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Effect of Anti-Sclerostin Therapy and Osteogenesis Imperfecta on Tissue-level Properties in Growing and Adult Mice While Controlling for Tissue Age

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Abstract

Bone composition and biomechanics at the tissue-level are important contributors to whole bone strength. Sclerostin antibody (Scl-Ab) is a candidate anabolic therapy for the treatment of osteoporosis that increases bone formation, bone mass, and bone strength in animal studies, but its effect on bone quality at the tissue-level has received little attention. Pre-clinical studies of Scl-Ab have recently expanded to include diseases with altered collagen and material properties such as Osteogenesis Imperfecta (OI). The purpose of this study was to investigate the role of Scl-Ab on bone quality by determining bone material composition and tissue-level mechanical properties in normal wild type (WT) tissue, as well as mice with a typical OI Gly→Cys mutation (Brtl/+) in type I collagen. Rapidly growing (3-week-old) and adult (6-month-old) WT and Brtl/+ mice were treated for 5 weeks with Scl-Ab. Fluorescent guided tissue-level bone composition analysis (Raman spectroscopy) and biomechanical testing (nanoindentation) were performed at multiple tissue ages. Scl-Ab increased mineral to matrix in adult WT and Brtl/+ at tissue ages of 2–4wks. However, no treatment related changes were observed in mineral to matrix levels at mid-cortex, and elastic modulus was not altered by Scl-Ab at any tissue age. Increased mineral-to-matrix was phenotypically observed in adult Brtl/+ OI mice (at tissue ages >3wk) and rapidly growing Brtl/+

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Author Contributions

BPS and KMK conceived and designed the study, and the Raman experiment was planned in conjunction with WRL and MDM. BPS performed animal work and sample preparation. Raman spectroscopy and nanoindentation data collected and analyzed by WRL, BPS, and JDS. Data interpreted by BPS, WRL, JDS, JCM, MSC, MDM, and KMK. Manuscript was written by BPS, and critically reviewed and revised by all authors.

(at tissue ages > 4wk) mice compared to WT. At identical tissue ages defined by fluorescent labels adult mice had generally lower mineral to matrix ratios and a greater elastic modulus than rapidly growing mice, demonstrating that bone matrix quality can be influenced by animal age and tissue age alike. In summary, these data suggest that Scl-Ab alters the matrix chemistry of newly formed bone while not affecting the elastic modulus, induces similar changes between *Brtl/+* and WT mice, and provides new insight into the interaction between tissue age and animal age on bone quality.

Keywords

Sclerostin antibody; osteogenesis imperfecta; Raman spectroscopy; nanoindentation; anabolic therapy; bone quality

1.0 Introduction

Bone fragility is regulated across multiple hierarchical scales. In addition to bone mass, changes in material-level composition and biomechanics are critical determinants of whole bone strength [1]. Sclerostin Antibody (Scl-Ab) is a novel anabolic candidate therapy for the treatment of osteoporosis. Experiments in a variety of animal models, as well as Phase I and Phase II clinical trials, demonstrate that Scl-Ab stimulates bone formation and increases bone mass [2–9]. However, the effect of Scl-Ab on tissue-level changes to bone composition and mechanics has received limited attention, with little, or no, change in mineralization (Backscattered scanning electron microscopy) and material composition (Fourier transform infrared spectroscopy) reported in rats, primates, or OI mice treated with Scl-Ab [10, 11]. While SOST-KO mice and patients with sclerosteosis represent extreme examples of lifelong absence of sclerostin unlikely to be replicated by periodic Scl-Ab dosing, data from these mice and human patients support the potential for sclerostin-related alterations in bone material composition [12].

Osteogenesis imperfecta (OI), also known as “brittle bone disease,” is a genetic collagen-related disorder which causes fragile bones and frequent fractures in children [13]. The increased fracture risk of OI is often a product of reduced bone mass and altered material composition. At the matrix level, despite decreases in overall bone mass, OI patients and animal models typically reflect an increased level of tissue mineralization [14–17] which is believed to be associated with tissue brittleness characteristic of the disease. To study the potential anabolic efficacy of Scl-Ab treatment in OI, pre-clinical studies in the *Brtl/+* [18–20], Amish [21], and JRT [11] mouse models of OI have been performed. The *Brtl/+* model of Type IV OI is heterozygous for a typical Gly→Cys substitution on *coll1a1* (G349C) and recapitulates many features of the OI phenotype including reduced bone mass, reduced bone strength, and increased bone resorption relative to bone formation [22–24]. We previously showed that Scl-Ab increased bone formation and bone mass in *Brtl/+*, and mechanical four-point bending revealed that Scl-Ab significantly reduced bone brittleness in young WT, and adult *Brtl/+* and WT mice [19, 20]. These findings suggested potential alterations at the material level that have yet to be fully explored.

The purpose of this study was to determine the effect of Scl-Ab on tissue-level mechanical properties and material composition in normal WT tissue, as well as Brl/+ OI mice using nanoindentation and Raman spectroscopy. As bone material properties and OI fracture risk change with age, both rapidly growing (3-week-old) and adult (6-month-old) ages of WT and Brl/+ were studied. Fluorescent-guided bone material analysis was used to rigorously control for tissue age and to determine any differences in mineralization dynamics. Analyzing samples at the same tissue ages in both rapidly growing and adult animals enabled insight into the direct interaction between tissue age and animal age on tissue level bone properties. This study adds new data describing Scl-Ab effects on tissue level material composition and biomechanics, investigates Scl-Ab effects on OI tissue, and highlights the interaction between animal age and tissue age during bone formation.

2.0 Materials and Methods

2.1 Animals

Wildtype (WT) and Brl/+ [22] mice are maintained on a mixed background of Sv129/CD-1/C57BL/6S, and all Brl/+ animals were the product of breeding male heterozygous Brl/+ with female WT. 3 week and 6 month old male WT and Brl/+ mice were randomly assigned to Scl-Ab (Scl-Ab VI, Amgen, Thousand Oaks, CA) treatment or vehicle injection (PBS). Sclerostin antibody was injected subcutaneously at 25 mg/kg, two times per week, for five weeks. Calcein (30mg/kg, i.p. injection) was injected at the start of experiment (5 weeks before euthanasia), after 2 weeks of treatment (3 weeks before euthanasia), and after 4 weeks of treatment (1 week before euthanasia). A final alizarin label (30mg/kg, i.p. injection) was given 1 day before euthanasia. A summary of the experimental design is shown in Figure 1A. The multiple fluorescent labels were used to visualize the growth pattern during the entire course of therapy. Body weights were recorded with each injection. Right femurs were harvested and stored at -20°C in lactated Ringers solution (LRS) soaked gauze until testing or further specimen preparation. All protocols and procedures involving animals were approved by the University of Michigan's Committee on Use and Care of Animals.

2.2 Specimen Preparation

Two distinct cohorts of animals were used for this study. Nanoindentation was performed on the same animals previously described [19, 20]. These samples underwent 2–4 freeze cycles in the course of harvesting and specimen preparation, but this was not rigorously controlled for. Nanoindentation group sizes are $n=7-9/\text{group}$, before accounting for testing errors. All Raman spectroscopy tests were performed on a second cohort of animals (both 3-week-old and 6-month-old) that were treated as described above except that femora had only one freeze/thaw cycle prior to imaging to avoid confounding the results [25]. All Raman Spectroscopy data was $n=5-7/\text{group}$.

Right femora were thawed, encased in a quick setting ($\sim 30\text{min}$) epoxy (Kold Mount, Vernon-Benshoff, Albany, NY) without dehydration, and cut transversely at the mid-diaphysis with a low-speed saw (IsoMet, Beuhler, Lake Bluff, IL). The distal section of tissue was polished using progressive grades of silicon carbide abrasive paper (1200, 2400,

and 4000 grit) under water irrigation for two minutes at each grade. Each specimen was polished to a plane just distal to the third trochanter, identical to that performed by dynamic histomorphometry, and similar to a plane included in our cortical microCT and mechanical 4pt testing data previously reported [19, 20].

To further decrease surface roughness for nanoindentation, the encased specimens were polished on a felt pad for 5 minutes with a $\frac{1}{4}$ μ m diamond suspension (Struers Inc., Cleveland, OH). Specimens were then ultrasonically cleansed in a water bath for 10 minutes to remove surface debris, and glued to a glass slide specimen plate for nanoindentation testing in a custom-made hydration chamber.

2.3 Nanoindentation

A custom TI 950 TriboIndenter (Hysitron, Minneapolis, MN) instrumented with a fluorescent light-source and FITC filter allowed for simultaneous visualization of calcein labeling in specimens and accurate positioning of indents to locations matched for tissue age and treatment status with 0.5 μ m spatial resolution. Five (rapidly growing) or six (adult) regions of interest were mechanically tested in the posterior aspect of the femoral cross section (Fig. 1B): the mid-cortex (defined as midway between the first calcein label on the periosteal and endosteal surfaces, if any), 15 μ m endosteal to (“inside”) the first 5 wk old label, along the first calcein label on 5 wk old bone, along the second calcein label on 3 wk old bone, along the third calcein label on 1 wk old bone, and along the final alizarin label on 1 day old bone. Due to technical constraints on the nanoindentation fluorescent imaging hardware, the outer alizarin label positions were identified by images taken on a separate microscope (Zeiss Axiovert) and measuring the distance from the 1 wk old calcein label. The mid-cortex and 15 μ m endosteal to the 5 wk label positions were nearly identical locations in rapidly growing 3 week animals. As such, nanoindentation values were only collected at one location and characterized as “mid-cortex” in all analysis. As Veh treated 6 mo old animals grow much slower than 3 wk old animals, it was not always possible to delineate the outer 1 wk and 1 day old labels in adult 6 mo mice as they appeared in the same location. Moreover, nanoindentation requires spacing between indents to not indent on the tissue deformed by a previous indent. Therefore, in these instances, data was only collected in one location, and it was treated as a tissue age of 1 wk. As a result, there is reduced statistical power with $n=5$ for Adult WT Veh at a tissue age of 1 and an $n=3$ for Adult Brtl/+ Veh at a tissue age of 1 day, instead of the $n=7-9$ for all other nanoindentation outcomes.

Indentation consisted of a 10 μ N pre-load, with a diamond Berkovich indenter tip into samples, followed by loading the sample at 300 μ N/s, hold at a maximal load of 3,000 μ N for 10 s, and unloading at 300 μ N/s. All indents were performed on samples fully hydrated with saline using a custom hydration chamber. The indentation modulus E was calculated from the load-displacement curves using the standard Oliver-Pharr method [26]. Quartz ($E=69.6$ GPa) was used to define the tip-area function, and was also used throughout testing to ensure consistent calibration of the machine. Eight indents, 10 μ m apart, were made along each fluorescent label and region of interest, with values averaged for each site and mouse.

2.4 Raman Spectroscopy

Raman measurements were performed with a custom microscope outfitted for Raman spectroscopy as previously described [27]. Briefly, Raman spectra were collected by directing a 785-nm stabilized diode laser (Invictus, Kaiser Optical Systems, Inc., Ann Arbor, MI) through a Nikon E600 microscope (Nikon Instruments Inc., Melville, NY) with a 40×/0.90 NA objective (S Fluor, Nikon Instruments, Inc., Melville, NY) operated in epi-illumination/ collection mode. Collected light from the sample was passed through an imaging spectrograph with a 50- μm slit (Holospec f/1.8i, Kaiser Optical Systems, Inc., Ann Arbor MI) and detected with a deep depletion, back-illuminated CCD camera (DU 401-BR-DD, Andor Technologies, South Windsor, CT). Estimated spectral resolution is $\sim 8\text{ cm}^{-1}$. After data preprocessing, the estimated imaging spot size is 2.3 by 69.1 μm .

For each specimen, periosteal bone was mapped in a 9 by 4 (adult) or 8 by 4 (rapidly growing) grid from the map origin. The map origin was defined as the upper left corner of a relatively flat and uniform region of the bone. The measurement rows were determined by the fluorescent labels. The 4 measurement columns were equally spaced 75 μm apart starting from the origin. The 9 (adult) or 8 (rapidly growing) measurement locations, as guided by the fluorescent labels, were the mid-cortex, 15 μm endosteal to the 5 week old bone label (only for adult 6 mo), atop the 5 weeks old bone label, halfway between the labels marking 5 and 3 weeks old bone, atop the 3 weeks old bone label, halfway between the labels marking 3 weeks and 1 week old bone, atop the 1 week old bone label, halfway between the 1 week and 1 day old bone label, and atop the 1 day old bone label (Fig. 1). At each location, a spectrum was collected in 120 s with $\sim 45\text{ mW}$ laser power. Custom-built Matlab (The Mathworks, Inc., Natick, MA) software was employed for data processing. The processing routine included cosmic spike correction, dark subtraction, correction for spectrograph slit curvature, calibration for wavelength-dependent changes in the CCD's quantum efficiency, and wavenumber calibration. Spectra were also processed to remove background signal contributed from epoxy. Samples had minimal epoxy interference as they were not placed in a vacuum during embedding which would promote epoxy integration into cortical bone.

Raman spectroscopic parameters were computed with locally written scripts to assess changes in the mineral and matrix components of the cortical bone. Prior to ratio analysis, Gaussian band fitting was performed to resolve Raman peaks in bands with multiple contributing species. Bands fitted included $\nu_1\text{ PO}_4$ and amide I bands. Least-squares fitting routine was executed with locally written scripts to identify the contribution of each band. The mineral to matrix ratio was assessed, and defined as the resolved intensity of the phosphate ν_1 band at 959 cm^{-1} divided by the maximum intensity of the proline and hydroxyproline bands between 850 and 880 cm^{-1} . Four spectra were collected at each tissue age on every sample, and the average of these four spectra for a specific outcome (e.g. mineral to matrix) was the value used for all analysis.

We note that most Raman bands are polarization sensitive, including parameters reported in this study such as phosphate ν_1 [28]. However, by employing a non-confocal microscope (line focused) with a slit (rather than a pinhole) prior to the detector, polarization effects are

minimized. Previous work demonstrated that special conditions (including a 25 μm slit rather than the 50 μm slit in this study) were required to observe polarization effects [28].

The Raman microprobe uses a greyscale video camera to position the specimen for data collection. Raman spectroscopy collection sites were co-located with fluorescence labels by aligning greyscale video images with previously acquired fluorescence images (Zeiss Axiovert, following nanoindentation protocol). Scratches made in embedding medium and unique features of edge of bone aided alignment.

2.5 Paraffin Sections

Differences in tissue microstructures were qualitatively observed from contralateral femurs of animals used for Raman spectroscopy. Femurs were fixed in 4% PFA, decalcified in 10% EDTA, embedded in paraffin, and sectioned transversely at 7 μm . The transverse anatomic plane used for the paraffin sections was the same as that used for the Raman spectroscopy and nanoindentation analysis.

2.6 Statistics

Comparisons between tissue ages were made with a two-factor general linear model with repeated measures. Specifically, within each animal age, genotype (WT and Brl/+) and treatment (Veh and Scl-Ab) were between-subjects factors, and tissue age identified as a within-subjects factor. All pairwise tissue age comparisons were performed with a conservative Bonferroni correction to a reference point of 5 weeks of tissue age. A tissue age of 5 weeks was chosen as the reference, instead of mid-cortex, because this allowed us to control for tissue age across two animal ages and directly contrast findings between rapidly growing and adult animals. These data are shown in panel A of Fig. 2–3 as well as Suppl. Fig. 1.

To assess the overall effect of genotype and treatment within a specific tissue age, a two factor multivariate model was performed with genotype (WT and Brl/+) and treatment (Veh and Scl-Ab) as the between-subjects factors that were analyzed at each tissue age. These data are shown in panels B and C of Fig. 2–3, as well as Suppl. Fig 1.

To assess the overall effect of animal age on material property and composition when controlling for tissue age, a three factor multivariate model was employed, with animal age (rapidly growing 3 wk and adult 6 mo), genotype (WT and Brl/+), and treatment (Veh and Scl-Ab) as the between-subjects factors that were analyzed for each outcome at all tissue ages. Figure 4 shows the data isolating the animal age effect. $p < 0.05$ was considered significant in all cases. Mean $\pm$ standard deviation is shown unless otherwise noted.

3.0 Results

3.1 Treatment Effect: Scl-Ab Increases the Mineral to Matrix Ratio in Adult Mice at Specific Tissues ages, and Does Not Affect the Elastic Modulus

In adult 6-month-old animals, Scl-Ab significantly increased the Mineral to Matrix ratio at tissue ages of 4 wks, 3 wks, and 2 wks on the periosteal posterior surface of the diaphyseal

femur in both WT and *Brtl/+* (Fig. 2C). In rapidly growing (3-week-old) animals, Scl-Ab did not significantly alter the mineral to matrix ratio (Fig. 2B).

The elastic modulus measured by nanoindentation was not significantly affected by Scl-Ab at any tissue age in rapidly growing or adult animals (Fig. 3B,C). Hardness data was similar, with no effect of Scl-Ab (Suppl. Fig. 1B,C).

3.2 Genotype Effect: *Brtl/+* Has Increased Mineral to Matrix in both Rapidly Growing and Adult Mice

Increases in *Brtl/+* mineral to matrix ratio relative to WT were observed in both rapidly growing and adult animals. Specifically, in 6-month-old adult animals, increased mineral to matrix ratios were observed at tissue ages of 3 weeks, 4 weeks, 5 weeks, and 15 μ m inside the 5week label (Fig. 2C). In rapidly growing animals, increased mineral to matrix ratio was observed between a tissue age of 4 weeks and mid-cortex (Fig. 2B).

No significant changes in elastic modulus with genotype were observed in rapidly growing or adult animals (Fig 3B,C). Similar data for hardness also revealed no significant genotype effects in *Brtl/+* (Suppl. Fig 1B,C).

3.3 Tissue Age Effect: Elastic Modulus and Mineralization Increase with Tissue Age in Adult Mice, but Plateau in Rapidly Growing Mice

In 6 mo old WT and *Brtl/+*, increasing tissue age is associated with increasing mineral to matrix ratio, with the highest levels found at mid-cortex (Fig 2A). In rapidly growing animals, the mineral to matrix ratio increased rapidly until peak values were achieved at ~3 wks of tissue age. At tissue ages greater than 3 wks in rapidly growing animals, increasing tissue age is correlated with decreased mineral to matrix ratios (Fig 2A).

The pattern of elastic modulus was similar to that observed in the mineral to matrix ratio. Specifically, in adult 6-month-old animals, increasing tissue age was associated with increasing elastic modulus, and the highest levels were observed at mid-cortex (Fig 3A). By contrast, in rapidly growing animals, the elastic modulus reached plateaus at ~3 wks of tissue age. The pattern observed for hardness across tissue age mimicked that of elastic modulus, with continued increases observed in adult, but not rapidly growing animals (Suppl. Fig 1A).

3.4 The Age of the Animal at Which a Tissue was Formed, Independent of the Age of the Tissue, Influences Material Composition and Elastic Modulus

Figure 4 shows the estimated marginal means across all groups within an animal age. The estimated marginal means are not the simple average across all groups for animals of a specific age, but rather reflect the mean response for each factor adjusted for by treatment status and animal genotype.

The differences in mineral to matrix ratio between adult and rapidly growing animals depend on tissue age (Fig 4). Between tissue ages of 1 and 4 weeks, rapidly growing animals had a greater mineral to matrix ratio than adult animals. At the very newest tissue age of 1 day, 6 mo animals had a greater mineral to matrix ratio. Adult animals also had a greater

mineral to matrix ratio than rapidly growing animals at mid-cortex, where tissue age is almost certainly greater in adult animals.

Elastic modulus in adult animals was significantly greater than rapidly growing animals at tissue ages of 3 weeks and 5 weeks, and at mid-cortex (Fig 4).

4.0 Discussion

This study provides new insights regarding the role of sclerostin-antibody on bone quality in both normal and OI tissue. As bone composition and biomechanics at the tissue-level are important regulators of whole bone strength, observed changes may relate to treatment efficacy. In addition to Scl-Ab effects, use of fluorescence-guided Raman spectroscopy and nanoindentation at multiple tissue ages in both rapidly growing and adult animals allowed for direct quantification of the interaction between tissue age and animal age on tissue-level bone properties.

Tissue elastic modulus and mineral to matrix levels showed strong correlation across tissue ages within each animal age cohort, although the pattern of material composition and elastic modulus with tissue age was different between rapidly growing (3 wk), and adult (6 mo) animals. In both groups, a rapid increase in the mineral-to-matrix ratio and elastic modulus from tissue ages of 1 day through 3 wks was observed. However, after 3 wks tissue age the patterns diverged. Adult animals continued to increase the mineral to matrix ratio and elastic modulus from 3 wks of tissue age through 5 wks of tissue age (Fig 2A, 3A), whereas rapidly growing animals maintained or decreased over this same period. To follow up on this result, additional histology assays were performed and found that the observed changes in mineral to matrix and elastic modulus corresponded with the presence of woven bone in rapidly growing mice (Fig 1B, 5). Specifically, in rapidly growing mice, a significant area of woven bone persisted at mid-cortex and at tissue ages of 5wks. However, adult mice displayed primarily lamellar bone and previously present woven bone at midcortex was likely removed due to cortical drift.

In this study, Scl-Ab increased the mineral to matrix ratio in adult animals at 2 wks, 3 wks and 4 wks tissue age (Fig 2C). This finding corroborates Scl-Ab treatment effects on mineral to matrix ratio patterns at the intracortical compartment of 4–5 year old cynomolgus monkeys [10]. Despite the mineral to matrix changes observed with Scl-Ab in this study, nanoindentation did not reveal comparable changes in elastic modulus with Scl-Ab therapy. However, nanoindentation was much more sensitive to the greater magnitude changes observed over the entire range of mineral to matrix deviations across tissue age.

The finding of small but significant increases in mineral to matrix in *Brtl/+* vs. WT animals (Fig 2B,C) is in agreement with other studies that have found increased mineralization and TMD in the *Brtl/+* OI mouse model [23, 29, 30], and in studies demonstrating increased tissue mineralization of human patient biopsies [14–16]. Because the present study controls for tissue age we were able to determine if increased *Brtl/+* mineralization was present in newly formed tissue, or occurred over time. No increases in mineral to matrix at tissue ages <3 wks were found in adult *Brtl/+* vs. WT mice. Similar findings were observed in rapidly

growing animals and significant differences were not observed at tissue ages <4 weeks. Thus, it appears that increased mineralization of OI tissue in this model is a result of continued increases over time, but is not significantly greater from the outset. This finding may be responsible for restoration of Brl/+ biomechanical phenotype with age, despite retention of smaller bone size and shape [23].

Notably, at two different animal ages, bone of equivalent tissue ages showed different material properties and composition (Fig 4). For example, adult mice tended to have a lower mineral-to-matrix ratio than rapidly growing mice at identical tissue ages (1wk, 2wk, 3wk, and 4wk, Fig 4B). This data directly shows that matrix properties at the same tissue age vary with the age of animal in which the tissue is formed. Similar mineral-to-matrix findings have recently been observed in a study of human biopsies from subjects 1 to 84 years old [31]. In addition, we note that several studies have looked at the effect of tissue age within a single animal age [32, 33], or less directly studied the interaction between the age of the animal and tissue age by examining numerous animal ages [34, 35], or inferring tissue age by osteonal morphology [36]. Despite our observation that adult animals had a lower mineral-to-matrix ratio than newly formed bone in young animals, they had a greater elastic modulus. This interesting observation supports the notion that factors other than mineral-to-matrix ratios regulate stiffness and moreover, the contribution of these additional factors varies with animal age.

Previously, we observed large (~500%) and significant increases in post-yield displacement, or reductions in bone brittleness, by whole bone four-point bending of rapidly growing and adult mice treated with Scl-Ab [19, 20]. Specifically, reduced brittleness was observed with Scl-Ab treatment in WT and Brl/+ adult mice [19], but only in rapidly growing WT mice [20]. Raman spectroscopy data did not reveal a definitive or highly suggestive effect to correlate with our previous whole bone brittleness findings, as mineral to matrix was, if anything, increased by Scl-Ab at specific tissue ages in these animals. Additional changes in collagen, such as cross-linking or organization not reported here might correlate with these observed changes in bone brittleness.

In this study, the observed changes in material level composition with Scl-Ab did not manifest in mechanical changes in the elastic modulus. The reasons for this are not immediately clear, but may be regulated by several factors. First, our study primarily focused on newly formed tissue, and dogmas derived from mature bone may not apply to rapidly mineralizing tissue. A previously discussed example is a case in point, as mineral-to-matrix comparisons between rapidly growing and adult mice were inversely correlated with elastic modulus (Fig. 4), suggesting that factors other than the ratio of mineral to collagen regulate the observed differences in material stiffness in newly formed tissue. Second, elastic modulus is a parameter describing material level stiffness, but does not describe post-yield properties or fracture toughness. Put simply, the absence of significant changes in the elastic modulus does not generally exclude the presence of mechanical differences in important outcomes such as fracture toughness. Thus, the differences in material composition may correlate with changes in mechanical properties not measured in this study. This was most evident in our prior results showing substantial changes in post-yield behavior in adult Brl/+ and WT and young WT mice [19, 20] despite no obvious explanation

provided by the spectroscopic and nanoindentation data measured in this study. A final consideration related to the impact of these findings centers around the hierarchical organization of bone. Bone is a complex tissue with a unique organization at its many length scales, and the present study focused on changes present at the micron to nanoscale. The observed changes at the tissue level in this study may be manifested in, or compensated by, changes at the greater ultrastructural and whole bone level. While prior work has examined whole bone mechanics by four-point bending in this same model, additional exploration of mechanical properties such as fracture toughness and response to fatigue loading are important to further define the effect of Scl-Ab on mechanical properties.

There are several limitations to this study. First, Raman spectroscopy and nanoindentation were performed on different animals in order to minimize freeze/thaw cycles that could damage tissue and corrupt Raman spectra. While this prevented direct correlations from data taken at identical locations, similar trends observed in different animals provide independent confirmation of main effects. Second, our data are limited to cortical bone of the posterior femur in a mouse model to maintain consistency with prior biomechanical analysis and because the persistent bone formation at this location allows for anatomically consistent measurements in untreated groups. As OI is a disease of many mutations, the results from one OI type may not extend to another. Therefore, these data should not be generalized to all types of OI, and require testing in other dominant and recessive OI types that feature unique mutations and phenotypes. Finally, as nanoindentation primarily quantifies pre-yield mechanical effects, future studies should examine post-yield effects at the tissue level.

In conclusion, our data suggests that Scl-Ab increased mineral to matrix ratios at specific tissue ages in adult animals. However, these changes were not associated with changes in the elastic modulus. By controlling for tissue age with fluorescence-guided analysis, this study directly shows that bone of identical tissue age has different material composition and elastic modulus in rapidly growing vs. adult animals. For the material properties and compositional parameters we analyzed in this study, the data do not suggest that there are unique concerns of Scl-Ab use in a *Brtl*^{+/+} model of OI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Sclerostin antibody effects on bone composition and tissue level biomechanics were assessed in a mouse model of osteogenesis imperfecta.
2. Tissue age was controlled for by sequential fluorochrome labeling in young and adult mice undergoing treatment.
3. Sclerostin antibody increased mineral-to-matrix ratios in adult and young mice at some tissue ages
4. Sclerostin antibody did not change tissue elastic modulus
5. Mouse age influences matrix composition and elastic modulus independent of tissue age.

Figure 1.

A) Experimental design and calcein labeling scheme for these experiments is shown above. The final alizarin label was given 1 Day before euthanasia. B) Location of fluorescent labels and data acquisition at the mid-diaphyseal femur of rapidly growing 3wk–8wk and adult 6mo–6mo+5wk animals. Calcein and alizarin labels were given as shown in A. Nanoindentation was not performed between labels due to resolution issues associated with the technique. In rapidly growing animals, mid-cortex was nearly identical in location to the “15 µm inside 5wk” location and data was only collected once, and labeled as “mid-cortex”.

In some adult 6mo Veh specimens, the 1wk and 1 Day labels were located in the nearly same place due to a low mineral apposition rate. As indents for nanoindentation cannot be placed on top of one another, in some cases data was only collected for 1 week old tissue. The fluorescent labels of the adult animal shown are from a mouse treated with Scl-Ab. Separate animals were used for nanoindentation (n=7–9/group) and Raman spectroscopy (n=5–7/group) in order to limit freeze-thaw cycles.

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Figure 2.

A) Mineral to Matrix ($958 \text{ cm}^1/\text{max}(840 \text{ cm}^{-1} - 900 \text{ cm}^{-1})$) ratio at nine tissue ages on the periosteal posterior surface of the mid-diaphyseal femur. All differences from a 5 week tissue age (orange bars) are shown within each experimental group. Lines indicate $p < 0.05$ from a repeated measures pairwise comparison with conservative Bonferonni correction.

B,C) This is the same mineral to matrix data as shown in A, but plotted to show the effect of genotype (WT vs. Brtl/+) and treatment (Veh vs. Scl-Ab) within each animal age. P-values listed represent the result of a two-factor analysis and show the overall effect of genotype and treatment (as well as the interaction between these two factors) at each tissue age. All data are mean $\pm$ stdev, $n=5-7/\text{group}$.

Figure 3.

A) Elastic Modulus at nine tissue ages on the periosteal posterior surface of the mid-diaphyseal femur. All differences from a 5 week tissue age (orange bars) are shown within each experimental group. Lines indicate $p < 0.05$, repeated measures pairwise comparison with Bonferroni correction. B,C) This is the same Elastic Modulus data as shown in A, but plotted to show the effect of genotype (WT vs. Brl/+) and treatment (Veh vs. Scl-Ab) within each animal age. P-values listed represent the result of a two-factor analysis and show the overall effect of genotype and treatment (as well as the interaction between these two factors) at each tissue age. All data are mean $\pm$ stdev, $n=7-9$ /group with two exceptions. Due to low MAR in Adult Veh animals (C) and insufficient label separation, Adult WT Veh at a tissue age of 1 Day is $n=5$, and Adult Brl/+ Veh at a tissue age of 1 day is $n=3$.

Figure 4.

At identical tissue ages, we found differences between the Mineral to Matrix ratio and Elastic Modulus exist between rapidly growing and adult animals. This implies that animal age, independent of tissue age, can influence the properties of recently formed bone. This effect is visually apparent in Figures 2A and 3A when comparing the left side of the graph (rapidly growing) to the right (adult). Figure 4, shown here, seeks to more clearly present this difference and statistically compare bone of equivalent tissue ages in rapidly growing mice vs. adult. Specifically, Figure 4 plots the estimated marginal means across all four experimental groups for the mineral to matrix ratio and elastic modulus at two animal ages. A significant difference indicates that on average, across all four experimental groups, animal age significantly influenced the matrix composition or material property despite analysis being carried out at the same tissue age. Note that tissue age was not controlled for at the “mid-cortex” location, and these represent different tissue ages between the rapidly growing and adult animals. The p-values shown are the animal age factor from a three factor ANOVA for genotype, treatment, and animal age, within each tissue age. Data are mean $\pm$ stddev. $n=5-7$ /group for Raman spectroscopy measures, and $n=7-9$ /group for nanoindentation.

Rapidly Growing 3wk-8wk Posterior Femur **Adult 6mo-6mo+5wk Posterior Femur** **Figure 5.**

Woven bone was observed at mid-cortex of the femur in rapidly growing, but not adult animals. Paraffin sectioned femurs were contralateral to those analyzed by Raman spectroscopy. Brightfield images on decalcified paraffin sections shown above are representative of the presence of woven bone in rapidly growing, but not adult, animals at the skeletal site analyzed (periosteal posterior femur). This was also observed by dynamic histomorphometry in Figure 1B on undecalcified sections. No differences were observed with *Brtl*/+ or with *Scl*-Ab.