Early Intervention with Low-Dose Sclerostin Antibody Therapy can Reduce Osteocyte-mediated Secondary Mineralization after initial bone loss in Ovariectomised Rats

Syeda Masooma Naqvi[1], Hollie Allison[1], Laoura M. O'Sullivan[1], Gill Holdsworth[3], Juan Alberto Panadero-Perez[1], Jessica Schiavi-Tritz[1, 2], Laoise M. McNamara[1]*

[1] Mechanobiology and Medical Devices Research Group (MMDRG), Biomedical Engineering, University of Galway, Ireland.
[2] Laboratory in Reactions and Chemical Engineering, CNRS-Lorraine University, UMR 7274, Vandœuvre-lès-Nancy, France.
[3] Early Solutions, UCB Pharma, Slough, UK

* Corresponding Author
Prof. Laoise M. McNamara,
Mechanobiology and Medical Devices Research Group (MMDRG),
Department of Biomedical Engineering,
School of Engineering,
College of Science and Engineering,
University of Galway,
Galway,
Ireland.
Phone: (353) 91-492251
Fax: (353) 91-563991
Email: laoise.mcnamara@universityofgalway.ie

Abstract

Neutralizing antibodies targeting sclerostin (Scl-Ab) mitigate bone loss and promote bone formation, thereby reducing fracture risk in postmenopausal osteoporosis. Clinical use of Scl-Ab is indicated for postmenopausal women with severe osteoporosis who are at high risk of, or have a history of, fragility fractures, which can occur many years after menopause. However, in early stages of estrogen deficiency there is rapid bone loss, which abates thereafter, whereas in the longer-term changes in bone mineralization occur. This study seeks to investigate whether administering intermittent low-dose monthly sclerostin antibody during early estrogen deficiency could mitigate osteoclastogenesis but also prevent osteocyte-driven secondary mineralization, due to apoptosis, perilacunar remodelling and osteocyte mechanosensitivity. Female Wistar rats that had completed their breeding cycles underwent bilateral ovariectomy as a model of postmenopausal osteoporosis. After 3 weeks of bone loss, one group received monthly low-dose Scl-Ab injections (2 mg/kg/month) and a control group remained untreated. We report that early treatment with intermittent low-dose Scl-Ab mildly increased bone formation, reduced TRAP+ osteoclasts and suppressed catabolic and matrix degradation gene expression (Sost, Ctsk, Mmp9) compared to untreated rats. There was a decrease in the percentage of empty lacunae and MMP14+ osteocytes, along with decreased gene expression associated with osteocyte-mediated mineralization (DMP1, PHEX, OPN, ALP) and mechanotransduction (Vcl, integrins α5, αV, β1, CX43, Axin2, IFT88, Adcy6, Pkd1, and Cav1). Thus, we propose that early intervention with intermittent low-dose Scl-Ab therapy can promote surface bone formation but reduce osteocyte-mediated secondary mineralization after initial bone loss.

Keywords

Sclerostin Antibody (Scl-Ab), Postmenopausal Osteoporosis, Ovariectomized Rats, Bone Remodelling, Secondary Mineralization, Early Intervention

1.1 Introduction

Postmenopausal osteoporosis is characterized by an imbalance in bone remodelling, primarily driven by excessive resorption of bone tissue by osteoclasts, coupled with insufficient formation of new bone tissue by osteoblasts [1]. The early phase of estrogen deficiency is characterized by a rapid deterioration of trabecular bone volume and microarchitecture within the first few weeks [2]. Subsequently, there is secondary bone tissue mineralization and thickening of the remaining trabecular bone over time [2, 3]. Moreover, estrogen deficiency induces site-specific alterations in mineral distribution, in addition to changes in the osteocyte microenvironment, and an increase in osteocyte apoptosis and perilacunar remodelling [4-7].

Antiresorptive therapies (bisphosphonates) have been widely used in treating osteoporosis to prevent further bone loss, yet they only mitigate fracture risk by 50% [8]. Neutralizing antibodies targeting sclerostin, a protein secreted by mature osteocytes that acts as negative regulator of bone formation, have demonstrated promising efficacy in osteoporosis treatment [9-11]. By inhibiting sclerostin, these antibodies promote osteogenic differentiation of progenitors and activate bone lining cells, stimulating bone formation at trabecular, endocortical, and periosteal surfaces [9-15], whilst indirectly inhibiting the formation and activity of bone-resorbing osteoclasts [16-18]. Animal studies have investigated the efficacy of sclerostin antibody (Scl-Ab) in restoring bone mass in ovariectomized (OVX) animals [14, 15, 19-30]. Administration of twice-weekly high-dose (25 mg/kg) Scl-Ab injections to OVX animals, untreated for up to 16 months, restored bone mass to control levels within 4 to 15 weeks of treatment [25, 26, 28]. Weekly Scl-Ab treatment for up to 26 weeks demonstrated sustained increases in trabecular and cortical bone volume and thickness, associated with endocortical and periosteal bone formation rates [31, 32]. The doses used in these studies exceed typical clinical exposure in humans, resulting in robust pharmacology. In humans, romosozumab is given in monthly doses of 210 mg, which is approximately equivalent to weekly Scl-Ab injections of 3 mg/kg in animal studies, based on area under the curve [18, 33, 34]. A study in cynomolgus monkeys indicated that weekly low-dose (3 mg/kg, approximately 1.2 times clinical exposure) treatment initiated 4 months post-OVX reversed bone loss and improved bone strength [33, 34]. Low dose (3 mg/kg) weekly Scl-Ab administration has also been shown to increase trabecular bone volume fraction (BVF) in the lumbar vertebrae of ovariectomised rats [18]. In OVX rats, weekly administration of low and high-dose (3 mg/kg and 50 mg/kg, respectively) Scl-Ab for up to 26 weeks resulted in dose-dependent increases in bone mass, with sustained benefits observed in both groups during and after the treatment period [18]. Extended dosing intervals, with 4-week administration-free periods, were adequate to completely restore Scl-Ab-induced increases in mineralization in gonadal-intact mice [35].

Clinical trials have shown that monthly injections of sclerostin antibody romosozumab (210 mg), results in a significant increase in bone mineral density (BMD) within the first year of treatment [12, 13, 36]. However, after six months of continuous treatment, markers of bone formation such as procollagen type 1 amino-terminal propeptide (P1NP), osteocalcin, and bone-specific alkaline phosphatase return to nearly baseline levels, indicating a dampening in the bone-forming response with repeated administration [12, 13, 37, 38]. This is consistent with animal models, where prolonged use of sclerostin antibody is associated with decreasing levels of serum P1NP and osteocalcin [31, 35]. Extended treatment with sclerostin antibody (Scl-Ab) normalizes Wnt target genes, while elevating the expression of secreted Wnt antagonists, including SOST [18, 35]. Additionally, prolonged Scl-Ab treatment induces changes in osteocyte gene expression, particularly in genes involved in inhibiting cell cycle progression and

mitogenesis [18]. These alterations may suggest a self-regulating mechanism in the bone-forming response with prolonged Scl-Ab administration. Interestingly, a treatment-free period restores the bone-forming response in rodent models [18, 35] and in humans [39]. Thus, intermittent dosing schedules, allowing periods without the antibody, could mitigate the dampening effect of continuous administration and maintain treatment efficacy. Immunoglobulin G (IgG) clearance is faster in rodents compared to humans, for this reason weekly dosing of antibodies in rodents is roughly equivalent to dosing every four weeks in humans. This difference in clearance rates suggests that dosing schedules need careful consideration for optimal outcomes.

Clinical use of Scl-Ab is indicated for postmenopausal women with severe osteoporosis who are at high risk of, or have a history of, fragility fractures [36, 40], which can occur many years after menopause. In postmenopausal women with estrogen deficiency, rapid bone loss occurs within the first two years after menopause, slowing gradually to around 1% per year thereafter [41]. Administration of a single dose of romosozumab to patients with low bone mass who had not previously received anti-resorptive therapy, demonstrated an increase in serum PINP by 66-147%, a decrease in serum C-telopeptide (sCTX) by 15-50%, and an increase in lumbar spine BMD by 4-7%. These effects were observed rapidly, with significant improvements in BMD noted as early as 3 months post-administration [42]. Preclinical studies indicate that OVX or estrogen deficiency leads to early bone loss within the first 4 weeks, followed by longer-term secondary mineralization [2] and increased microporosity associated with perilacunar remodelling and micropetrosis [7]. Apoptotic osteocytes synthesise and release sclerostin, inhibiting the Wnt/β-catenin pathway crucial for bone formation by binding to LRP5/6 proteins involved in Wnt signalling and osteoblastogenesis [43-49]. Sclerostin antagonizes the normal response to mechanical loading by osteocytes, resulting in micropetrosis processes [50, 51]. In vitro, osteocytes exhibit altered mechanosensitivity during estrogen deficiency, with reduced intracellular calcium oscillations and attenuated responses to mechanical loading [52] leading to exacerbated osteocyte-mediated osteoclastogenesis and resorption [53]. Interestingly, sclerostin inhibition reverts these affects [54], regulating osteoblast and osteocyte activity via the mechanosensitive Wnt/β-catenin signalling pathway [55, 56]. Microarray analysis of bone tissue from ovariectomised rats treated with Scl-Ab two months post-OVX confirmed increased expression of Wnt target genes and extracellular matrix genes, whereas cortical bone from mice treated with Scl-Ab for 2 weeks demonstrates upregulation of genes related to ECM in addition to *Twist1* and *Cgref* [57]. The efficacy of Scl-Ab therapy in the early stages of bone loss, prior to the occurrence secondary mineralization, has not yet been fully evaluated. We hypothesized that early administration of Scl-Ab may prevent temporal changes in mineralization, targeting perilacunar resorption and osteocyte apoptosis induced during post-menopausal osteoporosis.

The primary objective of this study was to assess the efficacy of initiating treatment with intermittent low-dose monthly sclerostin antibody early on, specifically three weeks after ovariectomy surgery. This timing coincides with the initial phase of rapid bone loss but precedes the onset of secondary mineralization changes. The study aimed to assess its efficacy in mitigating changes in (1) markers of osteoclastogenesis and bone resorption, (2) perilacunar remodelling, (3) secondary mineralization, and (4) osteocyte mechanosensitivity that arise in estrogen deficiency.

1.2 Materials and methods

1.2.1 Animal model

Female Wistar rats (6 months old, Charles River, Ireland) that had completed their breeding cycles underwent bilateral ovariectomy underwent bilateral ovariectomy (n=8). The success of ovariectomy was confirmed during necropsy by the absence of ovaries and determining atrophy of the uterine horns. A rodent version of romosozumab (Scl-Ab; r13c7, [34]) was kindly provided by UCB Pharma (UCB Pharma, Slough, UK / Amgen Inc., Slough, UK) for this study.

To investigate subcutaneous delivery of intermittent low-dose Scl-Ab, ovariectomised animals were randomly assigned to receive either (a) no treatment (CONTROL, n=4, body weight: 382 ± 39 g) or (b) a subcutaneous injection of Scl-Ab once every 4 weeks (2 mg/kg/month) (Scl-Ab, n=4, body weight: 399 ± 49g) (Figure 1) starting 3 weeks after ovariectomy surgery. This timepoint is during the initial phase of rapid bone loss but prior to the secondary mineralisation changes [58]. The animals were euthanised 3 weeks after the last injection of Scl-Ab.

All animal work was carried out under license from the Animal Care and Research Ethics Committee (ACREC) of the University of Galway and the Health Products Regulatory Authority (HPRA), the national authority for scientific animal protection in Ireland.

1.2.2 In vivo micro-CT imaging and analysis

In vivo micro-CT scanning was conducted to investigate the effect of Scl-Ab administration on morphometry and mineralisation during long-term estrogen deficiency. In vivo micro-CT scans were taken at Day 0 and week 14. At each time point, the right tibia of each animal (CONTROL, n=9; Scl-Ab, n=6) was scanned with an isotropic resolution of 15 µm, using an in vivo micro-CT system (VivaCT40, Scanco Medical AG, Basserdorf Switzerland). The in vivo micro-CT X-ray settings, region of interest, and segmentation threshold were used according to a protocol previously developed in our laboratory [58]. A series of microarchitectural parameters were quantified from the three-dimensional reconstruction of the trabecular volume of interest (VOI), using evaluation scripts in the Scanco Image Processing Language (IPL). Bone volume fraction (BV/TV), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm) and trabecular spacing (Tb.Sp, mm) were analysed to complete a detailed comparison between the trabecular structure in the proximal tibia of the treatment groups at each time point.

1.2.3 Calcein Labelling

Animals were injected intraperitoneally with calcein (20 mg/kg) ten days prior to the expected day of euthanization in order to label the mineralizing bone surface. The 10-day labelling interval was chosen to capture a sufficient number of sites and calculate the mineral apposition rate (MAR) over an appropriate duration to facilitate comparison between groups. Vertebrae were isolated from CONTROL and Scl-Ab rats after sacrifice at week 14 and fixed in 4% paraformaldehyde. A total of six animals were used for this analysis, with six images captured per animal to ensure robust data collection. Sections (1.5 mm) were cut using a diamond blade low-speed saw (Isomet™, Buehler, IL, USA). These sections were incubated in 10% di- and tetra-sodium ethylenediaminetetraacetic acid (EDTA) for 14 days until fully decalcified. The decalcification endpoint was determined using an oxalate test and a physical probing test. After decalcification, samples were rinsed in PBS overnight. The samples were then processed using a tissue processor (Leica ASP300) following a routine protocol (formalin, ascending grades of ethanol, xylenes), and then embedded in paraffin (Leica EG1150H). Paraffin sections (8 µm

thick) were generated using a microtome (Leica RM2235). Slices were collected on SuperFrost® Plus slides (Menzel Glaser), and stored at room temperature, until imaging. The mineral apposition rate was calculated as described previously [59].

1.2.4 Histological and Immunohistochemical Analysis

Femurs were isolated from CONTROL and Scl-Ab rats after sacrifice at week 14 and fixed in 4% paraformaldehyde. Sections of metaphyseal bone (1.5 mm) were cut from the fixed distal femurs using a diamond blade low-speed saw (Isomet™, Buehler, IL, USA). These sections were processed as described for the vertebrae above.

TRAP and H&E staining were performed to visualise osteoclast number and osteocytes present in lacunae, respectively. Slides were imaged on a light microscope (Olympus BX43, Olympus, Tokyo, Japan). Images were collected from n=4 femurs from each group. The number of TRAP positive (+) cells/mm and the percentage of empty lacunae were quantified on ImageJ. For immunohistochemistry, paraffin-embedded sections were incubated with the primary antibody anti-MMP14 (1:100; Abcam, ab38971) overnight at 4°C. This was followed by incubation with the secondary antibody (Goat Anti-Rabbit IgG H&L (HRP); Abcam, ab6721) for 1 hour at room temperature. Slides were then incubated with DAB substrate chromogen solution for 5 minutes. To evaluate specificity, controls containing antibody diluent instead of primary antibodies were used with the secondary antibody. The prevalence of MMP14 positively stained osteocytes, normalised to total bone area, was assessed using ImageJ [60].

1.2.5 Gene Expression Analysis

Tibiae were dissected (CONTROL, n=4; Scl-Ab, n=4), washed with DNase/RNase-free phosphate-buffered saline, and cortical bone was isolated. The samples were washed to remove the bone marrow, snap frozen in liquid nitrogen and stored at -80°C for gene expression analysis. Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen), and cDNA was obtained using a SuperScript VILO cDNA synthesis kit from RNA (Invitrogen). Hybridization was performed using a TaqMan® Fast Advanced Master Mix and customised microarrays (Applied Biosciences), and the StepOnePlus™ Real-Time PCR System was used for amplification and detection of nucleic acids in the samples, with analysis by the Pfaffl method using Rpl13a as the reference gene. Results are expressed as relative quantitative changes to samples from aged-matched OVX controls (CONTROL). Bone catabolism and matrix degradation markers (RANKL, OPG, Sost, NFATc-1, CtsK, MMP9, MMP13, Ccr2, ATG7, Raptor), apoptosis-related markers (Tp53, Casp3, Lamp1), bone anabolism and matrix formation markers (OPN, Col1α1, Col1α2, OCN, Alp, DMP1, PHEX, Ager, TGFβ1, Fn1 and Klf10), and mechanosensation and mechanotransduction markers (VCL, TRPV4, ADCY6, AXIN2, ITGβ1, ITGα5, ITGαV, CX43, RYR, PKD1, CAV1) were studied. The selection and categorization of genes were carried out based on specific research objectives and the nature of the custom microarray used in the study. The rationale for categorizing the genes in this manner was to align them with the primary biological processes they are associated with. Although these categories may not encompass the full complexity of gene functions, the categorization aimed to facilitate the analysis and interpretation of data related to bone health, remodelling, and

mechanosensitivity within the context of the study objectives. Independent batches were used to run a total of eight microarrays, with four microarrays each for CONTROL and Scl-Ab groups.

1.2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8) software. Student's t-tests analysed statistical significance of the differences between the CONTROL and Scl-Ab. The results are displayed as mean ± standard deviation. Significance was accepted at a level of $p \leq 0.05$.

All micro-CT imaging parameters (BV/TV, Tb.N, Tb.Th, Tb.Sp) were tested for normal distribution using the Kolmogorov-Smirnov normality test. For parameters which passed the normality test, Student's t-tests were used to analyse the statistical significance of the differences at each time point between the untreated controls (CONTROL) and the low monthly systemic Scl-Ab treatment group (Scl-Ab). For parameters which failed the normality test (Tb.N and Tb.Th), a nonparametric Mann-Whitney rank sum test was used for group comparisons at each timepoint. The main effects of time and the interaction between time and treatment were tested using a repeated measures two-way ANOVA. This analysis was divided into two sections to assess the effectiveness of intermittent low-dose systemic Scl-Ab treatment by comparing CONTROL and Scl-Ab. For each parameter where the two-way ANOVA indicated a significant interaction between the time and treatment group effects, Tukey's post hoc multiple comparison tests were performed to detect the differences between the parameter values at the different timepoints within a single group.

1.3 Results

1.3.1 Bone formation recovery in Scl-Ab treatment of ovariectomy-induced bone loss.

Morphometric analysis from baseline (day 0) and week 14 indicated no significant differences in bone volume fraction, trabecular microarchitecture or mineral distribution between Scl-Ab and CONTROL groups (Figure 2A and 2B). However, between day 0 and week 14, there was a significant decrease in bone volume fraction and trabecular number and a significant increase in trabecular spacing in week 14 compared to day 0 for both CONTROL and Scl-Ab groups [58].

Fluorochrome labelling of mineral apposition (Figure 2C) confirmed that Scl-Ab therapy in OVX rats promoted bone formation. The mean mineral apposition in the Scl-Ab treated groups was 11.54± 2.194, whereas in the non-treated OVX controls the range was 6.162± 2.347.

1.3.2 Markers of osteoclastogenesis and bone resorption are reduced in OVX rats treated early with intermittent low-dose Scl-Ab.

In this study, we investigated the influence of early administration of intermittent low-dose Scl-Ab (2 mg/kg/month) on markers of osteoclastogenesis and bone resorption. By week 14, there was a significant reduction in TRAP+ staining in Scl-Ab treated OVX animals compared to untreated OVX animals (Figure 3A). Moreover, there was a significant downregulation in gene expression associated with bone catabolism and matrix degradation in Scl-Ab treated OVX animals compared to untreated OVX animals; NFATc-1 (6-fold), CtsK (12-fold), MMP13,

Raptor, Sost (11-fold), MMP9 (25-fold) and ATG7 (10-fold) (Figure 3B (highlighted in blue) and 3C). There was a non-significant decrease (p=0.06) in Rankl/OPG in OVX animals treated with Scl-Ab (Scl-Ab) in comparison to untreated animals (CONTROL) (Figure 2C). C-C chemokine receptor type 2 (CCR2) expression was upregulated in Scl-Ab treated OVX animals (31-fold) compared to untreated OVX animals (Figure 3B (highlighted in red) and 3C).

1.3.3 Osteocyte apoptosis and perilacunar remodelling are reduced in OVX rats treated early with intermittent low-dose Scl-Ab .

In this study, we investigated whether early injection of intermittent low-dose Scl-Ab (2 mg/kg/month) reduces the incidence of empty lacunae, typically low in non-ovariectomised retired breeder female rats aged between 6-8 months with a mature skeleton, osteocyte apoptosis, perilacunar remodelling and secondary mineralisation, relative to control animals that had undergone ovariectomy-induced bone loss for 14 weeks. We report a significant decrease in the percentage of empty lacunae in OVX animals treated with an injection of intermittent low-dose Scl-Ab when compared to untreated OVX animals (Figure 4A). There was a significant downregulation of gene expression associated with apoptosis (Tp53 (11-fold), Casp3 (11-fold), Lamp1 (100-fold)) in OVX animals treated with intermittent low-dose Scl-Ab when compared to untreated OVX animals (Figure 4B). There was a reduction in the number of MMP14+ osteocytes in OVX animals treated with an intermittent low-dose Scl-Ab when compared to untreated OVX animals (Figure 4C).

1.3.4 Osteogenic gene expression is reduced in OVX rats early treated with intermittent low-dose Scl-Ab.

There was a significant downregulation in gene expression related to bone anabolism and matrix formation: Klf10 (6-fold), DMP1 and PHEX (10-fold), OPN (30-fold), Col1α1 (43-fold), Col1α2 (65-fold), ALP (100-fold), TGFβ1 (50-fold), and Fn1 (55-fold) in Scl-Ab treated OVX animals (Scl-Ab) compared to untreated OVX animals (CONTROL) (Figure 5A (highlighted in blue) and 5B). There was a significant upregulation in the expression of Advanced Glycation End Product Receptor (AGER) in Scl-Ab (82-fold) compared to CONTROL (Figure 5A (highlighted in red) and 5B), with no significant change in OCN.

1.3.5 Downregulation in mechanotransduction gene expression in OVX rats early treated with intermittent low-dose Scl-Ab.

The heatmap illustrates the expression patterns of genes related to mechanotransduction and mechanosensation, showing a downregulation (in blue) in the gene expression of markers Vcl, integrins α5, αV, β1, CX43, Axin2, Adcy6, IFT88, Pkd1 and Cav1 in Scl-Ab treated OVX animals (Scl-Ab) compared to untreated OVX animals (CONTROL) (Figure 5A). Statistical comparisons reveal a significant downregulation of genes, with Vcl (55-fold), integrin α5 (5-fold), integrin αV (25-fold), integrin β1 (50-fold), CX43 (33-fold), Axin2 (11-fold), Adcy6 (10-fold), IFT88 (6-fold), Pkd1 (4-fold), and Cav1 (16-fold) (Figure 6B). In comparison to controls, a significant upregulation of RyR, a protein crucial for regulating calcium release from intracellular stores, was detected in Scl-Ab compared to CONTROL, where it demonstrated a significant 22-fold increase (Figure 6B), whilst TRPV4 was unchanged.

324

1.4 Discussion:

The primary objective of this study was to assess the effectiveness of administering intermittent low-dose Sclerostin Antibody (Scl-Ab, 2 mg/kg/month) during the initial phase of rapid bone loss but prior to the onset of secondary mineralization changes [58]. Our findings indicate that early administration of intermittent low-dose Scl-Ab can reduce markers of osteoclastogenesis, osteocyte apoptosis, perilacunar remodelling, secondary mineralization and changes in osteocyte mechanosensitivity associated with estrogen deficiency (see Figure 6). Although fluorochrome labelling revealed a mild increase in bone formation on trabecular surfaces following Scl-Ab therapy in OVX rats, the extent of bone formation and accompanying suppression of osteoclastogenesis with intermittent low-dose Scl-Ab may still be insufficient to counteract bone loss, as there was no significant change in overall bone volume/total volume (BV/TV). Nonetheless, our data suggest that early intervention with intermittent low-dose Scl-Ab therapy shows promise for restoration by mitigating cellular activity and gene expression changes implicated in bone loss and osteocyte-mediated secondary mineralization. Further research into alternative dosing regimens or combination therapies may enhance the therapeutic efficacy of low-dose intermittent Scl-Ab in preserving bone mass and preventing osteoporosis in estrogen-deficient conditions.

Several limitations need to be considered in this study. Firstly, the estrogen-deficient rat model may not reflect the pathogenesis of human osteoporosis, as the depletion of estrogen is sudden, resulting in rapid bone loss. However, the ovariectomised rat is widely used as a model for studying post-menopausal osteoporosis, due to similarities in bone loss patterns between ovariectomized rats and humans [61-63]. Specifically, ovariectomized rats experience an initial period of bone loss in response to sudden estrogen deficiency, followed by a return to a more balanced bone remodelling process as bone loss subsides [64]. Secondly, the highest isotropic resolution achievable on the in vivo micro-CT system (vivaCT 40) is 15 μm, which may not suffice to detected changes in mineral due to micropetrosis or perilacunar remodelling. Indeed, while mild bone formation was reported here by calcein labelling, the mineral apposition was less than 15 μm and so such changes would not be detectable by micro-CT at the resolution. This might explain why bone formation assessed by micro CT (by BV/TV) was not significant. Previous animal studies, including those on ovariectomized rats and non-human primates, have reported that sclerostin antibody treatment significantly enhances mineral apposition rates (MAR), ranging from 2-4 μm/day over 7 to 10 days (~18 - 30 μm total mineral apposition), for short-term treatment periods (5 to 8 weeks) of frequent high-dose sclerostin antibody in rats and primates [22, 65, 66]. In comparison, the clinical impact of the low-dose investigated here was not evident, as the treatment did not effectively restore bone mass in the context of rapid bone resorption. However, it is important to note that micro-CT analysis revealed the net effect, whereas the opposing processes of reduced bone resorption and mild bone formation may cancel each other out. This underscores the importance of further studies on Scl-Ab dose and treatment frequency during the rapid resorption phase. However, mineralisation changes were captured in long term estrogen deficient animals compared to day 0 whereas our gene expression analysis confirms a decrease in expression of mineralization markers (OPN, DMP1, ALP, PHEX) after 14 weeks of Scl-Ab administration. Lastly, the effect of monthly Scl-Ab injection on bone loss was negligible, this could be due to the low dose administered (2 mg/kg/month) compared to the average dose of 25 mg/kg/week administered in many other rat OVX studies and also the frequency of administration which is typically once/twice a week in other rat studies [67-70]. It is important to note that we did not assess serum bone turnover markers (BTMs), which could be

assessed through a follow-up study to provide a more comprehensive understanding of treatment effects. Nevertheless, a 2 mg/kg/month dose of Scl-Ab is sufficient to reduce osteoclast number, MMP14 osteocyte expression and the incidence of empty lacunae in ovariectomised rats, despite no significant impact on bone loss as detected by in vivo microCT.

In this study, we report that early administration of intermittent low-dose Scl-Ab in estrogen-deficient rats reduces markers of osteoclastogenesis, which is consistent with its known dual effects on bone formation and resorption at higher doses [16-18]. A previous in vitro study demonstrated a decrease in osteocyte-induced osteoclastogenesis in response to 300 ng/mL Scl-Ab administration [54]. In the current study, the observed reductions in expression of NFATc-1, which controls osteoclast function [71], CtsK, essential for bone resorption [72], and Raptor, part of the mTOR pathway affecting osteoclast function [73], further confirm that early administration of intermittent low-dose Scl-Ab mitigates osteoclast formation and activity. However, we did not find any significant differences in trabecular bone mass and microarchitecture by week 14, whereas the antibody mitigates bone loss in younger ovariectomized rat models at higher and more frequent doses (25 and 50 mg/kg weekly) when administered at a later stage (8 weeks post-OVX) or in severe osteoporosis with unloading [18, 74]. It is important to note that we started the antibody treatment at 3 weeks, which is towards the end of the rapid phase of bone loss, by which time 10-30% of the bone loss has occurred. Thus, the indirect effect of Scl-Ab on later stage bone resorption is not investigated here.

Here we report that early administration of intermittent low-dose Scl-Ab in estrogen deficient rats can reduce osteocyte apoptosis and empty lacunae, which is consistent with reported effects in a glucocorticoid-induced osteoporosis rat model [75]. In ovariectomized rats it has been reported that there is an increase in osteocyte canalicular diameter [7], and load induced movement of small molecules within the lacunar-canalicular network [76]. Such changes might explain reported increased mechanical strain on osteocytes [77] and render them susceptible to apoptosis [7, 78, 79]. The current study reveals a significant downregulation of apoptosis markers and lysosomal activity (Tp53, Casp3 and Lamp1), indicating a potential mechanism by which Scl-Ab treatment protects from osteocyte apoptosis. These changes may initiate hypermineralisation by micropetrosis, a process involving mineral infilling of empty lacunae and canaliculi after osteocyte apoptosis. Interestingly, lacunar porosity and canalicular diameter are decreased in estrogen deficiency [7, 80], and there is an increased prevalence of empty and circular osteocyte lacunae with mineralised borders [7] and MMP14+ osteocytes in long term estrogen deficiency (14 weeks), indicative of both micropetrosis and perilacunar remodelling by osteocytes [7]. Sclerostin is a Wnt antagonist implicated in regulating perilacunar remodelling [50, 51]. Here we report that early treatment with intermittent low-dose Scl-Ab decreased the percentage of empty lacunae, but also reduced the number of MMP14+ osteocytes and MMP gene expression. Thus, we propose that early treatment with intermittent low-dose sclerostin antibody could prevent secondary mineralisation driven by osteocyte apoptosis and micropetrosis, which is also associated with glucocorticoid-induced osteoporosis.

Although we observed surface bone formation, explained by osteoblast activity and new mineral apposition, this process differs from osteocyte-mediated secondary mineralization, which involves mineral deposition within the existing bone matrix by osteocytes. Notably, our study is the first to report that early treatment with intermittent low-dose sclerostin antibody (Scl-Ab) significantly downregulates mineralization markers such as Klf10, DMP1, PHEX, OPN, Col1α1, Col1α2, ALP, TGFβ1, and Fn1, suggesting its potential to mitigate secondary pathological mineralization associated with estrogen This differs from the bone formation effects

of high doses of Scl-Ab administered weekly after a longer post-ovariectomy interval [33, 81, 82], which exceed typical clinical exposure in humans (210 mg) [18, 33]. Interestingly, short-term Scl-Ab treatment has been linked to reduced osteocyte mechanosensitivity [83], possibly due to the presence of immature osteocytes following new bone formation. However, in the longer-term osteocyte signalling reverted to normal, and were proposed to explain reduced bone formation [83]. Our study also found significant downregulation of mechanotransduction genes, including ItgαV, Itgβ1, and Cav1, which are associated with osteocyte mechanosensation, ciliary dynamics, calcium signaling, and Wnt signaling. We propose that the preventative effect of early, intermittent low-dose Scl-Ab treatment on osteocyte-mediated secondary mineralization may be due to its regulation of osteocyte mechanotransduction in response to altered mechanical strain following bone loss [77]. While fluorochrome labelling indicated increased surface bone formation, BV/TV remained unchanged, raising important questions about the impact of different Scl-Ab doses and frequencies in Ovx rats, which warrant further investigation.

1.5 Conclusion:

In summary, the study findings suggest that monthly systemic treatment of intermittent low-dose sclerostin antibody reduces markers of osteoclastogenesis, osteocyte apoptosis, perilacunar remodelling, secondary mineralization and osteocyte mechanosensitivity in ovariectomised rats. The results suggest that early intervention with intermittent low-dose sclerostin antibody therapy can promote surface bone formation but reduce osteocyte-mediated secondary mineralization after initial bone loss in ovariectomised rats.

Acknowledgement:

The authors would like to acknowledge Servier Medical Art (www.servier.com) for their image bank used to produce the figures and UCB Pharma and Amgen Inc. for providing the Scl-Ab via a materials transfer agreement.

Author contribution:

Conceptualization SMN, HA, LOS, JAPP, JS, LMcN; Data Curation SMN, HA, LOS; Formal Analysis SMN, HA, LOS; Funding Acquisition LMcN; Investigation SMN, HA, LOS, JAPP, JS, LMcN; Methodology SMN, HA, LOS, JAPP, JS, LMcN; Project Administration LMcN; Resources LMcN; Supervision LMcN; Validation SMN, HA, LOS, JAPP, JS; Visualization SMN, HA, LOS; Writing – Original Draft SMN, HA, LOS, LMcN; Writing – Review & Editing SMN, HA, LOS, JAPP, JS, GH, LMcN; All authors have read and agreed to the submitted version of the manuscript.

Funding Sources:

This publication has emanated from research conducted with the financial support of SFI co-funded under the European Regional Development fund (14/IA/2884) and the ERC Consolidator grant (MEMETic: 863795).

References:

[1] N.E. Lane, J.M. Thompson, D. Haupt, D.B. Kimmel, G. Modin, J.H. Kinney, Acute Changes in Trabecular Bone Connectivity and Osteoclast Activity in the Ovariectomized Rat In Vivo, Journal of Bone and Mineral Research 13(2) (1998) 229-236.

[2] L.M. O'Sullivan, H. Allison, E.E. Parle, J. Schiavi, L.M. McNamara, Secondary alterations in bone mineralisation and trabecular thickening occur after long-term estrogen deficiency in ovariectomised rat tibiae, which do not coincide with initial rapid bone loss, Osteoporosis International (2019).

[3] J.H. Waarsing, J.S. Day, J.A.N. Verhaar, A.G.H. Ederveen, H. Weinans, Bone loss dynamics result in trabecular alignment in aging and ovariectomized rats, Journal of Orthopaedic Research 24(5) (2006) 926-935.

[4] A. Tomkinson, E.F. Gevers, J.M. Wit, J. Reeve, B.S. Noble, The Role of Estrogen in the Control of Rat Osteocyte Apoptosis, Journal of Bone and Mineral Research 13(8) (1998) 103-113.

[5] M. Almeida, L. Han, M. Martin-Millan, L.I. Plotkin, S.A. Stewart, P.K. Roberson, S. Kousteni, C.A. O'Brien, T. Bellido, A.M. Parfitt, R.S. Weinstein, R.L. Jilka, S.C. Manolagas, Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids, Journal of Biological Chemistry 282(37) (2007) 27285-27297.

[6] K.B. Emerton, B. Hu, A.A. Woo, A. Sinofsky, C. Hernandez, R.J. Majeska, K.J. Jepsen, M.B. Schaffler, Osteocyte apoptosis and control of bone resorption following ovariectomy in mice, Bone 46(3) (2010) 577-583.

[7] H. Allison, L.M. O'Sullivan, L.M. McNamara, Temporal changes in cortical microporosity during estrogen deficiency associated with perilacunar resorption and osteocyte apoptosis: A pilot study, Bone Reports 16 (2022) 101590.

[8] D.M. Black, S.R. Cummings, D.B. Karpf, J.A. Cauley, D.E. Thompson, M.C. Nevitt, D.C. Bauer, H.K. Genant, W.L. Haskell, R. Marcus, S.M. Ott, J.C. Torner, S.A. Quandt, T.F. Reiss, K.E. Ensrud, Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group, Lancet 348(9041) (1996) 1535-41.

[9] D. Padhi, M. Allison, A.J. Kivitz, M.J. Gutierrez, B. Stouch, C. Wang, G. Jang, Multiple doses of sclerostin antibody romosozumab in healthy men and postmenopausal women with low bone mass: A randomized, double-blind, placebo-controlled study, Journal of Clinical Pharmacology 54(2) (2014) 168-178.

[10] P. Nioi, S. Taylor, R. Hu, E. Pacheco, Y.D. He, H. Hamadeh, C. Paszty, I. Pyrah, M.S. Ominsky, R.W. Boyce, Transcriptional Profiling of Laser Capture Microdissected Subpopulations of the Osteoblast Lineage Provides Insight Into the Early Response to Sclerostin Antibody in Rats, J Bone Miner Res 30(8) (2015) 1457-67.

[11] H.Z. Ke, W.G. Richards, X. Li, M.S. Ominsky, Sclerostin and Dickkopf-1 as therapeutic targets in bone diseases, Endocr Rev 33(5) (2012) 747-83.

[12] F. Cosman, D.B. Crittenden, J.D. Adachi, N. Binkley, E. Czerwinski, S. Ferrari, L.C. Hofbauer, E. Lau, E.M. Lewiecki, A. Miyauchi, C.A. Zerbini, C.E. Milmont, L. Chen, J. Maddox, P.D. Meisner, C. Libanati, A. Grauer, Romosozumab Treatment in Postmenopausal Women with Osteoporosis, N Engl J Med 375(16) (2016) 1532-1543.

[13] M.R. McClung, A. Grauer, S. Boonen, M.A. Bolognese, J.P. Brown, A. Diez-Perez, B.L. Langdahl, J.Y. Reginster, J.R. Zanchetta, S.M. Wasserman, L. Katz, J. Maddox, Y.C. Yang, C. Libanati, H.G. Bone, Romosozumab in postmenopausal women with low bone mineral density, N Engl J Med 370(5) (2014) 412-20.

[14] S. Costa, H. Fairfield, M. Farrell, C.S. Murphy, A. Soucy, C. Vary, G. Holdsworth, M.R. Reagan, Sclerostin antibody increases trabecular bone and bone mechanical properties by increasing osteoblast activity damaged by whole-body irradiation in mice, Bone 147 (2021).

[15] Y. Yao, F. Kauffmann, S. Maekawa, L.V. Sarment, J.V. Sugai, C.A. Schmiedeler, E.J. Doherty, G. Holdsworth, P.J. Kostenuik, W.V. Giannobile, Sclerostin antibody stimulates periodontal regeneration in large alveolar bone defects, Scientific Reports 10(1) (2020).

[16] R.W. Boyce, Q.T. Niu, M.S. Ominsky, Kinetic reconstruction reveals time-dependent effects of romosozumab on bone formation and osteoblast function in vertebral cancellous and cortical bone in cynomolgus monkeys, Bone 101 (2017) 77-87.

[17] M.S. Ominsky, R.W. Boyce, X. Li, H.Z. Ke, Effects of sclerostin antibodies in animal models of osteoporosis, Bone 96 (2017) 63-75.

[18] S. Taylor, M.S. Ominsky, R. Hu, E. Pacheco, Y.D. He, D.L. Brown, J.I. Aguirre, T.J. Wronski, S. Buntich, C.A. Afshari, I. Pyrah, P. Nioi, R.W. Boyce, Time-dependent cellular and transcriptional changes in the osteoblast lineage associated with sclerostin antibody treatment in ovariectomized rats, Bone 84 (2016) 148-159.

[19] X. Tian, R.B. Setterberg, X. Li, C. Paszty, H.Z. Ke, W.S. Jee, Treatment with a sclerostin antibody increases cancellous bone formation and bone mass regardless of marrow composition in adult female rats, Bone 47(3) (2010) 529-33.

[20] X. Li, K.S. Warmington, Q.T. Niu, F.J. Asuncion, M. Barrero, M. Grisanti, D. Dwyer, B. Stouch, T.M. Thway, M. Stolina, M.S. Ominsky, P.J. Kostenuik, W.S. Simonet, C. Paszty, H.Z. Ke, Inhibition of sclerostin by monoclonal antibody increases bone formation, bone mass, and bone strength in aged male rats, J Bone Miner Res 25(12) (2010) 2647-56.

[21] F. Agholme, X. Li, H. Isaksson, H.Z. Ke, P. Aspenberg, Sclerostin antibody treatment enhances metaphyseal bone healing in rats, J Bone Miner Res 25(11) (2010) 2412-8.

[22] M.S. Ominsky, C. Li, X. Li, H.L. Tan, E. Lee, M. Barrero, F.J. Asuncion, D. Dwyer, C.Y. Han, F. Vlasseros, R. Samadfam, J. Jolette, S.Y. Smith, M. Stolina, D.L. Lacey, W.S. Simonet, C. Paszty, G. Li, H.Z. Ke, Inhibition of sclerostin by monoclonal antibody enhances bone healing and improves bone density and strength of nonfractured bones, J Bone Miner Res 26(5) (2011) 1012-21.

[23] M.S. Virk, F. Alaee, H. Tang, M.S. Ominsky, H.Z. Ke, J.R. Lieberman, Systemic administration of sclerostin antibody enhances bone repair in a critical-sized femoral defect in a rat model, J Bone Joint Surg Am 95(8) (2013) 694-701.

[24] A.D. Taut, Q. Jin, J.H. Chung, P. Galindo-Moreno, E.S. Yi, J.V. Sugai, H.Z. Ke, M. Liu, W.V. Giannobile, Sclerostin antibody stimulates bone regeneration after experimental periodontitis, J Bone Miner Res 28(11) (2013) 2347-56.

[25] J. Wu, X.H. Cai, X.X. Qin, Y.X. Liu, The effects of sclerostin antibody plus parathyroid hormone (1-34) on bone formation in ovariectomized rats, Z Gerontol Geriatr 51(5) (2018) 550-556.

[26] M. Liu, P. Kurimoto, J. Zhang, Q.T. Niu, M. Stolina, P.C. Dechow, J.Q. Feng, J. Hesterman, M.D. Silva, M.S. Ominsky, W.G. Richards, H. Ke, P.J. Kostenuik, Sclerostin and DKK1 Inhibition Preserves and Augments Alveolar Bone Volume and Architecture in Rats with Alveolar Bone Loss, J Dent Res 97(9) (2018) 1031-1038.

[27] X. Tian, W.S. Jee, X. Li, C. Paszty, H.Z. Ke, Sclerostin antibody increases bone mass by stimulating bone formation and inhibiting bone resorption in a hindlimb-immobilization rat model, Bone 48(2) (2011) 197-201.

[28] A.S. Virdi, J. Irish, K. Sena, M. Liu, H.Z. Ke, M.A. McNulty, D.R. Sumner, Sclerostin antibody treatment improves implant fixation in a model of severe osteoporosis, J Bone Joint Surg Am 97(2) (2015) 133-40.

[29] A.S. Virdi, M. Liu, K. Sena, J. Maletich, M. McNulty, H.Z. Ke, D.R. Sumner, Sclerostin antibody increases bone volume and enhances implant fixation in a rat model, J Bone Joint Surg Am 94(18) (2012) 1670-80.
[30] S.H. Yu, J. Hao, T. Fretwurst, M. Liu, P. Kostenuik, W.V. Giannobile, Q. Jin, Sclerostin-Neutralizing Antibody Enhances Bone Regeneration Around Oral Implants, Tissue Eng Part A 24(21-22) (2018) 1672-1679.
[31] M. Stolina, D. Dwyer, Q.T. Niu, K.S. Villasenor, P. Kurimoto, M. Grisanti, C.Y. Han, M. Liu, X. Li, M.S. Ominsky, H.Z. Ke, P.J. Kostenuik, Temporal changes in systemic and local expression of bone turnover markers during six months of sclerostin antibody administration to ovariectomized rats, Bone 67 (2014) 305-13.
[32] X. Li, Q.T. Niu, K.S. Warmington, F.J. Asuncion, D. Dwyer, M. Grisanti, C.Y. Han, M. Stolina, M.J. Eschenberg, P.J. Kostenuik, W.S. Simonet, M.S. Ominsky, H.Z. Ke, Progressive increases in bone mass and bone strength in an ovariectomized rat model of osteoporosis after 26 weeks of treatment with a sclerostin antibody, Endocrinology 155(12) (2014) 4785-97.
[33] M.S. Ominsky, S.K. Boyd, A. Varela, J. Jolette, M. Felx, N. Doyle, N. Mellal, S.Y. Smith, K. Locher, S. Buntich, I. Pyrah, R.W. Boyce, Romosozumab Improves Bone Mass and Strength While Maintaining Bone Quality in Ovariectomized Cynomolgus Monkeys, J Bone Miner Res 32(4) (2017) 788-801.
[34] J.R. Turk, A.M. Deaton, J. Yin, M. Stolina, M. Felx, G. Boyd, J.G. Bienvenu, A. Varela, M. Guillot, G. Holdsworth, A. Wolfreys, D. Dwyer, S.V. Kumar, E.M. de Koning, Y. Qu, M. Engwall, K. Locher, L.D. Ward, C. Glaus, Y.D. He, R.W. Boyce, Nonclinical cardiovascular safety evaluation of romosozumab, an inhibitor of sclerostin for the treatment of osteoporosis in postmenopausal women at high risk of fracture, Regul Toxicol Pharmacol 115 (2020) 104697.
[35] G. Holdsworth, K. Greenslade, J. Jose, Z. Stencel, H. Kirby, A. Moore, H.Z. Ke, M.K. Robinson, Dampening of the bone formation response following repeat dosing with sclerostin antibody in mice is associated with up-regulation of Wnt antagonists, Bone 107 (2018) 93-103.
[36] K.G. Saag, J. Petersen, M.L. Brandi, A.C. Karaplis, M. Lorentzon, T. Thomas, J. Maddox, M. Fan, P.D. Meisner, A. Grauer, Romosozumab or Alendronate for Fracture Prevention in Women with Osteoporosis, N Engl J Med 377(15) (2017) 1417-1427.
[37] D. Padhi, M. Allison, A.J. Kivitz, M.J. Gutierrez, B. Stouch, C. Wang, G. Jang, Multiple doses of sclerostin antibody romosozumab in healthy men and postmenopausal women with low bone mass: a randomized, double-blind, placebo-controlled study, J Clin Pharmacol 54(2) (2014) 168-78.
[38] R.R. Recker, C.T. Benson, T. Matsumoto, M.A. Bolognese, D.A. Robins, J. Alam, A.Y. Chiang, L. Hu, J.H. Krege, H. Sowa, B.H. Mitlak, S.L. Myers, A randomized, double-blind phase 2 clinical trial of blosozumab, a sclerostin antibody, in postmenopausal women with low bone mineral density, J Bone Miner Res 30(2) (2015) 216-24.
[39] M.R. McClung, J.P. Brown, A. Diez-Perez, H. Resch, J. Caminis, P. Meisner, M.A. Bolognese, S. Goemaere, H.G. Bone, J.R. Zanchetta, J. Maddox, S. Bray, A. Grauer, Effects of 24 Months of Treatment With Romosozumab Followed by 12 Months of Denosumab or Placebo in Postmenopausal Women With Low Bone Mineral Density: A Randomized, Double-Blind, Phase 2, Parallel Group Study, J Bone Miner Res 33(8) (2018) 1397-1406.
[40] E.M. Lewiecki, T. Blicharski, S. Goemaere, K. Lippuner, P.D. Meisner, P.D. Miller, A. Miyauchi, J. Maddox, L. Chen, S. Horlait, A Phase III Randomized Placebo-Controlled Trial to Evaluate Efficacy and Safety of Romosozumab in Men With Osteoporosis, J Clin Endocrinol Metab 103(9) (2018) 3183-3193.

[41] M.R. Sowers, H. Zheng, M.L. Jannausch, D. McConnell, B. Nan, S. Harlow, J.F. Randolph, Jr., Amount of bone loss in relation to time around the final menstrual period and follicle-stimulating hormone staging of the transmenopause, J Clin Endocrinol Metab 95(5) (2010) 2155-62.
[42] D. Padhi, G. Jang, B. Stouch, L. Fang, E. Posvar, Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody, J Bone Miner Res 26(1) (2011) 19-26.
[43] F. Fontani, G. Marcucci, T. Iantomasi, M.L. Brandi, M.T. Vincenzini, Glutathione, N-acetylcysteine and lipoic acid down-regulate starvation-induced apoptosis, RANKL/OPG ratio and sclerostin in osteocytes: involvement of JNK and ERK1/2 signalling, Calcif Tissue Int 96(4) (2015) 335-46.
[44] G. Marcucci, V. Domazetovic, C. Nediani, J. Ruzzolini, C. Favre, M.L. Brandi, Oxidative Stress and Natural Antioxidants in Osteoporosis: Novel Preventive and Therapeutic Approaches, Antioxidants, 2023.
[45] R. Sapir-Koren, G. Livshits, Osteocyte control of bone remodeling: is sclerostin a key molecular coordinator of the balanced bone resorption-formation cycles?, Osteoporos Int 25(12) (2014) 2685-700.
[46] F.G.F. Tresguerres, J. Torres, J. López-Quiles, G. Hernández, J.A. Vega, I.F. Tresguerres, The osteocyte: A multifunctional cell within the bone, Ann Anat 227 (2020) 151422.
[47] T. Bellido, Osteocyte-driven bone remodeling, Calcif Tissue Int 94(1) (2014) 25-34.
[48] M. Notsu, I. Kanazawa, A. Takeno, M. Yokomoto-Umakoshi, K.I. Tanaka, T. Yamaguchi, T. Sugimoto, Advanced Glycation End Product 3 (AGE3) Increases Apoptosis and the Expression of Sclerostin by Stimulating TGF-β Expression and Secretion in Osteocyte-Like MLO-Y4-A2 Cells, Calcif Tissue Int 100(4) (2017) 402-411.
[49] L.F. Bonewald, The amazing osteocyte, J Bone Miner Res 26(2) (2011) 229-38.
[50] M. Kogawa, K.A. Khalid, A.R. Wijenayaka, R.T. Ormsby, A. Evdokiou, P.H. Anderson, D.M. Findlay, G.J. Atkins, Recombinant sclerostin antagonizes effects of ex vivo mechanical loading in trabecular bone and increases osteocyte lacunar size, Am J Physiol Cell Physiol 314(1) (2018) C53-C61.
[51] M. Kogawa, A.R. Wijenayaka, R.T. Ormsby, G.P. Thomas, P.H. Anderson, L.F. Bonewald, D.M. Findlay, G.J. Atkins, Sclerostin Regulates Release of Bone Mineral by Osteocytes by Induction of Carbonic Anhydrase 2, Journal of Bone and Mineral Research 28(12) (2013) 2433-2435.
[52] V. Deepak, P. Kayastha, L.M. McNamara, Estrogen deficiency attenuates fluid flow-induced [Ca2+]i oscillations and mechanoresponsiveness of MLO-Y4 osteocytes, FASEB J (2017).
[53] H. Allison, L.M. McNamara, Inhibition of osteoclastogenesis by mechanically stimulated osteoblasts is attenuated during estrogen deficiency, Am J Physiol Cell Physiol (2019).
[54] H. Allison, G. Holdsworth, L.M. McNamara, Scl-Ab reverts pro-osteoclastogenic signalling and resorption in estrogen deficient osteocytes, BMC Molecular and Cell Biology 21(1) (2020) 78.
[55] L.F. Bonewald, M.L. Johnson, Osteocytes, mechanosensing and Wnt signaling, Bone 42(4) (2008) 606-15.
[56] X.L. Tu, Y. Rhee, K.W. Condon, N. Bivi, M.R. Allen, D. Dwyer, M. Stolina, C.H. Turner, A.G. Robling, L.I. Plotkin, T. Bellido, Sost downregulation and local Wnt signaling are required for the osteogenic response to mechanical loading, Bone 50(1) (2012) 209-217.

[57] R. Kedlaya, S. Veera, D.J. Horan, R.E. Moss, U.M. Ayturk, C.M. Jacobsen, M.E. Bowen, C. Paszty, M.L. Warman, A.G. Robling, Sclerostin inhibition reverses skeletal fragility in an Lrp5-deficient mouse model of OPPG syndrome, Sci Transl Med 5(211) (2013) 211ra158.
[58] L.M. O'Sullivan, H. Allison, E.E. Parle, J. Schiavi, L.M. McNamara, Secondary alterations in bone mineralisation and trabecular thickening occur after long-term estrogen deficiency in ovariectomised rat tibiae, which do not coincide with initial rapid bone loss, Osteoporosis International 31(3) (2020) 587-599.
[59] A.M. Parfitt, M.K. Drezner, F.H. Glorieux, J.A. Kanis, H. Malluche, P.J. Meunier, S.M. Ott, R.R. Recker, Bone histomorphometry: Standardization of nomenclature, symbols, and units: Report of the asbmr histomorphometry nomenclature committee, Journal of Bone and Mineral Research 2(6) (1987) 595-610.
[60] N.S. Dole, C.M. Mazur, C. Acevedo, J.P. Lopez, D.A. Monteiro, T.W. Fowler, B. Gludovatz, F. Walsh, J.N. Regan, S. Messina, D.S. Evans, T.F. Lang, B. Zhang, R.O. Ritchie, K.S. Mohammad, T. Alliston, Osteocyte-Intrinsic TGF-β Signaling Regulates Bone Quality through Perilacunar/Canalicular Remodeling, Cell Reports 21(9) (2017) 2585-2596.
[61] D.N. Kalu, The ovariectomized rat model of postmenopausal bone loss, Bone and Mineral 15(3) (1991) 175-191.
[62] J.J. Štěpán, J. Musilová, V. Pacovský, Bone demineralization, biochemical indices of bone remodeling, and estrogen replacement therapy in adults with turner's syndrome, Journal of Bone and Mineral Research 4(2) (2009) 193-198.
[63] J.C. Gallagher, The pathogenesis of osteoporosis, Bone and Mineral 9(3) (1990) 215-227.
[64] P.P. Lelovas, T.T. Xanthos, S.E. Thoma, G.P. Lyritis, I.A. Dontas, The laboratory rat as an animal model for osteoporosis research, Comparative medicine 58(5) (2008) 424-430.
[65] M.S. Ominsky, F. Vlasseros, J. Jolette, S.Y. Smith, B. Stouch, G. Doellgast, J. Gong, Y. Gao, J. Cao, K. Graham, B. Tipton, J. Cai, R. Deshpande, L. Zhou, M.D. Hale, D.J. Lightwood, A.J. Henry, A.G. Popplewell, A.R. Moore, M.K. Robinson, D.L. Lacey, W.S. Simonet, C. Paszty, Two doses of sclerostin antibody in cynomolgus monkeys increases bone formation, bone mineral density, and bone strength, J Bone Miner Res 25(5) (2010) 948-59.
[66] X. Li, M.S. Ominsky, K.S. Warmington, S. Morony, J. Gong, J. Cao, Y. Gao, V. Shalhoub, B. Tipton, R. Haldankar, Q. Chen, A. Winters, T. Boone, Z. Geng, Q.T. Niu, H.Z. Ke, P.J. Kostenuik, W.S. Simonet, D.L. Lacey, C. Paszty, Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis, J Bone Miner Res 24(4) (2009) 578-88.
[67] F. Zhao, D. Zhang, Y. Zhao, W. Wang, H. Yang, F. Tai, C. Li, X. Hu, The Difference of Physiological and Proteomic Changes in Maize Leaves Adaptation to Drought, Heat, and Combined Both Stresses, Front Plant Sci 7 (2016) 1471.
[68] X. Li, Q.T. Niu, K.S. Warmington, F.J. Asuncion, D. Dwyer, M. Grisanti, C.Y. Han, M. Stolina, M.J. Eschenberg, P.J. Kostenuik, W.S. Simonet, M.S. Ominsky, H.Z. Ke, Progressive increases in bone mass and bone strength in an ovariectomized rat model of osteoporosis after 26 weeks of treatment with a sclerostin antibody, Endocrinology 155(12) (2014) 4785-4797.
[69] X. Li, M.S. Ominsky, K.S. Warmington, S. Morony, J. Gong, J. Cao, Y. Gao, V. Shalhoub, B. Tipton, R. Haldankar, Q. Chen, A. Winters, T. Boone, Z. Geng, Q.T. Niu, H.Z. Ke, P.J. Kostenuik, W.S. Simonet, D.L. Lacey, C. Paszty, Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis, Journal of Bone and Mineral Research 24(4) (2009) 578-588.

[70] X. Li, M.S. Ominsky, K.S. Warmington, Q.T. Niu, F.J. Asuncion, M. Barrero, D. Dwyer, M. Grisanti, M. Stolina, P.J. Kostenuik, W.S. Simonet, C. Paszty, H.Z. Ke, Increased bone formation and bone mass induced by sclerostin antibody is not affected by pretreatment or cotreatment with alendronate in osteopenic, ovariectomized rats, Endocrinology 152(9) (2011) 3312-3322.
[71] J.H. Kim, N. Kim, Regulation of NFATc1 in Osteoclast Differentiation, J Bone Metab 21(4) (2014) 233-41.
[72] R. Dai, Z. Wu, H.Y. Chu, J. Lu, A. Lyu, J. Liu, G. Zhang, Cathepsin K: The Action in and Beyond Bone, Front Cell Dev Biol 8 (2020) 433.
[73] Q. Dai, F. Xie, Y. Han, X. Ma, S. Zhou, L. Jiang, W. Zou, J. Wang, Inactivation of Regulatory-associated Protein of mTOR (Raptor)/Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling in Osteoclasts Increases Bone Mass by Inhibiting Osteoclast Differentiation in Mice*, Journal of Biological Chemistry 292(1) (2017) 196-204.
[74] D.Y. Zhang, M.Y. Hu, T. Chu, L.J. Lin, J.Y. Wang, X.D. Li, H.Z. Ke, Y.X. Qin, Sclerostin antibody prevented progressive bone loss in combined ovariectomized and concurrent functional disuse, Bone 87 (2016) 161-168.
[75] Z. Achiou, H. Toumi, J. Touvier, A. Boudenot, R. Uzbekov, M.S. Ominsky, S. Pallu, E. Lespessailles, Sclerostin antibody and interval treadmill training effects in a rodent model of glucocorticoid-induced osteopenia, Bone 81 (2015) 691-701.
[76] C. Ciani, D. Sharma, S.B. Doty, S.P. Fritton, Ovariectomy enhances mechanical load-induced solute transport around osteocytes in rat cancellous bone, Bone 59 (2014) 229-34.
[77] S.W. Verbruggen, M.J. Mc Garrigle, M.G. Haugh, M.C. Voisin, L.M. McNamara, Altered mechanical environment of bone cells in an animal model of short- and long-term osteoporosis, Biophys J 108(7) (2015) 1587-98.
[78] M.A. Brennan, M.G. Haugh, F.J. O'Brien, L.M. McNamara, Estrogen Withdrawal from Osteoblasts and Osteocytes Causes Increased Mineralization and Apoptosis, Horm Metab Res (2014).
[79] A. Tomkinson, E.F. Gevers, J.M. Wit, J. Reeve, B.S. Noble, The Role of Estrogen in the Control of Rat Osteocyte Apoptosis, 13(8) (1998) 1243-1250.
[80] D. Sharma, C. Ciani, P.A.R. Marin, J.D. Levy, S.B. Doty, S.P. Fritton, Alterations in the osteocyte lacunar-canalicular microenvironment due to estrogen deficiency, Bone 51(3) (2012) 488-497.
[81] X.D. Li, M.S. Ominsky, K.S. Warmington, S. Morony, J.H. Gong, J. Cao, Y.M. Gao, V. Shalhoub, B. Tipton, R.J. Haldankar, Q. Chen, A. Winters, T. Boone, Z.P. Geng, Q.T. Niu, H.Z. Ke, P.J. Kostenuik, W.S. Simonet, D.L. Lacey, C. Paszty, Sclerostin Antibody Treatment Increases Bone Formation, Bone Mass, and Bone Strength in a Rat Model of Postmenopausal Osteoporosis, Journal of Bone and Mineral Research 24(4) (2009) 578-588.
[82] X.D. Li, Q.T. Niu, K.S. Warmington, F.J. Asuncion, D. Dwyer, M. Grisanti, C.Y. Han, M. Stolina, M.J. Eschenberg, P.J. Kostenuik, W.S. Simonet, M.S. Ominsky, H.Z. Ke, Progressive Increases in Bone Mass and Bone Strength in an Ovariectomized Rat Model of Osteoporosis After 26 Weeks of Treatment With a Sclerostin Antibody, Endocrinology 155(12) (2014) 4785-4797.
[83] A.E. Morrell, S.T. Robinson, H.Z. Ke, G. Holdsworth, X.E. Guo, Osteocyte mechanosensing following short-term and long-term treatment with sclerostin antibody, Bone 149 (2021) 115967.

Figures

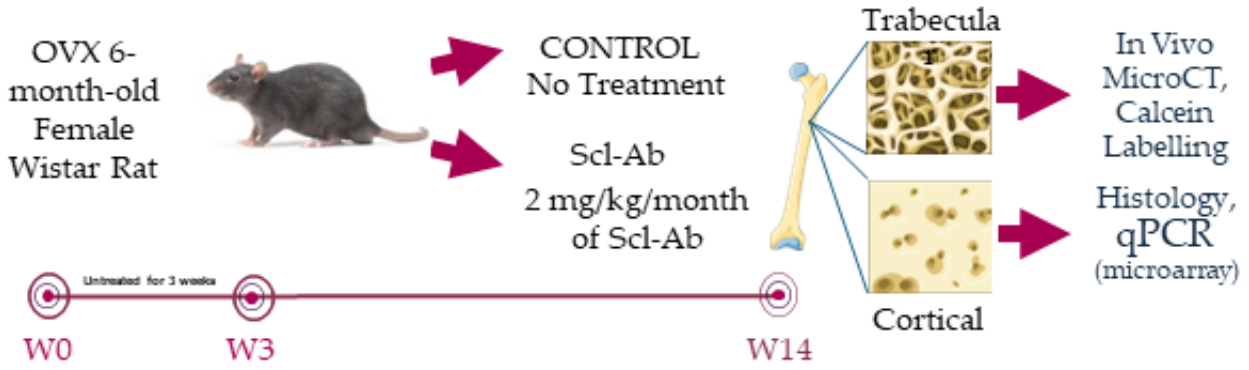
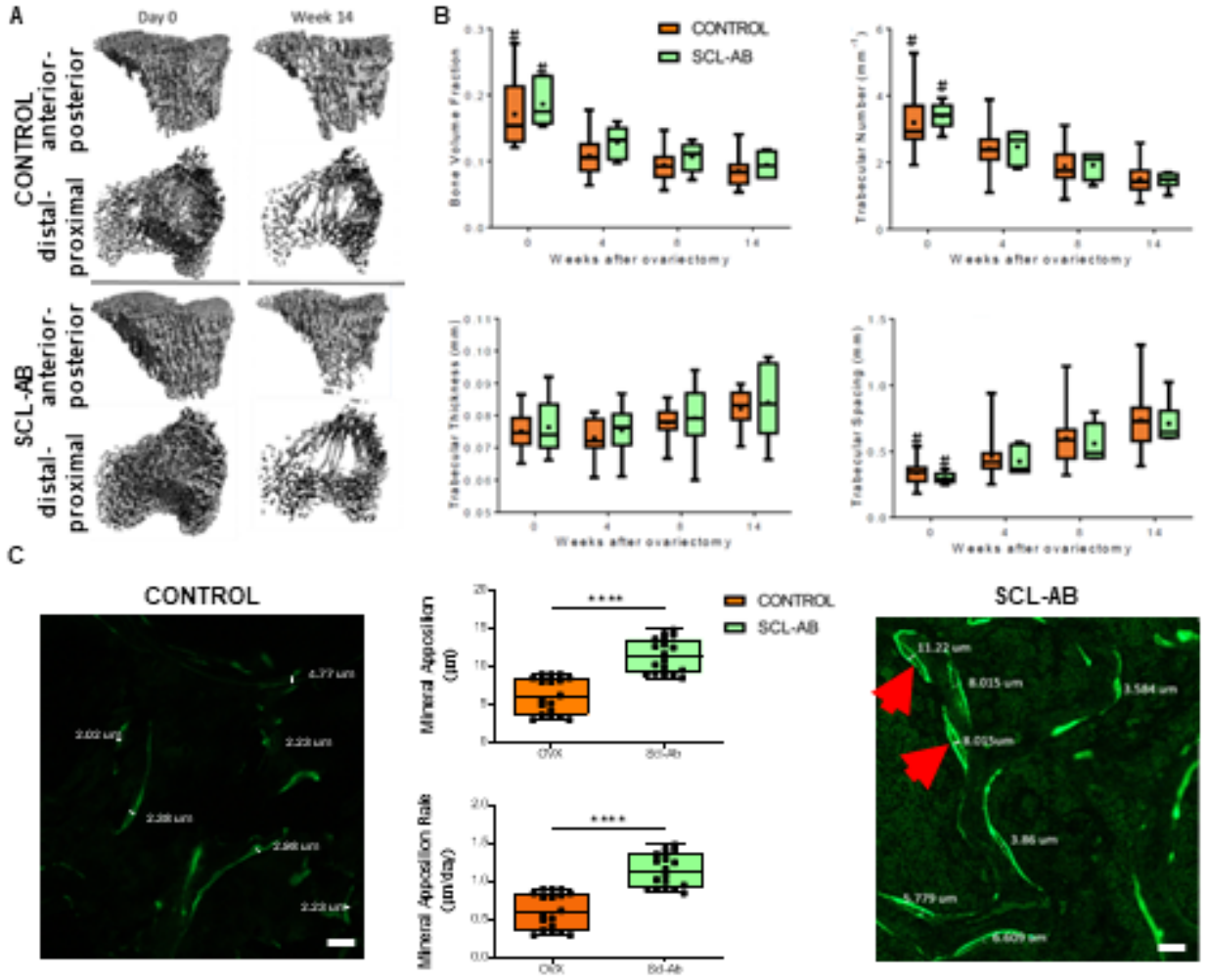


Figure 1: Schematic representation of treatment study design: Female retired breeder Wistar rats were ovariectomised at 6 months old. At 3 weeks post-ovariectomy, animals were randomly assigned to one of two treatment groups (a) CONTROL, no treatment, (b) Scl-Ab, systemic delivery of 2 mg/kg/month Scl-Ab.



743

744 ***Figure 2: (A) 3D micro-CT images of metaphyseal trabecular bone from the proximal tibia of***
745 ***a representative CONTROL and Scl-Ab at day 0 and after 14 weeks of estrogen deficiency in***
746 ***two orthogonal views; anterior-posterior and distal-proximal. (B) Comparison of bone mass***
747 ***and microarchitectural parameters in CONTROL and Scl-Ab groups, at day 0 and after 4, 8***
748 ***and 14 weeks post ovariectomy. Box and whiskers display the following: whiskers extend***
749 ***between min and max values of data set, the box extends from 25th to 75th percentiles,***
750 ***horizontal line across the box is the median value and the black dot represents the mean of the***
751 ***data. # indicates significance (p<0.05) vs. all other timepoints. (C) Images representing the***
752 ***fluorochrome labelling (calcein) for surface bone formation in CONTROL and Scl-Ab with***
753 ***measured thickness of new mineral apposition in micrometres (arrows indicate new mineral***
754 ***apposition), mineral apposition rate in □m/day, (n = 18). * indicates significance (p<0.05;***
755 ***Scale bar = 50 µm).***

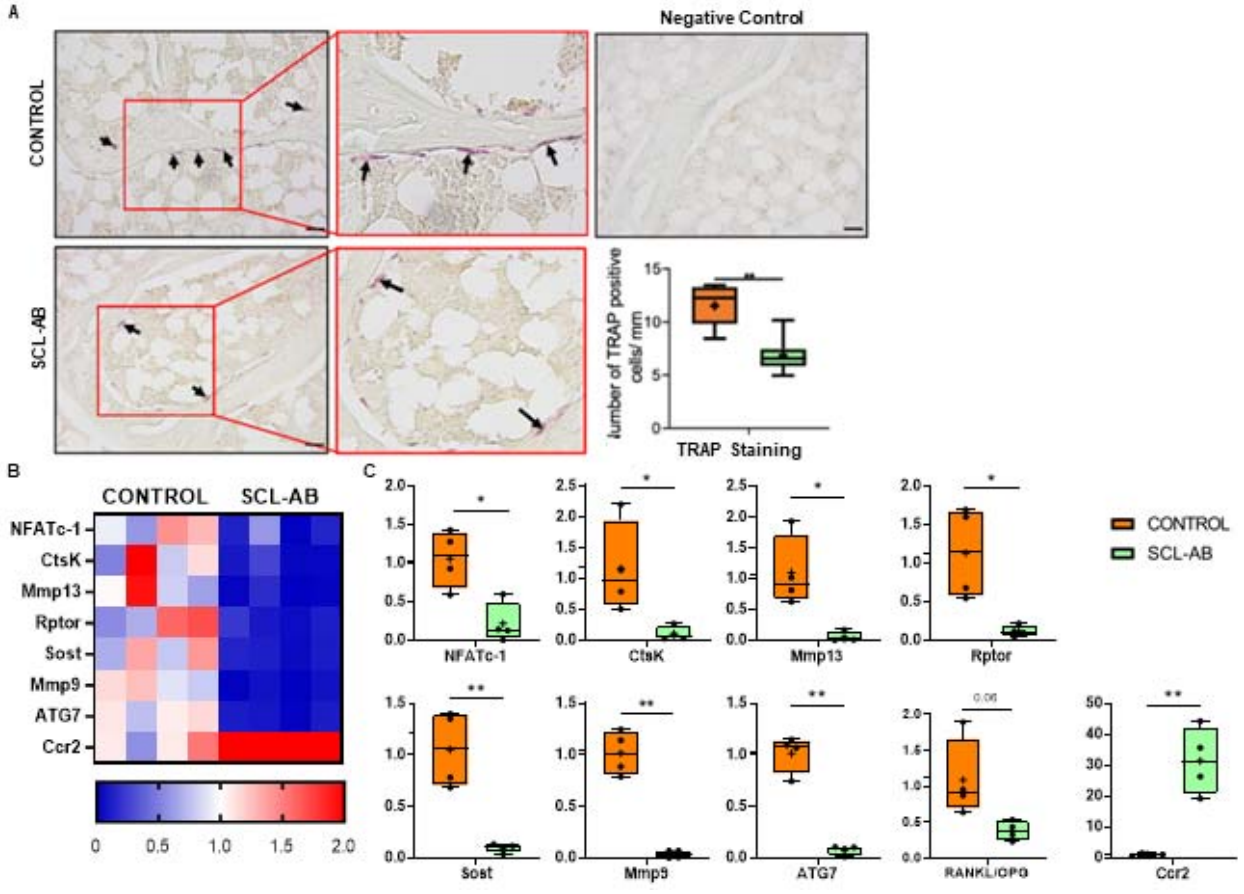


Figure 3: (A) TRAP+ staining is reduced in week 14 OVX animals that received subcutaneous sclerostin antibody (Scl-Ab) when compared to untreated week 14 OVX animals (CONTROL). Representative images showing TRAP staining (black arrows indicate TRAP+ osteoclasts) and fast green counterstain of cortical CONTROL (magnified image (red box) of CONTROL) and Scl-Ab (magnified image (red box) of Scl-Ab). ImageJ analysis was performed to quantify number of TRAP+ cells per mm. (B) Heat map showing catabolic and matrix degradation gene expression of cortical bone from week 14 OVX animals (CONTROL) and week 14 OVX animals that received injection of 2 mg/kg/month sclerostin antibody (Scl-Ab) starting from 3 weeks post-OVX. (C) Statistical comparisons reveal a decrease in Sost, Nfatc1, Ctsk, Mmp9, Ccr2, Rankl/Opg expression upon Scl-Ab administration. n= 4 rats / group, Scale bar = 20µm. * = p<0.05 and ** = p<0.01.

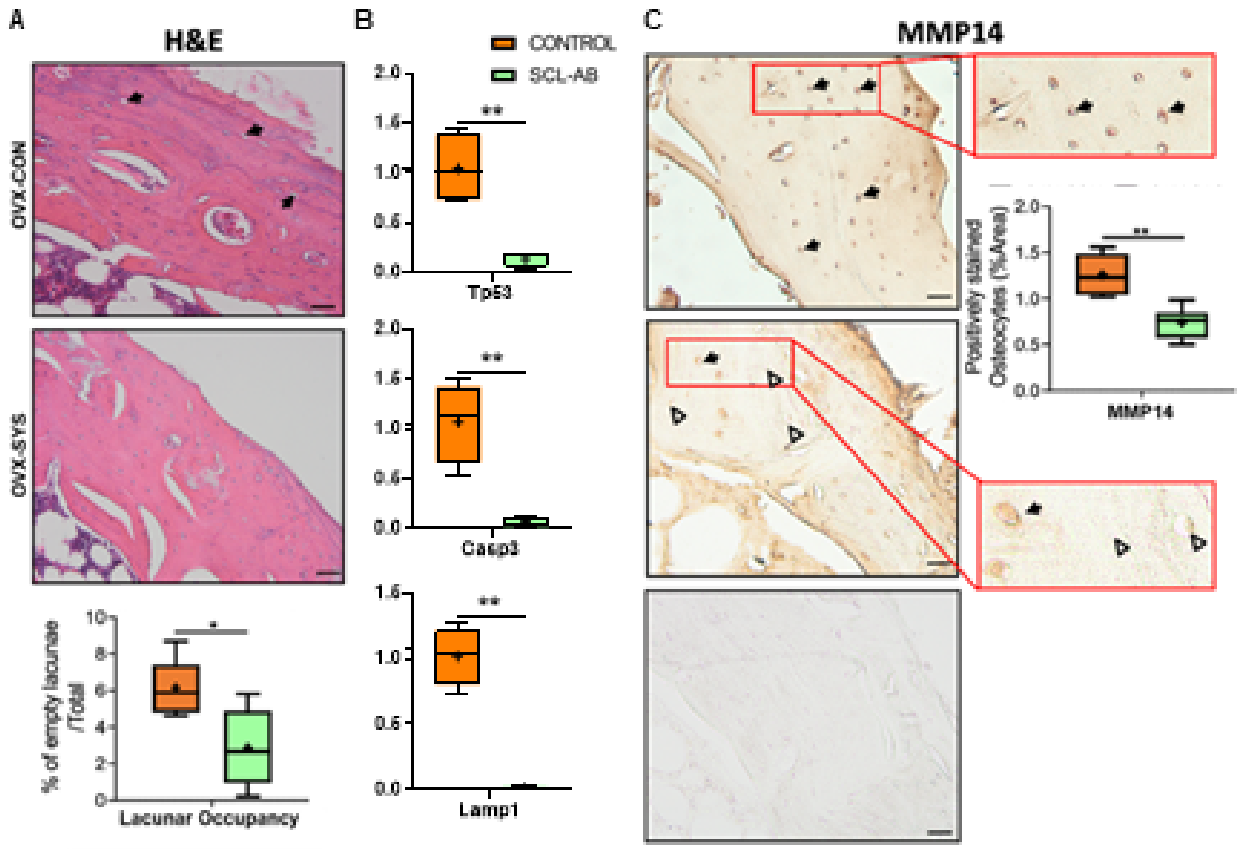


Figure 4: **(A)** ***Scl-Ab administration results in a decrease in unoccupied lacunae. Representative image showing Hematoxylin and Eosin (H&E) staining of cortical bone from week 14 OVX animals (CONTROL) and week 14 OVX animals that received injection of 2 mg/kg/month sclerostin antibody (Scl-Ab) starting from 3 weeks post-OVX. ImageJ analysis was performed to quantify the percentage of empty lacunae (Black arrows indicate empty lacunae).*** **(B)** ***Scl-Ab administration results in a decrease in gene expression associated with apoptosis (Tp53, Casp3 and Lamp1).*** **(C)** ***Scl-Ab administration results in a decrease in MMP14+ osteocytes. Representative images showing MMP14 staining in CONTROL (magnified image (red box) of CONTROL) and Scl-Ab (magnified image (red box) of Scl-Ab). Black arrows = MMP14+ osteocytes, white triangle = osteocyte that is not positive for MMP14. ImageJ analysis was performed to assess the prevalence of positively stained osteocytes, normalised to total bone area. n= 4 rats / group. Scale bar = 20µm. * = p<0.05 and ** = p<0.01.***

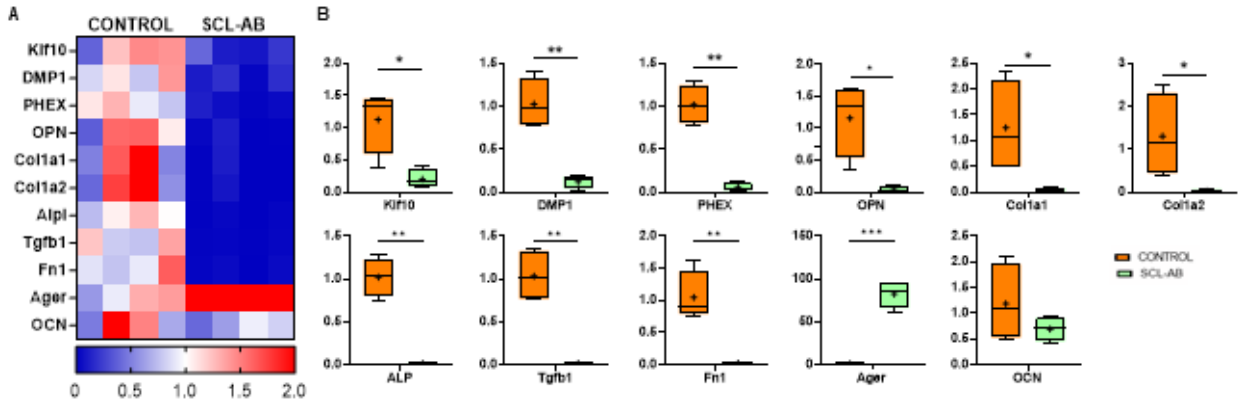


Figure 5: Scl-Ab administration results in a decrease in anabolic and bone matrix formation markers.* (A) *Heat map showing anabolic and bone matrix formation gene expression of cortical bone from week 14 OVX animals (CONTROL) and week 14 OVX animals that received injection of 2 mg/kg/month sclerostin antibody (Scl-Ab) starting from 3 weeks post-OVX.* (B) *Statistical comparisons reveal a decrease in markers of anabolism and bone matrix formation (OPN, ALP, DMP1, PHEX) upon Scl-Ab administration. n= 4 rats / group. * = p<0.05, ** = p<0.01, and *** = p<0.001.

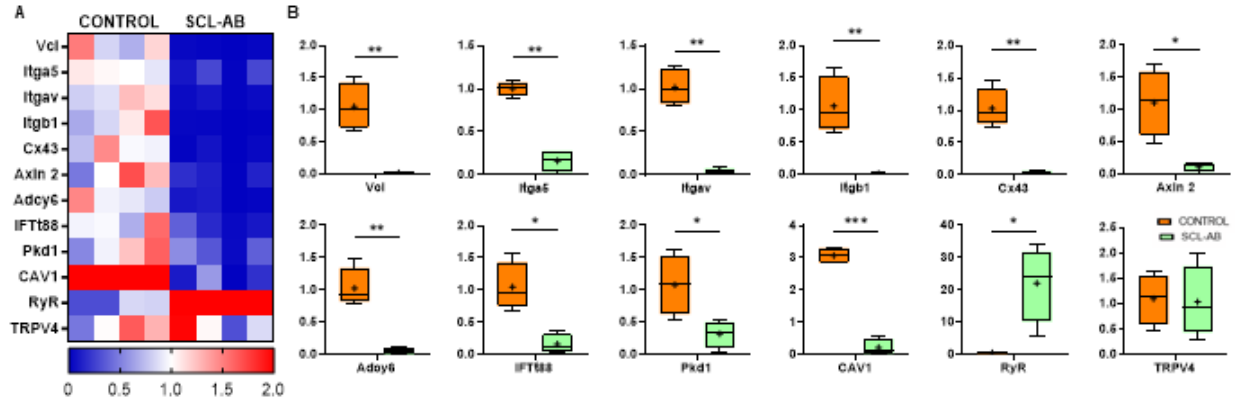


Figure 6: Scl-Ab administration results in a decrease in mechanotransduction and mechanosensation markers. (A) Heat map showing gene expression of cortical bone from week 14 OVX animals (CONTROL) and week 14 OVX animals that received injection of 2 mg/kg/month sclerostin antibody (Scl-Ab) starting from 3 weeks post-OVX. (B) Statistical comparisons reveal a decrease in markers of mechanotransduction and mechanosensation upon Scl-Ab administration. n= 4 rats / group. * = p<0.05 and ** = p<0.01.

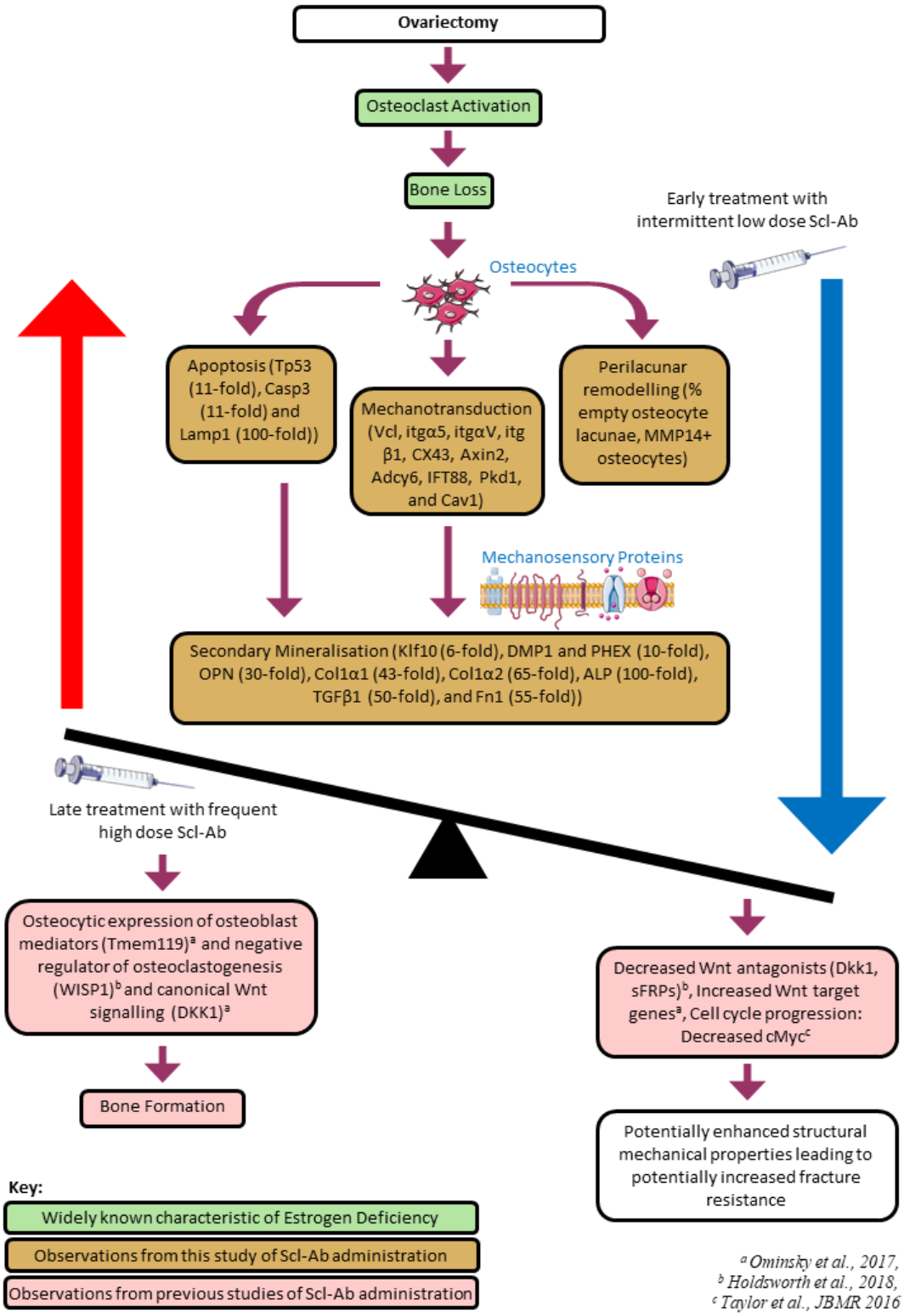


Figure 7: *Flowchart depicting the research from this study in the context of a proposed theory of the sequence of event during estrogen deficiency with (A) early administration of intermittent (monthly) low dose Scl-Ab antibody and (B) late administration of frequent (weekly) high dose Scl-Ab.*