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Rapidly Growing *Brtl/+* Mouse Model of Osteogenesis Imperfecta Improves Bone Mass and Strength with Sclerostin Antibody Treatment

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Abstract

Osteogenesis imperfecta (OI) is a heritable collagen-related bone dysplasia, characterized by brittle bones with increased fracture risk that presents most severely in children. Anti-resorptive bisphosphonates are frequently used to treat pediatric OI and controlled clinical trials have shown bisphosphonate therapy improves vertebral outcomes but has little benefit on long bone fracture rate. New treatments which increase bone mass throughout the pediatric OI skeleton would be beneficial. Sclerostin antibody (Scl-Ab) is a potential candidate anabolic therapy for pediatric OI and functions by stimulating osteoblastic bone formation via the canonical wnt signaling pathway. To explore the effect of Scl-Ab on the rapidly growing OI skeleton, we treated rapidly growing 3 week old *Brtl/+* mice, harboring a typical heterozygous OI-causing Gly->Cys substitution on *coll1a1*, for 5 weeks with Scl-Ab. Scl-Ab had anabolic effects in *Brtl/+* and led to new cortical bone formation and increased cortical bone mass. This anabolic action resulted in improved mechanical strength to WT Veh levels without altering the underlying brittle nature of the material. While Scl-Ab was anabolic in trabecular bone of the distal femur in both genotypes, the effect was less strong in these rapidly growing *Brtl/+* mice compared to WT. In conclusion, Scl-Ab was able to stimulate bone formation in a rapidly growing *Brtl/+* murine model of OI, and represents a potential new therapy to improve bone mass and reduce fracture risk in pediatric OI.

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Keywords

Osteogenesis Imperfecta; Sclerostin Antibody; Collagen; Bone Mass; Anabolic Therapy; Dynamic Histomorphometry

1. Introduction

Osteogenesis imperfecta (OI) is a heritable collagen-related dysplasia that results in bone fragility (1). This condition presents with a wide range of clinical severity, depending upon the type of OI and specific mutation. While OI persists throughout life, the symptoms and fracture risk are greatest during childhood (2).

Numerous therapies have been tried to reduce fracture risk in pediatric OI. The most widely used treatment are anti-resorptive bisphosphonates. Multiple clinical trials with pediatric OI and bisphosphonates have demonstrated their efficacy at increasing vertebral BMD and surrogate measures of vertebral strength; however, the effect on long bone fracture risk appears equivocal (3-8). Treatment of pediatric OI with growth hormone (rGH) has shown encouraging results for an anabolic therapy, but was not consistently effective among all patients (9). In positive responders, bone histology and mass were improved, and long bone fracture rates decreased, along with increased linear growth in some mildly and moderately severe pediatric OI patients (9,10). Intermittent PTH is not used in children before growth plate fusion because of a reported osteosarcoma risk in rats, and as a result is not available as a treatment for pediatric OI (11).

Neutralizing antibodies to sclerostin are a candidate anabolic therapy for children with OI. Sclerostin is a potent inhibitor of bone formation which is secreted primarily by osteocytes (12). Broadly, sclerostin functions by inhibiting canonical Wnt signaling through its binding to the Wnt signaling co-receptors LRP5 and LRP6 present on cells of the osteoblast lineage (13). Neutralizing antibodies have been developed which functionally reduce sclerostin activity and thereby prevent sclerostin inhibition of bone formation. An anabolic response to sclerostin antibodies has been demonstrated in numerous pre-clinical studies (14–17) as well as phase I and phase II trials of osteoporosis (18,19).

The *Brtl/+* mouse is heterozygous for a G349C mutation on *coll1a1* found in an OI patient, and is a model for moderately severe Type IV OI (20). *Brtl/+* recapitulates many of the phenotypic features of pediatric OI including small size, impaired remodeling, reduced trabecular and cortical bone mass, altered bone matrix structure, and impaired fracture mechanics (20–23). As such, *Brtl/+* has been used to explore multiple clinically relevant questions including fracture repair (24), bisphosphonate treatment (25), and cell therapies (26).

Treatment of the rapidly growing skeleton of children with OI is the most clinically relevant time point. Previously, we reported that a short-term, 2 week treatment of *Brtl/+* with Scl-Ab successfully induced an anabolic response in 8 week old *Brtl/+* mice (27). While this proof-of-concept study demonstrated anabolic efficacy, it was not a direct correlate to the pediatric clinical condition, as mice only gained 2-5% body mass over the 2 weeks of treatment. In

contrast, 3wk old Brl/+ mice in the present study more than doubled their body mass over the 5wk treatment duration, highlighting the large difference between these two ages. To directly address whether Scl-Ab can elicit an anabolic response in an OI model during periods of rapid bone growth and body mass accrual, we treated rapidly growing 3 week old WT and Brl/+ mice for 5 weeks with Scl-Ab and hypothesized that therapy would lead to increased bone mass and strength by stimulating bone formation.

2. Materials and Methods

2.1 Animals

Wildtype (WT) and Brl/+ (20) 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 old male WT and Brl/+ mice were randomly assigned to Scl-Ab (Scl-Ab VI, Amgen, Thousand Oaks, CA) treatment or vehicle injection (PBS) with WT Veh n=9, WT Scl-Ab n=9, Brl/+ Veh n=8, Brl Scl-Ab n=9. Sclerostin antibody was injected subcutaneously at 25mg/kg, two times per week, following our previously described protocol (27,28), for five weeks. Calcein (30mg/kg, i.p. injection) was injected at the start of experiment (3 wks animal age), after 2 weeks of treatment (5 wks animal age), and after 4 weeks of treatment (7 wks animals age). A final alizarin label (30mg/kg, i.p. injection) was given 1 day before euthanasia (7wks + 6 days of animal age). The multiple fluorescent labels were used to visualize the growth pattern during the entire course of therapy. Body weights were recorded with each injection. Blood samples were collected at sacrifice by intracardiac puncture, serum separated by centrifuge, and stored at -80°C until analyzed by ELISA.

Left femurs were collected for microCT and mechanical testing, and right femurs for dynamic histomorphometry. Both were 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 Bone Length and Growth

Left femur and tibia lengths were measured longitudinally using whole body radiographs taken at the beginning and end of experiments on Scl-Ab or Veh treated animals. At 3 weeks of age, animals were briefly anesthetized with isoflurane, and microradiographs were taken in the coronal plane at 3x magnification (Faxitron MX-20, Faxitron X-Ray LLC, Lincolnshire, IL). Limbs were immobilized with tape during imaging to minimize motion and keep the leg bones flat. At 8 weeks of age, the radiograph protocol was repeated immediately after sacrifice. A lead ruler was used for calibration of the field of view, and bone lengths were measured with calipers.

2.3 Serum Assays

To measure osteoblast activity, serum osteocalcin (OCN) was quantified with a commercially available ELISA kit (BT-470, BTI, Stoughton, MA). As a measure of osteoclast number, serum TRACP5b was measured with a commercially available solid

phase immunofixed enzyme activity assay (MouseTRAP, IDS, Fountain Hills, AZ). Both serum tests were performed in duplicate. In several animals, inadequate serum volume was available to perform the assay, thus resulting in a modest reduction in group size.

2.4 MicroCT

Left femora were scanned in water using cone beam computed tomography (eXplore Locus SP, GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Scan parameters included a 0.5 degree increment angle, 4 frames averaged, an 80kVp and 80 μ A x-ray source with a 0.508mm Al filter to reduce beam hardening artifacts, and a beam flattener around the specimen holder (29). All images were reconstructed and calibrated at an 18 μ m isotropic voxel size to a manufacturer-supplied phantom of air, water and hydroxyapatite. Regions of interest (ROI) were located for both cortical and trabecular parameters. A diaphyseal cortical ROI spanning 15% of total femur length was located midway between the distal growth plate and third trochanter. Cortical bone was isolated with a fixed threshold of 2000 Hounsfield Units for all experimental groups. Parameters including cortical thickness, cross sectional area, marrow area, total area, anterior-posterior bending moment of inertia and tissue mineral density (TMD) were quantified with commercially available software (MicroView v2.2 Advanced Bone Analysis Application, GE Healthcare Pre-Clinical Imaging, London, ON, Canada). A trabecular ROI 10% of total femur length was located immediately proximal to the distal femoral growth plate and defined along the inner cortical surface with a splining algorithm. Due to the different morphology induced by Scl-Ab treatment, a fixed threshold could not be utilized without bias. Trabecular metaphyseal bone was isolated with a more conservative autothresholding algorithm for each specimen based on the bimodal distribution between marrow and bone (30). Parameters including bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N), and trabecular bone mineral density (Tb.BMD) were quantified using standard stereology algorithms (MicroView v2.2).

A qualitative difference in femoral trabecular bone morphology was noticed as a function of distance from the growth plate. To quantify this, the 10% of bone length region of interest was sub-divided into the 5% of bone length region that was more distal (closer to the growth plate), and the 5% region that was more proximal. The same threshold determined for the entire 10% of bone length ROI was used for each of the 5% of bone length subdivided regions. In this way, the relative contribution of each sub-region could be assessed.

2.5 Whole Bone Mechanical Four-Point Bending

Following μ CT scanning, left femora were loaded to failure in four-point bending using a servohydraulic testing machine (MTS 858 MiniBionix, Eden Prairie, MN). All specimens were kept hydrated in LRS-soaked gauze until mechanical testing. In the same mid-diaphyseal region analyzed by μ CT, the femur was loaded in four point bending with the posterior surface oriented under tension. The distance between the wide, upper supports was 6.26mm, and span between the narrow, lower supports 2.085mm. The vertical displacement rate of the four-point bending apparatus in the anterior-posterior direction was 0.5mm/sec. Force was recorded by a 50 lb load cell (Sensotec) and vertical displacement by an external linear variable differential transducer (LVDT, Lucas Schavitts, Hampton, VA), both at

2000Hz. A custom MATLAB script was used to analyze the raw force-displacement data and calculate all four-point bending parameters. Combining anterior-posterior bending moment of inertia data from μ CT with mechanical stiffness from four point bending, the estimated elastic modulus was calculated using standard beam theory as previously described (21).

2.6 Dynamic Histomorphometry

Right femora were encased in epoxy (Kold Mount, Vernon-Benshoff, Albany, NY), and cut transversely 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) to a plane immediately distal to the third trochanter. Specimens were further polished on a felt pad for 5 minutes with a $\frac{1}{4}$ μ m diamond suspension (Struers Inc., Cleveland, OH).

Fluorescent images were acquired using a Zeiss Axiovert 200M inverted microscope equipped with Apotome imaging system to minimize out-of-plane light, negating the need for several micron thin sections. Multiple fluorescent images were taken with a 10 $\times$ objective of the calcein and alizarin labels around the entire cortex. The 10 $\times$ images of the cortex were merged into a single image (Photoshop, Adobe), and these merged images were analyzed using commercially available software (Bioquant Osteo v7.20.10, Nashville, TN). Bone surface (BS), mineral apposition rate (MAR), mineralizing surface / bone surface (MS/BS), and bone formation rate / bone surface (BFR/BS) were quantified on both the periosteal and endosteal surfaces. The final two labels, given 1 week (calcein) and 1 day (alizarin) before sacrifice, were analyzed according to ASBMR standards (31). The highly conserved cortical drift pattern found during analysis made the calcein labels easy to identify with confidence, and we assumed the final calcein label to be that closest to the alizarin label on a given surface.

In order to examine the effect of Scl-Ab superimposed over a strong posterior shifting cortical drift pattern, dynamic histomorphometry outcomes were also assessed in anatomic regions around the cortex with a method similar to Bivi et. al (32). The fluorescent images were oriented anatomically by using the contralateral microCT femoral 3D images as a reference. The centroid of the fluorescent image was identified in ImageJ, and 110 degree angles from the centroid were used to define the anterior and posterior regions (Fig. 6). MAR, MS/BS, and cortical thickness were quantified in each of these anterior and posterior subregions.

In any regions where there was no dual label, MAR was treated as a missing value. For the purpose of calculating BFR/BS, a value of 0.3 μ m/day was assigned to MAR where missing values were present. This value was determined from both looking at the biological limit observed in our specimens as well as values established in the literature (33).

Missed calcein or alizarin injections occurred in five animals (2 animals in WT Veh group, and 1 each in WT Scl-Ab, Brtl/+ Veh, and Brtl/+ Scl-Ab). These animals were excluded from analysis and reduced dynamic histomorphometry group sizes to WT Veh n=7, WT Scl-Ab n=8, Brtl/+ Veh n=7, Brtl Scl-Ab n=8.

2.7 Statistics

A Kruskal-Wallis ANOVA and non-parametric Mann-U Whitney post-hoc tests were performed and $p < 0.05$ was considered significant. Values between $p = 0.05$ and $p = 0.10$ are reported in all instances. Comparisons between WT Veh and Brtl/+ Scl-Ab were made for the cortical microCT and mechanical four-point bending outcomes to assess the ability of Scl-Ab to restore the Brtl/+ phenotype to WT control levels. Data is presented as mean $\pm$ S.D.

3. Results

3.1 Scl-Ab Does Not Change Body Mass or Linear Growth Rates in Rapidly Growing Mice

Three week old mice are rapidly growing (Fig. 1) Throughout the five weeks of treatment, from 3 to 8 weeks of age, body mass was increased significantly by an average of 87% in WT Veh and 121% in Brtl/+ Veh. Consistent with previously reported phenotypic differences, Brtl/+ mice weigh less than WT (Fig. 1). Brtl/+ Veh weighed 30% less than WT Veh (11.0 ± 2.3 g vs. 15.8 ± 2.0 g) at 3 weeks of age, and 22% less than WT Veh (23.9 ± 3.8 g vs. 29.2 ± 3.0 g) at 8 weeks of age. Both genotypes gained a similar amount of weight on an absolute basis (WT Veh 13.4 ± 2.9 g; Brtl/+ Veh 12.9 ± 2.9 g) over the 5 weeks of treatment. Scl-Ab did not change the rate of body mass accrual throughout the treatment as weight gain was similar between Veh and Scl-Ab groups in both WT and Brtl/+. As a result, body weight was similar at the end of experiment in both WT (WT Veh 29.2 ± 3.0 g vs. WT Scl-Ab 27.8 ± 1.3 g) and Brtl/+ (Brtl/+ Veh 23.9 ± 3.8 g vs. Brtl/+ Scl-Ab 24.1 ± 2.5 g) with therapy.

Reduced stature is a characteristic phenotype of OI patients. To determine if Scl-Ab affected linear growth rate in animals that are rapidly growing, bone length was measured on X-rays taken at the beginning and end of treatment. (Table 1). In the left tibia and femur, no difference in linear bone growth during treatment or overall endpoint bone length was observed with Scl-Ab in either WT or Brtl/+. Consistent with previously reported phenotypic differences, Brtl/+ bones were shorter than WT.

3.2 Serum Osteocalcin is Elevated in Rapidly Growing Brtl/+

Brtl/+ Veh had increased serum osteocalcin relative to WT Veh at 8 weeks of age (Fig. 2). Scl-Ab treatment did not significantly increase mean serum osteocalcin levels in either WT and Brtl/+. No effect on serum TRACP5b levels was observed.

3.3 Anabolic Scl-Ab Effect Depends on Distance of Trabecular Bone From Growth Plate

Scl-Ab treatment increased femoral trabecular BV/TV by 57% in WT, but a significant effect was not observed in Brtl/+ ($p = 0.321$) over the entire distal femoral 10% of bone length ROI (Fig. 3). The Scl-Ab BV/TV gains in WT were a result of increases in both Tb.Th (+28%) and Tb.N (+22%). Interestingly, despite the lack of Scl-Ab effect on BV/TV in Brtl/+, Tb.Th was significantly increased by a small amount (+8.7%). Tb.BMD demonstrated a similar pattern as BV/TV, with a significant ($p < 0.001$) 67% increase observed in WT with Scl-Ab, but only a marginal 33% increasing trend observed in Brtl/+ ($p = 0.06$).

Despite this lack of effect over the entire distal femoral region, we observed a qualitative difference in the action of Scl-Ab as a function of distance from the distal femoral growth plate. To quantify this, we performed a post-hoc analysis whereby we separated the 10% of bone length trabecular ROI into the 5% region closer to the growth plate and the 5% of bone length region farther from the growth plate (Fig 4).

In the region closest to the growth plate we observed a significant gain in WT BV/TV with Scl-Ab treatment, but not in Brl/+ ($p=0.89$). The BV/TV gains in WT were a result of significant gains in Tb.Th (+49%), while Brl/+ only showed a modest trend towards increased Tb.Th ($p=0.097$). There were no Tb.N changes with Scl-Ab observed in either genotype in this 5% of bone length region closest to the growth plate.

In contrast, in the proximal 5% sub region further from the growth plate, Scl-Ab treatment resulted in a significant increase in BV/TV in both WT (126%) and Brl/+ (69%) mice. In WT, BV/TV gains in this subregion were a result of significant gains in both Tb.Th (49%) and Tb.N (51%), while Brl/+ only increased Tb.Th (29%), but not Tb.N.

3.4 Scl-Ab Increases Cortical Bone Mass in Brl/+ and WT by Similar Amounts

Phenotypically, Brl/+ Veh femoral bone had reduced cortical thickness (−18%), cross sectional area (−18%), and anterior-posterior bending moment of inertia (−31%) relative to WT Veh (Table 2, Fig. 5). The marrow area was not significantly different in Brl/+ Veh animals, but total area was less than observed in WT Veh.

Treatment with Scl-Ab significantly increased cortical thickness (WT +26%; Brl/+ +24%) and cortical cross sectional area (WT +31%; Brl/+ +25%) in both genotypes, leading to significantly increased anterior-posterior bending moment of inertia in both WT (+49%) and Brl/+ (+30%). These increases returned the Brl/+ cortical geometric phenotype to levels not significantly different from WT Veh. Neither marrow area nor total area were significantly altered by Scl-Ab in Brl/+; WT yielded only a modest increasing trend ($p=0.077$) in total area with treatment. Cortical TMD was increased in WT with Scl-Ab, and an increasing trend was observed in Brl/+.

3.5 Scl-Ab Rescues Reduced Brl/+ Femoral Bone Strength to WT Veh Levels

Phenotypically, Brl/+ Veh had reduced mechanical properties compared to WT Veh (Table 2, Fig. 5). These included reduced strength outcomes such as Yield Load (−24%), Ultimate Load (−38%), Stiffness (−35%), and Energy to Failure (−60%). In addition, Brl/+ Veh also failed in a more mechanically brittle manner than WT Veh. This is evident in the decreased plastic energy (−75%), post yield displacement (−61%), and failure/yield displacement ratio (−39%).

Scl-Ab therapy significantly increased most mechanical outcomes in both WT and Brl/+. Specifically, Scl-Ab treatment increased Yield Load (WT +47%; Brl/+ +39%), Ultimate Load (WT +68%; Brl/+ +51%) and Stiffness (WT +55%; Brl/+ +61%) in both genotypes, and in Brl/+, restored these three parameters to the level of WT Veh (Fig. 5). Energy-To-Failure was significantly increased by Scl-Ab in WT (+303%), but only trended ($p=0.093$) with treatment in Brl/+ (+42%) (Table 2). This differential gain is caused by a genotype specific

effect of Scl-Ab on bone brittleness. In WT, the large increase in Energy-to-Failure with Scl-Ab was primarily due to a substantial increase in Plastic Energy (+386%). The increased plastic energy was mostly due to increased Post Yield Displacement (+164%) in WT with Scl-Ab. In contrast, Scl-Ab did not alter Plastic energy or Post Yield Displacement in Brtl/+. Yield Displacement was not altered by Scl-Ab in either WT or Brtl/+. The estimated elastic modulus was not significantly different.

3.6 Regional Dynamic Histomorphometry Analysis Reveals Scl-Ab Action Superimposed over a Strong Cortical Drift Pattern

In both WT Veh and Brtl/+ Veh there was a strong posterior shifting cortical drift pattern (Fig. 6). Specifically, we found high BFR/BS on the periosteal posterior and endosteal anterior bone, with over 90% of these surfaces forming bone in both genotypes. Conversely, low bone formation rates were found on the periosteal anterior and endosteal posterior surfaces in both WT and Brtl/+ (Table 3).

On the low bone forming posterior endosteal surface, Scl-Ab significantly increased MS/BS (WT +610%, Brtl/+ +243%), which lead to increased BFR/BS (WT + 829%, Brtl/+ +542%). On the low bone forming periosteal anterior surface, Brtl/+ trended towards an increase in MS/BS, but this did not significantly change BFR/BS. The Scl-Ab response was highly variable in WT animals on the low forming posterior endosteal surface.

On the high bone forming surfaces, Scl-Ab had no effect on dynamic histomorphometry outcomes in either genotype. Specifically, on the periosteal posterior surface, no significant differences were observed for MS/BS, MAR, or BFR/BS in either WT or Brtl/+. On the high bone forming endosteal anterior surface, Scl-Ab resulted in an unexpected trend towards reduced MAR ($p=0.094$) during the last week of treatment, but this did lead to a change in BFR/BS. No changes in WT endosteal anterior surface were observed with Scl-Ab.

These regional changes with sclerostin antibody were sufficient to significantly increase cortical thickness in both WT and Brtl/+ on the anterior (WT +19%; Brtl/+ +23%) and posterior (WT +25%; Brtl/+ +32%) surfaces after 5 weeks of treatment. However, due to the regional nature of the underlying cortical drift pattern, Scl-Ab treatment did not result in any significant changes in overall periosteal MAR, MS/BS, or BFR/BS in either WT or Brtl/+ when summed over the entire periosteal surface (Table 3). The total endosteal surface showed a more robust response to therapy. In Brtl/+, treatment with Scl-Ab significantly increased total endosteal MS/BS (28%). In WT, Scl-Ab had opposing effects of significantly reducing total endosteal MAR (-24%) while increasing total endosteal MS/BS (+61%). The relatively larger gains in WT MS/BS led to an increasing trend ($p=0.094$) in total endosteal BFR/BS with treatment.

4. Discussion

This study demonstrated that Scl-Ab increased bone formation leading to improved bone mass and strength in a rapidly growing Brtl/+ mouse model of moderately severe Type IV OI. Impressively, treatment with Scl-Ab significantly improved long bone strength to levels not significantly different than WT and rescued the Brtl/+ phenotype of reduced bone mass.

This potent effect of Scl-Ab on cortical bone mass and long bone strength starkly contrasts with the existing bisphosphonate data reported in both animal models and clinical studies. Previously, we examined the effect of alendronate treatment of the *Brtl/+* mouse during growth (25). While alendronate treatment of *Brtl/+* between 2-8 weeks of age resulted in extremely large gains in femoral metaphyseal bone mass, alendronate had little effect on cortical bone mass and strength, which required a full 12 weeks of treatment to show a modest effect. Furthermore, alendronate appeared to exacerbate brittleness in WT, and had a trend toward worsening brittleness in *Brtl/+* (25). The absence of a strong alendronate effect on OI cortical bone is not specific to the *Brtl/+* mouse model and similar data has been observed in other OI mouse models such as *oim* (34). This observation of a reduced or absent effect of bisphosphonate on OI cortical bone mass is also reflected in clinical data. The controlled clinical trials of bisphosphonates in pediatric OI suggest a beneficial effect on vertebral trabecular bone but an equivocal effect on long bone strength (3–7). Therefore, a therapy which consistently increases long bone strength in pediatric OI is currently lacking. The current study suggests that Scl-Ab may provide a novel and unique therapeutic option for pediatric OI by reducing long-bone susceptibility to fracture.

The underlying cause of classical OI fragility is that a collagen structural defect produces bone of both reduced material quality as well as reduced bone mass. In this study, Scl-Ab was able to significantly improve femoral strength by increasing bone mass without altering the underlying brittle nature of the material. Specifically, Scl-Ab was able to increase cortical bone mass in *Brtl/+* by significantly increasing cortical thickness (+24%) and cortical area (+25%). Although the unchanged post-yield displacement in *Brtl/+* with Scl-Ab treatment suggests that Scl-Ab did not improve the inherent brittle material behavior of *Brtl/+* bone, Scl-Ab did significantly improve long bone strength by increasing cortical bone mass, making the bone less fragile. A trend towards increased cortical TMD with Scl-Ab in *Brtl/+* could be suggestive of increased mineralization, but this finding did not correlate with the estimated elastic modulus as measured by four-point bending. Rather, this increase could be a result of porosity changes below the resolution of our microCT, or partial volume effects, both of which could artificially increase this index of bone mineralization.

In this study, the *Brtl/+* trabecular response to Scl-Ab in the distal femur was notably less robust than in WT. Trabecular thickness increased after 5 weeks of Scl-Ab treatment in both WT and *Brtl/+*, confirming our previous work where *Brtl/+* was treated between 8-10 weeks of age (27). In both studies, BV/TV was not significantly increased in *Brtl/+*. However, the extended 5 week treatment duration of the present study allowed us to discriminate more subtle treatment effects, and further analysis revealed a significant anabolic BV/TV response in the *Brtl/+* Scl-Ab group in a more proximal subregion of the femur metaphysis. The lesser gains in bone mass near the growth plate in both genotypes may be related to the shorter duration of Scl-Ab exposure to this newly formed bone. While a proximal-distal effect is likely not a unique consideration for *Brtl/+*, the reduced trabecular response to Scl-Ab in *Brtl/+* may be amplified due to a result of increased bone resorption levels in *Brtl/+* animals (22) which may mask equivalent bone formation responses, although we did not observe significant serum TRACP5b differences in this study. Alternatively, there may be a reduced

anabolic response to Scl-Ab in Brl/+ trabecular bone compared to WT which may be a result of differential sclerostin levels or impaired osteoblast function.

The reduced Brl/+ femoral trabecular response observed in this study contrasts with the robust Scl-Ab increases in cortical bone mass in both WT and Brl/+. Furthermore, these findings also contrast with our observations studying adult 6 month old Brl/+ mice that were treated for 5 weeks with Scl-Ab (28). In these adult mice, we observed significant BV/TV gains in both WT and Brl/+ using an identical Scl-Ab treatment period and microCT methodology. Furthermore, serum results from the present study contrast with our prior reports of a strong anabolic serum response shown in short term treatment (27) and 5 weeks of Scl-Ab treatment in 6 month old Brl/+ mice (28). This suggests a potential age dependent response to Scl-Ab that must be considered in greater detail before undertaking the translation of Scl-Ab to pediatric OI patients.

Patients with OI often have severely reduced stature. To examine if Scl-Ab treatment altered linear growth rates during this phase of rapid growth of the Brl/+ mouse we examined pre- and post-treatment bone lengths by X-ray. Here, we found no significant effects of treatment on long bone length after 5 weeks of therapy, consistent with prior reports of neutral effects of Scl-Ab on bone length in 7 week old mice (35). While Scl-Ab appears promising for increasing bone mass, it does not appear to increase the short stature phenotypic of Brl/+ OI mice. However, the lack of a bone length increase in mice does not necessarily preclude an effect in humans; SOST KO mice do not have longer bones (36) but patients with sclerosteosis do have increased stature (37).

We observed a mechanistic difference between increasing MAR on existing bone formation surfaces, and stimulating new bone formation on otherwise quiescent or resorbing bone surfaces. The regional anterior/posterior dynamic histomorphometry analysis performed in this study helps to separate these two effects by analyzing the superposition of a strong underlying cortical drift pattern characteristic of young animals (38,39) with a potent anabolic therapy. Scl-Ab activated bone formation surfaces on quiescent or low bone forming surfaces in Brl/+ and WT mice, with minimal additional effect on increasing osteoblast activity on surfaces with already high levels of bone formation. These findings may have important clinical impact when considering where Scl-Ab may have its most potent effects during growth and development, and where its effect may be limited, perhaps due to already high levels of bone formation.

The regional dynamic histomorphometry responses to Scl-Ab treatment were not always consistent within experimental group. For example, on the anterior periosteal surface where no bone formation was observed in WT Veh controls, the bone formation response to Scl-Ab was either robust or non-existent. Specifically, Scl-Ab activated bone formation in only 3 of the 8 mice in the WT Scl-Ab group. Moreover, these three mice had MS/BS values that were greater than 0.95, highlighting the strong anabolic response in this subset of animals. The 5 out of 8 mice that did not respond had no fluorescent labeling on this surface, and an MS/BS of zero.

The changes in cortical thickness in the posterior compartment of the femoral cortex are likely due to the strong endosteal BFR/BS response to therapy (Table 3). However, the significant increases in anterior femoral thickness are not easily explained by regional response differences, as no significant differences in BFR/BS occurred in either genotype with Scl-Ab. It is possible that Scl-Ab treatment reduced bone resorption on the periosteal anterior surface, which would increase cortical thickness. Another possibility is that significant gains in bone formation with Scl-Ab occurred in the anterior compartment before the last week of treatment. Finally, we were able to measure cortical thickness in all specimens (n=8-9/group) allowing for greater statistical power, but had slightly smaller group sizes for the dynamic histomorphometry outcomes due to missed injections of fluorescent label (n=7-8).

This study has several limitations. First, despite the fact that the *Brtl/+* mouse was made to have a mutation identical to that found in a Type IV OI patient, it does not fully recapitulate the phenotype of humans and suffer spontaneous fractures. In addition, as OI is a disease of many mutations, the results of this study may not extend to all types of OI. Before considering translation to humans, future studies need to analyze the potential effect of sclerostin inhibition on skeletal overgrowth in undesired locations apparent in human diseases of sclerostin deficiency (40)

In summary, Scl-Ab significantly increased cortical bone formation, long bone mass, and long bone strength without affecting brittleness in growing mice with a typical OI-causing mutation in type I collagen during a period of rapid bone mass accrual. These results contrast with existing bisphosphonate treatment strategies which have not demonstrated convincing improvements in long bone fragility in pre-clinical models or clinical trials. On bone surfaces where osteoblasts were highly active in rapidly growing animals in this study, Scl-Ab had a minimal additional anabolic effect. Rather, increased anabolic activity resulted from activation of quiescent or resorbing surfaces. These observations, along with regional improvements in trabecular bone mass associated with tissue age reflect the necessity of testing anabolic agents during periods of rapid growth to prepare for pediatric indications. The current data suggest that Scl-Ab may be a candidate pharmacologic for the treatment of pediatric osteogenesis imperfecta.

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Highlights

- We treated a rapidly growing Brl/+ mouse model of OI for 5 weeks with Sclerostin Antibody (Scl-Ab)
- Brl/+ had increased bone mass with Scl-Ab treatment.
- Cortical bone strength in Brl/+ mice was increased with Scl-Ab and restored to WT levels.
- Scl-Ab induced bone formation on surfaces that are normally quiescent or resorptive in rapidly growing mice.

Figure 1.

Body mass curves throughout the course of experiment from 3 wks of age to 8 wks of age confirm that Brtl/+ animals are lighter than WT and reveal Scl-Ab does not effect body mass. Data are WT Veh n=9, WT Scl-Ab n=9, Brtl/+ Veh n=8, Brtl Scl-Ab n=9.

Figure 2.

Serum osteocalcin is significantly greater in Brtl/+ Veh vs. WT Veh, but no significant effect of Scl-Ab on serum osteocalcin is observed in either genotype. The inability to obtain serum samples from all animals, and order in which assays were performed, result in the following group sizes. For OCN: WT Veh n=6, WT Scl-Ab n= 9; Brtl/+ Veh n =6, Brtl/+ Scl-Ab n=6. For TRACP5b: WT Veh n=9, WT Scl-Ab n= 9; Brtl/+ Veh n=7, Brtl/+ Scl-Ab n=7.

Figure 3.

Micro computed tomography of the distal femur metaphysis reveals anabolic changes with Scl-Ab therapy. The ROI (A) was 10% of bone length and placed immediately proximal to the growth plate. Representative median BV/TV isosurface images (B) reveal a qualitative reduction in Brtl/+ bone mass and the anabolic effect of Scl-Ab therapy. Quantitative analysis (C) reveals a reduction in Brtl/+ BV/TV as a result of decreased trabecular number. Scl-Ab significantly increased trabecular number and thickness in WT, leading to increased BV/TV. In Brtl/+, Scl-Ab increased trabecular thickness but did not alter trabecular number

or BV/TV. There was a trend ($p=0.06$) towards increased trabecular BMD in Brl/+ with Scl-Ab. * $p<0.05$ Scl-Ab vs. Veh; # $p<0.05$ WT Veh vs. Brl/+ Veh. Data are WT Veh $n=9$, WT Scl-Ab $n=9$, Brl/+ Veh $n=8$, Brl Scl-Ab $n=9$

Figure 4.

Differential response of Scl-Ab on trabecular bone mass as a function of distance from the growth plate. The trabecular 10% ROI shown in Fig 3A was separated into proximal (A) and distal (C) 5% on bone length subregions. In the proximal compartment (B), we found that Scl-Ab significantly improved trabecular BV/TV and thickness in both WT and Brtl/+. In the distal ROI (D), there were few genotype differences and Scl-Ab increased BV/TV and trabecular thickness in WT, but had little effect on Brtl/+. * $p < 0.05$ Scl-Ab vs. Veh; # $p < 0.05$ WT Veh vs. Brtl/+ Veh. Data are WT Veh $n=9$, WT Scl-Ab $n=9$, Brtl/+ Veh $n=8$, Brtl Scl-Ab $n=9$.

Figure 5.

Selected cortical microCT (A) and mechanical 4pt bending (B) outcomes from Table 2 are shown here in graphical form. Brtl/+ had reduced cortical bone geometry and whole bone strength relative to WT. For each outcome, Scl-Ab increased both WT and Brtl/+ cortical bone geometry and whole bone strength. * $p < 0.05$ Scl-Ab vs. Veh; # $p < 0.05$ WT Veh vs. Brtl/+ Veh. Data are WT Veh $n = 9$, WT Scl-Ab $n = 9$, Brtl/+ Veh $n = 8$, Brtl Scl-Ab $n = 9$

Figure 6.

Representative dynamic histomorphometry images of a Brtl/+ Veh animal showing a strong posterior shifting cortical drift pattern and the bone formation induced by Scl-Ab. In all samples, the centroid of the bone was located, and 110° angles drawn to define the anterior and posterior regions of bone. These subregions were used to define the anterior-posterior cortical drift dynamic histomorphometry response shown in Table 3.

Table 1
Linear Bone Growth as Measured by Pre and Post Treatment X-ray

Linear bone growth of the femur and tibia from 3 wks to 8 wks of age as assessed by microradiograph.

	<i>WT Veh</i>	<i>WT Scl-Ab</i>	<i>Brtl Veh</i>	<i>Brtl/+ Scl-Ab</i>
Left Femur				
Length at 3 weeks of age (mm)	11.43 ± .43	11.34 ± .42	10.25 ± .80 [#]	10.49 ± .39
Length at 8 weeks of age (mm)	14.00 ± .43	14.25 ± .33	13.03 ± .90 [#]	13.10 ± .58
Paired percent bone growth (%)	22.5 ± 3.4	25.8 ± 4.1	27.3 ± 3.3	24.9 ± 4.1
Left Tibia				
Length at 3 weeks of age (mm)	14.72 ± .37	14.67 ± .57	13.44 ± .75 [#]	13.55 ± .77
Length at 8 weeks of age (mm)	17.50 ± .42	17.31 ± .45	16.29 ± .70 [#]	16.44 ± .42
Paired percent bone growth (%)	19.0 ± 3.1	18.1 ± 2.8	21.4 ± 4.0	21.6 ± 5.1

* p<0.05 Scl-Ab vs. Veh

[#] p<0.05 WT Veh vs. Brtl/+ Veh. Data are WT Veh n=9, WT Scl-Ab n=9, Brtl/+ Veh n=8, Brtl Scl-Ab n=9

Table 2
Cortical MicroCT and Mechanical Properties

Femoral cortical microCT and mechanical four-point bending.

	<i>WT Veh</i>	<i>WT Scl-Ab</i>	<i>Brtl/+ Veh</i>	<i>Brtl/+ Scl-Ab</i>
Cortical Micro CT				
Thickness (mm)	0.20 ± .01	0.25 ± .03 [*]	0.16 ± .02 [#]	0.20 ± .01 [*]
Cross Sectional Area (mm ²)	0.91 ± .07	1.19 ± .22 [*]	0.71 ± .10 [#]	0.89 ± .08 [*]
Marrow Area (mm ²)	1.30 ± .17	1.33 ± .18	1.20 ± .08	1.17 ± .15
Total Area (mm ²)	2.21 ± .22	2.53 ± .40 ^{p=0.077}	1.91 ± .16 [#]	2.06 ± .21
Bending Moment of Inertia (mm ⁴)	0.17 ± .03	0.26 ± .09 [*]	0.12 ± .02 [#]	0.16 ± .03 [*]
Tissue Mineral Density (mg/cm ³)	1017 ± 35	1046 ± 33 [*]	1004 ± 48	1054 ± 47 ^{p=0.059, +}
Mechanical Four Point Bending				
Yield Load (N)	19.7 ± 3.2	28.8 ± 5.3 [*]	15.0 ± 3.1 [#]	20.8 ± 3.3 [*]
Ultimate Load (N)	27.6 ± 2.4	46.4 ± 10.8 [*]	17.0 ± 3.6 [#]	25.6 ± 3.9 [*]
Stiffness (N/mm)	198 ± 25	308 ± 71 [*]	129 ± 30 [#]	208 ± 32 [*]
Energy to Failure (mJ)	4.6 ± 1.5	18.4 ± 12.4 [*]	1.8 ± 1.0 [#]	2.6 ± 1.0 ^{p=0.093, +}
Elastic Energy (mJ)	1.10 ± .30	1.55 ± .41 [*]	0.95 ± .22	1.19 ± .36
Plastic Energy (mJ)	3.5 ± 1.5	16.8 ± 12.3 [*]	0.88 ± .92 [#]	1.4 ± 1.1 ⁺
Yield Displacement (mm)	0.109 ± .013	0.112 ± .022	0.129 ± .027	0.116 ± .023
Post Yield Displacement (mm)	0.144 ± .058	0.380 ± .198 [*]	0.056 ± .059 [#]	0.062 ± .042 ⁺
Fail/Yield Displacement Ratio	2.32 ± .51	4.65 ± 2.37 [*]	1.42 ± .44 [#]	1.59 ± .46 ⁺
Estimated Elastic Modulus (GPa)	4.46 ± .73	4.79 ± 1.07	4.05 ± .48	5.16 ± 1.09

* p<0.05 Scl-Ab vs. Veh

p<0.05 WT Veh vs. Brtl/+ Veh

+ p<0.05 WT Veh vs. Brtl/+ Scl-Ab. Data are WT Veh n=9, WT Scl-Ab n=9, Brtl/+ Veh n=8, Brtl Scl-Ab n=9

Table 3
Dynamic Histomorphometry on Femoral Cortical Subregions

Dynamic histomorphometry of femoral cortical bone. Regional anterior-posterior femoral cortical dynamic histomorphometry analyses are shown on top, with traditional analysis from the total periosteal and endosteal surface at the bottom. Due to missed injections, group sizes for total endosteal and periosteal surface were WT Veh n=9, WT Scl-Ab n=9, Brtl/+ Veh n=8, Brtl Scl-Ab n=9. MAR was treated as a missing value when no dual label was present, and led to further reduced group sizes on low forming surfaces: Periosteal Anterior MAR (WT Veh n=0, WT Scl-Ab n=3). Endosteal Posterior MAR (WT Veh n=2, Brtl/+ Veh n=5).

	<i>WT Veh</i>	<i>WT Scl-Ab</i>	<i>Brtl/+ Veh</i>	<i>Brtl/+ Scl-Ab</i>
Anterior				
Cortical Thickness (μm)	182 ± 17	217 ± 27*	158 ± 21 [#]	194 ± 17*
Periosteal MS/BS	0.00 ± .00	0.36 ± .51	0.43 ± .25 [#]	0.73 ± .28 ^{p=0.094}
Periosteal MAR	NA ± NA	1.31 ± .56	1.18 ± .98	1.14 ± .48
Periosteal BFR/BS	0.00 ± .00	0.48 ± .73	0.59 ± .78 [#]	0.85 ± .46
Endosteal MS/BS	0.91 ± .20	0.96 ± .04	0.93 ± .09	0.86 ± .09
Endosteal MAR	2.06 ± .29	1.66 ± .61	1.36 ± .51 [#]	0.96 ± .39 ^{p=0.094}
Endosteal BFR/BS	1.76 ± .75	1.59 ± .56	1.28 ± .52	0.81 ± .40
Posterior				
Cortical Thickness (μm)	213 ± 15	267 ± 41*	178 ± 29 [#]	235 ± 19*
Periosteal MS/BS	0.94 ± .09	0.99 ± .01	0.99 ± .01	0.98 ± .02
Periosteal MAR	1.90 ± .54	2.38 ± .77	2.11 ± .50	2.55 ± .61
Periosteal BFR/BS	1.79 ± .56	2.37 ± .78	2.10 ± .49	2.55 ± .61
Endosteal MS/BS	0.07 ± .08	0.71 ± .16*	0.32 ± .19 ^{p=0.053}	0.91 ± .18*
Endosteal MAR	1.65 ± 1.2	0.92 ± .29	0.63 ± .18	1.03 ± .45
Endosteal BFR/BS	0.08 ± .13	0.72 ± .33*	0.15 ± .15	0.96 ± .48*
Total Endosteal				
MS/BS	0.53 ± .17	0.86 ± .06*	0.69 ± .07 ^{p=0.097}	0.89 ± .07*
MAR	1.92 ± .24	1.46 ± .31*	1.20 ± .24 [#]	1.13 ± .37
BFR/BS	1.13 ± .12	1.25 ± .24 ^{p=0.094}	0.83 ± .17 ^{p=0.053}	0.99 ± .30
Total Periosteal				
MS/BS	.43 ± .03	.64 ± .27	.71 ± .09 [#]	.84 ± .11
MAR	2.23 ± .71	2.18 ± .43	2.34 ± .53	2.40 ± .62
BFR/BS	.97 ± .31	1.49 ± .86	1.71 ± .61 [#]	1.99 ± .49

* p<0.05 Scl-Ab vs. Veh

[#] p<0.05 WT Veh vs. Brtl/+ Veh.