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A Xenograft Model to Evaluate the Bone Forming Effects of Sclerostin Antibody in Human Bone Derived from Pediatric Osteogenesis Imperfecta Patients

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Abstract

Osteogenesis imperfecta (OI) is a rare and severe skeletal dysplasia marked by low bone mass and poor bone quality which is especially burdensome during childhood. Since clinical trials for pediatric OI are difficult, there is a widespread reliance on genetically modified murine models to understand the skeletal effects of emerging therapeutics. However a common model does not yet exist to understand how patient-specific genotype may influence treatment efficacy. Recently, sclerostin antibody (SclAb) has been introduced as a novel putative anabolic therapy for diseases of low bone mass, but effects in pediatric patients remain unexplored. In this study, we aim to establish a direct xenograft approach using OI patient-derived bone isolates which retain patient-specific genetic defects and cells residing in their intrinsic extracellular environment to evaluate the bone-forming effects of SclAb as a bridge to clinical trials.

OI and age matched non-OI patient bone typically discarded as surgical waste during corrective orthopaedic procedures were collected, trimmed and implanted subcutaneously (s.c.) on the dorsal surface of 4–6-week athymic mice. A subset of implanted mice were evaluated at short (1 week), intermediate (4 week), and long-term (12 week) durations to assess bone cell survival and presence of donor bone cells in order to determine an appropriate treatment duration. Remaining implanted mice were randomly assigned to a two or four-week SclAb-treated (25 mg/kg s.c. 2QW) or untreated control group. Immunohistochemistry determined osteocyte and osteoblast donor/host relationship, TRAP staining quantified osteoclast activity, and TUNEL assay was used to understand rates of bone cell apoptosis at each implantation timepoint. Longitudinal changes of *in vivo* μ CT outcomes and dynamic histomorphometry were used to assess treatment response and ex

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in vivo μ CT and dynamic histomorphometry of host femora served as a positive internal control to confirm a bone forming response to SclAb.

Human-derived osteocytes and lining cells were present up to 12 weeks post-implantation with nominal cell apoptosis in the implant. Sclerostin expression remained donor-derived throughout the study. Osterix expression was primarily donor-derived in treated implants and shifted in favor of the host when implants remained untreated. μ CT measures of BMD, TMD, BV/TV and BV increased with treatment but response was variable and impacted by bone implant morphology (trabecular, cortical) which was corroborated by histomorphometry. There was no statistical difference between treated and untreated osteoclast number in the implants. Host femora confirmed a systemic bone forming effect of SclAb.

Findings support use of the xenograft model using solid bone isolates to explore the effects of novel bone-targeted therapies. These findings will impact our understanding of SclAb therapy in pediatric OI tissue through establishing the efficacy of this treatment in human cells prior to extension to the clinic.

Keywords

Anabolic therapy; Xenograft model; Osteogenesis imperfecta; Sclerostin antibody; Immunohistochemistry; Micro computed tomography; Bone formation

1. Introduction

Osteogenesis imperfecta (OI) is a heritable collagen-related bone dysplasia characterized by low bone mass and poor bone quality with increased pathological fracture risk presenting most severely in childhood. OI occurs equally in males and females with an overall incidence of ~1:10,000–20,000 births [1]. The disease can be classified into 18 genetically unique types differing in modes of inheritance (dominant, recessive, X-linked) and affected gene loci resulting in a range of phenotypic presentation ranging in severity both within and between type [2, 3]. The hallmark genetic heterogeneity of OI may not only contribute to its growing variable clinical classifications but may also impact patient response to treatment.

The goal of OI treatment is to reduce fracture risk and increase bone mass and density. Numerous treatment options for OI have been proposed with inconsistent treatment response across patients where efficacy appears to be based, in part, on the underlying disease pathology [4–8]. Bisphosphonates, a class of drugs which reduce osteoclastic activity and thereby decrease resorption, represent the predominant treatment strategy over the past decade for OI [9], yet bisphosphonates only partially correct the bone phenotype [10, 11]. The advent of monoclonal sclerostin antibody (SclAb) has gained interest as a bone-forming therapeutic suggesting a different treatment strategy through inhibition of endogenous sclerostin, a negative regulator of bone formation [12]. Clinical trials of SclAb have led to positive increases in bone quality and mechanical strength in post-menopausal osteoporosis [13–18] and results are corroborated in OI animal models supportive of its use in OI [19–24]. In a phase II clinical trial in adults with a moderate phenotype of OI, SclAb stimulated markers of bone formation, reduced bone resorption and increased lumbar spine areal bone

mineral density (aBMD) [25], yet results in pediatric OI patients across all types and severity remains unknown.

Clinical trials in the pediatric OI population are especially challenging due to low patient number and high disease variability coupled with a desire for treatment-naïve individuals [26]. As such there is wide-use and reliance on genetically modified mouse models to understand the impact of emerging bone therapeutics, yet a mouse model does not exist to parallel each unique OI mutation. Furthermore, studies have revealed divergent phenotypes in patients with identical, or near-identical genetic mutations [27]. Specifically for SclAb, varying magnitude of positive bone-forming response to therapy has been reported pre-clinically between mild, moderate, and severe phenotypic murine models of OI suggesting the impact of genotypic and phenotypic variability [19, 22–24] including baseline fragility [24], pre-existing trabecular phenotype [28], or other yet undescribed mechanisms, on treatment response. As new antiresorptive and anabolic agents are being investigated, the efficacy of these drugs within- and across all OI types and patients in the pediatric population should be better understood prior to clinical extension.

Based on these limitations, there exists a need for a model which represents the inherent phenotypic tissue-level, cellular and genetic variability of OI to safely evaluate emerging therapeutics prior to translation to patients. Human xenograft models in the form of heterotransplantation of human immortalized cancer cell lines into immunodeficient mice were first documented by Isaacson and Cattanaach in 1962 and have continually been employed with success over the past five decades [29]. The advent of patient-derived xenograft systems represents a bench-to-bedside approach in cancer research to generate more clinically relevant preclinical models. Solid tumor isolates can be surgically harvested from the patient and subcutaneously implanted into the nude mouse to explore treatment in humanized tumors thus more closely recapitulating human disease [30, 31]. The use of solid tissue isolates has been extrapolated to bone where McCauley and colleagues evaluated the effects of anabolic intermittent parathyroid hormone (iPTH) on implanted neonate mouse tissue drawing on the interaction of host-derived osteoclasts and donor-derived osteoblasts in the model [32, 33]. Together, these earlier works motivate the exploration of a similar transplantation model to evaluate therapeutic interventions in OI. Here, we describe a patient-derived xenograft approach as an alternative to genetically modified OI mouse models through the procurement of pediatric OI bone tissue that is implanted directly into a biologically rich host-derived microenvironment where we can evaluate treatment efficacy of a bone-forming therapeutic in its target tissue.

2. Materials and Methods

2.1. Study design

Eight pediatric OI patients undergoing corrective orthopaedic surgery (osteotomy, open reduction-internal fixation, rodding, fracture intervention, implant exchange) were prospectively enrolled and provided informed consent for this Internal Review Board approved study. Tissue from three age-matched non-OI patients with unaffected collagen status undergoing anterior cruciate ligament (ACL) reconstructive surgery were collected as tissue which was considered exempt by the IRB. Patient demographics can be found in Table

1. These orthopaedic procedures yield bone fragments typically discarded as surgical waste which were collected at surgery (OI n=8/non-OI n=3), immediately transferred to media (α MEM/10% fetal bovine serum) on ice and trimmed to roughly 3 mm³. Samples were implanted subcutaneously (s.c.) on the dorsal surface of a 4–6 week athymic mouse (Foxn^{nu} [002019]; The Jackson Laboratory, Bar Harbor, ME, USA) through a 1 cm posterior incision adjacent to the spinal column (Figure 1). Each mouse received up to three implants depending on patient yield with one non-implanted sample per patient fixed (10% neutral buffered formalin (NBF)) for baseline analysis. In total, 67 mice were implanted with 84 samples and 14 additional samples were fixed for baseline analysis. Donor OI bone tissue was either trabecular-like or cortical-like in morphology while healthy non-OI tissue always originated from metaphyseal tibial tunnel samples and was received as morselized trabecular bone pieces approximately 1–2 mm³ in size and implanted as a “conglomerate” of the smaller morselized pieces. Due to the nature of the OI procedures and how the bone samples were procured, we had no control over selection of anatomical site and bone type. Two mice implanted with one bone sample each died before the end of the implantation period due to formation of a bulbourethral gland cyst which was strain-dependent and unrelated to treatment, and one implant fell out from the surgical incision site before the end of the implantation period and thus were excluded from analysis. All experimental animal procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

Bone was randomly divided for this three-stage study. In the first stage, implanted mice were randomly assigned to 1, 2, 4 and 12 week untreated groups to evaluate donor implant viability in the host during short and long-term implantation durations. Second, additional implanted mice were assigned to 2 and 4 week untreated and treated groups used to assess the bone-forming effects of SclAb treatment in the human explants. Mice received SclAb (SclAb VI, [Amgen Inc, Thousand Oaks, CA, USA; UCB, Brussels, Belgium] 25 mg/kg s.c. 2QW) beginning one day following implantation for 2 or 4 weeks and compared to 2 and 4 week untreated controls. All mice received calcein (30 mg/kg, intraperitoneal (i.p.) injection), administered seven days before sacrifice and alizarin (30 mg/kg, i.p.) administered one day prior to sacrifice, to follow new bone formation. At sacrifice, implanted human bone and mouse right femora (RF) were dissected free of soft tissue and fixed overnight (10% NBF), rinsed for 10 minutes in dH₂O and placed in 70% ethanol at 4°C for in preparation for tissue processing. Implants and RF were divided for immunohistochemical and histological analysis (paraffin processed) or histomorphometry (plastic processed). The mouse (host) RF served as an internal control for all subsequent analyses described below.

To confirm efficacy of drug delivery to the implanted human bone at the initiation of treatment on day one following implantation, one mouse received calcein (30 mg/kg) and SclAb (25 mg/kg) s.c. 24 hours post-implantation. The mouse was euthanized 24 hours following the calcein and SclAb injections (48 hours post-implantation) and the human bone implant was prepared for non-decalcified fluorescence histology.

Finally, to provide information on the effects of bone tissue storage for the third stage of the study, tissue from two additional OI patients were collected to media and immediately stored

at 4°C with one sample from each patient fixed immediately for baseline analysis. At five time points during a 48 hour period, bone fragments were removed from media and fixed to evaluate donor bone cell survival following storage.

2.2. Tissue preparation

Bone samples prepared for paraffin processing were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) with constant shaking for 15–20 days and complete decalcification was ensured by Faxitron imaging. Bone tissue was dehydrated using graded alcohols (Leica ASP 300 paraffin tissue processor), embedded and cut into 5 µm thick sections (Leica RM2255 microtome).

A subset of undecalcified bone samples and mouse RF were prepared for plastic processing using a Leica ASP300 tissue processor and placed in a series of methyl methacrylate (MMA) and dibutyl phthalate with progressively higher concentrations of benzoyl peroxide. Samples were manually embedded in partially polymerized polymethyl methacrylate (PMMA) and allowed to cure at room temperature for up to ten days followed by hardening in a 37° C oven overnight. Bone explants were sectioned to 5 µm thickness (Leica RM2255 fitted with a disposable tungsten carbide blade) and slides were placed under compression for two days in a 37° C oven to allow section fixation to the slide. Embedded mouse RF were sectioned transversely with a low-speed saw (IsoMet, Beuhler, Lake Bluff, IL), polished using progressive grades of silicon carbide abrasive paper (1200, 2400, and 4000 grit) followed by a felt pad with a ¼ µm diamond slurry suspension (Struers Inc., Cleveland, OH) on the plane distal to the lateral third trochanter. All images were acquired using a Nikon Eclipse Ni-U microscope (Nikon Instruments Inc., Melville, NY).

2.3. Micro-computed tomography (µCT)

Bone morphology and microarchitecture of the implanted bone sample were analyzed using high resolution µCT (Bruker, Skyscan 1176) at two time points; a pre- scan acquired 24 hours following implantation under anesthesia and a post- scan acquired immediately following euthanasia. A third *ex vivo* scan of the implant and RF was acquired following removal from the host. *Ex vivo* scans of the excised implant and RF were acquired on the same system by placing the sample of interest in a 0.5 mL or 1.5 mL tubule filled with deionized water, respectively. All pre- and post- images were obtained at an X-ray voltage of 75 kV and current of 333 µA and *ex vivo* at an X-ray voltage of 50 kV and 500 µA current and all acquisitions used a 0.5 mm aluminum filter to ensure uniform beam energy. Reconstructed scans were calibrated with the use of two manufacturer-provided hydroxyapatite standards at either 18 µm (pre- and post-) or 9 µm (*ex vivo*) isotropic voxel size. Images were analyzed using the Skyscan CT-Analyzer software (CTAn, Bruker, Version 1.15.4.0).

For the implant, area surrounding the bone was segmented manually followed by several automated processes to extract only the implant from the image allowing for morphometric 3D analysis to be performed on the donor bone without assumptions on underlying bone structures of the host mouse (e.g. mouse spine, rib, sacrum). Due to the differential morphology of the donor bone tissue, the implant was subject to a specimen-specific

threshold to obtain densitometry values [34]. Parameters including bone mineral density (BMD), tissue mineral density (TMD), bone volume fraction (BV/TV) and bone volume (BV) were quantified using manufacturer supplied algorithms (CTAn, Bruker, Belgium). Analysis was performed using percent change from pre- to post- *in vivo* scans for BMD (%changeBMD), TMD (%changeTMD), BV/TV (%changeBV/TV) and BV (%changeBV). Baseline values derived from pre- *in vivo* scans of BMD, TMD, BV/TV and BV were used to describe bone morphology at implantation.

To evaluate a systemic treatment response to SclAb, host right femora were analyzed for femoral geometry (trabecular thickness, cortical area and cortical thickness) and volumetric trabecular BMD (g/cm^3) by first isolating cortical and trabecular bone with fixed attenuation coefficient derived densitometry threshold values of 110 and 90 μ , respectively. The volumes of interest (VOIs) were created and assessed using an auto-segmentation method (CTAn) which separates cortical and trabecular bone automatically using a series of morphological and bitwise operations. A trabecular VOI was created proximal to the end of the distal growth plate spanning 1 mm in the z-direction. The cortical VOI was selected at the mid diaphysis immediately distal to the lateral third trochanter spanning 2 mm in the z-direction.

2.4. Immunohistochemical analysis

Immunohistochemistry with fluorescence (IHC-F) was used to evaluate the presence of human-derived bone cells through staining for human mitochondria (hMito) at baseline and upon removal from the host following implantation durations of 1, 4 and 12 weeks and a subset of 2 and 4 week treated and untreated implants. Detection of host/donor contributions of sclerostin protein (the gene is SOST) and osterix (Osx) was performed using a dual IHC-F staining protocol where primary antigens of sclerostin/hMito, and on serial sections, Osx/hMito were immunolocalized. Staining of hMito was used in all instances to indicate donor-derived cells with the addition of sclerostin or Osx (using antibodies with validated sensitivity to both mouse and human antigens) in order to detect all instances of sclerostin and Osx expression (both mouse (host) and human (donor) derived) to derive host/donor relationship.

In brief, paraffin sections were deparaffinized in xylene and rehydrated through a descending alcohol series. For dual stained hMito/Osx, heat-mediated antigen retrieval was achieved by placing slides in 0.01 mol/L sodium citrate (pH 8.0) in a steamer (Black and Decker) for 15 minutes and sections were cooled to room temperature. For dual stained hMito/sclerostin, antigen retrieval using Proteinase K (2.5 μ l in 100 mm Tris, pH 9.0, and 50 mm EDTA, pH 8.0) for 10 minutes at 37 °C followed by three washes with 0.1 m Tris-buffered saline (pH 7.4) containing 0.02% Tween-20. In all cases, endogenous peroxidase activity was quenched by a 10 minute immersion in 0.3% hydrogen peroxide. hMito/Osx sections were blocked with 5% donkey blocking serum mixed with 1% bovine serum albumin and 1xPBS plus 0.1% triton detergent. hMito/sclerostin sections were blocked in the same manner except for Tween-20 used in the place of triton.

Sections were incubated with the primary anti-hMito antibody (MAB1273, EMD Millipore) at a 1:200 dilution and either a primary polyclonal rabbit anti-Osx antibody (ab22552, Abcam; 1:400) or primary polyclonal rabbit anti-sclerostin antibody (bs-10200r, Bioss;

1:200) overnight at 4°C. For all cases, hMito was developed with a biotinylated mouse anti-rabbit secondary antibody (715-066-150, Jackson ImmunoResearch) at a 1:400 dilution, incubated at ambient temperature for one hour and followed by the addition for Osx of the donkey anti-rabbit IgG secondary antibody conjugated to Alexafluor 488, (A-21206, Invitrogen; 1:400) or, for sclerostin, of the goat anti-rabbit IgG secondary antibody conjugated with Alexafluor 488 (ABIN400260, antibodies-online; 1:250) for an additional hour at 37°C. Following washing, slides were incubated with an avidin conjugated peroxidase system (Vectastain Elite ABC Kit; Vector Laboratories) and developed with a tyramide signal amplification (TSA) substrate (Perkin Elmer) per the manufacturer's instructions to amplify the hMito signal. All slides were washed with a DAPI dilution and mounted using Prolong Gold Antifade Mountant (Life Technologies). Negative control sections underwent the same procedure, but primary antibodies were omitted. Species control sections were used to evaluate the specificity of the primary antibodies. Serial sections were stained with hematoxylin and eosin (H&E) for morphology guiding IHC-F findings using established procedures [35]. Dual, positive-stained cells indicate that either Osx or sclerostin is human (donor) derived while single Osx or sclerostin stained cells suggest host (mouse) contributions.

2.5. Dynamic and static histomorphometry

Undecalcified plastic-embedded implant sections were assessed for the presence and labeling characteristics of calcein and alizarin fluorochrome labeling on newly-formed bone surfaces in 2 and 4 week treated and untreated implants. Analysis of labeling was qualitative in nature due to the heterogeneity of the bone tissue (size, orientation, bone type). Serial implant sections were stained with Goldner's Masson Trichrome (GMT) for static histomorphometry assessment of cellular populations guiding fluorochrome labeling findings.

To further confirm bone-forming activity due to treatment in the host, dynamic histomorphometry of mouse RF including bone surface (BS), mineral apposition rate (MAR), mineralizing surface to bone surface (MS/BS) and bone formation rate (BFR) were performed at the mid-diaphysis on the periosteal and endosteal surfaces using the calcein and alizarin label according to standard nomenclature [36]. Fluorescent images of the prepared cross sections were acquired with a 10x objective of calcein (excitation 485/20 nm, emission: 540/25 nm) and alizarin (excitation: 557/55 nm, emission: 615 nm) and merged (NIS Elements Br, Nikon Instruments Inc., Melville, NY). Quantitative dynamic measurements were performed using ImageJ. Regions where a dual label was not present, MAR was treated as a missing value. To calculate BFR/BS in the cases with a missing MAR value, a value of 0.3 $\mu\text{m}/\text{day}$ was assigned. This value has been used previously in our lab and is established in the literature for cases of missing MAR values [21, 37].

2.6. Tartrate-resistant acidic phosphatase (TRAP) staining

A subset of decalcified paraffin embedded baseline (time-0), and 2 and 4 week treated and untreated implant sections were stained for tartrate-resistant acidic phosphatase (TRAP) activity, a marker of bone resorption, and counterstained with Fast Green according to manufacturer protocol (387A-1KT, Sigma, St. Louis, MO, USA). Slides were imaged using

a bright-field light microscope (10x objective) and analysis was performed in ImageJ where osteoclast detection comprised TRAP-positive cells [38]. For each slide, the entire implant was treated as the ROI where the bone surface was measured followed by quantification of TRAP-positive cells. Measurements were expressed as the number of positive TRAP-stained osteoclastic cells per millimeter of bone surface (Oc.N./B.pm.) of the implant.

2.7. TUNEL assay for bone cell apoptosis

For the third stage of the study, a subset of OI bone samples were evaluated for cell survival by initiating TdT-mediated dUTP nick end labeling (TUNEL) assay for apoptosis. To evaluate the effects of tissue storage and determine the optimal time course for retrieval and implantation, trabecular bone from one OI patient and cortical bone from a separate OI patient (5 samples per patient, ~3 mm³ each in size for a total of 10 bone samples) were collected to α MEM/10% FBS and immediately stored at 4°C. One bone sample per patient was formalin fixed immediately for baseline analysis (time-0) and the remaining bone tissue was removed from storage and formalin fixed at the following time points: 18, 24, 32, and 48 hours. The procedure was performed with a TUNEL staining kit (In Situ Cell Death Detection Kit, Roche, 11684817910) following instructions from the manufacturer. All fluorescent images were acquired at 20x using a Nikon Eclipse Ni-U microscope. Analysis was performed in ImageJ using the analyze particle function combined with the watershed pre-processing algorithm to better separate areas of dense cells (e.g. marrow cells) [39]. In short, quantification of all nucleated cells (DAPI) and positive stained cells (TUNEL) were performed in a total tissue region of interest (ROI) that contained bone and bone marrow, a bone-only ROI, and a marrow-only ROI to determine if cell apoptosis rate differs between the marrow and bone. Results were presented as a percentage of total TUNEL/total DAPI. Serial standard H&E stained sections were used to morphologically guide ROI selection.

To determine the presence of bone cell apoptosis in our implanted human bone, excised implants 1, 4 and 12 weeks (untreated) were evaluated in the same manner using TUNEL assay to ensure bone cells remained viable throughout the proposed implantation duration.

2.8. Statistical methods

Descriptive and inferential statistics for μ CT implant outcomes, presented as a relative percent change derived from pre- and post- image acquisitions, were analyzed using SPSS v. 22.0 (IBM, Armonk, NY). First, we describe the demographic, clinical and related characteristics of the implant including patient type (OI or non-OI), implant duration (2 or 4 week), treatment status (treated or untreated), baseline μ CT values (BMD, TMD, BV/TV and BV) and bone type (trabecular, cortical or morselized) by using raw counts, measures of central tendency (e.g., mean, median or mode) and measures of data dispersion (e.g., 95% confidence intervals, standard deviations (SD)) where appropriate. Comparisons of baseline μ CT BMD, TMD, BV/TV and BV between randomly assigned treated and untreated OI and treated and untreated non-OI groups using Student's t tests were undertaken to ensure group equality at implantation. Direct comparisons of μ CT outcome measures were carried out with paired t-test for 2 and 4 week data within patient type (OI and non-OI). To understand the impact of bone type, univariate ANOVAs for 2 and 4 week analysis of %changeBMD, %changeTMD, %changeBV/TV, and %changeBV were used to detect a differential

response in implant bone type (cortical, trabecular, morselized) to treatment (treated, untreated). This analysis also served to detect differential response due to implantation (using the untreated condition) between OI and non-OI control tissue at each time point. Bonferroni post-hoc analyses were conducted as appropriate.

Analysis of positive TRAP activity using the Oc.N./B.Pm. ratio was conducted on a subset of baseline (time-0), treated and untreated 2 and 4 week OI bone implants. The Oc.N./B.Pm. were analyzed using a regular one-way ANOVA with a Holm-Sidak test for multiple comparisons between implant groups. Results from the TUNEL assay for cell apoptosis following tissue storage were presented as a percentage of total positive TUNEL cells/total DAPI nucleated cells from trabecular- and cortical-derived bone and reported in the total tissue ROI, bone-only ROI and marrow-only ROI at five time points.

Mouse (host) dynamic histomorphometry outcomes (periosteal and endosteal MAR, MS/BS, and BFR/BS) and *ex vivo* μ CT derived trabecular (BMD, trabecular thickness) and cortical (area, thickness) measurements were analyzed using a Two-Way ANOVA with treatment (treated vs. untreated) and duration (2 vs. 4 week) as factors to confirm a systemic effect of SclAb. Post-hoc t tests with a Tukey correction was conducted as appropriate (GraphPad Prism v7, GraphPad Software, La Jolla, CA, USA). In all cases, alpha was set at $p = 0.05$.

3. Results

3.1. Donor bone is bioaccessible, viable, and donor-derived bone cells are present for up to 12 weeks

A definitive calcein fluorochrome label was present on the OI bone surface (Figure 2, A, white arrows) following one dose of calcein administered 24 hours following s.c. implantation. This suggests that an injectable dose of drug given to the host mouse, such as SclAb, could plausibly reach the target implant 24 hours following implantation. Results from TUNEL assay for apoptosis demonstrated minimal TUNEL-positive apoptotic cells following short (1 week) and intermediate (4 week, Figure 2, B) durations. Following long-term implantation, nominal positive TUNEL cells were observed in osteocytes, marrow cells and bone lining cells (Figure 2, C). The existence of viable, human-derived osteocyte-rich bone which expressed human-derived bone lining cells was observed upon harvest and maintained at intermediate durations (Figure 2, D) through 12 weeks (Figure 2, E) in OI and non-OI untreated implants (positive for hMito, red). Together, these results suggest that SclAb treatment can reach the target implant 24 hours following s.c. implantation, and xenografts present with human-derived bone cells at short, intermediate and long-term implantation durations with nominal bone cell apoptosis.

3.2. Balance of host-to-donor bone cell contribution is altered with duration and treatment

Qualitative analysis of hMito IHC-F revealed the presence of human-derived bone cells, but their occurrence decreased slightly with longer implantation durations (up to 12 weeks) (Figure 2D–E). Trabecular-derived bone presented with more positive hMito cells, including a greater number of hMito positive bone lining cells that remained present longer (12 weeks)

compared to cortical-derived bone which had the fewest instances of viable hMito cells at 12 weeks. Similar to trabecular-derived implants, hMito positive bone cells were observed in greater quantities (both osteocyte and lining cells) in the morselized bone compared to cortical bone. No specific staining was observed in negative control sections and no species cross-reactivity was observed in mouse tissue controls ensuring the specificity of the hMito antibody.

A subset of baseline (time-0), and 2 and 4 week treated and untreated OI and non-OI implants were dual IHC-F stained to probe for Osx (green) and hMito (red), and on serial sections, dual sclerostin (yellow) and hMito (red) to determine donor/host cell populations. Following both 2 and 4 week treatment durations, implants exhibited bone surfaces rich with human-derived Osx-expressing cells (Figure 3, B and D) which became more robust with increasing treatment duration. Untreated implants expressed Osx on bone surfaces; however, expression was principally host-derived (i.e. positive for Osx but not hMito) (Figure 3, A–C). In untreated implants, these host-derived Osx-expressing lining cells decreased between 2 and 4 weeks (Figure 3, C) implantation duration regardless of bone type (trabecular, cortical, morselized), and in some cases, no Osx expressing lining cells were present by 4 weeks. Non-OI bone implants behaved similarly to OI as they contained more instances of human-derived Osx-expressing bone lining cells with treatment compared to untreated xenografts at the same time point (data not shown). While most treated OI implants expressed primarily human-derived Osx, cortical xenografts from patient OI4 demonstrated surfaces which were rich with a combination of mouse-derived Osx-expressing cells, along with human-derived Osx-expressing cells, at both treated time points. Patients with less immunolocalized Osx expression at baseline generally demonstrated more robust Osx-expression, primarily human-derived, by 4 weeks of treatment (Supplemental Fig 1, A). Conversely, high baseline Osx expression led to fewer Osx-expressing cells on implant bone surfaces for patient OI4 who also had a mix of mouse and human-derived Osx-expressing cells (reported, above) following four weeks of treatment (Supplemental Fig 1, A).

Donor sclerostin is expressed at baseline and throughout the 4 week period for all conditions (Figure 3, E–H). Varying levels of sclerostin staining surrounding the osteocyte was observed at baseline and generally demonstrated an increase in staining following SclAb treatment (Figure 3, F and H). Baseline sclerostin expression surrounding the osteocyte varied by patient ranging from minimal (OI4, non-OI1), moderate (OI5, OI6) to robust (non-OI1, non-OI3). Implants with minimal sclerostin expression at baseline generally demonstrated the greatest increase in staining levels surrounding the osteocyte following treatment compared to the untreated condition (e.g. OI4 implants, Supplemental Fig 1, B). This observed increase in staining appeared less robust in implants presenting with moderate and robust baseline sclerostin expression prior to the initiation of treatment. In all cases, osteocytes were human-derived with the exception of OI6 cortical-like implants, which demonstrated several instances of mouse-derived, sclerostin expressing osteocytes near bone surfaces at 4 week treated and untreated conditions (Supplemental Fig 1, C example in treated condition).

3.3. *In vivo* μ CT confirms a variable bone-forming treatment effect

Baseline *in vivo* μ CT confirmed there were no significant differences between baseline values of BMD, TMD, BV/TV and BV between treated and untreated two week + four week OI or treated and untreated two + four week non-OI groups (Supplemental Fig 2, A–D). This ensured equality between randomized groups and that no unintended bias was introduced at the time of implantation.

When OI implants were combined into treatment groups regardless of baseline morphology, treated OI implants were not significantly different from untreated OI implants in any percent change μ CT outcome at two or four weeks despite numerically higher increases in all measures with SclAb treatment (Figure 4, A–D). Non-OI implants elicited a significant treatment response for two week %changeTMD, %changeBV/TV, and four week %changeBV.

Given the large variation in the data, we performed a post-hoc analysis based on bone type. OI implants were both trabecular and cortical-derived while non-OI implants were all morselized trabecular bone. SclAb treatment response varied in magnitude by bone type at both two and four weeks (Figure 5, A–D, bolded p-values indicate significant effect of bone type). In some conditions, implantation alone (untreated) continued to increase in bone formation which was differential by bone type (Figure 5, A–D). Results from follow-up Bonferonni post-hoc analysis are denoted by brackets and stars when significance was reached between bone type. While not statistically significant, we observed a 7-fold increase in mean %changeBMD for trabecular OI groups between two and four weeks of treatment (2.24 vs. 18.47), a 0.5-fold increase for cortical OI groups (7.38 vs. 11.19) and a decrease in morselized non-OI groups (65.42 vs 40.61).

Untreated xenografts were not significantly different following two weeks of implantation. At four weeks, trabecular and morselized trabecular implant %changeBMD was significantly increased over cortical implants (Figure 5, A). Interestingly, untreated trabecular implants trended for an increase in %changeBMD, TMD and BV/TV between two and four weeks while untreated cortical implants decreased between 2 and 4 weeks (Figure 5, A–C).

3.4. Implant histomorphometry findings corroborate μ CT results

Analysis of implant histomorphometry was qualitative due to tissue heterogeneity including variation in bone size, orientation and bone type and an inability to define a common sectioning plane. OI and non-OI SclAb-treated implants demonstrated positive formation of new bone presenting with more definitive calcein and alizarin labeling and greater inter-label width (Figure 6, E and M) compared to weaker and non-specific labeling observed in untreated implants (Figure 6, A and I). In general, labeling became more robust following 4 weeks of SclAb treatment compared to two weeks of treatment.

Osteoid seams were observed on serial sections stained with GMT and closely matched locations of calcein and alizarin-labeled surfaces in all implants (Figure 6, B, F, J, N). Evaluation of Osx expression in the implants corroborated fluorochrome and GMT data by revealing a concurrent increase in osteoblast activity. In general, Osx expression on

implanted bone surfaces was stronger in treated implants compared to untreated implants and increased in expression with treatment duration (Figure 6, C,G,K,O).

3.5. No significant changes in bone resorption following SclAb was observed in the implants

TRAP analysis was conducted on a subset of baseline, treated and untreated 2 and 4 wk implants. No significant changes in Oc.N./B.Pm., a measure indicative of bone resorption, were observed across all groups. (Supplemental Fig 3, A and B).

3.6. Host mouse femora confirmed systemic bone-formation response to SclAb

Ex vivo μ CT analysis of mouse RF confirmed a systemic bone-forming effect of treatment in the host (Figure 7, A–E). Two-Way ANOVA results revealed a significant effect of treatment and duration for cortical area (treatment: $p<0.0001$, duration: $p<0.01$) and cortical thickness (treatment: $p<0.0001$, duration: $p<0.0001$) and a significant treatment effect for trabecular BMD ($p<0.001$) and trabecular thickness ($p=0.022$). Specifically, cortical area increased by +10% following 2 weeks ($p=0.06$) and +18% following 4 weeks ($p=0.0001$) of treatment. Cortical thickness increased by +9% ($p=0.02$) and +15% ($p<0.0001$) following 2 and 4 weeks of treatment, respectively. Trabecular BMD increased by +25% following treatment at 2 weeks ($p=0.06$) and +20% at 4 weeks ($p=0.008$) and trabecular thickness increased by +5% following treatment at 2 weeks ($p=0.89$) and +21% ($p=0.05$) at 4 weeks.

Dynamic histomorphometry measures of MAR, MS/BS, and BFR/BS on the periosteal and endosteal surfaces of the host RF (Figure 7,F–G) further corroborated a systemic bone-forming effect of SclAb.

3.7. Bone tissue morphology dictates donor cell apoptosis rates following harvest and during storage

Trabecular and cortical OI bone revealed dissimilar rates of donor cell apoptosis following removal from the patient (baseline, time-0) through storage up to 48 hours at 4°C. TUNEL assay results from two patients can be appreciated in Figure 8, A–K. Patient OI7 yielded trabecular-like bone while patient OI 8 yielded cortical-like bone (Table 1 for detailed patient demographics; both patients Type III OI). At baseline following harvest from the patient, trabecular bone experienced fewer positive apoptotic cells compared to the cortical bone (5% vs. 12%). Following 18 hours in storage, cortical bone underwent cell apoptosis in 54% of the total tissue including 63% of the bone and 38% of the marrow. At the same time point, trabecular bone demonstrated apoptosis in 19% of the total tissue with 14% of the bone and 20% of the marrow donor cells undergoing apoptosis. Both trabecular- and cortical-like bone demonstrated a linear increase in total tissue cell apoptosis with increasing storage time except for trabecular bone at 32 hours and cortical bone at 24 hours. In general, trabecular bone underwent a greater amount of apoptosis in the marrow ROI while cortical bone experienced a greater amount of apoptosis in the bone ROI following storage.

4. Discussion

There is currently no US Food and Drug Administration- or European Medicines Agency-approved therapy for the treatment of OI. Rather, current clinical OI management relies, in part, on off-label use of therapies targeting osteoporosis. Osteoporosis represents a metabolic disease of bone fragility, and therefore, patient response to therapy is not fully predictive of the clinical response in pediatric OI patients using the same interventions [4, 40]. Divergent clinical therapeutic responses in OI highlights the need for an appropriate model to evaluate bone drug efficacy prior to clinical extension. To address this deficiency, we describe a novel xenograft model using solid, OI patient-derived bone tissue harvested during corrective orthopaedic procedures which maintains human-derived bone cells within their native extracellular environment and preserves the genetic mutation unique to each patient. The model provides a controlled host-derived micro-environment to evaluate the *in vivo* bone-forming effects of SclAb in its target tissue. Following bone implantation, TUNEL assays demonstrated that donor bone cells remain viable at short (1 week), intermediate (2–4 weeks) and long-term (12 week) durations. Implanted donor-derived bone tissue became bioaccessible to the host following 24 hours, indicating that systemic treatment is accessible by the xenograft tissue as early as day one in the model. Taken together, we administered SclAb systemically beginning 24 hours after implantation and evaluated implant treatment response at 2 and 4 weeks durations to maximize the likelihood that treatment targeted the human-derived bone cells.

We used immunohistochemistry to evaluate the donor/host relationship of the implants with and without SclAb treatment. Most notably, there was a shift in donor/host origin of Osx expression with SclAb treatment in the human OI implants. We observed that treated implant surfaces were rich with human-derived Osx expressing cells while untreated implants demonstrated surfaces primarily expressing mouse-derived Osx expressing cells. This observation was determined using a dual-IHC approach where human-derived Osx was confirmed by the presence of cells positively stained for both hMito and Osx antibodies. TUNEL results reveal that nominal apoptosis occurs in the implant at 2 and 4 weeks, therefore the lack of human-derived Osx expressing cells in the untreated implant is likely not attributable to cell death. We hypothesize that SclAb treatment may have been able to preserve and differentiate the human-derived cells of the osteoblast lineage leading to the human-derived Osx expression observed in the treated xenografts. It is possible that untreated implant osteoblast precursors were present in the implant but did not differentiate to Osx-expressing lining cells and instead, mouse-derived osteoblasts were recruited to the untreated implant. Baseline Osx expression also appeared to dictate the magnitude of osteoblast response to treatment in the implants. For instance, bone from patient OI6 had very little Osx expression on bone surfaces prior to implantation; as soon as 2 weeks of SclAb treatment, all OI6 implant surfaces were rich in Osx expressing cells which were primarily human-derived. Conversely, patient OI4 had surfaces rich with Osx at baseline (Supplemental Fig 1,A). Of the two bone samples from patient OI4 treated for 2 weeks, one bone sample maintained some surfaces of strong Osx expression while the other bone sample had less human-Osx expression compared to baseline.

Human-derived sclerostin was expressed by osteocytes in both 2 and 4-week treated and untreated implants. At baseline, sclerostin expression was variable ranging from minimal positive sclerostin-expressing osteocytes to moderate and even robust in a few cases. Following treatment, immunolocalization of sclerostin surrounding the osteocyte appeared more robust compared to the positive immunolocalized levels surrounding untreated control osteocytes at the same timepoint. SclAb treatment acts by inhibiting sclerostin which is a negative regulator of bone formation. The drug itself possesses a post-translational effect; it blocks the effects of the protein rather than halting sclerostin gene (SOST) expression by the osteocytes. Holdsworth et al. reported a significant upregulation, or compensatory response, of SOST expression as measured by RT-qPCR (TaqMan) analysis following both a single dose and long-term treatment with SclAb [41]. We postulate that this compensatory response following SclAb treatment may explain the increased levels of immunolocalized sclerostin surrounding the osteocytes in the treated OI implants. Future studies should evaluate sclerostin gene (SOST) expression using qPCR approaches in the human bone following treatment to confirm upregulation of SOST expression.

Osteoblast differentiation into matrix-embedded osteocytes is a complex and not well understood process which includes a gradual transition to osteoid-osteocytes before differentiating fully into osteocytes [42]. By two weeks we confirmed the presence of mouse-derived *Osx*-expressing lining cells on some of the human implants; while human osteoblasts remain active up to three months [43], the lifespan of a murine osteoblast is ~10–20 days [44]. While numerous studies report bone deposition rates, the time between osteoblast-osteocyte transformation is still not well understood and range from three days in young rabbits [45], 2–5 days in newborn rats [46, 47] and 10–19 days on periodontal surfaces in newborn mice [44]. Expression of sclerostin is indicative of a mature osteocyte phenotype and is expressed as early as the onset of mineralization of the osteoid [48, 49]. Expression of sclerostin has been confirmed at low levels in osteoblasts [48] yet its presence in the osteoid-osteocyte in humans and mice remains in question [49–51]. We postulate that it is feasible for mouse osteoblasts to begin differentiation and express sclerostin by our 4 week timepoint (14 days after we observed mouse *Osx* expression) where immunolocalization of sclerostin in patient OI6 treated and untreated implants near the bone surface appeared to be mouse-derived (absence of positive hMito, Supplemental Fig 1,C). Future studies using transgenic mice tagging cells of the osteoblast lineage could evaluate the host-osteoblast fate in the human xenograft bone tissue to validate our findings.

Similar to our model, Pettway and McCauley et al. described an ectopic ossicle bone model system utilizing donor bone marrow stromal cells (BMSCs), expanded *in vitro*, and implanted into an athymic murine host. The model represents a system where mesenchymal components are from the donor and hemopoietic cells are from the host [32, 33, 52, 53]. The authors observed an increase in marrow cellularity following 1 week of iPTH treatment, an anabolic response by 3 weeks and plateau in treatment response following 7 weeks. The authors hypothesized that the plateau was indicative of a timepoint where the host was incapable of supplying viable mesenchymal cells. In our study, SclAb stimulated new bone formation in the implanted bone tissue which significantly differed in both magnitude and rate when implant bone type was considered. Specifically, trabecular and morselized implants continued to respond in μ CT markers of bone formation with longer treatment

forming therapeutic being considered. We observed a significant increase in μ CT and dynamic histomorphometry markers of bone formation in treated mice compared to untreated using the host right femora for analysis. Results in the mice were consistent with prior work performed in our lab evaluating the effects of SclAb treatment [63].

The time between bone harvest and implantation is an important consideration for donor bone cell survival. We evaluated the effects of tissue storage at 4°C over the course of 48 hours to guide decision making regarding implantation latency duration and the potential for tissue banking and shipping tissue between OI centers for future diagnostic and treatment studies. At baseline, the time human bone was implanted in the proposed xenograft model, trabecular bone experienced less apoptosis in the total tissue and the marrow ROI compared to cortical bone. Further, trabecular bone tissue (from the bone-only ROI) demonstrated fewer instances of apoptotic cells over the 48 hours compared to cortical bone. Over time, however, marrow space for trabecular bone did demonstrate greater amount of positive TUNEL cells compared to the cortical bone by the 24 and 48 hour time points. Taken together, it appears that 18 hours of storage at 4°C may be the upper limit following removal from the patient and that trabecular derived bone may have a greater capacity for bone cell survival through 18 hrs.

Limitations

There are several limitations to the study. First, the study was limited by low pediatric patient numbers (eight OI patients were enrolled) which was inevitable due to the rarity of the disease [1]. This inhibited us to reach sufficient statistical power to statistically compare results by OI type. However, each patient yielded up to 20 implantable bone samples which allowed us to evaluate numerous treated and untreated implantation durations. When patient bone yield was low, it limited our ability to allocate bone to every outcome measure (varying implantation durations and treatment durations) while maintaining an adequate size of the sample to implant ($\sim 3 \text{ mm}^3$). To mitigate this, outcome measures were prioritized to address our primary hypothesis that human OI bone can respond to SclAb *in vivo*, by focusing on μ CT, IHC-F, and histomorphometry in 2 and 4-week treated and untreated groups. Evaluation of the effects of tissue storage has shed light on the appropriate latency period between harvest and implantation. To increase patient numbers in future studies using this model, it may be possible to receive bone samples from other institutions within an 18 hour period following removal from the OI patient. Due to the rarity of the disease and our interest in investigating the impact of heterogeneity on treatment response, bone was harvested from OI patients undergoing orthopaedic surgical intervention which ranged from fracture repair, osteotomy and hardware removal and included tissue from various locations in the body (humerus, radius, femur, tibia) which intraskeletally undergo varying rates of remodeling [64, 65]. The non-OI group contained morselized trabecular bone tissue harvested from otherwise healthy individuals during tibial tunnel reamings during ACL reconstructive procedure and implanted as a morselized trabecular bone mass. The bone collected for the study was bone that would have typically been discarded as surgical waste therefore it was not possible to standardize harvest location and bone type (cortical/trabecular). Future work should attempt to standardize both diseased and control bone tissue in order to reduce heterogeneity of the bone implants. It should be noted that the model itself

could be adaptable in two ways. First, while we are using tissue from OI patients and healthy controls, it would be reasonable that tissue could come from patients with different bone dysplasia to evaluate how that diseased tissue would respond to therapy. Secondly, the model is not limited to SclAb; the method described could be extrapolated to other pharmaceuticals particularly in cases where response in human tissue is unknown.

5. Conclusion

In summary, we propose a xenograft model using solid bone tissue derived from OI patients as a means to evaluate treatment response to novel therapeutics. We demonstrate that patient bone remains viable during implantation, contains human-derived bone cells for up to 12 weeks of implantation, is bioaccessible by the host as early as 24 hours and systemic SclAb treatment in the host elicits a bone-forming effect in the implant. Response to treatment was variable; bone morphology, treatment duration and baseline cellular phenotype likely play important contributing roles. When donor/host response was evaluated, donor sclerostin remains present in all conditions while SclAb treatment appeared to dictate *Osx* expression in the implants. We believe the promising findings from this proof of principle study could lead to the use of patient derived bone to investigate therapeutic strategies leading to a more personalized medicine approach in OI. We observed differences in response in this heterogeneous OI bone tissue which is of interest; however, variability in tissue collection must be standardized where findings from the model could then lead to understanding which patients would respond best to therapy. The successful implementation of the model may provide a safe approach to evaluate drug efficacy in a disease state without contraindications to the patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Osteogenesis imperfecta patient bone tissue used in a xenograft model can assess the effects of sclerostin antibody on bone cells harboring the causative disease defect.
- Treatment administered systemically to the host can be initiated as early as 24 hours following implantation where donor bone becomes bioaccessible.
- Patient-derived osteocytes expressing human sclerostin and lining cells are present and remain viable up to 12 weeks of implantation in the host.
- Sclerostin antibody recruits human-derived, osterix expressing lining cells to the implant surface while untreated implants had surfaces with host-derived osterix expressing cells.
- Osteogenesis imperfecta patients with low baseline osterix expression demonstrated robust human-derived osterix-expression after treatment while high baseline osterix expression demonstrated smaller expression gains with treatment.

Figure 1.

Radiograph from patient OI 1, open reduction internal fixation (ORIF) surgery, left humerus (A). Bone samples typically discarded during orthopaedic surgery were collected into media (B), trimmed, and implanted subcutaneously on the dorsal side of a 4–6 week nude mouse through a 1 cm posterior incision adjacent to the spinal column in the lumbar region (C,D). Upon removal (E), implanted bone is rich with host blood vessels (white arrows). Each patient yielded ~3–20 samples.

Figure 2.

Bioaccessibility, bone cell viability and presence of donor cells in the human-derived implant. One subcutaneous administration of calcein (30 mg/kg) to the host 24 hours following implantation demonstrated a definitive calcein label (green label, white arrow) present on the implant bone surface upon removal 24 hours after injection indicating that an injectable dose of drug administered to the host could plausible reach the donor implant at this timepoint (A, left). Representative fluorescent image acquired in grayscale (A, inset) of the implant. TUNEL assay results following intermediate (B) and long-term (C) implantation duration (4 weeks and 12 weeks untreated) demonstrate minimal positive TUNEL cells (green; DAPI=blue) at each time point; insets (1) positive apoptotic osteocyte(s) and (2) example of positive apoptotic progenitor and lining cell populations. Representative H&E stained implant from the patient at baseline can be appreciated in (D). Immunolocalization (IHC) of donor derived cells using human mitochondria primary antigen (hMito=red; DAPI=blue) at 4 weeks (E) and 12 weeks (F) demonstrate human-derived osteocytes (inset 1) and bone rich with human-derived lining cells (inset 2) in the OI implant. Representative H&E stained implant from the patient at baseline can be appreciated in (G). A, E-G from patient OI 3 (Type III OI) and patient OI 2 (Type I OI), respectively; A, E and F acquired at 40x (50 μ m scale bar) and G acquired at 20x (250 μ m scale bar). B-D from patient OI 1 (Type III OI) acquired at 20x (100 μ m scale bar for fluorescent images and 250 μ m scale bar for H&E image). OI= osteogenesis imperfecta.

Figure 3.

Immunohistochemistry with fluorescence (IHC-F) following removal from the host at 2 and 4 weeks treated (SclAb) and untreated. Human osteogenesis imperfecta (OI) implants were dual IHC-F stained to probe for the presence of Osterix (Osx; green) and human mitochondria (hMito; red) (A-D) and on serial sections, Sclerostin (yellow) and hMito (red) (E-H). In all cases, hMito was used to indicate donor derived cells and Osx or sclerostin primary antibody (validated sensitivity to both mouse and human antigens) were used to probe all instances of expression (both host and donor). Zoomed insets (1) depict lining cells expressing Osx (A-D) and osteocytes expressing sclerostin (E-H). Representative Hematoxylin and Eosin stained bone acquired at baseline (I). Images were acquired at 40x (50 μ m scale bar). DAPI= nuclear stain (blue). Panel represents data from one OI patient (OI 6, Type III/IV OI) who yielded cortical-derived bone samples (I), hematoxylin and eosin (H&E) stained implant acquired at 20x with a 250 μ m scale bar.

Figure 4.

Mean $\pm$ standard deviation of *in vivo* μ CT derived %changeBMD (A), %changeTMD (B), %changeBV/TV (C) and %changeBV (D) for treated and untreated 2 and 4 week OI and non-OI implants. %change values were derived as the change from pre- μ CT acquisition acquired 24 hours following implantation (baseline) and post- μ CT acquired immediately following sacrifice. Direct comparisons of μ CT outcome measures were carried out with paired test for 2 and 4 week data within patient type (OI and non-OI) where significance is denoted by brackets and stars. In all cases, statistical significance was set to $p < 0.05$. OI = osteogenesis imperfecta; BMD = bone mineral density; TMD = tissue mineral density; BV/TV = bone volume fraction; BV = bone volume.

Figure 5.

ANOVA results with Bonferonni correction of *in vivo* implant μ CT results by bone type for two and four week treated and untreated implants. Mean $\pm$ standard deviation of *in vivo* μ CT derived parameters of %changeBMD (A), %changeTMD (B), %changeBV/TV (C) and %changeBV (D) for trabecular-derived implants, cortical-derived implants and morselized trabecular implants are plotted. ANOVA p-values are reported and bolded when a significant effect of bone type was detected at $p < 0.05$. Significance between bone type within condition, assessed via post-hoc analysis with a Bonferonni correction, is denoted by brackets and stars. Trabecular and cortical implants originated from OI patients while morselized trabecular implants originated from non-OI patients. OI= osteogenesis imperfecta; TRAB= trabecular; CORT= cortical; MORS= morselized; BMD = bone mineral density; TMD = tissue mineral density; BV/TV = bone volume fraction; BV = bone volume.

Figure 6.

Calcein (green) and alizarin (red) fluorochrome bone labeling, serial Goldner's Masson trichrome (GMT), and immunohistochemistry (IHC) with fluorescence of osterix (green) and corresponding DAPI (blue) nuclear stained images for one patient at 2 (A-D) and 4 week (I-L) untreated, 2 (E-H) and 4 week (M-P) treated. SclAb treated implants presented with a definitive calcein and alizarin label and greater inter-label width (E,M) compared to weaker and non-specific labeling observed in untreated conditions (A,I) which can be appreciated in the image insets. GMT osteoid seams corresponded to areas of fluorochrome labeling (B,F,J,N). Osterix expression on implant bone surfaces was strong in treated implants and increased with treatment duration (G, O). Untreated implants at 2 weeks displayed osterix on bone surfaces which diminished by 4 weeks untreated (C, K). Calcein/alizarin images and GMT were acquired at 10x (100 μ m scale bar) and IHC images at 20x (250 μ m scale bar). Panel represents data from one OI patient (OI 4, Type III OI, cortical-derived implant). Due to the tissue processing requirements for histomorphometry (undecalcified plastic processing) and immunohistochemistry (decalcified paraffin processing) images are parallel implants harvested from the same OI patient (OI 4).

Figure 7.

Quantitative μ CT analysis host (mouse) femora reveal a significant bone-forming response to SclAb treatment compared to untreated controls in measures of cortical area (A), thickness (B) and trabecular bone mineral density (BMD) (C) and thickness (D) at 2 and 4 weeks. Significance is indicated by brackets and stars (*). Representative *ex vivo* μ CT images of mouse femora at 2 and 4 week treated with SclAb (top) and untreated (bottom) (E). Treated 2 week n=14, untreated 2 week n=10, treated 4 week n=13, untreated 4 week n=11. Representative cross-sectional dynamic histomorphometry images of femora demonstrate stronger calcein (green) and alizarin (red) labeling, including increased inter-label width, following 2 and 4 weeks of SclAb treatment compared to untreated controls (F). Results from endosteal and periosteal mineralizing surface/bone surface (MS/BS), mineral

apposition rate (MAR) and bone formation rate/bone surface (BFR/BS) for all groups is presented as mean $\pm$ SD with statistical significance indicated (G). Dual label was not present on all surfaces (preventing MAR measurements in some cases) and was treated as a missing value. Treated 2 week n=16, untreated 2 week n=10, treated 4 week n= 13, untreated 4 week n=8. In all cases, statistical significance was p 0.05. Images acquired at 20x (500 μ m scale bar).

Figure 8.

OI patient trabecular-derived and cortical-derived bone was evaluated for donor cell apoptosis using a TUNEL assay at baseline and following storage four time points (18, 24, 32 and 48 hrs) in α MEM/10% FBS at 4° C. TUNEL assay results from trabecular bone (A-E) and cortical bone (F-J) (left) and serial hematoxylin and eosin (H&E, right) are displayed. Detailed TUNEL results, presented as a percent of total TUNEL positive cells/total DAPI stained cells for a total tissue ROI, bone only ROI and marrow only ROI for the trabecular and cortical bone at each timepoint is depicted (K). Positive TUNEL apoptotic cells are shown in green and DAPI nuclear stain (highlighting all cells) in blue. Trabecular bone was

harvested from OI7 (Type III OI) and cortical from OI8 (Type III OI). All images acquired at 20x (500 μm scale bar).

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Table 1.

Patient Demographics.

Patient	OI Type	Analysis Type	Surgical Indication	Drug TR History	Harvest Location	Age	Sex	Bone Sample Yield (baseline/implant)
OI								
OI 1	III	Xenograft	Open Reduction Internal Fixation	Depo-testosterone	L Humerus	17	M	1/9
OI 2	I	Xenograft	Implant Exchange	Vitamin D2	R Tibia	14	M	2/18
OI 3	III	Xenograft	Fracture	Ca Carbonate, Vitamin D2	R Humerus	17	F	1/11
OI 4	III	Xenograft	Bowing of Femur over Rod	Ca Carbonate, Vitamin D2	R Metaphyseal Femur	18	F	1/6
OI 5	III/IV	Xenograft	Bilateral, Multiple Osteotomies	None	R&L Femur	16 mo (	F	1/2
OI 6	III/IV	Xenograft	Osteoplasty	Ca Citrate-Vitamin D3	R Femur	23	F	1/8
OI 7	III	Storage Pilot	Revision	Depo-testosterone	L Ulna/Radius	17	M	6 (Storage Pilot)
OI 8	III	Storage Pilot	Osteotomy	None	R Tibia/Fibia	21	M	6 (Storage Pilot)
Non-OI								
Non-OI 1	Unaffected	Xenograft	ACL Reconstruction	N/A	Tibial Reaming	15	F	4/9
Non-OI 2	Unaffected	Xenograft	ACL Reconstruction	N/A	Tibial Reaming	14	M	1/9
Non-OI 3	Unaffected	Xenograft	ACL Reconstruction	N/A	Tibial Reaming	10	M	2/12