Published in final edited form as:

Bone. 2015 April ; 0: 98–104. doi:10.1016/j.bone.2014.12.015.

HIF-1 α Regulates Bone Formation after Osteogenic Mechanical Loading

Ryan E. Tomlinson^a and Matthew J. Silva^a

^aDepartments of Orthopaedic Surgery and Biomedical Engineering, Musculoskeletal Research Center, Washington University in St. Louis, 425 S. Euclid Avenue, St. Louis MO 63110

Abstract

HIF-1 is a transcription factor typically associated with angiogenic gene transcription under hypoxic conditions. In this study, mice with HIF-1 α deleted in the osteoblast lineage (HIF-1 α) were subjected to damaging or non-damaging mechanical loading known to produce woven or lamellar bone, respectively, at the ulnar diaphysis. By microCT, HIF-1 α mice produced significantly less woven bone than wild type (WT) mice 7 days after damaging loading. This decrease in woven bone volume and extent was accompanied by a significant decrease in vascularity measured by immunohistochemistry against vWF. Additionally, osteocytes, rather than osteoblasts, appear to be the main bone cell expressing HIF-1 α following damaging loading. In contrast, 10 days after non-damaging mechanical loading, dynamic histomorphometry measurements demonstrated no impairment in loading-induced lamellar bone formation in HIF-1 α mice. In fact, both non-loaded and loaded ulnae from HIF-1 α mice had increased bone formation compared to WT ulnae. When comparing the relative increase in periosteal bone formation in loaded vs. non-loaded ulnae, it was not different between HIF-1 α mice and controls. There were no significant differences observed between WT and HIF-1 α mice in endosteal bone formation parameters. The increases in periosteal lamellar bone formation in HIF-1 α mice are attributed to non-angiogenic effects of the knockout. In conclusion, these results demonstrate that HIF-1 α is a pro-osteogenic factor for woven bone formation after damaging loading, but an anti-osteogenic factor for lamellar bone formation under basal conditions and after non-damaging loading.

Keywords

Hypoxia Inducible Factor; HIF-1 α ; Mechanical Loading; Osteogenesis

© 2014 Elsevier Inc. All rights reserved.

Corresponding Author: Ryan E. Tomlinson, 720 N. Rutland Avenue, Ross 209C, Baltimore, MD 21211, +1-410-502-9443 (phone), +1-443-287-4428 (fax), ryan.tomlinson@jhmi.edu, Matthew J. Silva, 425 S. Euclid Avenue, Campus Box 8233, St. Louis MO 63110, silvam@wustl.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 Introduction

Hypoxia-inducible factor 1 (HIF-1) was initially reported as a nuclear factor for transcriptional activation in response to reduced O₂ concentration (hypoxia) [1]. Subsequently, it was identified as a basic helix-loop-helix heterodimeric protein consisting of two subunits designated HIF-1 α and HIF-1 β [2, 3]. HIF-1 α is expressed ubiquitously throughout the body. In normoxic conditions, HIF-1 α is rapidly degraded by the ubiquitin-proteasome pathway, but in conditions of hypoxia it remains stable [4]. HIF-1 stability is one of the methods by which all nucleated cells in the body sense and respond to O₂ availability [5]. As a result, HIF-1 has been called the “highly involved factor” [6], since it has a role in a wide variety of hypoxia-related processes required for development and homeostasis, including angiogenesis, erythropoiesis, and vasomotor control [7].

HIF-1 α plays an important role in coupling angiogenesis and osteogenesis, particularly in skeletal healing and development [8–11]. During fracture healing, HIF-1 target genes such as VEGF, PGF, and SDF-1 are both spatially and temporally regulated [12–15]. VEGF in particular is also responsible for orchestrating the vascular invasion of hypertrophic cartilage that establishes the primary ossification center in developing mouse long bones [16, 17]. Mice lacking HIF-1 α in the osteoblast lineage have narrow, poorly vascularized long bones at 3 weeks of age, despite normal expression of HIF-1 α in the surrounding tissues [18]. Moreover, these mice do not produce adequate vascularity to support woven bone induction during distraction osteogenesis [19], consistent with the requirement of angiogenesis in this process [20]. However, by 24 weeks of age, bones from mice lacking HIF-1 α in the osteoblast lineage have normal cortical thickness and moment of inertia as well as significantly increased bone area compared to wild type controls [21]. Consistent with increased bone apposition in adult mice, these mice have enhanced periosteal lamellar bone formation when subjected to mild tibial mechanical loads [21]. Taken together, these results indicate that HIF-1 α plays a complex role in postnatal osteogenesis.

In general, repetitive mechanical loading of the skeleton is a potent stimulus of bone formation. When applied at hyperphysiological strain levels for many cycles, mechanical loading produces fatigue damage that can progress to a non-displaced stress fracture and stimulate periosteal woven bone formation [22]. In this setting, woven bone formation is associated with the upregulation of angiogenic genes (*Vegf*, *Pecam1*, *Hif1 α*) and a dramatic downregulation of the Wnt antagonist Sclerostin (*Sost*) [23, 24]. Robust expression of HIF-1 α has been observed in the inflammatory cells located in the expanded periosteal region as soon as 1 day after loading, with a peak in gene expression at day 7 [22, 25]. Additionally, woven bone formation after stress fracture is preceded by increased periosteal vascularity [25, 26] and is impaired by angiogenic inhibition [27]. In contrast to fatigue loading, mechanical loading applied near physiological strain levels for fewer cycles does not produce damage, and stimulates lamellar bone formation. Loading-induced lamellar bone formation is preceded by modest upregulation of angiogenic genes (*Vegf*, *Hif1 α*) [25]. A small increase in vascularity is detectable after lamellar bone formation has occurred, but loading-induced lamellar bone formation does not depend on angiogenesis [28].

In this study, mice with HIF-1 α selectively removed from the osteoblast lineage were subjected to either damaging or non-damaging ulnar mechanical loading that triggered the formation of woven or lamellar bone, respectively. Bone formation was assessed using microCT and dynamic histomorphometry, and vascularity was quantified by immunohistochemistry. The overall goal of this study was to determine the influence of osteoblastic HIF-1 α on the postnatal formation of woven and lamellar bone using a single mechanical loading model.

2 Materials and Methods

2.1 HIF-1 α Mice

Mice with HIF-1 α selectively removed from the osteoblast lineage with high efficiency (>90% by mRNA) have been previously described [18]. Briefly, mice with the second exon of HIF-1 α flanked with loxP sites (floxed) [29] were crossed with mice expressing cre recombinase under the control of the osteocalcin (OC) promoter [30]. HIF-1 $\alpha^{fl/fl}$; OC-cre⁺ mice are referred to as HIF-1 α mice, and HIF-1 $\alpha^{fl/fl}$; OC-cre⁻ mice are considered wild type (WT) controls. PCR was used to determine expression of OC-cre using 5'-CAAATAGCCCTGGCAGATTC-3' (forward) and 5'-TGATACAAGGGACATCTTCC-3' (reverse) primers. Additionally, PCR was also used to identify the loxP site on the second exon of HIF-1 α with 5'-TGATGTCCCTGCTGGTGTC-3' (forward) and 5'-TTGTGTTGGGGCAGTACTG-3' (reverse) primers.

2.2 Study Design

Female mice were housed under standard conditions until 18–22 weeks of age, to ensure skeletal maturity [31, 32] and allow approximate normalization of any cortical bone phenotype [21]. At the time of loading, there was no significant difference in weight between WT (23.1 ± 1.4 g) and HIF-1 α (22.6 ± 2.0 g) mice. The right forelimb of each mouse was mechanically loaded using one of two loading protocols designed to induce woven bone formation (WBF) or lamellar bone formation (LBF). For each animal, the contralateral (left) forelimb was used as a non-loaded control. Euthanasia was by CO₂ asphyxiation. WBF forelimbs were analyzed by microCT to assess woven bone volume and density, then decalcified and embedded in paraffin for histological analysis. LBF forelimbs were embedded in PMMA and sectioned for dynamic histomorphometry to assess measures of lamellar bone formation. All animal protocols were approved by the Animal Studies Committee of Washington University in St. Louis. Results are given as fold changes (loaded limb / non-loaded limb) and plotted as mean $\pm$ standard deviation. Statistical evaluation was performed using t-tests (Statview 5.0, SAS Institute Inc.) with p-value < 0.05 considered significant.

2.3 Mechanical Loading Protocols

Mechanical loading of the right ulna of each animal was performed as previously described [33]. Briefly, the right forelimb was axially compressed by placing the olecranon process and the flexed carpus into specially designed fixtures. Animals were anesthetized using isoflurane gas (1–3%) during loading. A material testing system (Instron Electropuls 1000) was used to apply force and monitor displacement. Similar to previous studies in mice [22]

and rats [34], loading parameters were selected as a function of ultimate force and total displacement to fatigue fracture as measured during preliminary work. First, ultimate force was determined using axial monotonic compression to failure by displacement ramp (0.1 mm/s). Next, total displacement to fatigue fracture was determined using a cyclic haversine waveform of 3.5 N (80% of the ultimate force) at 2 Hz. Animals were euthanized immediately following either procedure. There were no significant differences between WT and HIF-1 α mice in ultimate force (4.38 ± 0.17 N vs 4.45 ± 0.21 N) or average total displacement to failure (0.89 ± 0.08 mm vs. 0.90 ± 0.13 mm), so loading parameters for subsequent survival experiments were the same for both genotypes.

For WBF loading of experimental animals, a 0.3 N compressive pre-load was applied followed by a cyclic haversine waveform of 3.5 N at 2 Hz until a total displacement of 0.5 mm relative to the 10th cycle was achieved. In this loading model, the magnitude of displacement increase is an external index of internal ulnar damage [22]. This amount of displacement was equivalent to 55% of the average total displacement to fatigue fracture (0.9 mm), reliably producing an ulnar stress fracture centered at 1 mm distal to the ulnar midpoint. Although the number of cycles required to reach 0.5 mm of displacement varied widely between animals (range: 743 to 10257 cycles), there was no significant difference between WT (6066 ± 2771 cycles) and HIF-1 α (4358 ± 3239 cycles) mice. For LBF loading, a 0.3 N compressive pre-load was applied followed by a cyclic rest-inserted trapezoidal waveform with a peak force of 3.0 N at 0.1 Hz for 100 cycles, similar to multi-day ulnar loading protocols used previously in the mouse [35–38]. The single loading bout protocol used here was modified from a similar procedure in rats [25], and stimulates strain-adaptive bone modeling via lamellar apposition. After loading, mice were given an intramuscular injection of analgesic (0.05 mg/kg buprenorphine) and allowed unrestricted cage activity. Mice were euthanized 3, 7, or 10 days after loading for subsequent analysis.

2.4 MicroCT Analysis

Woven bone formation was analyzed using *ex vivo* micro computed tomography (μ CT40, Scanco Medical AG) in WBF loaded animals 7 days after damaging loading, a timepoint when abundant woven bone is observed in this model [22]. The central 9 mm of each loaded ulna was scanned separately at 70 kV and 114 μ A with 200 msec integration time. The scan tube diameter was 12.3 mm, and medium resolution was used to obtain a 12 μ m voxel size. Scan slices were acquired in the transverse plane by placing the forelimb parallel to the z-axis of the scanner. Hand drawn contours (sigma = 1.2, support = 2, lower/upper threshold = 150/1000) were used to manually segment bone with Scanco imaging software. Woven bone volume was calculated by subtracting the original cortical bone volume from the total bone volume in the entire scan. Woven bone extent was quantified by measuring the axial length of woven bone formation along the ulna. Woven bone BMD was calculated by analyzing only woven bone in the middle 20 slices of the woven bone extent. Finally, the crack extent following WBF loading was measured as the axial length of apparent cortical cracking. Previous studies have demonstrated that microCT assessment of woven bone corresponds well with dynamic histomorphometric assessment [22], so separate histomorphometry was not performed for WBF groups.

2.5 Immunohistochemistry

HIF-1 α expression and vascularity was visualized using immunohistochemistry in WBF loaded limbs at 3 and 7 days. Intact forelimbs were harvested and fixed overnight in 10% NBF, then decalcified in 14% EDTA for 14 days. Following this, each bone was embedded in paraffin to generate thin (5 μ m) sections from 1 mm distal to the ulnar midpoint. Sections were deparaffinized in xylenes and rehydrated in graded ethanol solutions. Antigen retrieval was performed by overnight incubation in 0.33 M boric acid (Sigma, B6867) at 55 °C. A 20-minute incubation in 3% H₂O₂ was used to block endogenous peroxidase activity, then sections were incubated in normal goat serum (sc-2043, Santa Cruz – 1.5% in PBS) to reduce nonspecific background staining. Following this, slides were incubated in 1:200 dilution of rabbit polyclonal antibody against HIF-1 α (sc-10790, Santa Cruz) or vWF (AB7356, Millipore) at 4 °C overnight. Negative control slides were prepared by substituting normal goat serum for the primary antibody. To visualize binding, biotinylated goat anti-rabbit (sc-2018, Santa Cruz) secondary antibody was applied for 30 minutes followed by avidin-biotin-peroxidase complex for 30 minutes. Finally, slides were developed using diaminobenzidine for 60 seconds, dehydrated, and mounted. Digital images of these sections were captured using bright field microscopy (Olympus BX-51) with a 20X or 40X objective. Imaging stitching and manual quantification was performed using FIJI [39].

2.6 Dynamic Histomorphometry

Lamellar bone formation rates in LBF loaded animals were quantified by dynamic histomorphometry. Mice were given two intraperitoneal injections of fluorescent bone formation markers. Calcein (10 mg/kg, Sigma C0875) was administered 3 days after loading, and Alizarin Complexone (30 mg/kg, Sigma A3882) was administered 8 days after loading; animals were euthanized on day 10. Following fixation, forelimbs were embedded in poly-(methyl methacrylate). 100 μ m thick transverse sections were cut (SP 1600, Leica Microsystems) at 1 mm distal to the ulnar midpoint, then polished to 30 μ m and mounted on glass slides. Digital images of these sections were captured using fluorescence microscopy (Olympus IX-51) with fluorescein isothiocyanate (FITC – excitation 465–495 nm, emission 515–555 nm) and tetramethylrhodamine isothiocyanate (TRITC – excitation 515–565 nm, emission 550–660 nm) filters for visualization of calcein (green) and alizarin (red), respectively. Images were analyzed in Bioquant OSTEO for endocortical (Ec) and periosteal (Ps) bone formation rate (BFR/BS), mineral apposition rate (MAR), and mineralizing surface (MS/BS), as defined by the ASBMR committee for histomorphometry nomenclature [40].

3 Results

3.1 Woven bone formation after damaging loading is impaired in HIF-1 α mice

Immunohistochemistry was used to localize HIF-1 α expression following damaging (WBF) loading as well as verify the efficacy of the knockout. Previous work using qPCR has shown that HIF-1 α expression is maximal on days 3 and 7 following WBF loading [22]. Here, HIF-1 α was strongly expressed at day 3 in the inflammatory and stromal cells present in the expanded periosteal region in both WT and HIF-1 α mice (Figure 1a,b - bracket). HIF-1 α

was also expressed by bone cells, with $63 \pm 4\%$ of osteocytes in the original cortical bone positively stained (Figure 1a). As expected, less than half as many osteocytes in HIF-1 α mice were positively stained ($29 \pm 8\%$, $p < 0.05$ vs. WT, Figure 1b). Similarly, $51 \pm 6\%$ of the osteocytes in the original cortical bone of WT mice had strong expression of HIF-1 α at day 7, with some osteoblasts in the newly formed woven bone also positive for HIF-1 α (Figure 1c). Again, less than half as many osteocytes in HIF-1 α mice were positively stained for HIF-1 α at day 7 ($19 \pm 3\%$, $p < 0.05$ vs. WT, Figure 1d). By day 7, limited HIF-1 α expression was observed in the skeletal muscle adjacent to bone in both WT and HIF-1 α mice, a stark contrast to the inflammatory cells present at day 3, and similar to HIF-1 α expression routinely observed in normoxic skeletal muscle [41, 42].

MicroCT and immunohistochemistry were used to quantify woven bone formation and vascularity, respectively, in WT and HIF-1 α mice 7 days after WBF loading. The extent of the cortical crack was not significantly different between WT (0.37 ± 0.08 mm) and HIF-1 α (0.42 ± 0.13 mm) mice, indicating each genotype received a similar level of fatigue damage by WBF loading. In response to this injury, both WT (Figure 2a) and HIF-1 α (Figure 2b) mice produced abundant woven bone, but HIF-1 α mice had significantly less (-22%) woven bone volume compared to WT mice (Figure 2c). Similarly, woven bone extent was significantly less (-21%) in HIF-1 α mice compared to WT mice (Figure 2d). On the other hand, there was no difference in woven bone BMD between WT and HIF-1 α mice (Figure 2e), indicating normal mineralization. WBF loading stimulated significant angiogenesis in both HIF-1 α and WT mice (Figure 3a). However, HIF-1 α mice had significantly fewer (-35%) blood vessels in the expanded periosteal region compared to WT mice (Figure 3b).

3.2 Lamellar bone formation after non-damaging loading is enhanced in HIF-1 α mice

Dynamic histomorphometry was used to quantify bone formation rates in WT and HIF-1 α mice after a single bout of non-damaging (LBF) loading (Figure 4). No woven bone was observed in the LBF groups. Periosteal lamellar bone formation was greatly increased as a result of mechanical loading in both genotypes. Ps.MS/BS, Ps.MAR, and Ps.BFR/BS were all significantly increased in both WT and HIF-1 α loaded limbs compared to the non-loaded control limbs (Figure 5). Comparing genotypes, HIF-1 α loaded limbs generated more lamellar bone than WT loaded limbs, with increased Ps.MAR and Ps.BFR/BS compared to WT loaded limbs. HIF-1 α non-loaded limbs also had significantly increased periosteal bone formation (Ps.MS/BS, Ps.MAR, and Ps.BFR/BS) compared to WT non-loaded limbs, in which no double labels were observed. The differences in bone formation parameters between WT and HIF-1 α non-loaded limbs indicate a different baseline rate of bone formation. Therefore, relative (r) parameters were also considered, computed as the difference between loaded and non-loaded limbs (Table 1). By this measure, no periosteal bone formation parameters were significantly different between genotypes, although rPs.BFR/BS was still increased ($+9\%$) in HIF-1 α mice compared to WT. On the endocortical surface of the bone, loaded limbs had significantly increased Ec.MS/BS, but there were no differences between WT and HIF-1 α mice. No other endocortical parameters were significantly different between loaded and non-loaded limbs.

4 Discussion

In this study, the role of HIF-1 α in loading-induced osteogenesis was explored using mice with a targeted deletion of HIF-1 α in the osteoblast lineage. Each animal was subjected to a single bout mechanical loading protocol of either damaging or non-damaging axial compression of the forelimb. The results demonstrate that HIF-1 α mice have attenuated woven bone formation following damaging mechanical loading (stress fracture), but enhanced lamellar bone formation following non-damaging mechanical loading (strain adaptive modeling). Thus, this study shows that HIF-1 α is a pro-osteogenic factor for woven bone formation following damaging loading, but an anti-osteogenic factor for lamellar bone formation after non-damaging mechanical loading.

The diminished response to damaging mechanical loading is attributed to the role of HIF-1 as a transcription factor associated with angiogenesis. One of the major targets of HIF-1 is vascular endothelial growth factor (VEGF), a well-characterized pro-angiogenic factor that plays an important role in the vascularization of bone [43] and is upregulated after damaging bone loading [23, 25]. Previous studies have shown that HIF-1 α is critical for angiogenesis and subsequent bone formation during murine distraction osteogenesis [19, 44]. Additionally, inhibition of angiogenesis led to diminished woven bone formation following damaging mechanical loading in rats [27]. Here, a significant decrease in periosteal vascularity due to decreased HIF-1 expression was associated with less woven bone formation. This result demonstrates that HIF-1 α expression in the osteoblast lineage is specifically required for complete angiogenesis, despite robust expression of HIF-1 α in inflammatory cells and tissues immediately adjacent to the site of bone formation.

In contrast, lamellar bone formation was not impaired in HIF-1 α mice following non-damaging osteogenic forelimb loading, consistent with previous work that found loading-induced lamellar bone formation to be independent of angiogenesis in rats [28]. The significant increases (+50%) in HIF-1 α gene expression observed 3 days after loading [25] suggested that HIF-1 α may be a positive regulator in the accrual of lamellar bone, but the results from the current work illustrate that mice with HIF-1 α removed in the osteoblast lineage have increased baseline bone formation. When challenged with non-damaging forelimb compression, HIF-1 α mice respond normally, resulting in a greater absolute level of bone formation in HIF-1 α loaded limbs compared to WT. This finding is similar to results from a previous study that showed HIF-1 α mice generated more bone following 3 weeks of tibial bending than WT mice, although they found no differences in bone formation in sham limbs [21]. The increased bone formation in non-loaded limbs observed in this study is consistent with the age-related changes in bone size previously reported –

HIF-1 α mice begin life with small bones that become comparable to their wild type littermates in adulthood [21].

Increased bone formation in HIF-1 α mice has previously been attributed to HIF-1 α negatively regulating Wnt signaling [21], a pathway central in the differentiation and function of osteoblasts [45]. Although HIF-1 α has been shown to bind to β -catenin, potentially inhibiting canonical Wnt signaling [46], Wnt target genes are upregulated following osteogenic mechanical loading along with HIF-1 α , whereas known Wnt inhibitors

such as SOST and Dkk are downregulated [24, 47, 48]. These observations suggest that a strong negative effect of HIF-1 α on canonical Wnt signaling in bone following loading is unlikely. In addition, the increase in bone apposition in non-loaded limbs of HIF-1 α mice was only observed here on the periosteal surface – in scenarios of increased Wnt signaling, bone formation is also dramatically increased on the endocortical surface [49–51]. Thus, the mechanism of the non-angiogenic effects of HIF-1 α on periosteal bone formation remains unclear, although we have shown here that osteoblastic HIF-1 α is not required for a strong induction of lamellar bone formation by mechanical loading.

Previous studies of damaging mechanical loading of the mouse ulna observed 3-fold increases in HIF-1 α expression on day 3 after loading, based on qPCR of whole bone with attached periosteum [22]. Here, immunohistochemistry indicates that HIF-1 α is primarily expressed in osteocytes and inflammatory cells adjacent to the bone, whereas few osteoblasts present at the site of woven bone formation in WT mice expressed HIF-1 α . Thus, the effect of HIF-1 α in the osteoblast lineage may be mediated largely by osteocytes. A role for osteocytes in periosteal angiogenesis and woven bone formation after bone injury has not yet been established. On the other hand, osteocytes have been shown to play an important role in lamellar bone formation [48, 52].

5 Conclusion

In this study, mice with HIF-1 α deleted from the osteoblast lineage were subjected to damaging and non-damaging osteogenic mechanical loading. Following damaging loading, HIF-1 α deficiency results in decreased angiogenesis and reduced woven bone formation. In contrast, HIF-1 α deficiency does not impair lamellar bone formation induced by non-damaging loading, with overall bone formation increased in knockout mice. Immunohistochemistry against HIF-1 α suggests that osteocytes are the osteoblast lineage cells driving these results. In summary, this study demonstrates that HIF-1 α is a factor that regulates both damaging and non-damaging osteogenic mechanical loading.

Acknowledgements

Founding members of the mouse colony used in this study were generously donated by Drs. Thomas Clemens and Ryan Riddle (Johns Hopkins University). The authors are grateful to Dr. Deborah Novack for assistance with histology. This study was funded by a grant from the National Institutes of Health (R01 AR050211). This work was performed in facilities supported by the Washington University Center for Musculoskeletal Research (P30 AR057235).

References

1. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and cellular biology*. 1992; 12:5447–5454. [PubMed: 1448077]
2. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92:5510–5514. [PubMed: 7539918]
3. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *The Journal of biological chemistry*. 1995; 270:1230–1237. [PubMed: 7836384]
4. Salceda S, Caro J. Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on

- redoxinduced changes. *The Journal of biological chemistry*. 1997; 272:22642–22647. [PubMed: 9278421]
5. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *Journal of applied physiology*. 2000; 88:1474–1480. [PubMed: 10749844]
 6. Semenza GL. HIF-1 and human disease: one highly involved factor. *Genes & development*. 2000; 14:1983–1991. [PubMed: 10950862]
 7. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*. 2001; 107:43–54. [PubMed: 11595184]
 8. Riddle RC, Khatri R, Schipani E, Clemens TL. Role of hypoxia-inducible factor-1alpha in angiogenic-osteogenic coupling. *Journal of Molecular Medicine*. 2009; 87:583–590. [PubMed: 19415227]
 9. Glowacki J. Angiogenesis in fracture repair. *Clinical Orthopaedics and Related Research*. 1998 Oct.;S82–S89. [PubMed: 9917629]
 10. Wan C, Shao J, Gilbert SR, Riddle RC, Long F, Johnson RS, Schipani E, Clemens TL. Role of HIF-1alpha in skeletal development. *Annals of the New York Academy of Sciences*. 2010; 1192:322–326. [PubMed: 20392254]
 11. Shomento SH, Wan C, Cao X, Faugere MC, Bouxsein ML, Clemens TL, Riddle RC. Hypoxia-inducible factors 1alpha and 2alpha exert both distinct and overlapping functions in long bone development. *Journal of cellular biochemistry*. 2010; 109:196–204. [PubMed: 19899108]
 12. Ferguson C, Alpern E, Miclau T, Helms JA. Does adult fracture repair recapitulate embryonic skeletal formation? *Mechanisms of development*. 1999; 87:57–66. [PubMed: 10495271]
 13. Liu L, Marti GP, Wei X, Zhang X, Zhang H, Liu YV, Nastai M, Semenza GL, Harmon JW. Agedependent impairment of HIF-1alpha expression in diabetic mice: Correction with electroporation-facilitated gene therapy increases wound healing, angiogenesis, and circulating angiogenic cells. *Journal of cellular physiology*. 2008; 217:319–327. [PubMed: 18506785]
 14. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nature medicine*. 2004; 10:858–864.
 15. Le AX, Miclau T, Hu D, Helms JA. Molecular aspects of healing in stabilized and non-stabilized fractures. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2001; 19:78–84. [PubMed: 11332624]
 16. Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mechanisms of development*. 2001; 100:245–250. [PubMed: 11165481]
 17. Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *Journal of cell science*. 2000; 113(Pt 1):59–69. [PubMed: 10591625]
 18. Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, Bouxsein ML, Faugere MC, Guldberg RE, Gerstenfeld LC, Haase VH, Johnson RS, Schipani E, Clemens TL. The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. *The Journal of clinical investigation*. 2007; 117:1616–1626. [PubMed: 17549257]
 19. Wan C, Gilbert SR, Wang Y, Cao X, Shen X, Ramaswamy G, Jacobsen KA, Alaqi ZS, Eberhardt AW, Gerstenfeld LC, Einhorn TA, Deng L, Clemens TL. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:686–691. [PubMed: 18184809]
 20. Jacobsen KA, Al-Aqil ZS, Wan C, Fitch JL, Stapleton SN, Mason ZD, Cole RM, Gilbert SR, Clemens TL, Morgan EF, Einhorn TA, Gerstenfeld LC. Bone formation during distraction osteogenesis is dependent on both VEGFR1 and VEGFR2 signaling. *Journal of Bone and Mineral Research*. 2008; 23:596–609. [PubMed: 18433297]

21. Riddle RC, Leslie JM, Gross TS, Clemens TL. Hypoxia-inducible factor-1 α protein negatively regulates load-induced bone formation. *The Journal of Biological Chemistry*. 2011; 286:44449–44456. [PubMed: 22081627]
22. Martinez MD, Schmid GJ, McKenzie JA, Ornitz DM, Silva MJ. Healing of non-displaced fractures produced by fatigue loading of the mouse ulna. *Bone*. 2010; 46:1604–1612. [PubMed: 20215063]
23. Wohl GR, Towler DA, Silva MJ. Stress fracture healing: fatigue loading of the rat ulna induces upregulation in expression of osteogenic and angiogenic genes that mimic the intramembranous portion of fracture repair. *Bone*. 2009; 44:320–330. [PubMed: 18950737]
24. McKenzie JA, Bixby EC, Silva MJ. Differential gene expression from microarray analysis distinguishes woven and lamellar bone formation in the rat ulna following mechanical loading. *PLoS ONE*. 2011
25. McKenzie JA, Silva MJ. Comparing histological, vascular and molecular responses associated with woven and lamellar bone formation induced by mechanical loading in the rat ulna. *Bone*. 2011; 48:250–258. [PubMed: 20849995]
26. Matsuzaki H, Wohl GR, Novack DV, Lynch JA, Silva MJ. Damaging fatigue loading stimulates increases in periosteal vascularity at sites of bone formation in the rat ulna. *Calcified Tissue International*. 2007; 80:391–399. [PubMed: 17551770]
27. Tomlinson RE, McKenzie JA, Schmieder AH, Wohl GR, Lanza GM, Silva MJ. Angiogenesis is required for stress fracture healing in rats. *Bone*. 2013; 52:212–219. [PubMed: 23044046]
28. Tomlinson RE, Schmieder AH, Quirk JD, Lanza GM, Silva MJ. Antagonizing the α β Integrin Inhibits Angiogenesis and Impairs Woven but Not Lamellar Bone Formation Induced by Mechanical Loading. *J Bone Miner Res*. 2014
29. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS. Hypoxia-inducible factor-1 α is a positive factor in solid tumor growth. *Cancer research*. 2000; 60:4010–4015. [PubMed: 10945599]
30. Zhang M, Xuan S, Boussein ML, von Stechow D, Akeno N, Faugere MC, Malluche H, Zhao G, Rosen CJ, Efstratiadis A, Clemens TL. Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. *The Journal of biological chemistry*. 2002; 277:44005–44012. [PubMed: 12215457]
31. Ferguson VL, Ayers RA, Bateman TA, Simske SJ. Bone development and age-related bone loss in male C57BL/6J mice. *Bone*. 2003; 33:387–398. [PubMed: 13678781]
32. Brodt MD, Ellis CB, Silva MJ. Growing C57Bl/6 mice increase whole bone mechanical properties by increasing geometric and material properties. *J Bone Miner Res*. 1999; 14:2159–2166. [PubMed: 10620076]
33. Lee KC, Maxwell A, Lanyon LE. Validation of a technique for studying functional adaptation of the mouse ulna in response to mechanical loading. *Bone*. 2002; 31:407–412. [PubMed: 12231414]
34. Uthgenannt BA, Kramer MH, Hwu JA, Wopenka B, Silva MJ. Skeletal self-repair: stress fracture healing by rapid formation and densification of woven bone. *Journal of Bone and Mineral Research*. 2007; 22:1548–1556. [PubMed: 17576168]
35. Liedert A, Mattaesch L, Rontgen V, Blakytyn R, Vogele D, Pahl M, Bindl R, Neunaber C, Schinke T, Harroch S, Amling M, Ignatius A. Midkine-deficiency increases the anabolic response of cortical bone to mechanical loading. *Bone*. 2011; 48:945–951. [PubMed: 21185956]
36. Xiao Z, Dallas M, Qiu N, Nicolella D, Cao L, Johnson M, Bonewald L, Quarles LD. Conditional deletion of Pkd1 in osteocytes disrupts skeletal mechanosensing in mice. *The FASEB Journal*. 2011; 25:2418–2432.
37. Sugiyama T, Saxon LK, Zaman G, Moustafa A, Sunter A, Price JS, Lanyon LE. Mechanical loading enhances the anabolic effects of intermittent parathyroid hormone (1–34) on trabecular and cortical bone in mice. *Bone*. 2008; 43:238–248. [PubMed: 18539556]
38. Robling AG, Turner CH. Mechanotransduction in bone: genetic effects on mechanosensitivity in mice. *Bone*. 2002; 31:562–569. [PubMed: 12477569]
39. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nature Methods*. 2012; 9:676–682. [PubMed: 22743772]

40. Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *Journal of Bone and Mineral Research*. 2013; 28:2–17. [PubMed: 23197339]
41. Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J*. 2001; 15:2445–2453. [PubMed: 11689469]
42. Milkiewicz M, Pugh CW, Egginton S. Inhibition of endogenous HIF inactivation induces angiogenesis in ischaemic skeletal muscles of mice. *J Physiol*. 2004; 560:21–26. [PubMed: 15319416]
43. Chappell JC, Wiley DM, Bautch VL. How blood vessel networks are made and measured. *Cells, tissues, organs*. 2012; 195:94–107. [PubMed: 21996655]
44. Carvalho RS, Einhorn TA, Lehmann W, Edgar C, Al-Yamani A, Apazidis A, Pacicca D, Clemens TL, Gerstenfeld LC. The role of angiogenesis in a murine tibial model of distraction osteogenesis. *Bone*. 2004; 34:849–861. [PubMed: 15121017]
45. Soltanoff CS, Yang S, Chen W, Li YP. Signaling networks that control the lineage commitment and differentiation of bone cells. *Critical reviews in eukaryotic gene expression*. 2009; 19:1–46. [PubMed: 19191755]
46. Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nature cell biology*. 2007; 9:210–217.
47. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, Kharode Y, Sauter L, Babij P, Brown EL, Hill AA, Akhter MP, Johnson ML, Recker RR, Komm BS, Bex FJ. Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. *The Journal of biological chemistry*. 2006; 281:31720–31728. [PubMed: 16908522]
48. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, Mantila SM, Gluhak-Heinrich J, Bellido TM, Harris SE, Turner CH. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *The Journal of biological chemistry*. 2008; 283:5866–5875. [PubMed: 18089564]
49. Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, D'Agostin D, Kurahara C, Gao Y, Cao J, Gong J, Asuncion F, Barrero M, Warmington K, Dwyer D, Stolina M, Morony S, Sarosi I, Kostenuik PJ, Lacey DL, Simonet WS, Ke HZ, Paszty C. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J Bone Miner Res*. 2008; 23:860–869. [PubMed: 18269310]
50. Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Boussein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B, Marzolf J, Moran RA, Bex F. High bone mass in mice expressing a mutant LRP5 gene. *J Bone Miner Res*. 2003; 18:960–974. [PubMed: 12817748]
51. Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. *J Clin Invest*. 2006; 116:1202–1209. [PubMed: 16670761]
52. Javaheri B, Stern A, Lara N, Dallas M, Zhao H, Liu Y, Bonewald LF, Johnson ML. Deletion of a single beta-catenin allele in osteocytes abolishes the bone anabolic response to loading. *Journal of Bone and Mineral Research*. 2013

Highlights

- HIF-1 α deletion in osteoblasts and osteocytes leads to decreased woven bone formation and vascularity after damaging loading.
- Osteocyte is primary bone cell that expresses HIF-1 α following damaging loading.
- HIF-1 α deletion did not impair lamellar bone formation following non-damaging loading.

Figure 1. HIF-1 α Expression following Damaging Mechanical Loading

Immunohistochemistry against HIF-1 α was performed 3 and 7 days after damaging (WBF) loading. In WT mice (A, C), expression of HIF-1 α was robust in osteocytes on days 3 and 7 (black arrows) as well as the inflammatory and stromal cells present in the expanded periosteum on day 3 (bracket), though relatively few osteoblasts were positive. In contrast,

HIF-1 α mice (B, D) had diminished expression of HIF-1 α in bone cells (white arrows), but expression in the expanded periosteum remained strong (bracket). Images are representative of 5–8 samples per group.

Figure 2. Woven Bone Formation after Damaging Mechanical Loading

7 days after loading, A) WT mice and B) Δ HIF-1 α mice were imaged using microCT to quantify woven bone formation. The original cortical bone (cb - red), the newly formed woven bone (wb - green), and the cortical crack (arrow) are marked in each cross section. C) Δ HIF-1 α mice had significantly less woven bone volume (-22%) and D) woven bone extent (-21%). * $p < 0.05$ vs. WT. (n = 8 per group)

Figure 3. Blood Vessel Formation after Damaging Mechanical Loading

A) 7 days after loading, vascularity was quantified using immunohistochemistry against von Willebrand Factor. B) HIF-1 α mice had significantly fewer vessels (~35%) compared to WT mice. * $p < 0.05$ vs. WT. (n = 8 per group)

Figure 4. Dynamic Histomorphometry after Non-Damaging Mechanical Loading

Calcein (green) and Alizarin (red) fluorescent bone formation markers were administered at days 3 and 8, respectively, following non-damaging mechanical loading. Wild type loaded (A) and non-loaded (B) limbs were quantitatively compared to Δ HIF-1 α loaded (C) and non-loaded (D) limbs. Inset is magnified 200%. (n = 6 per group)

Figure 5. Lamellar Bone Formation after Non-Damaging Mechanical Loading

Bone formation at the ulnar mid-diaphysis was quantified using dynamic histomorphometry.

Loading was associated with increases in Ec.MS/BS, Ps.MS/BS, Ps.MAR, and Ps.BFR/BS

in both WT and Δ HIF-1 α mice. * $p < 0.05$ vs. non-loaded, + $p < 0.05$ vs. WT. (n = 6 per

group)

Table 1

Relative (loaded – non-loaded) periosteal bone formation parameters.

	rPs.MS/BS (%)	rPs.MAR (μm/day)	rPs.BFR/BS (μm/day)
WT	25.4 ± 5.7	0.67 ± 0.28	0.35 ± 0.15
HIF-1α	20.2 ± 4.1	0.68 ± 0.38	0.38 ± 0.10
<i>p-value</i>	0.23	0.49	0.43

There are no significant differences between WT and HIF-1α mice by this measure.