5 fetuses and cultured for 3 days. The explants were then treated with rhVEGF (10 ng/ml) for another 5, 7, or 10 days. Cultures were fixed and processed for the detection of endothelial sprouting by immunostaining with anti-CD31 monoclonal antibody. Representative images are shown. Magnification, ×25.
Supplemental Figure 3. PHD inhibitors do not affect osteoblastic differentiation of cultured mouse MSCs. When cultures became confluent, MSCs were treated with DFO (50 and 200 µM) or L-mim (300 and 500 µM) for 24 hours before adding the osteogenic supplements. Alkaline phosphatase (ALP) staining was performed 14 days later. Representative images are shown.
CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation project, I have presented genetic evidence that the HIF 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 during development, and this increased bone formation primarily due to VEGF-mediated angiogenesis. Conversely, disruption of Hif1α in osteoblasts results in the opposite phenotype, i.e., thinner and less vascularized bones. These results enable the construction of a model for the role of the HIF pathway in bone formation. In this model (Figure 1), osteoblasts sense reduced oxygen tension and upregulate HIFα subunits. HIF-1α and HIF-2α then translocate into the nucleus and transactivate HIF target genes such as Vegf to stimulate new blood vessel formation and invasion into bone. In one possible scenario, the increasing numbers of new blood vessels introduce more osteoblast progenitors, which then mature and function to form more individual bone formation units. Alternatively, it is also possible that VEGF might act in an autocrine or paracrine mode to directly stimulate osteoblast differentiation, thereby accelerating bone mineralization (23;25). However, the precise mechanisms underlying VEGF-stimulated osteogenesis remain to be established.

As discussed in the Introduction, VEGF can function independently of angiogenesis on chondrocyte differentiation, osteoclast recruitment, and osteoblast differentiation (18). Therefore, as a first step in determining whether VEGF can directly influence os-
Figure 1. Working model of the role of the HIF pathway in bone formation. The HIF pathway is activated in the resident osteoblasts, leading to the upregulation of VEGF, which works through two possible modes to promote bone formation. In one mode, VEGF level increases vessel growth into bone, bringing in more osteoblast progenitors, which mature and function to form bone. In a second possible mode, VEGF directly stimulates proliferation of endothelial cells, from which BMPs and other factors are produced and function to promote osteoblast differentiation and mineralization to form new bone.


I examined the effect of VEGF on osteoblastic differentiation using the in vitro system. These preliminary results show that cultured mouse primary MSCs have a low level of expression of VEGFR-1 and -2 mRNA, which may explain why VEGF does not affect osteoblastic differentiation in these cells. These results suggest that VEGF produced by the osteoblast may act on endothelial cells to indirectly promote osteoblastic differentiation. It is known that VEGF can induce the production of BMP-2 by endothelial cells (53). This possibility will be further tested in an endothelial cell-MSC coculture.
system, in which E17.5 metatarsals will be cocultured with MSCs. In one set of experiments, normal metatarsals will be cultured together with MSCs and treated with VEGF. In a parallel set of experiments, metatarsals from \( \Delta Vhl \) mice that express elevated levels of endogenous VEGF in bone, will be cocultured with MSCs and examined for osteoblastic differentiation without treatment of VEGF. Since VEGF does not directly affect osteoblastic differentiation of MSCs, any observed changes in differentiation should solely result from an effect of endothelial cells.

In the first part of my dissertation study, I presented evidence for a functional redundancy between HIF-1\( \alpha \) and HIF-2\( \alpha \). Analysis of bone in double mutant mice lacking both \( Hif1 \alpha \) and \( Vhl \) revealed a bone phenotype intermediate between \( \Delta Vhl \) and \( \Delta Hif1 \alpha \) single mutants, but a vessel phenotype similar to \( \Delta Vhl \) single mutants. To further elucidate the role of HIF-2\( \alpha \) in bone development, we are generating another mouse strain with osteoblast-specific deletion of \( Hif2 \alpha \). We plan to characterize the \( Hif2 \alpha \) deficient mice to determine whether they have a bone phenotype which is distinct from that seen in \( \Delta Hif1 \alpha \) mice. We also plan to generate double knockout mice lacking both \( Hif1 \alpha \) and \( Hif2 \alpha \) in osteoblasts. A triple mutant lacking \( Vhl \), \( Hif1 \alpha \) and \( Hif2 \alpha \) will also be created. Phenotypes of \( Hif1 \alpha \) and \( Hif2 \alpha \) double mutants will be compared with that from \( Hif1 \alpha \) and \( Hif2 \alpha \) single mutants to confirm the role of HIF-1\( \alpha \) and HIF-2\( \alpha \), as well as the relationship between these two isoforms during bone development. The phenotype of the triple mutant mice will provide information as to whether other pVHL-regulated factors, besides HIFs, function during skeletal development.

In the second part of my dissertation research, I demonstrated that tissue hypoxia which develops following a standard surgical distraction procedure in normal mice is ac-
accompanied by upregulation of the HIF/VEGF pathway and results in neoangiogenesis. Using the established genetic mouse models, we demonstrated that HIF-1α is required to stimulate VEGF production and to mount a normal angiogenic and osteogenic response in a mouse model of bone repair.

One piece of important information that has not been obtained from this dissertation research relates to the mechanism responsible for coupling angiogenesis and osteogenesis during embryonic skeletal development. Recently, pericytes, the microvascular smooth muscle cells, have been identified as an important osteoprogenitor (54-57). Pericytes have multilineage potential and are capable of differentiating into smooth muscle cells, osteoblasts, chondrocytes, and adipocytes (58). They are anatomically juxtaposed to the endothelial capillary network, and express features of early vascular smooth muscle cell lineage, including smooth muscle 22 kDa (SM22), α-smooth muscle actin (α-SMA), pericyte-associated antigen 3G5 and Nerve/Glial antigen 2 (NG2) proteoglycan. Interestingly, MSCs also possess features of pericytes (54;56). From this point of view, MSCs could be viewed as tissue-specific pericytes (59). During vasculogenesis, the pericyte is essential for normal blood vessel development and produces VEGF that acts to stabilize newly formed vessels (60). At the same time, pericytes may serve as a reservoir of primitive precursor cells, suggesting that pericyte-produced VEGF not only promotes early angiogenesis but also plays a role to stimulate osteoblastic differentiation of pericyte itself in an autocrine manner. Indeed, recent work using mice expressing a Col3.6-GFP reporter has shown that during de novo osteogenesis, preosteoblasts express pericyte markers SM22 and α-SMA, suggesting that skeletal osteoblasts do arise from the pericytes in vivo (61).
To begin to investigate the mechanism for angiogenesis-osteogenesis coupling, I plan to use mice heterozygous for the \textit{LacZ} gene under control of the VEGF promoter (VEGF reporter mice) established by Miquerol et al. (62). These mice will enable us to monitor VEGF expression throughout embryonic development. Long bones, such as femur, will be isolated at different developmental stages, and fixed for immunolabeling. Three antibodies including anti-\textit{βGal}, anti-NG2, and anti-osteocalcin will be used to localize VEGF, pericytes, and osteoblasts, respectively. Confocal analysis will reveal the spatial relationship between pericytes and VEGF, as well as pericytes and osteoblasts.

As discussed in the first portion of my dissertation research, osteoblast progenitors could also derive from circulation (63). To explore this possibility, we have obtained an Osx-cre-ER\textsuperscript{T2} transgenic mouse that selectively expresses the tamoxifen-inducible cre-ER\textsuperscript{T2} recombinase under the control of a mouse osterix (Osx) promoter fragment in osteoblasts. In this mouse line, the Cre recombinase is linked to a mutant form of the ligand-binding domain of the estrogen receptor (ER), which can only be activated by tamoxifen, and not by estradiol (64). We also established a reporter mouse line by cross-breeding Osx-cre-ER\textsuperscript{T2} mouse with a Cre-dependent \textit{LacZ} reporter strain (ROSA26). The ROSA26 mouse has a target-inserted \textit{LacZ} gene which was preceded by a floxed strong transcriptional termination sequence, so the transcription of \textit{LacZ} is prematurely terminated. In the Osx-cre-ER\textsuperscript{T2}-\textit{LacZ} reporter mice, when Cre recombinase activity is induced, the Cre-mediated excision of the floxed termination sequence leads to constitutive \textit{LacZ} expression. Thus, these double transgenic mice express \textit{LacZ} only in osteoblasts that express Cre, which can be visualized by X-galactosidase staining. Distrac-
tion osteogenesis will be performed on these Osx-cre-ER\textsuperscript{T2}-\textit{LacZ} reporter mice to investigate the efficiency and selectivity of tamoxifen-induced Cre expression.

Following the establishment of efficient tamoxifen induction of Cre expression in osteoblasts, we will join the circulation from Osx-cre-ER\textsuperscript{T2}-\textit{LacZ} reporter mice with that from nude mice through a process called parabiosis. This allows the circulating cells in the Osx-cre-ER\textsuperscript{T2}-\textit{LacZ} reporter mice present in the circulation of nude mice. By doing distraction on the nude mice, the presence of Osx-positive cells can be monitored in the distraction gap. If labeled cells appear in the distraction gap, it is possible to conclude that osteoblast progenitors derive from circulation. In order to avoid possible effects of immune rejection, we will perform local irradiation on tibiae prior to distraction osteogenesis, and then check the arrival of osteoblasts that required for new bone formation in the distraction gap.

In summary, I believe these follow-up studies will contribute to a better understanding of angiogenic-osteogenic coupling during bone development and following bone injury, and aid in the design of new therapies to accelerate bone healing following injury.
GENERAL LIST OF REFERENCES


APPENDIX

IACUC APPROVAL FORM
NOTICE OF APPROVAL

DATE: April 4, 2007

TO: Thomas L. Clemens, Ph.D.
   VH-0001A 0019
   FAX: 934-1775

FROM: Judith A. Kapp

Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Oxygen Sensing and Osteogenesis
         Sponsor: NIH
         Animal Project Number: 070307044

On March 28, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>1387</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from March 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070307044 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1630 3RD AVE S
BIRMINGHAM AL 35294-0019
MEMORANDUM

DATE: April 4, 2007

TO: Thomas L. Clemens, Ph.D.
VH-G001A 0019
FAX: 934-1775

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on March 28, 2007.

Title of Application: Oxygen Sensing and Osteogenesis
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).