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# Time-dependent cellular and transcriptional changes in the osteoblast lineage associated with sclerostin antibody treatment in ovariectomized rats

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ABSTRACT

Inhibition of sclerostin with sclerostin antibody (Scl-Ab) has been shown to stimulate bone formation, decrease bone resorption, and increase bone mass in both animals and humans. To obtain insight into the temporal cellular and transcriptional changes in the osteoblast (OB) lineage associated with long-term Scl-Ab treatment, stereological and transcriptional analyses of the OB lineage were performed on lumbar vertebrae from aged ovariectomized rats. Animals were administered Scl-Ab 3 or 50 mg/kg/wk or vehicle (VEH) for up to 26 weeks (d183), followed by a treatment-free period (TFP). At 50 mg/kg/wk, bone volume (BV/total volume [TV]) increased through d183 and declined during the TFP. Bone formation rate (BFR/bone surface [BS]) and total OB number increased through d29, then progressively declined, coincident with a decrease in total osteoprogenitor (OP) numbers from d29 through d183. Analysis of differentially expressed genes (DEGs) from microarray analysis of mRNA isolated from laser capture microdissection samples enriched for OB, lining cells, and osteocytes (OCy) revealed modules of genes that correlated with BFR/BS, BV/TV, and osteoblastic surface (Ob.S)/BS. Expression change of canonical Wnt target genes was similar in all three cell types at d8, including upregulation of *Twist1* and *Wisp1*. At d29, the pattern of Wnt target gene expression changed in the OCy, with *Twist1* returning to VEH level, sustained upregulation of *Wisp1*, and upregulation of several other Wnt targets that continued into the TFP. Predicted activation of pathways recognized to integrate with and regulate canonical Wnt signaling were also activated at d29 in the OCy. The most significantly affected pathways represented transcription factor signaling known to inhibit cell cycle progression (notably p53) and mitogenesis (notably c-Myc). These changes occurred at the time of peak BFR/BS and continued as BFR/BS declined during treatment, then trended toward VEH level in the TFP. Concurrent with this transcriptional switch was a reduction in OP numbers, an effect that would ultimately limit bone formation. This study confirms that the initial transcriptional response in response to Scl-Ab is activation of canonical Wnt signaling and the data demonstrate that there is induction of additional regulatory pathways in OCy with long-term treatment. The interactions between Wnt and p53/c-Myc signaling may be key in limiting OP populations, thus contributing to self-regulation of bone formation with continued Scl-Ab administration.

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*Abbreviations:* BPs, biological processes; BFR, bone formation rate; BS, bone surface; BV, bone volume; DEGs, differentially expressed genes; ECM, extracellular matrix; GO, gene ontology; LCM, laser capture microdissection; LC, lining cells; Ma.Op, marrow osteoprogenitor; MS, mineralizing surface; OB, osteoblast; Ob.N, osteoblast number; Ob.S, osteoblastic surface; OCy, osteocytes; OP, osteoprogenitor; OVX, ovariectomized; pQCT, peripheral quantitative computed tomography; PTb.OP, peritrabecular osteoprogenitor; s.c., subcutaneous; Scl-Ab, sclerostin antibody; TV, total volume; TFP, treatment-free period; URA, upstream regulator analysis; VEH, vehicle; vBMD, volumetric bone mineral density.

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## 1. Introduction

Sclerostin is a secreted protein of the osteocyte (OCy) and an inhibitor of bone formation [1]. The effects of sclerostin on bone metabolism are based on its actions as an extracellular inhibitor of the canonical Wnt signaling pathway, resulting in decreased bone formation and increased bone resorption [2,3]. Inhibition of sclerostin with sclerostin antibody (Scl-Ab) has been shown to stimulate bone formation, decrease bone resorption, and increase bone mass in both animals and humans [4,5]. The rapid increases in bone formation with Scl-Ab have been acutely associated with the activation of bone lining cells (LC) to form new bone [6].

In contrast to acute Scl-Ab administration, which stimulates bone formation on trabecular, endocortical, and periosteal surfaces, long-term Scl-Ab administration exhibits a time-specific profile of osteoblast (OB) stimulation. In aged ovariectomized (OVX) rats, Li et al. [7] showed self-regulation of bone formation in vertebral cancellous bone. Bone formation rate per bone surface (BFR/BS) returned toward control values after 6 months of continuous Scl-Ab administration, while decreases in bone resorption were sustained through month 6. In young rats administered Scl-Ab, self-regulation of vertebral BFR/BS was associated with reductions in osteoprogenitor (OP) populations [8]. These histomorphometry data align with similar phenomena observed with serum biomarkers in rats [9] and humans [10], where early increases in serum bone formation markers with Scl-Ab were followed by attenuation of this response over continuous treatment periods of 6–12 months. Human studies also showed that bone resorption markers were consistently decreased over a 12-month Scl-Ab treatment period.

Associated with the early activation of bone formation in response to Scl-Ab treatment, the acute downstream signaling in vivo has been shown to involve upregulation of selected canonical Wnt target genes coincident with upregulation of extracellular matrix genes in all mature OB subpopulations, i.e., OB, LC, and OCy after enrichment with laser capture microdissection (LCM) [6]. It is unknown how the transcriptional profile changes with long-term treatment with Scl-Ab, or whether there is a relationship between transcriptional changes and quantitative changes in BFR/BS and the OB lineage. To investigate these potential relationships, we utilized LCM-enriched subpopulations of the mature OB lineage to study the temporal transcriptional responses of mature OB lineage to long-term Scl-Ab treatment and examined the association of these responses with changes in bone formation and resorption by histomorphometry and OB and OP numbers by stereological methods.

## 2. Material and methods

### 2.1. Study design

Six-month-old Sprague–Dawley female rats (SD®IGS; Charles River Laboratories, Hollister, CA) were OVX and left untreated for 8 weeks, at which time they weighed approximately 400 g. Rats were assigned to three treatment groups in a manner to achieve body weight balance across treatment groups. Rats were administered by s.c. injection vehicle (VEH); or 3 or 50 mg/kg/wk of a Scl-Ab (Scl-AbVI) once weekly up to day (d)183 (Suppl Fig. 1). Scl-AbVI was engineered to be less immunogenic in rats (rat Fc construct) than humanized antibody. The 50 mg/kg/wk Scl-Ab dose, which matches the high dose used in a rat lifetime pharmacology study, provided an approximate 18-fold margin to clinical exposure; the 3 mg/kg/wk dose approximated clinical exposure (Amgen internal data). Up to 15 rats per group were euthanized at d8, 29, 85, and 183. To assess effects following treatment withdrawal, additional groups were maintained through a treatment-free period (TFP) and euthanized at d197 and 267 for VEH and 3 mg/kg groups, and at d237 and 309 for VEH and 50 mg/kg groups. For euthanasia, animals were anesthetized with isoflurane/oxygen and then exsanguinated.

To label active bone-forming surfaces for dynamic histomorphometry and to facilitate OB and LC enrichment during LCM as described by Nioi et al. [6], 10 mg/kg calcein green (in 2% sodium bicarbonate in saline) was administered s.c. 13 and 3 days prior to scheduled euthanasia. At necropsy, the first and second lumbar vertebral (L1 and L2) bodies were isolated for histomorphometry and peripheral quantitative computed tomography (pQCT) analyses, respectively. At 4 °C, L6 body was fixed in 4% paraformaldehyde for 48 h then decalcified in 10% EDTA with 2% paraformaldehyde, processed to paraffin for stereological analyses. L3 body was isolated and embedded in Tissue-Tek® O.C.T. (Sakura Finetek, Torrance, CA) in Peel-A-Way® disposable histology molds (Ted Pella, Inc., Redding, CA), snap frozen in liquid nitrogen, and stored at −80 °C until sectioning.

Animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals, 8th Edition* [11]. All research protocols were approved by the Institutional Animal Care and Use Committee. Animals were group-housed (two per cage) at an Association for Assessment and Accreditation of Laboratory Animal Care, international-accredited facility in nonsterile, ventilated, microisolator housing with corn cob bedding. Animals had ad libitum access to pelleted feed (Rodent Diet 2020X, Teklad Diets, Madison, WI) and water (reverse osmosis purified) using an automatic watering system. Animals were maintained on a 12:12 h light:dark cycle in rooms with controlled temperature (72 °F ± 2 °F) and humidity (30%–70%), and access to enrichment opportunities (Nylabones and Rat Retreats or Rat Tunnels). Further details on study design and animal care are provided in Supplemental Material 1.

### 2.2. Histomorphometry and pQCT

L1 vertebral bodies were fixed in 10% neutral buffered formalin for 72 h and processed undecalcified in methyl methacrylate for histomorphometry. Undecalcified stained and unstained sections of L1 were prepared and evaluated for static and dynamic histomorphometry as described previously [12]. L2 vertebral bodies were scanned and analyzed on a Stratec XCT Research M instrument (Norland Medical Systems, Fort Atkinson, WI, software version 5.40). Additional details on histomorphometry and pQCT are provided in Supplemental Material 1.

### 2.3. Stereological analyses of OB subpopulations

Vertebrae L6 from animals at d8, 29, and 183, of the dosing phase, and d267 and 309, the last TFP time point for 3 and 50 mg/kg, respectively, were used for estimation of total number of subpopulations of the Ob lineage. Due to small group size of d267 controls, three VEH animals from d237 were combined with d267 VEH to increase the group size. The stereological estimator, the physical fractionator [13], was used to provide estimates of total number of cell subpopulations (OB and OP) contained within the entire vertebral body. Number estimates by these methods are independent of size, shape, distribution, or orientation. The physical fractionator uses physical disectors (i.e., paired consecutive thin sections that constitute a three-dimensional [3-D] probe [volume] in which cells are counted). The disector counting principle uses a unique counting feature of the cell, in this case the nucleus, and a cell is only counted when the nucleus is present in a sampled field in one section of the disector (counting field) and not the other (lookup field) [14]. A combination of immunophenotyping, morphology, and location was used to classify cells for estimation. Sets of disector sections were stained for runt-related transcription factor 2 (Runx2) to identify subpopulations of OP, including peritrabecular OP (PTb.OP) and marrow OP (Ma.Op) in cancellous bone. PTb.Op were identified as Runx2-positive spindle-shaped cells located typically one cell layer from the bone surface, frequently in the canopy overlying Ob. Ma.Op were identified as a Runx2-positive cell with a round to ovoid nucleus associated with thin-walled vessels

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