An Akt-dependent Increase in Canonical Wnt Signaling and a Decrease in Sclerostin Protein Levels Are Involved in Strontium Ranelate-induced Osteogenic Effects in Human Osteoblasts*

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Sclerostin is an important regulator of bone homeostasis and canonical Wnt signaling is a key regulator of osteogenesis. Strontium ranelate is a treatment for osteoporosis that has been shown to reduce fracture risk, in part, by increasing bone formation. Here we show that exposure of human osteoblasts in primary culture to strontium increased mineralization and decreased the expression of sclerostin, an osteocyte-specific secreted protein that acts as a negative regulator of bone formation by inhibiting canonical Wnt signaling. Strontium also activated, in an apparently separate process, an Akt-dependent signaling cascade via the calcium-sensing receptor that promoted the nuclear translocation of β-catenin. We propose that two discrete pathways linked to canonical Wnt signaling contribute to strontium-induced osteogenic effects in osteoblasts.

Strontium ranelate is a treatment for osteoporosis that decreases the incidence of vertebral and femoral fracture risk in postmenopausal women (1–3). Strontium ranelate has been shown to modulate the physiological processes of bone formation and bone resorption (2), resulting in increased bone apposition rates (3) and bone mineral density (1, 2, 4–6), while maintaining the quality of bone mineral (6).

Sclerostin is exclusively expressed by osteocytes in adult life (7, 8) but is more widely expressed during development (7) and plays a physiological role as a negative regulator of bone formation by repressing bone morphogenetic protein-induced osteogenesis (9–11). The importance of sclerostin in bone-loss disorders has been described in several in vivo studies. Positional cloning studies identified loss of function mutations in the SOST gene that cause sclerostosis and van Buchem disease, bone dysplasias characterized by progressive skeletal overgrowth (12). In contrast, transgenic mice overexpressing sclerostin had significant reductions in bone mass and mineral apposition rate compared with wild type (7). A sclerostin knock-out mouse model was shown to have increased osteoblast activity and enhanced osteoblast/osteocyte viability and was resistant to mechanical unloading-induced bone loss. This phenotype was associated with an increase in canonical Wnt signaling when compared with wild-type mice (8).

Sclerostin functions as an antagonist of canonical Wnt signaling, whereby GSK-3β-stimulated, ubiquitin-mediated breakdown of β-catenin is alleviated, resulting in its nuclear translocation, and binding to transcription factors of the T-cell factor/lymphoid enhancer factor family, to induce the transcription of growth-associated genes (13). Non-canonical Wnt signaling does not involve β-catenin translocation to the nucleus (11). Sclerostin binds to the extracellular domains of the Wnt co-receptors LRP5, LRP6, and LRP4 and disrupts extracellular Wnt-induced Frizzled/LRP complex formation thus providing a molecular mechanism by which loss of sclerostin function may lead to conditions such as sclerostosis (12, 14). In addition to Frizzled/LRP-mediated activation of canonical Wnt signaling, β-catenin also translocates to the nucleus following phosphorylation by Akt at Ser552 (15) and in response to Akt-mediated deactivation of GSK-3β at Ser373 (16). This Akt-signaling mechanism represents an alternative activation pathway for canonical Wnt signaling. The mammalian target of rapamycin (mTOR) complex 2 (mTORC2) has been shown to be the upstream kinase responsible for Akt phosphorylation at Ser473 (17), whereas mTOR complex 1 (mTORC1) lies downstream of Akt (18) and responsible for the control of p70 S6 kinase (19). Although mTORC2 activation arises in response to a variety of stimulatory factors (20), the mechanism is unclear.

We and others have previously reported that treatment of primary human osteoblasts with strontium increases replication as well as expression of differentiation markers RUNX2 and alkaline phosphatase (21–23). These results complement in vivo studies in animals (24) and human subjects (6), which show increases in bone formation after treatment with strontium ranelate. Because stimulation of the canonical Wnt pathway is also osteogenic in vivo (25) we investigated whether...

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‡ The abbreviations used are: GSK-3β, glycogen synthase kinase-3β; mTOR, mammalian target of rapamycin; mTORC1, mTORC1 complex 1; mTORC2, mTORC complex 2; BCA, bicinchoninic acid; HOB, human osteoblast; ARS, Alizarin Red S; HDC, heat-denatured casein; dHOB, differentiated osteoblast; CaSR, calcium-sensing receptor.
strontium ranelate affected canonical Wnt signaling in primary human osteoblasts.

**EXPERIMENTAL PROCEDURES**

**Materials**

All chemicals, including culture media and fetal calf serum (FCS) were obtained from Sigma-Aldrich unless otherwise specified. Sodium ranelate was supplied by Les Laboratoires Servier (Neuilly, France). ThermaNox™ coverslips were obtained from Thermo Fisher Scientific (Rochester, NY). Opti-MEM™ was obtained from Invitrogen. Antibodies were obtained as follows: β-catenin-Ser552, Akt, Akt-Ser473, Akt-Thr308, GSK-3β, and GSK-3β-Ser9 monoclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA), a sclerostin monoclonal antibody, a sclerostin biotinylated polyclonal antibody, recombinant human sclerostin were obtained from R&D Systems (Minneapolis, MN), and β-actin and β-catenin monoclonal antibodies were obtained from Sigma-Aldrich. The bicinchoninic acid (BCA) assay was obtained from Thermo Scientific (Rockford, IL). The Akt kinase inhibitor AKT-XI was obtained from Merck KGaA (Darmstadt, Germany). The NPS2143 calcilytic was kindly donated by Dr. Arthur Christopoulos. All absorbance and fluorescence measurements were carried out on a Polarstar Galaxy plate reader (BMG Labtech GmbH, Offenburg, Germany).

**Methods**

**HOB Culture Conditions**—Human osteoblasts (HOB) were grown from the minced trabecular ends of fetal long bone in accordance with the National Health and Medical Research Council guidelines and with the approval of the University of Sydney Human Ethics Committee (approval number: 01/02/40), as described previously (26). The study was conducted using HOBs from several different donors. Each experiment was conducted using HOBs from at least two different donors. HOBs were routinely maintained in DMEM containing 10% (v/v) FCS supplemented with 150 μM L-ascorbic acid 2-phosphate (10% DMEM) and were plated in 6-well plates at 5 × 10⁵ HOBs/well for all experiments. HOBs were treated in serum-reduced Opti-MEM™ media (Invitrogen) containing 1 mM Ca²⁺ (defined as the “Vehicle” treatment for all experiments, to approximate the concentration of physiological Ca²⁺ for humans (27) with the addition of strontium ranelate at various concentrations as described for the individual experiments. For all experiments HOBs were adapted to the vehicle treatment for 3 h prior to the addition of strontium ranelate. For the purposes of the study, strontium ranelate consisted of a 100:1 molar ratio of Sr²⁺/Ca²⁺, derived from SrCl₂, and ranelate, sourced from sodium ranelate, because this ratio reflects the relative concentrations of strontium and ranelic acid in the serum of patients treated with strontium ranelate 2 g/day for 3 years. Previous studies have shown that Sr²⁺ is the active agent (28). Concentrations of strontium ranelate used in this study are expressed in terms of Sr²⁺ (millimolar). The concentrations of strontium ranelate used for treatment of HOBs in this study were 0.1 mm, intended to mimic the approximate strontium blood concentration of patients treated with strontium ranelate 2 g/day (29), and 2 mM, which may reflect increased strontium concentrations that may be present at sites of bone remodeling (30).

To permit HOBs to grow into a multilayer structure the cells were subcultured into 6-well plates containing Thermanox™ coverslips and grown using an adaptation of a previously established protocol (26). Briefly, cells were grown to confluence in 10% DMEM and the media was then changed to mineralization medium (Opti-MEM™ containing 2.5% (v/v) FCS, 2.5 mM β-glycerophosphate, and 150 μM L-ascorbic acid 2-phosphate, 100 units/ml penicillin, and 100 μg/ml streptomycin) with the addition of strontium ranelate for the indicated duration and concentration as described for the individual experiments. Mineralization media were routinely changed every 2 days for the course of the experiment.

**Quantification of Mineralization**—Alizarin Red S (ARS) was used to quantify the mineralization of HOB cultures in response to strontium ranelate treatment using an adaptation of a previously established protocol (31). Briefly, HOBs were grown to confluence on ThermaNox™ as previously described and then grown for a further 7 or 14 days in mineralization media in the presence or absence of strontium ranelate at the indicated concentration. Following treatment, mineralized HOBs were washed three times with PBS and fixed with 70% (v/v) ice-cold ethanol for 1 h. The fixed layers were washed three times with excess dH₂O before the addition of 2% (w/v) ARS (pH 4.2) for 30 min at room temperature with gentle agitation. Following removal of ARS the stained layers were washed five times for 5 min with excess dH₂O and three times with PBS for 5 min at room temperature. Mineral-bound ARS was solubilized by the addition of 1 ml of 10% (w/v) cetylpyridinium chloride in 10 mM NaH₂PO₄ (pH 7). The absorbance of the dye was measured at 560 nm.

**Preparation of HOB Multilayers for Ultrastructural Analysis**—Three sets of long-term cultures were processed for ultrastructural examination as follows. After removal from the well the ThermaNox™ coverslips bearing the HOB multilayers were cut into two equal strips and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h. They were then washed in 0.1 M cacodylate buffer for three changes of 10 min each, after which they were postfixed in 2% aqueous osmium tetroxide for 2 h. Next they were washed in 0.1 M cacodylate buffer for three changes of 10 min each. dehydration was carried out in 20-min changes of 30, 50, 70, and 90% (v/v) ethanol followed by two changes of water-free absolute ethanol (30 min each) and the two changes of water-free absolute acetone (30 min each). A mixture of 1:1 acetone:Spurr’s resin was prepared, and the tissue was transferred to it for 1 h, followed by 3 h and 1.9 acetone: Spurr’s resin. The tissue was then left in 100% Spurr’s resin overnight. At this stage the coverslips were placed in BEEM capsules filled with Spurr’s resin. Curing was carried out at 60 °C for 18 h.

**Solubilization of Mineralized HOB Multilayers for Protein Detection**—Because sclerostin has been reported to have a basic region, which causes it to bind to bone matrix proteins (32), extraction from the multilayers was tested as well as ELISA, because secreted sclerostin levels might be expected to be low. HOBs were grown to confluence on ThermaNox™ as described previously, and subsequently grown for a further 7,
14, or 28 days in mineralization medium in the presence or absence of strontium. Following treatment the wells were washed with PBS containing 1 mM EDTA. The mineralized matrix was then re-solubilized by the addition of 300 µl of 62.5 mM Tris-HCl, pH 6.8, 5% (v/v) SDS, 10% (v/v) glycerol, 0.25 mM DTT, 0.01% (w/v) bromophenol blue, with the protease inhibitors 2 mM PMSE, 1 mM EDTA, 20 µM leupeptin, 2 µM aprotinin, 10 µM pepstatin A, and 50 µM Bestatin-HCl, which had been pre-heated to 95 °C. The resultant slurry was scraped to the bottom of the well and sonicated using the micro-tip of a Branson Sonifier® 250 (Branson Ultrasonics Corp., Danbury, CT) for ~30 s or until the viscosity of the solution was such that it was able to be freely pipetted. Equal volumes of the sonicated solution were subjected to Western blot analysis and immunoblotting for sclerostin and β-actin as described above.

**Sclerostin ELISA**—A capture ELISA protocol was designed to measure the level of secreted sclerostin in the dHOB culture media. Unless otherwise stated stages between the ELISA protocol were separated by five washes with PBS, pH 7.4, containing 0.1% (v/v) Tween 20 (PBS-T), and all stages were carried out at room temperature. Briefly, a monoclonal antibody to sclerostin was used to coat a 96-well polystyrene plate overnight at 4 °C at 1 µg/ml in 0.1 mM NaHCO₃, pH 9. Nonspecific sites of the plate were blocked with 0.1% (w/v) heat-denatured casein (HDC) in PBS-T (HDC/PBS-T). Sclerostin from the supernatants from dHOB cultures or recombinant human sclerostin, at a known concentration, were incubated in the pre-adsorbed wells for the indicated time of strontium ranelate treatment. Cell lysates were centrifuged for 12,000 g for 30 min at 4 °C, and the resulting soluble solution represented the non-nuclear fraction. To isolate the nuclear fraction, HOB monolayers were lysed in a detergent buffer (10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) Nonidet P-40, with the protease inhibitors listed above) for 5 min on ice following the indicated time of strontium ranelate treatment. Cell lysates were centrifuged at 500 × g for 30 min at 4 °C. The pellet was washed two additional times with the same detergent buffer, and the resulting pellet was resuspended in a high salt buffer (10 mM Tris (pH 7.5), 0.4 mM NaCl with the protease inhibitors listed above) for 30 min on ice. The lysed nuclear suspension was centrifuged at 12,000 × g for 30 min at 4 °C, and the resulting supernatant represented the nuclear fraction. Protein concentrations from both fractions were normalized using a BCA assay (Pierce), and equal amounts of protein were subjected to Western blot analysis. The presence of β-actin and LaminB1 were used as loading controls for the cytosolic and nuclear fractions, respectively.

**Measurement of Caspase Activity**—HOBs were seeded in 6-well plates at a density of 1 × 10⁶ cells per well in 10% (v/v) DMEM for 24 h. Following a 24-h period of adaptation to serum-free conditions in Opti-MEM™ containing 1 mM Ca²⁺, strontium was added to the cells in the absence or presence of 50 nm wortmannin (10 × IC₅₀) for a further 24 h. Oxidative stress was subsequently induced by the addition of 50 µM H₂O₂, and the level of apoptosis in the cell population was assessed via caspase activity, based on a previously reported protocol utilizing the caspase-3 substrate Ac-DEVD-AFC (34). Protein levels used in the caspase assay for various treatment conditions were normalized following concentration determination by Bradford assay, carried out according to the manufacturer’s instructions.

**Measurement of Replication**—HOBs were seeded in 96-well plates at a density of 7.5 × 10³ cells per well in 10% (v/v) DMEM for 24 h. Following a 24-h period of adaptation to serum-free conditions in Opti-MEM™ containing 1 mM Ca²⁺, strontium was added to the cells in the absence or presence of 50 nm wortmannin (10 × IC₅₀) for a further 48 h. At this time point HOBs were lysed in PBS, pH 7.4, containing 0.1% (v/v) Triton X-100, and the total cell protein level in the lysates was determined by BCA assay according to the manufacturer’s instructions.

**Statistics and Data Analysis**—Experiments were performed in triplicate. Each experiment was repeated at least three times, and data are given as means ± S.D. One-way analysis of variance with Tukey-Kramer post test to determine significant dif-
Strontium Activates Canonical Wnt Signaling

FIGURE 1. A, transmission electron micrograph of the cell multilayer at 20 days post-confluence. In these sections the osteocyte-like cell layer (O) can be seen layered with sections of the extracellular matrix. The type I collagen was organized into layers that ran parallel to (P) or perpendicular to (T) the plane of the section. B, strontium (Sr²⁺) increased HOB mineralization as measured by ARS staining. ARS was measured in HOBs treated in the absence or presence of strontium at the indicated concentration for 7 days (clear bars) or 14 days (black bars) post-confluence. The vehicle treatment contained 1 mM Ca²⁺. **, p < 0.001 compared with vehicle; *, p < 0.05 compared with vehicle.

RESULTS

HOBs Adopted a Multilayered Structure in Long-term Culture under Mineralizing Conditions—When HOB monolayers were cultured on Thermax™ coverslips and then incubated in mineralization media in the absence or presence of strontium at the indicated concentration for 7 or 14 days. In these mature mineralizing cultures, treatment with strontium resulted in a concentration- and time-dependent increase in mineral deposits as measured by ARS staining when compared with the vehicle treatment (Fig. 1B). Significant increases in ARS staining were observed in cultures in which 2 mM strontium was present for 7 (p < 0.01) and 14 days (p < 0.001) of dHOB mineralization when compared with the vehicle treatment. 0.1 mM strontium also induced a significant increase in ARS staining after 14 days of mineralization (p < 0.05).

Strontium Increased the Mineralization Rate of HOBs—To assess the effect of strontium on the mineralization rate of dHOBs, the cells were permitted to grow to confluence on Thermax™ coverslips and then incubated in mineralization media in the absence or presence of strontium at the indicated concentration for 7 or 14 days. In these mature mineralizing cultures, treatment with strontium resulted in a concentration- and time-dependent increase in mineral deposits as measured by ARS staining when compared with the vehicle treatment (Fig. 1B). Significant increases in ARS staining were observed in cultures in which 2 mM strontium was present for 7 (p < 0.01) and 14 days (p < 0.001) of dHOB mineralization when compared with the vehicle treatment. 0.1 mM strontium also induced a significant increase in ARS staining after 14 days of mineralization (p < 0.05).

Strontium Activated an Akt-dependent Signaling Cascade in HOBs—The activation of Akt via phosphorylation at Thr³⁰⁸ and Ser³⁷³ plays a critical role in the control of cell survival (37–39). Akt has been shown previously to phosphorylate β-catenin at Ser⁵⁵² (15) and GSK-3β at Ser³⁷ (16) resulting in β-catenin accumulation in the nucleus and increases in its transcriptional activity. Treatment of monolayer HOB cultures with 0.1 or 2 mM strontium resulted in the phosphorylation of Akt at Thr³⁰⁸ and Ser³⁷³ as well as β-catenin phosphorylation at Ser⁵⁵² and GSK-3β at Ser³⁷ (Fig. 4A).

Strontium Promoted Canonical Wnt Signaling in HOBs—Canonical Wnt signaling induces a decrease in the ubiquitin-mediated breakdown of the cytosolic pool of β-catenin and its subsequent translocation to the nucleus. Treatment of monolayer HOB cultures with 2 mM strontium resulted in a time-dependent increase in the cytosolic level of β-catenin, with a maximum ~3-fold increase after 15 min (Fig. 3A). Strontium also induced an increase in the nuclear translocation of β-catenin, with a maximum ~2-fold increase after 45-min exposure to 2 mM (Fig. 3B).

Strontium Decreased the Expression of Sclerostin in Mineralized HOB Cultures—We were unable to detect sclerostin in monolayer cultures. This marker, localized to osteocytes in adult bone, was reliably detected when mineralized HOB cultures (Fig. 1A) were broken down using a combination of detergent treatment and sonication, and the resultant extractions were analyzed by Western blot for sclerostin protein. When strontium was present during the mineralization of HOBs for 7 or 14 days post-confluence, a concentration- and time-dependent decrease in the level of sclerostin was shown (Fig. 2, A–C). Significant decreases in sclerostin were observed in the presence of 0.1 mM strontium for 7 (p < 0.05) and 14 days (p < 0.001) when compared with vehicle. In the presence of 0.1 mM strontium a significant reduction in sclerostin was observed after 14 days (p < 0.001). After 14 days the magnitude of the effect of 2 mM strontium was significantly greater than that due to 0.1 mM strontium (p < 0.01). The level of sclerostin secreted into the supernatant of dHOB cultures was measured by capture ELISA. When dHOBs were permitted to mineralize for 14 days and subsequently exposed to medium in the absence or presence of strontium for 3 days, a strontium-induced decrease in secreted sclerostin was detected (Fig. 2D). Thus strontium causes a decrease in the amount of mineral-associated sclerostin (Fig. 2, A–C) as well as secreted sclerostin (Fig. 2D).

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In addition to a role in cell adhesion (43), β-catenin is a highly conserved component of the canonical Wnt signaling pathway (44). The nuclear translocation of β-catenin is induced by extracellular Wnt proteins but also occurs in response to other stimuli, including oxidative stress (34). Here we show that strontium induces canonical Wnt signaling in HOBs (Fig. 3) and provide evidence that two candidate pathways are involved. The first is an Akt-dependent mechanism (Fig. 4) previously described for canonical Wnt signaling (15, 16). The second arises from a strontium-induced decrease in the protein expression of sclerostin (Fig. 2), a known antagonist of canonical Wnt signaling, in dHOB multilayers.

The activation of Akt is modulated via extracellular Wnt proteins but also occurs in response to other stimuli, including oxidative stress (34). Here we show that strontium induces canonical Wnt signaling in HOBs (Fig. 3) and provide evidence that two candidate pathways are involved. The first is an Akt-dependent mechanism (Fig. 4) previously demonstrated for canonical Wnt signaling (15, 16). The second arises from a strontium-induced decrease in the protein expression of sclerostin (Fig. 2), a known antagonist of canonical Wnt signaling, in dHOB multilayers.

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Strontium induces phosphorylation at both of these sites in HOBs (Fig. 4A). Dual-phosphorylation of Akt may explain the prosurvival effect of strontium that was previously reported (21). Knock-out mouse studies have shown that of the three isoforms of Akt, Akt1 regulates the control of osteoblast survival (45), whereas Akt2 promotes induction of differentiation prior to Runx2 expression (46). Here we show that strontium induces the phosphorylation of Akt at Thr³⁰⁸ in HOBs in a PI3K-dependent manner (Fig. 4B). Interestingly, strontium-induced phosphorylation of Akt at Ser⁴⁷³ was relatively unaffected by wortmannin (Fig. 4B) distinct from its effects on Akt-Ser⁴⁷³ phosphorylation in some cell lines (38, 47). The results suggest that Akt signaling mechanisms may be differentially regulated in native cells.

We and others have previously reported that the CaSR is required for strontium-induced responses in HOBs (21, 48).
the current study, the finding that the calcilytic NPS2143 prevented strontium-induced phosphorylation of Akt at Thr\(^{308}\) as well as Ser\(^{373}\) (Fig. 4C) provides additional evidence that the CaSR mediates key elements of the strontium response, including the activation of PI3K and PDK1 (Akt-T\(^{308}\)) and mTORC2 (Akt-Ser\(^{473}\)). Wortmannin did not affect strontium-induced Akt-Ser\(^{473}\) phosphorylation (Fig. 4B). This finding implies that the CaSR-dependent activation of mTORC2, unlike PDK1, is independent of PI3K in HOBs. The differential effects of wortmannin on strontium-induced replication (inhibited) and on strontium-induced decreases in caspase activity after oxidative stress (no effect) (Fig. 5) are consistent with the proposal that stimulation of replication was dependent on phosphorylation of Akt at Thr\(^{308}\), but that protection from stress-induced apoptosis requires phosphorylation at Ser\(^{473}\), an mTORC2-dependent process (17). In regards to strontium-induced activation of mTOR complexes, it is relevant that we have also observed strontium-induced phosphorylation of mTOR at Ser\(^{2448}\), a marker of mTOR complex 1 activity (data not shown), however this is known to be a downstream element of Akt (18). We are currently investigating the activation of mTOR complexes by strontium in HOBs.

The phosphorylation of \(\beta\)-catenin at Ser\(^{552}\) by Akt has been shown to release \(\beta\)-catenin from cell-cell contacts leading to its accumulation in both the cytosol and the nucleus followed by an increase in its transcriptional activity (15). Here we found that \(\beta\)-catenin is phosphorylated at Ser\(^{552}\) by Akt in response to strontium (Fig. 4A). \(\beta\)-Catenin nuclear translocation was further promoted by Akt-dependent phosphorylation of GSK-3\(\beta\) at Ser\(^{9}\) (Fig. 4A), which alleviates constitutive breakdown of \(\beta\)-catenin, in the cytoplasm (16). Therefore, strontium promoted nuclear translocation of \(\beta\)-catenin via two Akt-dependent signaling mechanisms: direct phosphorylation of \(\beta\)-catenin at Ser\(^{552}\); and decreased constitutive breakdown of \(\beta\)-catenin via phosphorylation of GSK-3\(\beta\) at Ser\(^{9}\). AKT-XI, an inhibitor of Akt-kinase decreased strontium-induced phosphorylation of GSK-3\(\beta\)-Ser\(^{9}\) and \(\beta\)-catenin-Ser\(^{552}\) and suppressed strontium-induced \(\beta\)-catenin nuclear translocation in HOBs (Fig. 4D).

A recent study in murine MC3T3-E1 cells reported that strontium promoted calcineurin-mediated nuclear factor of activated T-cells (NFAT) signaling resulting in enhanced transcriptional activity of \(\beta\)-catenin (49). Here we found that strontium induced PI3K-mediated activation of Akt in HOBs (Fig. 4B). Because the nuclear factor of activated T-cell signaling is mediated, at least in part by GSK-3\(\beta\) activity (50), and Akt is recognized as a modulator of GSK-3\(\beta\) (15), activation of both signaling pathways may arise in response to strontium in HOBs.

The level of strontium-induced phosphorylation was greater for all proteins investigated in the presence of 2 mM when compared with 0.1 mM (Fig. 4A). Such an increase in Akt-dependent signaling may explain the increased effectiveness of strontium ranelate when used in studies at doses yielding serum concentrations higher than the \(\sim 0.1\) mM typically seen in patients on standard therapy (29, 51). Consistent with this, variations in the activation of intracellular signaling cascades and expression of sclerostin (Figs. 2 and 4) were dependent on the strontium concentration. The use of higher concentrations of strontium (2 mM) in the current study, than are commonly observed in the blood of patients (51), may also be pharmacologically relevant at sites of bone remodeling where strontium can accumulate (30).

To assess the effect of strontium on HOB differentiation we employed an established protocol that promotes the adoption of an “osteocyte-like” phenotype (Fig. 1A) (26). We and others have previously shown that strontium induces differentiation of osteoblasts, as measured by increases in alkaline phosphatase activity and RUNX2 mRNA expression (21, 22), as well as osteocalcin (23). Here we found that strontium induced an increase in mineralization of dHOBs in a time- and concentration-dependent manner (Fig. 1B). This mineral was shown to be an immature form of hydroxyapatite by energy-dispersive spectroscopy (26). The expression of the osteocyte-derived sclerostin protein (9) as early as 7 days post-confluence supports the notion that the mineralizing culture conditions had induced differentiation of HOBs to an osteocyte-like phenotype (Fig. 2A).

We evaluated sclerostin protein expression, because an earlier study on the effect of strontium on sclerostin mRNA expression reported variable results (23). Furthermore, discordant correlations between the levels of mRNA and protein have been observed (52–54), and the correlations between...
Strontium Activates Canonical Wnt Signaling

FIGURE 6. Strontium (Sr$^{2+}$) promotes canonical Wnt signaling in HOBs via two independent signaling pathways. In the absence of strontium canonical Wnt signaling operates at a basal level via the interaction of extracellular canonical Wnt proteins with the Frizzled/LRP complex leading to the translocation of β-catenin to the nucleus. Strontium has also been reported to increase the secretion of canonical Wnts from osteoblasts (49). Sclerostin expression by osteocytes into the cellular environment disrupts the Wnt-Frizzled/LRP complex and thus inhibits Wnt signaling. Strontium activates Akt (via phosphorylation at Thr308 and Ser473), inhibits GSK-3β (via phosphorylation at Ser9), and promotes phosphorylation of β-catenin at Ser552 to promote β-catenin translocation to the nucleus. Strontium also suppresses the expression of sclerostin by osteocyte-like cells further promoting canonical Wnt signaling via a decrease in the level of disruption of the Wnt-Frizzled/LRP complex formation by sclerostin. Inhibitors used in this study are highlighted in italics at their sites of action in the signaling cascade.

mRNA and secreted protein levels for various human genes are low (52, 55). Because the primary structure of sclerostin is known to contain a positively charged basic region (32), it is likely that this region strongly interacts with negatively charged proteoglycans present in dHOB multilayer cultures (56). For this reason we measured the level of sclerostin extracted from the dHOB multilayer. We have previously identified other secreted proteins, including insulin-like growth factors I and II, transforming growth factor-β1, and basic fibroblast growth factor associated with cultured dHOB multilayers (56). This could also be the reason for the relatively low level of secreted sclerostin. Despite detection levels for the ELISA as low as 150 pg/ml, dHOB supernatant levels were frequently below threshold. Where levels of sclerostin were detected by ELISA a statistically significant strontium-dependent decrease in the level of the protein was observed (Fig. 2D). In these osteocyte-like cells, strontium induced a time- and concentration-dependent decrease in sclerostin protein expression, following 7 or 14 days of mineralization (Fig. 2). Because sclerostin disrupts the Frizzled/LRP complex (57), a decrease in sclerostin expression promotes canonical Wnt signaling. This finding is of particular significance for osteoblasts, because Wnts have been shown to repress alternative differentiation pathways (e.g. adipocytes) and promote osteoblast survival, differentiation, proliferation, and mineralization (58).

In conclusion, strontium promotes canonical Wnt signaling in HOBs at pharmacologically relevant concentrations. This effect occurs at least in part via activation of an Akt-dependent signaling mechanism (Fig. 6). In an apparently separate process, strontium also suppressed sclerostin expression, an effect that would be expected to increase canonical Wnt signaling (Fig. 6). Taken together these findings provide a molecular mechanism for the increased bone formation rates seen with strontium ranelate treatment in vivo (3).

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