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Hypoxia Decreases Sclerostin Expression and Increases Wnt Signaling in Osteoblasts

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Abstract

Mutations in sclerostin function or expression cause sclerosing bone dysplasias, involving decreased antagonism of Wnt/Lrp5 signaling. Conversely, deletion of the VHL tumor suppressor in osteoblasts, which stabilize HIF- α isoforms and thereby enables HIF- α / β -driven gene transcription, increases bone mineral content and cross-sectional area compared to wild-type controls. We examined the influence of cellular hypoxia (1% oxygen) upon sclerostin expression and canonical Wnt signaling. Osteoblasts and osteocytes cultured under hypoxia revealed decreased sclerostin transcript and protein, and increased expression and nuclear localization of activated β -catenin. Similarly, both hypoxia and the hypoxia mimetic DFO increased β -catenin gene reporter activity. Hypoxia and its mimetics increased expression of the BMP antagonists gremlin and noggin and decreased Smad-1/5/8 phosphorylation. As a partial explanation for the mechanism of regulation of sclerostin by oxygen, MEF2 reporter assays revealed decreased activity. Modulation of VEGF signaling under normoxia or hypoxia revealed no influence upon *Sost* transcription. These data suggest that hypoxia inhibits sclerostin expression, through enhanced antagonism of BMP signaling independent of VEGF. *J. Cell. Biochem.* 110: 457–467, 2010.

Keywords

SCLEROSTIN; *Sost*; HYPOXIA; OSTEOBLAST; BONE MORPHOGENETIC PROTEIN; Wnt

The vertebrate skeleton has the capacity to alter its shape and size in response to biophysical and biomechanical cues. For example, bone apposition occurs in regions of high stress to reduce tissue strain, whereas net bone loss occurs under conditions of disuse with a resulting increase in tissue strain. Over the past decades the identity of humoral, paracrine, and mechanical factors, acting both in isolation and in concert, that govern the formation and activity of osteoblasts and osteoclasts have been revealed. Among those factors identified as key regulators of skeletal formation are bone morphogenetic proteins (BMPs), the RANK/RANKL/

OPG axis, ATP, and prostaglandins. Additional novel skeletal regulators were identified as candidates for genetic disorders producing osteoporotic or osteopetrotic phenotypes. For instance, individuals with the autosomal recessive disorder osteoporosis pseudoglioma (OPPG) demonstrate an osteoporotic phenotype [Gong et al., 2001] caused by mutations in the *Lrp5* Wnt co-receptor, thereby identifying a unique gene in bone accrual. Similarly, mutational analysis of the rare sclerosing bone dysplasias sclerosteosis (OMIM 269500) and van Buchem disease (OMIM 293100) implicated *Sost* as an inhibitor of bone formation [Balemans et al., 1999, 2001]. Sclerosteosis and van Buchem disease are both autosomal recessive disorders that result from loss-of-function mutations in the *Sost* gene or its distal enhancer, respectively. Sclerosteosis and van Buchem phenotypes are characterized by markedly increased bone mineral density at the lumbar spine, hip, and forearm [Gardner et al., 2005] and overall increased bone mass and strength [Wergedal et al., 2003]. Because no corresponding changes in markers of bone resorption are noted in those affected, this indicates that, in sclerosteosis and van Buchem disease, there is a shift of skeletal homeostasis in favor of bone formation. Transgenic mice that overexpress sclerostin reveal an osteoporotic phenotype [Winkler et al., 2003; Loots et al., 2005], and in vitro studies demonstrate that sclerostin increases osteoblast apoptosis, and decreases osteoprogenitor proliferation and matrix mineralization. Consistent with the clinical description, sclerostin knock-out mice demonstrate increased bone mineral density, bone volume, and strength [Li et al., 2008]. Thus, sclerostin is identified as an important regulator of bone formation.

While sclerostin has emerged as a potent inhibitor of bone formation, the cellular and molecular mechanisms whereby it functions remain to be elucidated. Sclerostin was initially characterized as a BMP antagonist because of sequence similarity to the DAN family of secreted BMP antagonists, and sclerostin was indeed shown to attenuate such BMP-induced responses in osteoblastic cells such as alkaline phosphatase activity and Smad phosphorylation [Winkler et al., 2003]. However, accumulating evidence shows that the primary effect of sclerostin on bone is not mediated via BMP antagonism, but instead by antagonism of the *Lrp5* co-receptor to prevent binding of Wnt glycoproteins. Sclerostin binds to *Lrp5* at the first two repeats of the YWTD-EGF region, a binding region of Wnt inhibitors [Li et al., 2005]. The *Lrp5*^{G171V} mutation, which recapitulates the high bone mass phenotype, binds sclerostin less avidly than does wild-type *Lrp5* [Ellies et al., 2006; Semenov and He, 2006]. Additionally, sclerostin has considerably lower binding affinity for BMPs compared to traditional BMP antagonists such as gremlin and noggin [Kusu et al., 2003], and higher concentrations of sclerostin are required to inhibit BMP- versus Wnt-induced alkaline phosphatase activity [Winkler et al., 2005].

Oxygen tension is another powerful stimulus for regulation of skeletal mass [Schipani et al., 2001], yet the net result of hypoxia—whether it is anabolic or catabolic to the skeleton—is inconclusive. In vitro studies demonstrate both stimulatory and inhibitory effects of hypoxia on osteoblast proliferation, differentiation, and bone formation [Tuncay et al., 1994; Park et al., 2002; Ontiveros et al., 2004; Salim et al., 2004; D'Ippolito et al., 2006; Utting et al., 2006; Zahm et al., 2008]. Recent work by Wang et al. [2007] however, provides compelling evidence that hypoxia stimulates bone formation, and therefore has a dominant anabolic effect. Targeted deletion of the tumor suppressor von Hippel-Landau (VHL) within osteoblasts, and the subsequent stabilization of hypoxia-inducible factor- α (HIF- α) and induction of HIF- α -responsive genetic repertoire produced mice expressing high levels of VEGF with enhanced vascularized tissue and denser long bones; in contrast, deletion of HIF-1 α produced an inverse phenotype, with low levels of VEGF, poor vascularization, and thinner bones compared to wild-type mice. This stimulatory effect of VHL deletion and subsequent HIF- α stabilization was not limited to skeletal development, as enhanced bone volume and vessel volume were also observed during fracture repair [Wan et al., 2008].

Because these pathways—Wnt/Lrp5 and HIF—reveal powerful effects upon skeletal homeostasis, we sought whether these pathways are coupled. We hypothesized that hypoxia may exert its stimulatory effect upon bone formation via reductions in sclerostin expression, to thereby mitigate its inhibitory influence upon bone formation. We observed that osteoblastic UMR 106.01 and osteocytic MLO-A5 cells cultured under 1% oxygen tension express significantly lower levels of both *Sost* transcript and sclerostin protein compared to cells cultured at 21% oxygen tension. Reductions in sclerostin levels by hypoxia or hypoxia mimetics were accompanied by increases in activated β -catenin expression, nuclear localization, and increased β -catenin-driven transcription. This work demonstrates that hypoxia also suppresses sclerostin transcription in osteocytic MLO-A5, but not in HEK293 kidney cells. The difference in regulation of sclerostin between the osteocytic and hepatic cell lines suggests that the van Buchem enhancer region, which is specific to bone, may be the target of hypoxic regulation of sclerostin. MEF2 transcription factors, which bind the van Buchem enhancer region, revealed reduced nuclear accumulation under hypoxia. Osteoblastic cells expressed higher levels of the BMP antagonists gremlin and noggin, as well as decreased Smad 1/5/8 phosphorylation, suggesting that reduced BMP signaling under hypoxia is responsible for the attenuation of *Sost* transcription. Neither VEGF₁₆₅ nor VEGF-inhibiting antibody affected *Sost* transcription at 21% nor 1% oxygen. These are the first descriptions of sclerostin modulation by oxygen tension and suggest that the mechanism may involve attenuated BMP signaling under hypoxia.

MATERIALS AND METHODS

CELL CULTURE

UMR 106.01 cells (kindly provided by Dr. Alexander G. Robling, Indiana University School of Medicine), which express phenotypic markers of mature osteoblasts [Partridge et al., 1983], were cultured in MEM with Earle's Salts (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (P&S; Invitrogen). MLO-A5 pre-osteocytic cells (kindly provided by Dr. Lynda F. Bonewald, School of Dentistry, University of Missouri-Kansas City) were cultured in α -MEM supplemented with 5% FBS, 5% FCS, and 1% P&S. Cells were routinely sub-cultured with 0.05% trypsin/EDTA when 80–90% confluent.

HYPOXIC CULTURE

Cells were cultured under normoxia (21% oxygen) in a standard humidified incubator at 37°C with 5% CO₂/95% air. For hypoxia experiments, cells were transferred into humidified incubators at 37°C with 5% CO₂ and oxygen tension reduced to 1% using supplemental N₂ (HERAcell® 150; Kendro).

CONFIRMATION OF CELLULAR HYPOXIA

Pimonidazole hydrochloride (Hypoxyprobe™-1; Chemicon) was used to qualitatively examine cellular hypoxia by indirect immunofluorescence. At pO₂<10 mmHg (1.4% oxygen) Hypoxyprobe™-1 forms protein adducts in cells that are visualized using a FITC-labeled monoclonal antibody. Cells were seeded at 4,000 cells/cm² onto glass coverslips and, 2 days later, were transferred to 21% or 1% oxygen incubators; immediately prior to transfer, cells were treated with media supplemented with or without 200 μ M Hypoxyprobe™-1 pre-conditioned in 1% or 21% oxygen for 24 h. One and 96 h thereafter, cells were washed in PBS, fixed in ice-cold 90% methanol for 10 min and blocked with 3% BSA/PBS for 1 h at room temperature. After blocking, cells were incubated with Hypoxyprobe™-1-Mab1 (1:200) in blocking solution for 45 min at room temperature. Coverslips were washed in PBS and mounted on glass slides using fluorescent safe mounting media (Biomed). Cells not treated with pimonidazole were fixed and stained in the same manner and served as negative controls.

Additional cultures received HypoxyprobeTM-1 only in the final 2 h of culture; these revealed no differences in staining compared to cells cultured in the presence of HypoxyprobeTM-1 for the entire culture period, indicating that stability of the hypoxic adducts was not an issue in determining cellular hypoxia with this method.

QUANTITATIVE PCR

Cells were washed with PBS and total RNA was collected using RNeasy Mini kit (Qiagen), which included on-column DNase treatment. Total RNA (200–1,000 ng) was reverse-transcribed with SuperScript First-Strand (Invitrogen). qPCR was performed using TaqMan[®] Universal PCR Master Mix (Applied Biosystems) on a Mastercycler[®] realplex2 (Eppendorf); all primers and probes were purchased from Applied Biosystems. Amplification conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 1 min. Quantitative PCR results were normalized to loading control (*Rpl13* or *ActB*) transcript level to yield ΔC_t , then normalized to control conditions (e.g., normoxia at the same time point) to generate $\Delta\Delta C_t$. Relative or fold change in expression was subsequently calculated using the formula $2^{-\Delta C_t}$ or $2^{-\Delta\Delta C_t}$, respectively. Alternately, data are presented as expression relative to loading control $2^{-\Delta C_t}$ [Livak and Schmittgen, 2001].

WESTERN IMMUNOBLOTTING

Cells were washed with PBS and whole cell protein lysates were collected in 0.1% Triton X-100, 10 mM Tris, pH 8, 1 mM EDTA, supplemented with a protease and phosphatase inhibitor cocktail (Calbiochem). Samples were electrophoresed on 7.5% or 10% Tris-HCl gels (Bio-Rad), resolved onto 0.2 μ m nitrocellulose membranes, and blocked in non-fat milk or BSA in Tris-buffered saline supplemented with 0.1% Tween-20. Proteins were detected with antibodies directed against sclerostin (1:100; R&D Systems), phospho-Smad 1/5/8 (1:1,000; Cell Signaling), or HIF-1 α (1:200; Cayman Chemical); detection of β -actin (1:1,000; Abcam) or α -tubulin (1:1,000; Cell Signaling) was performed under similar conditions and evaluated as a control for equal loading across lanes. Overnight primary antibody incubation was followed by incubation with appropriate HRP-conjugated antibodies (Jackson ImmunoResearch) and ECL Plus Western Blotting Detection Reagents (GE Healthcare). Densitometric analysis was performed with Quantity One software (Bio-Rad).

IMMUNOCYTOCHEMISTRY

UMR 106.01 cells were seeded onto 25mm glass coverslips at a density of 4,000 cells/cm². Twenty-four hours later, media was replaced with reduced serum (2%) medium. Forty-eight hours after seeding, reduced serum medium preconditioned in either 1% or 21% oxygen was added to cells, which were then placed in normoxic or hypoxic incubators. At indicated times, cells were fixed with 2% formaldehyde and permeabilized with 0.1% Triton X, or fixed in 90% methanol. Non-specific binding was reduced by incubation in 10% FBS in PBS (for activated β -catenin) at room temperature for 1 h. Cells were incubated overnight at 4°C with primary antibodies against activated β -catenin primary antibody (1:100; Millipore), washed in PBS, and incubated with TRITC-labeled secondary antibody (1:1,000; Jackson Immuno Research Laboratories) for 45 min at room temperature; nuclear staining was achieved with Hoechst 33258 (2 μ g/ml; Sigma-Aldrich). For controls, primary and/or secondary antibodies were omitted. Coverslips were mounted on slides using fluorescent-safe mounting medium (Biomed).

TRANSIENT TRANSFECTIONS AND LUCIFERASE ASSAY

To examine the influence of hypoxia mimetics upon Wnt/ β -catenin-mediated gene transcription, UMR 106.01 cells were seeded at a density of 40,000 per well in 24 well plates. On the following day, cells were transfected with 200 ng of TOPFlash (β -catenin reporter) and

100 ng of *Renilla* Luciferase (pRL-TK; control for transfection efficiency) per well using Lipofectamine Plus (Invitrogen). Transfectants were exposed to pharmacologic agents or hypoxia on the following day, and 24 h thereafter, lysates were collected and TCF-luciferase was quantitated using Dual-Luciferase Reporter Assay (Promega) in a luminometer (TD-20/20; Turner Systems). TOPFlash luciferase activity was divided by *Renilla* luciferase; each ratio was subsequently normalized to vehicle (DFO-free) control, defined as 1. Similarly, to analyze MEF2 signaling, UMR 106.01 cells were seeded at 80,000 per well in 48-well plates, and transfected with MEF2 reporter luciferase plasmids (SA Biosciences). Twenty-four hours later, cells were switched to 21% or 1% oxygen for an additional 24 h, after which time lysates were collected and analyzed. Expression was normalized to transfection efficiency, which was determined relative to appropriate transfection controls.

STATISTICAL ANALYSIS

Each data set was acquired a minimum of three times, in duplicate. Data were analyzed by Kruskal–Wallis or ANOVA followed by Dunnett or Tukey post-hoc tests where appropriate.

RESULTS

UMR 106.01 OSTEOBLASTS ARE HYPOXIC IN 1% OXYGEN

HypoxyprobeTM-1 staining and Western immunoblotting against HIF-1 α were used to confirm that UMR 106.01 osteoblastic cells experienced intracellular hypoxia. HypoxyprobeTM-1 staining revealed immunofluorescence in cells cultured in 1% oxygen for 1 h, indicating the presence of HypoxyprobeTM-1 protein adducts and an intracellular oxygen tension of < 1.4% (Fig. 1A); in contrast, no positive fluorescent signal was observed in cells cultured at 21% oxygen. Positive staining for HypoxyprobeTM-1 protein adducts was also observed in cells cultured at 1% oxygen for 96 h, indicating that the cells sustained intracellular hypoxia throughout the time course of the study. Cellular response to hypoxia was verified by observing the levels of HIF-1 α protein. Transfer to hypoxia significantly increased HIF-1 α protein expression (Fig. 1B). Increases in HIF-1 α were observed as soon as 1 h after transfer to 1% oxygen compared to 21% oxygen cultures. These data indicate that, not only the cells are experiencing cellular hypoxia, but also responding to it via the HIF-1 α pathway.

HYPOXIA DECREASES SCLEROSTIN EXPRESSION

UMR106.01 cells cultured under 1% oxygen tension for 48 or 96 h demonstrated decreased sclerostin expression compared to cells cultured under 21% oxygen tension (Fig. 2A). Cells cultured under 1% or 21% oxygen tension for 6–96 h revealed significant reductions in both sclerostin mRNA and protein after 24 h of hypoxic culture, and expression of both transcript and protein product continued to decrease throughout the remainder of the study, ultimately reaching a 75% and 66% decrease in transcript and protein, respectively, compared to cells cultured in 21% oxygen (Fig. 2B). These data indicate a continuous decrease in sclerostin expression that is mediated by reductions in *Sost* transcript in response to 1% oxygen tension.

We also examined whether the Wnt antagonists Dkk1 and sFRP1 were similarly influenced by hypoxia. In cells cultured under normoxia, *Sost* was expressed at approximately 150-fold higher levels than was *Dkk1* (Fig. 2C), while no expression of *sFrp1* was consistently observed. Similar to *Sost*, *Dkk1* revealed reductions in expression under 1% oxygen, although this was transient, with expression maximally reduced after 72 h of 1% oxygen, and which rebounded after 96 h of hypoxic culture (Fig. 2D).

HYPOXIA INCREASES NUCLEAR β -CATENIN ACCUMULATION AND TRANSCRIPTIONAL ACTIVITY

A hallmark of canonical Wnt signaling is stabilization and nuclear accumulation of β -catenin. In the absence of Wnt signaling, β -catenin is phosphorylated by GSK-3 β , which targets it for proteasomal degradation; in contrast, dephosphorylated β -catenin is stable and transcriptionally active. Once inside the nucleus, β -catenin binds to TCF/LEF elements, displaces transcriptional corepressors in favor of transcriptional coactivators p300 and CBP to induce expression of target genes. Having observed a decrease in sclerostin expression under hypoxia (Fig. 2A), we next examined whether this produced a functional effect on Wnt signaling. UMR 106.01 osteoblastic cells were cultured for 96 h at 1% or 21% oxygen; this time point was chosen because it revealed maximal suppression of sclerostin at 1% oxygen (Fig. 2B). Immunofluorescent staining for activated β -catenin revealed increases in both total expression as well as nuclear localization of activated β -catenin in cells cultured at 1% oxygen, suggesting that hypoxia-driven suppression of sclerostin drives canonical Wnt signaling (Fig. 3A). The addition of recombinant Dkk1 and sclerostin (each 250 ng/ml) reduced total expression and nuclear localization of activated β -catenin in cells cultured at 1% oxygen, although neither Dkk1 nor sclerostin alone (each 250 ng/ml) mimicked the effect of treatment with both Wnt antagonists (not shown).

Hypoxia-driven canonical Wnt signaling was confirmed using a TOPFlash plasmid reporter system. Addition of the hypoxia mimetic DFO to cells cultured at 21% oxygen for 24 h revealed dose-dependent increases in TOPFlash reporter activity (Fig. 3B). The increase in TOPFlash reporter activity in the presence of 100 μ M DFO was the same as that from cells treated with 100nM hPTH(1–34), a positive control for sclerostin suppression. Additionally, cells cultured in 1% oxygen for 24 h revealed similar increases in TOPFlash reporter activity compared to cells treated with DFO or PTH at 21% oxygen. These data indicate that hypoxia induces β -catenin transcriptional activity.

HYPOXIA DIFFERENTIALLY REGULATES SCLEROSTIN EXPRESSION IN OSTEOGENIC AND NON-OSTEOGENIC CELLS

We next sought whether the observed reduction in *Sost* was specific to UMR 106.01 cells. Pre-osteocytic ML0-A5 cells, which express *Sost* at low levels [Bellido et al., 2005] yet increase expression in response to BMP-2 [Papanicolaou et al., 2009], similarly attenuated BMP-2-induced *Sost* expression under hypoxic conditions. Despite characterization as an osteocyte-specific protein [van Bezooijen et al., 2004], sclerostin transcript and expression is observed within soft tissues [Keller and Kneissel, 2005; van Bezooijen et al., 2007] and hypertrophic chondrocytes [Winkler et al., 2003]. Indeed, *Sost* transcript is detected at high levels in the human embryonic kidney cell line 293 (HEK293) [Keller and Kneissel, 2005], although it is expressed approximately 700-fold lower than *Sost* in UMR 106.01 cells (Fig. 4A). Interestingly, we observed no reductions in *Sost* expression from HEK293 cells under hypoxic challenge, suggesting that the ability of hypoxia to regulate sclerostin expression may be specific to osteogenic cells and does not occur secondary to general reduction in protein synthesis under hypoxia.

Targeted sclerostin expression to bone, the absence of which causes van Buchem disease [Loots et al., 2005], involves the MEF2 family of transcription factors [Leupin et al., 2007]. We observed no alterations in *Mef2c* expression in UMR106.01 cells cultured at 1% oxygen compared to 21% oxygen (data not shown), suggesting that reductions in MEF2C were not the cause of decreased sclerostin expression under hypoxia. A MEF2 reporter assay was used to determine whether hypoxia altered MEF2 transcriptional activity. Cells cultured in 21% oxygen revealed luciferase activity in control samples, and this was decreased by 100nM hPTH (1–34), consistent with the work of Leupin et al. [2007], whereas addition of 20% FBS to

cultures increased MEF2 transcriptional activity (Fig. 4B). In contrast, cells cultured under hypoxia revealed decreased luciferase activity, which was not statistically different from 100nM hPTH(1–34)-treated cells maintained under hypoxia. As under 21% oxygen culture, the addition of 20% FBS increased luciferase activity, indicating that hypoxia did not eliminate the capacity for MEF2-driven transcription.

HYPOXIC SUPPRESSION OF SCLEROSTIN INVOLVES ALTERED BMP SIGNALING

Sclerostin expression is induced by BMPs in mesenchymal stem cells [Sutherland et al., 2004] and osteocyte-like cells [Papanicolaou et al., 2009]. BMP-2 induced *Sost* expression over a 3 h treatment interval at doses ranging from 50 to 250 ng/ml (Fig. 5A), demonstrating that *Sost* is also a target of BMP signaling in mature osteoblastic UMR 106.01 cells. Hypoxia has been shown to affect expression of BMP2 and BMP receptor expression [Salim et al., 2004; Takahashi et al., 2007], although we observed no discernible effect upon expression of *Bmp2*, *Bmp4*, or *Bmp-RII* (Fig. 5B), suggesting that reductions in sclerostin under hypoxic conditions result from neither decreased BMP expression nor BMP receptor availability. In contrast to reductions in sclerostin expression, we observed marked increases in the expression of the BMP antagonists noggin and gremlin in cells cultured in 1% oxygen compared to 21% oxygen (Fig. 5C). Significant increases in *Grem 1* and *Nog* expression were observed after 24 h in hypoxic culture, and their expression continued to increase compared to samples from normoxic culture throughout the remainder of the study. Consistent with increases in BMP antagonist expression under hypoxia, immunoblotting revealed reductions in Smad-1/5/8 phosphorylation under hypoxia (Fig. 5D), suggesting that reduced BMP signaling may be a causative factor contributing to sclerostin reduction under hypoxic culture.

THE INFLUENCE OF HYPOXIA UPON BMP AND Wnt SIGNALING IS MEDIATED VIA HIF- α

We sought to support the link between hypoxia and its effect upon *Sost* transcription. Addition of actinomycin D (2.5 μ g/ml) prevented increases in *Grem 1* and *Nog*, as well as decreases in *Sost*, when cells were cultured under hypoxic conditions (data not shown), indicating that hypoxia affects their expression at the level of transcription, and does not alter mRNA stability. Thus, we next utilized hypoxia mimetics to examine their effect upon *Sost*, *Nog*, and *Grem 1* expression. Stabilization of HIF- α isoforms with the iron chelator DFO, which inhibits prolyl hydroxylases that target HIF- α isoforms for ubiquitination and degradation, in cells cultured at 21% oxygen mimicked the effect of 1% oxygen tension upon *Sost*, *Grem 1*, and *Nog* (Fig. 6). Thus, these data support the role of HIF- α in modulating expression of these transcripts under hypoxia.

Sost EXPRESSION IS NOT MEDIATED BY VEGF SIGNALING

Wang et al. [2007] demonstrated that stabilization of HIF-1 α enhanced angiogenesis and endochondral bone formation in mice, providing the most compelling evidence to date that hypoxia-regulated gene expression enhances bone formation. A subsequent study by Wan et al. [2008] demonstrated that HIF-1 α stabilization increased bone regeneration under conditions of distraction osteogenesis through a mechanism involving increased VEGF. We next sought whether hypoxic reductions in *Sost* were mediated by VEGF. The addition of recombinant 10 ng/ml VEGF₁₆₅ for 48 h revealed no effects on *Sost* transcript levels in UMR 106.01 cells cultured at 21% or 1% oxygen (Fig. 7), nor did 50 ng/ml VEGF₁₆₅ influence transcript (data not shown). Culture of cells at 21% or 1% oxygen in the presence of a VEGF neutralizing antibody revealed no effect upon *Sost* transcript level. Similar results were observed after 72 h of VEGF₁₆₅ or VEGF antibody treatment (data not shown). These data indicate that alterations in *Sost* expression in response to hypoxia are not mediated by VEGF.

DISCUSSION

The clinical manifestations of sclerosing bone dysplasias sclerosteosis and van Buchem disease, coupled with phenotypic characterization of *Sost* knockout mice, have classified sclerostin as a potent negative regulator of bone formation. Thus, the capability to selectively regulate sclerostin expression pharmacologically could modify the course of osteoporosis, as well as hasten bone repair following trauma; indeed, bone therapeutics currently exist or are under development that regulate bone mass, at least in part, by modulating sclerostin. Parathyroid hormone (PTH) exerts an inhibitory influence upon sclerostin expression, whether administered intermittently in vivo [Keller and Kneissel, 2005; Silvestrini et al., 2007] or through constitutive activation of the PTHrP receptor [Bellido et al., 2005; O'Brien et al., 2008]. Similarly, ovariectomized rats treated with sclerostin-inhibiting antibody showed reversal of hormone-deficiency bone loss, even to the point of enhanced bone mass and strength above that of control [Li et al., 2009]. These treatments demonstrate that further study of sclerostin will likely be fruitful.

The similarity in skeletal phenotype between sclerostin knockout [Li et al., 2008] and *Lrp5*^{G171V} gain-of-function [Babij et al., 2003] mice supports the assertion that sclerostin antagonizes Wnt signaling, so that suppression or loss of sclerostin function enables upregulation of Wnt signaling, thereby increasing cellular differentiation during both embryonic development and post-natal homeostasis. Although Wnt-receptor interactions may promote multiple signaling pathways, the canonical Wnt pathway involves stabilization of cytosolic β -catenin and subsequent induction of gene transcription. We observed that hypoxia decreased both *Sost* transcript and sclerostin protein expression (Fig. 2A,B); additional cultures treated with the RNA transcription inhibitor actinomycin D revealed no further change in the *Sost* levels over hypoxia alone, ruling out the possibility that hypoxia was affecting mRNA stability (data not shown). We next sought the influence of hypoxia upon β -catenin and β -catenin reporter assays. Immunofluorescent staining for activated (dephosphorylated) β -catenin revealed little expression in cells cultured under 21% oxygen; in contrast, cells cultured at 1% oxygen revealed intense nuclear staining, indicating increased levels of activated β -catenin under hypoxia, and suggesting participation in gene transcription (Fig. 3A). Indeed, we observed a dose-dependent increase in TOPFlash reporter output in response to both 1% oxygen culture and 10–100 μ M DFO (Fig. 3B), an iron-chelating agent used to chemically mimic hypoxia [Qu et al., 2008], as well as increased TOPFlash activity in cells cultured under 1% oxygen. These data demonstrate that hypoxia, or chemical mimicry of hypoxia by iron chelation, stabilizes β -catenin and induces β -catenin-driven gene expression. These findings support work by Qu et al. [2008], wherein DFO treatment stabilized β -catenin in human mesenchymal stem cells and murine mesenchymal C3HT101/2 cells, which enhanced alkaline phosphatase activity and extracellular calcium deposition. Combined with earlier data indicating that DFO increases alkaline phosphatase activity and osteocalcin secretion in human osteosarcoma cells [Naves Diaz et al., 1998; Gonzalez Suarez et al., 2003], these findings in toto support a role for hypoxic stabilization of β -catenin stimulating or enhancing osteogenesis.

There is interaction between BMP and Wnt signaling in osteogenic cells. For example, BMPs exert a major influence on sclerostin function by inducing its expression. Sutherland et al. [2004] demonstrated dose-dependent increases in *Sost* expression in hMSC-derived osteoblasts in response to BMP-2, -4, and -6, and we have shown similar results in post-osteoblastic/pre-osteocytic MLO-A5 cells [Papanicolaou et al., 2009]; herein, we describe stimulatory effect of BMP-2 upon *Sost* expression in UMR 106.01 osteoblasts. Genetic manipulation reveals similar influence of BMPs upon *Sost* expression: using an inducible *Cre-loxP* system, Kamiya et al. [2008] demonstrated that osteoblasts from conditional *Bmpr1a*-deficient mice exhibited increased bone mass and low levels of sclerostin. In contrast, mice expressing constitutively active *Bmpr1a* in osteoblasts exhibited increased *Sost* expression, and, subsequently,

attenuated Wnt signaling. Thus, we sought whether hypoxia mitigated sclerostin expression through the BMP pathway, or via a novel pathway. Indeed, hypoxia has previously been shown to alter BMP signaling through induction of BMP antagonists chordin-like-1 [Kane et al., 2008] and gremlin [Costello et al., 2008], decreased *Bmp2* synthesis [Salim et al., 2004], or via reductions in BMPRII levels and Smad1/5/8 phosphorylation [Takahashi et al., 2007]. We observed no change in expression of *Bmp2*, *Bmp4*, or *BmpRII* under hypoxia, suggesting that neither absence of ligand nor receptor was a causative factor for reductions in sclerostin. Instead, we observed significant increases in the expression of the BMP antagonists gremlin and noggin under both hypoxia and hypoxia mimicry, and this increase in BMP antagonist expression was paralleled by reduced phosphorylation of the BMP-effector Smad 1/5/8. When conditioned media collected from cells exposed to 1% oxygen, but not 21% oxygen, was added to cells cultured at 21% oxygen, we observed a similar decrease in *Sost* transcript (data not shown). Thus, while further effort is required to refine this mechanism, our results suggest that hypoxia partially reduces sclerostin expression via increased antagonism of BMP signaling. This hypothesis is supported by the work of Kamiya et al. [2009] wherein 100 ng/ml recombinant noggin decreased sclerostin expression in primary calvarial osteoblasts.

Leupin et al. demonstrated that the van Buchem enhancer region, located downstream of *Sost* and which is deleted in van Buchem disease, is a target of MEF2 family of transcription factors. The binding of MEF2 transcription factors to this distal *Sost* regulatory element has been shown to enhance basal *Sost* transcription driven by the proximal promoter [Leupin et al., 2007], in UMR106.01 cells. Using a combination of siRNA and dominant-negative transfectants, they demonstrated that MEF2C and MEF2D were primarily responsible for controlling *Sost* expression in a synergistic manner. Although Leupin et al. did not examine the influence of BMPs upon MEF2 localization or transcriptional activity, our results tentatively link BMP signaling and MEF2 family members in controlling *Sost* expression: hypoxia reduced BMP signaling in UMR 106.01 cells but not in HEK293 cells, and altered both Smad-1/5/8 phosphorylation and MEF2 reporter assays. Further experiments are required to demonstrate whether such a signaling node is shared by both BMPs and MEF2s.

Although evidence strongly supports a role for oxygen tension in the regulation of bone formation, in vitro studies have yielded contradictory results. Recent in vivo studies demonstrate that deletion of the ubiquitin ligase VHL, the absence of which stabilizes HIF- α isoforms, increases bone mineral content compared to wild-type controls [Wang et al., 2007; Wan et al., 2008]. Whereas Wan et al. implicated VEGF in enhanced angiogenesis and, subsequently, increased bone formation in *Vhl*^{-/-} mice via systemic administration of VEGF receptor antibody, we did not observe any influence of VEGF₁₆₅ nor VEGF-inhibiting antibody upon *Sost* transcript in cells cultured at 21% or 1% oxygen (Fig. 7). These data suggest that hypoxia-driven gene transcription positively influences bone formation through two distinct pathways, one involving VEGF and the other involving sclerostin. Our results establish that hypoxia decreases sclerostin expression in UMR 106.01 cells and, as a result, increases both total expression and nuclear localization of β -catenin. Furthermore, we found that hypoxia did not suppress *Sost* transcription in sclerostin-expressing kidney cells, suggesting that osteoblasts have a mechanism, not present in the kidney cells that influences the regulation of sclerostin transcription under hypoxia. UMR 106.01 osteoblasts increased *Sost* expression in response to BMPs, and hypoxia attenuated Smad1/5/8 phosphorylation, perhaps resultant to increased gremlin and noggin expression under hypoxia (Fig. 5C) or hypoxia mimicry (Fig. 6).

We present as Figure 8 our results and suggestive model. Under normoxic conditions (Fig. 8A), when molecular oxygen is present at sufficient levels for prolyl hydroxylases to function, HIF- α isoforms are targeted for degradation; MEF2 family members and Smads are recruited to the distal van Buchem enhancer region, where they augment sclerostin expression. Because sclerostin is transcribed and secreted, it functions in an autonomous fashion to inhibit Wnt

binding to Lrp5 or Lrp6; thus, β -catenin is degraded, and there is no transcription of TCF-dependent genes. Under hypoxia (Fig. 8B), HIF-1 α isoforms are stabilized and induce HIF-dependent transcription of *Vegf*, *Gremlin*, and *Noggin*; gremlin and noggin are released from the cell, where they may antagonize BMP function and reduce Smad phosphorylation. Through an unknown mechanism, MEF2 isoforms are excluded from the nucleus and, thus, *Sost* transcription is reduced. Reductions in sclerostin protein enhance Wnt signaling, thereby stabilizing β -catenin and enabling gene transcription with TCF. Further work in vivo is required to confirm these findings and to elucidate the regulatory interplay between hypoxia, *Sost* and bone formation.

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Fig. 1.

UMR106.01 osteoblasts respond to pericellular hypoxia. A: Immunofluorescent staining for hypoxia in UMR106.01 osteoblasts after 1- or 96-h treatment under 1% or 21% oxygen tension. Positive immunofluorescence indicates the presence of HypoxyProbe-1 adducts, which form at oxygen <100 mm Hg (1.4% oxygen). B: Immunoblotting for HIF-1 α under 1% or 21% oxygen tension for 1–96 h. Abundant HIF-1 α protein is detected in UMR106.01 cells after 1–96h culture in 1% oxygen.

Fig. 2.

Hypoxic culture decreases Wnt antagonists sclerostin and Dkk1 transcript and protein expression. **A:** Representative Western blot demonstrating decreased sclerostin expression after 48 or 96 h culture under 1% oxygen, β-actin is shown as a loading control. **B:** Quantitation of *Sost* transcript (solid line) or sclerostin protein (dashed line) in UMR106.01 cells cultured for 6–96 h under 1% or 21% oxygen. **C:** UMR106.01 cells under normoxia demonstrate greater expression of *Sost* than *Dkk1*, while *sFrp1* is not detectable. **D:** Quantitation of *Dkk1* transcript in UMR106.01 cells cultured for 6–96 h under 1% or 21% oxygen tension. Data are normalized to *ActB* transcript or β-actin protein, and are presented as mean expression at 1% relative to 21% oxygen at the same time point ±SEM; n = 3–5 per time point per condition. **P* < 0.05, ***P* < 0.001.

Fig. 3.

Hypoxia activates β -catenin signaling. A: UMR 106.01 cells cultured for 96 h under 1% oxygen demonstrate increased total expression and nuclear localization of activated β -catenin compared to cells cultured under 21% oxygen. Addition of recombinant Dkk1 and sclerostin (250 ng/ml) reduced B: UMR 106.01 cells transfected with the β -catenin reporter assay TOPFlash were treated with the hypoxia mimetic DFO, or 100 nM hPTH(1–34) 21% oxygen, or cultured under 1% oxygen, for 24 h. The hypoxia mimetic DFO dose-dependently increased β -catenin transcriptional activity, as did 1% oxygen. Data are presented as Luciferase activity $\pm$ SEM, normalized to vehicle control, $n = 3$ –5 per time point per condition. * $P < 0.05$ compared to control or vehicle-treated cells; ** $P < 0.01$ compared to control or vehicle cells.

Fig. 4. Suppression of sclerostin under hypoxia occurs in osteogenic cells and involves MEF2 transcription factors. **A:** *Sost* expression was analyzed in total RNA from osteoblasts, osteocytes, and embryonic kidney cells cultured under 21% or 1% oxygen. Bars represent mean transcript expression relative to 21% oxygen cultures $\pm$ SEM. **B:** Mef2-luciferase activity is decreased under 1% oxygen culture compared to 21% culture in UMR 106.01 cells. * $P < 0.05$ compared to control or vehicle-treated cells.

Fig. 5.

Hypoxia alters BMP signaling in osteoblastic cells. A: *Sost* expression is induced in UMR 106.01 in response to 24 h culture with BMP-2 (10, 250, or 500 ng/ml). B: Hypoxia does not alter transcription of BMP signaling pathway components *Bmp2*, *Bmp4*, or *Bmp-R1A*. C: Expression of BMP antagonists *Gremlin* and *Noggin* increases under hypoxia. D: pSmad 1/5/8 phosphorylation is attenuated in osteoblastic cells cultured under 1% oxygen compared to 21% oxygen. Bars represent transcript or Smad phosphorylation and are presented as mean expression at 1% relative to 21% oxygen at the same time point $\pm$ SEM. $n = 3-5$ per time point per condition. * $P < 0.05$ compared to control or vehicle-treated, time-matched samples; *** $P < 0.001$ compared to control or vehicle-treated, time-matched samples.

Fig. 6.

HIF- α stabilization mimics the effect of hypoxia upon transcription of *Sost* and BMP antagonists. UMR106.01 cells cultured at 21% oxygen in the presence of the HIF- α stabilizing agent DFO (50 or 100 μ M) for 48 h reveal decreased *Sost* transcription and increased *Grem 1* and *Noggin* expression relative to control cultures. Bars represent mean transcript level $\pm$ SEM normalized to 21% oxygen, n = 3 per condition. * P <0.05 compared to control; ** P <0.01 compared to control.

Fig. 7.

Sost expression is unaltered by VEGF signaling. Manipulation of VEGF signaling with recombinant VEGF165 or VEGF-inhibiting antibody revealed no effect upon *Sost* expression, regardless of oxygen tension. Bars represent mean *Sost* transcript $\pm$ SEM normalized to 21% oxygen. $n = 4$ per condition. * $P < 0.05$ compared to 21% oxygen.

Fig. 8.

Proposed model for Wnt signaling under hypoxia. Under normoxic conditions (A), HIF- α isoforms are targeted for degradation; Mef2 and Smads are recruited to the distal van Buchem enhancer region, where they augment sclerostin expression. Sclerostin is transcribed and secreted, functions in an autonomous fashion and inhibit Wnt binding to Lrp5 or Lrp6. β -catenin is degraded, and there is no transcription of TCF-dependent genes. Under hypoxia (B), HIF-1 α isoforms are stabilized and induce HIF-dependent transcription of *Vegf*, *Greml*, and *Noggin*; gremlin and noggin are released from the cell, where they may antagonize BMP function and reduce Smad phosphorylation. Through an unknown mechanism, Mef2 isoforms are excluded from the nucleus and, thus, *Sost* transcription is reduced. Reductions in sclerostin

protein enhance Wnt signaling, thereby stabilizing β -catenin and enabling gene transcription with TCF.