Hypoxia Inducible Factor-2α Predominantly Contributes to Cortical Phenotype in *Vhl* cKO Mice

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INTRODUCTION: Hypoxia inducible factors (HIFs) are heterodimeric transcription factors that respond to changes in cellular oxygen levels. HIFs are composed of an oxygen-dependent α subunit (HIFα) and a constitutively expressed β subunit (HIFβ). Under normoxic conditions, HIFα is hydroxylated via prolyl hydroxylase domain protein (PHD) and targeted for 26S proteasomal degradation via an E3 ligase complex Von-Hippel Lindau (VHL). Under hypoxic conditions, hydroxylation via PHD is inhibited, allowing for HIF-α stabilization and accumulation in the cytosol. Stabilized HIFα complexes with HIFβ in the nucleus then binds HIF-response elements in target genes promoting angiogenesis, osteogenic differentiation and proliferation, glucose uptake, and anaerobic glycolysis (1). Thus, VHL serves as a master regulator of HIFα activity by targeting its degradation. Our studies have shown that deletion of *Vhl* in osteocytes but not osteoblasts (*Dmp1-cre; Vhl*$^{f/f}$) produces a robust skeletal phenotype, characterized by dramatic increases in both cortical and trabecular microarchitecture compared to age-matched wild-type mice (2). Our previous work deleting *Hif1a* or *Hif2a* in osteocytes showed no effect on skeletal microarchitecture, however, expressing a degradation resistant osteocytic HIF-2α, not HIF-1α, generated a high bone mass phenotype (3). Therefore, we hypothesize that osteocytes may only require HIF-2α to transduce *Vhl* signaling. The objective of this study was to evaluate whether HIF-2α is the predominant isoform required to transduce *Vhl* signaling in osteocytes, culminating in a high bone mass phenotype.

METHODS: *Hif1a*$^{f/f}$ (#007561), *Hif2a*$^{f/f}$ (#008407) and *Vhl*$^{f/f}$ (#004081) mice were purchased from Jackson Laboratories. To generate double knock-out mice, *Hif1a*$^{f/f}$ and *Hif2a*$^{f/f}$ mice were bred with *Vhl*$^{f/f}$ mice for several generations to produce homozygous, dual-floxed *Vhl*$^{f/f}$/*Hif1a*$^{f/f}$ and *Vhl*$^{f/f}$/*Hif2a*$^{f/f}$ mice, which were bred with mice expressing *cre*-recombinase driven by the 10 kb-*Dmp-1* promoter (*Dmp1-cre*) to generate *Dmp1-cre; Vhl*$^{f/f}$, *Hif1a*$^{f/f}$ and *Dmp1-cre; Vhl*$^{f/f}$, *Hif1a*$^{f/f}$ mice with osteocyte conditional deletion of *Vhl* and *Hif1a* (*Vhl; Hif1a cKO*) or *Vhl* and *Hif2a* (*Vhl; Hif2a cKO*). Twelve female mice were euthanized at 16 weeks of age (n = 3/group); the left femur was dissected, fixed, and scanned per (2) to evaluate femoral mid-diaphyseal cortical bone tissue mineral density distribution. Tissue mineral density distributions (TMDD) were obtained by distributing voxels into 100 evenly spaced bins (4–1,500 mg HA/cm^3). Mean calcium content (Ca_{MEAN}), peak calcium content (Ca_{PEAK}), and width at half-maximum (Ca_{WIDTH}) were calculated as per (4). Three-point bending mechanical testing (ElectroForce 3200; TA Instruments) was conducted on unfixed contralateral femora (3 mice per genotype) to evaluate structural and tissue mechanical properties. Data were analyzed using multiple comparison ordinary one-way ANOVA tests, where $p<0.05$ was statistically significant.

RESULTS: Our analysis of tissue-level calcium distribution revealed reductions in multiple cortical bone parameters in single *Vhl* or dual mutant *Vhl; Hif1a* cKO mice that was not observed in *Vhl; Hif2a* cKO mice. Hydroxyapatite-calibrated heatmap of TMDD (**Fig. 1A**) and TMDD profiles (**Fig. 1B**) of mid-diaphyseal cortical bone revealed marked heterogeneity as a function of *Vhl* and *Hif1a* deletion. Loss of *Vhl* with either *Hif1a* (*Vhl; Hif1a cKO*) or *Hif2a* (*Vhl; Hif2a cKO*) reduced TMDD heterogeneity to *cre*-negative levels (**Fig. 1C**). Both single (*Vhl* cKO) and double mutants (*Vhl; Hif1a cKO* and *Vhl; Hif2a cKO)* demonstrated reduced peak calcium compared to *cre*-negative controls (**Fig. 1D)**. Loss of *Vhl* and *Hif1a* dramatically reduced mean mineralization compared to *cre*-negative controls **Fig. 1E**). Elastic modulus of the *Vhl; Hif1a cKO* was reduced to *Vhl* cKO levels, whereas the *Vhl; Hif2a cKO* was indistinguishable from *cre*-negative controls (**Fig. 1F**).

DISCUSSION: The unique impacts of HIFα isoforms on skeletal health remain understudied. We have previously used an osteocytic model of stabilized HIFα- signaling (*Vhl* cKO) to investigate effects on bone development and strength, yet the relative contribution of unique HIF-α isoforms was not addressed. By generating mice with compound deletion of *Vhl* and *Hif1a* or *Hif2a*, we found that deletion of *Vhl; Hif1a* dramatically reduced cortical mean density and moderately reduced elastic modulus, indicating impaired cortical tissue and mineral bone quality. Dual deletion of *Hif2a* and *Vhl* generated mild reductions in cortical mean density and elastic modulus and reduced cortical peak mineral to *Vhl* cKO levels. Our studies suggest that osteocytic HIF-2α appears to predominantly determine cortical mineralization distribution in the *Vhl* cKO mouse.

SIGNIFICANCE/CLINICAL RELEVANCE: Osteoporotic fractures caused by weak and brittle bones are associated with significant decreases in mobility, quality of life, and in some cases, significant financial burden due to rehabilitative and/or long-term disability care. Osteocytic *Vhl* deletion drives a high bone mass phenotype, thus, identifying the mechanisms by which this increase in bone mass is generated can be used to identify novel therapeutic targets to subvert poor bone quality.

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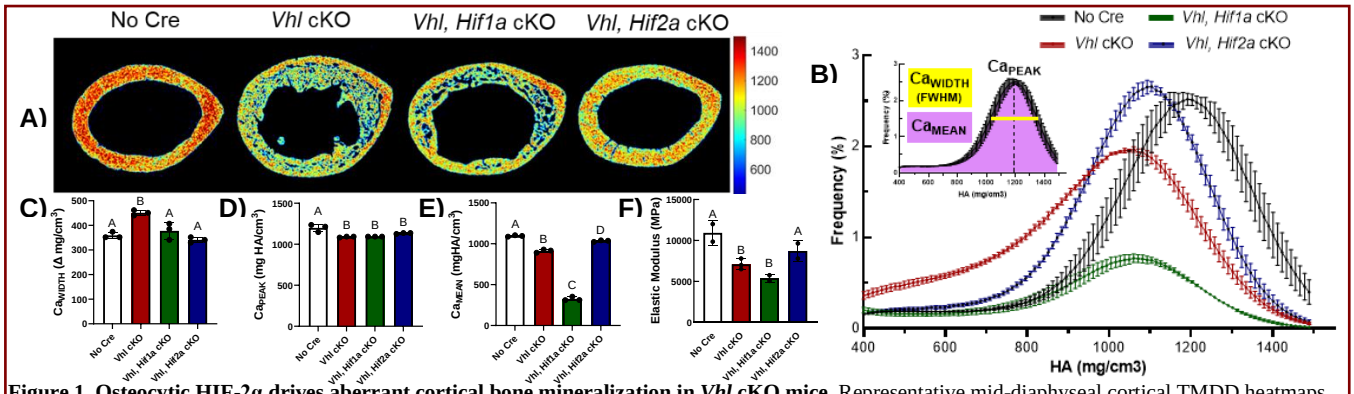

Figure 1. **Osteocytic HIF-2α drives aberrant cortical bone mineralization in *Vhl* cKO mice**. Representative mid-diaphyseal cortical TMDD heatmaps (**A**), TMDD profiles **(B)**, and quantification of full width at half maximum (**C**), peak mineral (**D**), mean density (**E**) and elastic modulus (**F)** from *cre*-negative, *Vhl; Hif1a cKO*, *Vhl; Hif2a cKO*, and *Vhl* cKO mice. Bars represent mean ± SD, groups with different letters are statistically distinct.