

Inhibiting the osteocyte specific protein sclerostin increases bone mass and fracture resistance in multiple myeloma

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Word Count: 4113

Abstract word count:245

Number of Figures: 7

Number of Supplemental Figures: 2

Number of Supplemental Tables: 1

Number of Supplemental Movies: 0

Number of references: 58

Running title: Anti-sclerostin increases bone strength in myeloma

Key Points: 1-2 key points summaries of paper:

- Anti-Sclerostin treatment increases bone mass and fracture resistance in multiple myeloma.
- Anti-Sclerostin in combination with zoledronic acid is superior to zoledronic acid alone in increasing fracture resistance.

Abstract

Multiple myeloma is a plasma cell cancer that develops in the skeleton causing profound bone destruction and fractures. The bone disease is mediated by increased osteoclastic bone resorption and suppressed bone formation. Treatment with bisphosphonates, which inhibit bone resorption, prevents bone loss but fail to influence bone formation and do not replace lost bone, so patients continue to fracture. Stimulating bone formation to increase bone mass and fracture resistance is a priority; however, targeting tumor-derived modulators of bone formation has had limited success. Sclerostin is an osteocyte-specific wnt antagonist that inhibits bone formation. We hypothesized that inhibiting sclerostin would prevent development of bone disease and increase resistance to fracture in multiple myeloma. Sclerostin was expressed in osteocytes from bones from naïve and myeloma-bearing mice. In contrast, sclerostin was not expressed by plasma cells from 630 patients with myeloma or 54 myeloma cell lines. Mice injected with 5TGM1-eGFP, 5T2MM or MM1.S myeloma cells demonstrated significant bone loss, which was associated with a decrease in the fracture resistance in the vertebrae. Anti-sclerostin antibody treatment increased osteoblast numbers and bone formation rate, but did not inhibit bone resorption or reduce tumour burden. Anti-sclerostin treatment prevented myeloma-induced bone loss, reduced osteolytic bone lesions and increased fracture resistance. Combination treatment with anti-sclerostin and zoledronic acid increased bone mass and fracture resistance when compared to zoledronic acid treatment alone. This study defines a therapeutic strategy superior to the current standard of care, which will reduce fractures for patients with multiple myeloma.

Introduction

Multiple myeloma (MM) is a neoplastic disease of B-cells that develops in the skeleton. 86,000 new cases of myeloma are diagnosed globally each year¹. 95% of patients develop bone disease, which leads to a 16-fold increase in the risk of skeletal fractures, most commonly in the vertebrae¹⁻⁶. The bone disease results from tumor cell production of paracrine factors that increase osteoclast mediated bone resorption and suppress bone formation by osteoblasts^{7,8}. Inhibiting the osteoclastic bone resorption component prevents development of myeloma bone disease⁹⁻¹³. The bisphosphonate, zoledronic acid, which inhibits osteoclasts directly, is now the standard of care for treating myeloma bone disease. Whilst this prevents further bone loss and can reduce skeletal related events (SREs), bisphosphonates do not stimulate bone formation and patients continue to suffer SREs, including fractures¹⁴. Thus, comprehensive treatment of multiple myeloma bone disease requires not only inhibition of osteoclastic resorption but also stimulation of osteoblastic bone formation.

Wnt signaling is important in regulating osteoblastic bone formation¹⁵. Soluble Wnt antagonists are critical components of this system and inhibit bone formation¹⁵. The Wnt antagonist Dickkopf-1 (DKK1) is expressed by myeloma cells and serum levels are elevated in some patients¹⁶⁻¹⁹. Furthermore, inhibiting DKK1 in experimental models of myeloma prevents bone disease, suggesting this soluble Wnt antagonist has a role in osteolysis²⁰⁻²². Activin, a member of the transforming growth factor beta superfamily, is also expressed by myeloma cells and inhibiting activin increases bone formation and prevents bone loss in models of myeloma²³⁻²⁵. However, these approaches that target tumor-derived factors have yet to be translated successfully into the clinic, with only a proportion of patients

responding to treatment²⁶, which likely reflects their heterogeneous expression within myeloma tumors and between patients. A superior strategy, that has yet to be explored, is to target molecules in the bone microenvironment that are tumor independent and more likely common to all patients. One clear candidate with expression restricted to osteocytes embedded within bone matrix is sclerostin, a soluble Wnt antagonist that controls bone formation^{27,28}. Concentrations of sclerostin are elevated in serum of patients with myeloma²⁹. Anti-sclerostin antibodies powerfully promote bone formation and increase bone mass in models of osteoporosis and bone repair³⁰⁻³⁴. In patients with osteoporosis, anti-sclerostin antibody treatment increases bone mass and reduces fracture incidence, across large clinical cohorts^{35,36}. We hypothesized that combining existing anti-resorptive therapy with inhibition of sclerostin will increase bone formation and bone mass, and thus decrease fracture susceptibility in multiple myeloma, thereby defining an important new therapeutic strategy to treat myeloma bone disease. To address this we determined whether sclerostin was expressed by myeloma cells, or restricted to osteocytes, and evaluated the effect of anti-sclerostin antibody alone and in combination with bisphosphonate therapy on the skeleton in a series of well characterized murine and human xenograft models of myeloma bone disease.

Methods

Human myeloma patient sample analysis

Patients with previously untreated, therapy-requiring or relapsed myeloma and healthy donors were included in the study approved by the University of Heidelberg ethics committees (#229/2003 and #S-152/2010) after written informed consent. Normal bone marrow plasma cells and myeloma cells were purified using anti-CD138³⁷⁻³⁹.

Myeloma cell lines and murine primary cell harvests

Murine 5TGM1-enhanced green fluorescent protein (5TGM1-eGFP), human MM1.S-luc-eGFP and OPM2 myeloma cells were cultured as previously described^{40,41}. Murine 5T2MM cells were maintained by serial passage in C57BL/KaLwRijHsd mice⁴².

Peripheral blood CD27⁺ memory B-cells were FACS-sorted and polyclonal plasmablasts generated as described⁴³. Human myeloma cell lines and sources are reported in supplemental methods. Primary osteocytes were isolated as previously described⁴⁴.

SOST and DKK1 gene expression

Gene expression profiling was performed using U133 2.0 plus arrays (Affymetrix)^{38,45}. For RNA-sequencing, cDNA was amplified using the SMARTer Ultra-low RNA Kit (Illumina). NEBNext Chip-Seq Library Prep protocol (New England BioLabs) was used for library preparation and sequenced on an Illumina HiSeq2000 (Illumina).

Osteocyte RNASeq analysis

Osteocyte-enrichment of gene expression was determined by comparing transcriptome sequencing data from osteocyte-enriched bones void of bone-marrow, to samples with intact bone marrow. Transcriptome analysis of osteocyte gene expression from tumor bearing bones relative to naive samples was performed following RNA extraction from homogenized (TriReagent-Sigma-Aldrich) osteocyte enriched bone, quality checks by Total RNA Pico Chip (Agilent Technologies) and depletion of ribosomal RNA using RNaseH (Epicentre) and ribosomal RNA targeting oligos⁴⁶. Total-RNA library preparation was performed using the TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina). Samples

were sequenced on a HiSeq 2500 sequencer (Illumina). Details are described in supplemental methods.

Measuring sclerostin protein

Secreted sclerostin (SOST) protein was assayed in media from 5TGM1, MM1.S OPM2 and primary osteocyte cultures by enzyme linked immunosorbent assay (ELISA) (R&D Systems).

Animal experiments

Animal experiments were performed in accordance with approved protocols from St Vincent's Hospital Animal Ethics committee (ARA 14/43) and Dana-Farber Cancer Institute Animal Care and Use Committee (14-001). C57BL/KaLwRijHsd mice (BKAL) (Harlan, Netherlands) were injected intravenously with syngeneic 5TGM1-eGFP or 5T2MM murine myeloma cells^{40,42}. SCID-beige or NOD.Cg-Prkdcscid/Il2rgtm1Wjl/SzJ (NSG) mice were injected intravenously with MM1.S-luc-eGFP human myeloma cells. Vehicle buffer or anti-sclerostin antibody (i.v. 100mg/kg weekly, BPS804, Novartis Pharma), PBS or zoledronic acid (i.p. 0.1mg/kg once) commenced on day 1. Bioluminescent imaging (BLI) for whole body tumor was performed on an IVIS imaging system (Xenogen, Caliper Life Sciences) or IVIS Spectrum (Perkin Elmer) and analysed using Living Image (Perkin Elmer). Bone marrow tumor burden was assessed by flow cytometry using GFP expression (for 5TGM1, MM1.S) or APC conjugated 5T2 idiotype antibody (5T2MM)⁴². See supplemental methods for details.

MicroCT analysis of bone structure in myeloma bearing mice

Femora and lumbar vertebra were imaged in a microCT scanner (Model 1172; Skyscan) and analysed using CTAn software (Skyscan)⁴⁷, and detailed in supplemental methods.

Histology and Immunohistochemistry of femora from myeloma bearing mice

To identify osteoclasts femora sections were stained for tartrate resistant acid phosphatase (TRAP). Immunohistochemistry was used to identify tumor cells with CD138 and osteocytes with sclerostin protein as described in supplemental methods ⁴⁷. Osteomeasure (Osteometrics) was used to quantify osteoblasts and osteoclast parameters on endosteal surfaces and dynamic osteoblast activity on trabecular surfaces using standard methods ^{20,23,48,49}.

Compression testing of L4 vertebra

L4 vertebrae were bio-mechanically tested by compression testing, on an Instron 5944 load frame (Instron, MA, USA) and data collected using BlueHill 3 (Instron).

Statistical analysis

Data was analyzed using Prism (GraphPad). One-way ANOVA and multiple comparisons were performed using Tukey's correction. Unpaired t-tests were performed when comparing two populations. A two-sample Student's t-test using the R function was performed when studies involved small groups of independent samples. All data are expressed as mean $\pm$ SEM. Details for RNA-seq and microarray analyses are listed in supplemental methods.

Results

Osteocytes but not myeloma cells express sclerostin

To demonstrate that sclerostin expression is restricted to bone tissue and not present in myeloma cells, we examined sclerostin expression in tumor cells from patients with myeloma. We analysed microarray datasets from CD138⁺ plasma cells isolated from 630 patients with untreated multiple myeloma, 82 individuals with relapsed myeloma and 54 myeloma cell lines⁵⁰ D138⁺ bone marrow plasma cells (BMPC) from 10 normal donors, memory B cells (MBC) and polyclonal plasmablasts (PPC) served as controls (Figure 1A). A cohort of CD138⁺ plasma cells isolated from 263 patients with myeloma and 19 myeloma cells lines along with BMPC, MBC and PPC were also examined by RNAseq analysis (Figure 1B). Dickkopf-1 (*DKK1*) was expressed by CD138⁺ myeloma cells isolated from patients with myeloma and human myeloma cell lines, but not normal bone marrow plasma cells nor B cell lineage cells (Figure 1A and B). Sclerostin was not expressed by myeloma cells, myeloma cell lines, or other B-lineage cells, (Figure 1A and B). Immunohistochemical analysis of 5TGM1-eGFP myeloma cells confirmed the absence of sclerostin in murine myeloma cells (Figure 1C). Furthermore, analysis of the sclerostin protein in media conditioned by human myeloma cells (MM1.S and OPM2), murine myeloma cells (5TGM1-eGFP) and primary osteocyte cultures demonstrated sclerostin production by osteocytes but not myeloma cells (Figure 1D). RNAseq analysis of osteocytes isolated from non-tumor bearing murine bones revealed osteocyte enrichment of genes encoding the soluble Wnt antagonists, sclerostin (*Sost*), dickkopf1 (*Dkk1*) soluble frizzled related proteins 1 and 2 (*Sfrp1* and *Sfrp2*) and frizzled-b (*Frzb*), compared to bone marrow (Figure 1E). These Wnt antagonists were also expressed in osteocytes isolated from mice bearing 5TGM1-eGFP cells. Despite a reduction in *Sost* and *Dkk1* mRNA expression in osteocytes isolated from the

bones of 5TGM1-eGFP-bearing mice compared to naïve non-tumor bearing mice (Figure 1F), immunohistochemical analysis demonstrated sclerostin protein was present in osteocytes from naïve and disease-bearing mice (Figure 1G). Together these data demonstrate that sclerostin is expressed by osteocytes but not myeloma cells, supporting sclerostin as a tumor-independent regulator of bone formation in myeloma.

Inhibiting sclerostin prevents myeloma bone disease

To investigate whether inhibition of sclerostin prevents the development of myeloma bone disease we treated non-myeloma bearing mice, and mice-bearing either murine 5TGM1-eGFP or human MM1.S myeloma cells, with an anti-sclerostin antibody and measured changes in bone mass by microCT analysis (Figure 2A). Treatment of naïve C57BL/KaLwRijHsd mice with an anti-sclerostin antibody increased trabecular bone volume and trabecular thickness, as well as the volume and thickness of cortical bone in the femur and lumbar vertebra (Figure 2B-E, supplemental Table 1). This was replicated in two independent experiments, confirming the anti-sclerostin antibody exerts direct anabolic effects on bone.

Injection of C57BL/KaLwRijHsd mice with 5TGM1-eGFP murine myeloma cells resulted in a significant decrease in femur trabecular volume and reduction in trabecular number (Figure 2B-E, supplemental Table 1). MicroCT analysis of the lumbar vertebra, a common site of fracture in patients with myeloma, showed that 5TGM1-eGFP cells also caused bone loss at this site (Figure 2B-E and supplemental Table 1). Anti-sclerostin treatment prevented 5TGM1-eGFP induced bone loss and increased trabecular and cortical bone thickness in both the femur and vertebrae (Figure 2B-E and supplemental Table I).

To determine if the inhibition of sclerostin also prevents bone loss induced by human myeloma cells we injected NOD.Cg-Prkdcscid/Il2rgtm1Wjl/SzJ (NSG) mice with either human MM1.S-Luc-eGFP myeloma cells, or saline, via the tail vein, and treated them with anti-sclerostin antibody or control buffer (Supplemental Figure 2A). Anti-sclerostin treatment increased trabecular bone volume and trabecular number in the femur and vertebra of, NSG non-myeloma bearing mice in a similar manner to C57BL/KaLwRijHsd mice (Supplemental Figure 2). Injection of human MM1.S-Luc-eGFP myeloma cells induced bone loss and a reduction in trabecular thickness and number (Supplemental Figure 2B, C). Anti-sclerostin treatment prevented the bone loss in both femur and vertebra (Supplemental Figure 2).

To determine the mechanism underlying the ability of anti-sclerostin to prevent myeloma bone disease we performed histomorphometric analysis of C57BL/KaLwRijHsd mice bearing 5TGM1-eGFP cells. Consistent with previous studies osteoclast number and surface were increased in C57BL/KaLwRijHsd mice bearing 5TGM1-eGFP cells (Figure 3A and 3B)¹¹. Furthermore, the presence of tumor cells resulted in a decreased number of osteoblasts and the extent of endosteal bone surface covered by osteoblasts (Figure 3A and 3C), consistent with the uncoupling of bone resorption and bone formation that occurs in patients with myeloma. Treatment with anti-sclerostin antibodies increased both osteoblast number and the surface occupied by osteoblasts compared to control, but did not affect osteoclastic resorption (Figure 3A-C). To investigate osteoblast function we injected mice with calcein 6 and 2 days before sacrifice. The distance between the two time-separated calcein labels was used to determine the mineral apposition rate (MAR) and bone formation rate (BFR). Consistent with the reduced osteoblast numbers in C57BL/KaLwRijHsd mice bearing 5TGM1-

eGFP cells osteoblast mineralizing surfaces (MS), MAR and BFR were all decreased (Figure 3D and E). Anti-sclerostin antibody treatment prevented 5TGM1-eGFP-induced suppression of MAR, MS and BFR, normalizing bone formation to levels seen in control, non-myeloma bearing mice. These data demonstrate that anti-sclerostin antibody treatment does not affect the increased osteoclastic resorption but does prevent myeloma-cell mediated inhibition of bone formation. This suggests that anti-sclerostin antibody treatment prevents myeloma bone disease by ensuring the coupling between bone resorption and formation.

Inhibiting sclerostin stops osteolytic lesion formation

Whilst generalized bone loss is a common feature in myeloma, one of the defining features in patients with multiple myeloma is the development of osteolytic bone lesions. To investigate whether inhibiting sclerostin can prevent development of osteolytic lesions we used the 5T2MM syngeneic model, which develops osteolytic bone lesions similar to those seen in patients⁴². Injection of 5T2MM murine myeloma cells into C57BL/KaLwRijHsd resulted in the development of defined osteolytic bone lesions (Figure 4A-D). Histological analysis demonstrated that TRAP positive osteoclasts on the cortico-endosteal surface were associated with multiple steps in the development of bone lesions. This included sites of initial cortical erosion (Figure 4Bii 1), lesion development (Figure 4Bii 2), and overt osteolytic lesions that penetrate the cortical bone (Figure 4Bii 3). Osteoblasts were absent from these sites. MicroCT analysis demonstrated the presence of clearly defined bone lesions not seen in naïve mice (Figure 4C). Importantly, anti-sclerostin treatment reduced the number of osteolytic bone lesions in the femora of 5T2MM-bearing mice (Figure 4D).

The impact of anti-sclerostin antibody treatment on generalized bone loss induced by 5T2MM myeloma cells was also examined. Injection of 5T2MM murine cells resulted in a significant decrease in trabecular and cortical bone in the femur. Anti-sclerostin treatment prevented the 5T2MM-induced bone loss in the femur and increased bone volume in 5T2MM bearing vertebra (supplemental Figure 3A and B and supplemental Table IIIA and IIIB). This is consistent with data from the 5TGM1-eGFP and MM1.S-Luc-eGFP models and confirms the robust anti-sclerostin response.

Inhibiting sclerostin increases resistance to fracture

Pathological fracture is a major cause of morbidity in multiple myeloma and whilst pharmacological interventions reduce further bone loss they do not increase bone strength. To investigate whether anti-sclerostin antibody treatment would increase bone strength and resistance to fracture we performed biomechanical compression testing of the vertebra from the 5TGM1-eGFP, 5T2MM mouse models and MM1.S human xenograft models after anti-sclerostin antibody treatment. In naïve non-tumor bearing C57BL/KaLwRijHsd mice and NSG mice anti-sclerostin antibody treatment increased the maximum load to failure sustained by lumbar vertebrae (L4) (Figure 5A-C). The myeloma induced bone loss in 5TGM1-eGFP, 5T2MM and MM1.S bearing mice was associated with a 37%, 30% and 51% reduction in maximum load to failure, respectively (Figure 5C). Importantly, anti-sclerostin treatment prevented the reduction in bone strength in each model (Figure 5C). In 5TGM1-eGFP and MM1.S anti-sclerostin treatment returned the maximum load to failure to naïve control levels, whereas, values were significantly higher in the 5T2MM model (Figure 5C).

Additive effects of anti-sclerostin and zoledronic acid

Bisphosphonates, which inhibit bone resorption are the standard of care for patients with symptomatic multiple myeloma; however the effect of combining an agent that stimulates bone formation with a bisphosphonate that inhibits bone resorption is unknown. The consequence of combining the anti-sclerostin antibody with the bisphosphonate, zoledronic acid, was investigated in the 5TGM1-eGFP model (Figure 6A). Anti-sclerostin and zoledronic acid alone, both prevented 5TGM1-eGFP-induced bone loss in the femur and vertebra, but likely by contrasting mechanisms (Figure 6B-D and Table IV). The combination of anti-sclerostin treatment and zoledronic acid resulted in a 4-fold increase in trabecular bone volume when compared to control treated 5TGM1-eGFP bearing mice and a 142% and 112% increase respectively, when compared to mice treated with anti-sclerostin or zoledronic acid alone (Figure 6C). The combination of anti-sclerostin and zoledronic acid treatment increased trabecular thickness when compared to zoledronic acid given alone but not anti-sclerostin, whereas trabecular number was increased relative to anti-sclerostin but not zoledronic acid (Figure 6C). This difference likely reflects the different mechanism of action of the two agents with anti-sclerostin antibody treatment promoting new bone formation on existing trabecular elements and zoledronic acid preventing osteoclastic resorption and loss of individual trabeculae. The combination of anti-sclerostin antibody and zoledronic acid also increased vertebral bone volume more than anti-sclerostin antibody (37%) or zoledronic acid alone (97%) (Figure 6D and Table IV).

To determine if the additive effects of anti-sclerostin antibody and zoledronic acid treatment on bone volume were associated with increased bone strength, biomechanical testing of the lumbar vertebrae was performed. Combined treatment with anti-sclerostin and zoledronic acid increased the maximum load to failure sustained by the lumbar

vertebrae when compared to zoledronic acid treatment alone, but did not differ from anti-sclerostin treatment alone (Figure 6E). Zoledronic acid treatment alone did not increase vertebral maximum load to failure compared to control. This indicates that the increase in bone formation has the predominant effect on bone strength. Together these data demonstrate that combining anti-sclerostin antibody treatment with zoledronic acid increases bone volume and bone structural parameters beyond that seen with zoledronic acid treatment alone and this improvement is associated with an increase in bone strength and resistance to fracture.

Inhibiting sclerostin does not promote myeloma burden

Wnt signaling promotes tumor growth^{51,52} and aberrant Wnt signaling has been reported to increase myeloma cell proliferation⁵³. Soluble Wnt antagonists inhibit endogenous Wnt signaling and therefore the anti-sclerostin antibody might promote tumor proliferation by stopping Wnt suppression in the local bone microenvironment. To confirm that anti-sclerostin antibody treatment did not alter myeloma growth directly we treated MM1.S-luc-eGFP and 5TGM1-eGFP cells *in vitro* with increasing concentrations of anti-sclerostin antibody (up to 2.25µg/ml). Anti-sclerostin antibody treatment had no effect on the proliferation of these myeloma cells (data not shown). To determine whether anti-sclerostin treatment had indirect effects on myeloma burden we measured myeloma burden in the skeleton, and spleen as an extra-osseous site, in all models (Figure 7). FACS analysis of 5TGM1-eGFP cells demonstrated the presence of myeloma cells in both bone marrow and spleen of 5TGM1-eGFP-bearing mice. Importantly, there was no difference in myeloma burden in either the bone marrow or spleen of anti-sclerostin antibody treated or control mice (Figure 7A-C). Furthermore, anti-sclerostin treatment did not alter the proportion of

idiotype positive cells in either the bone marrow or spleen of the 5T2MM murine model (Figure 7D). Finally, we evaluated the effect of anti-sclerostin antibody treatment on myeloma burden in the MM1.S human xenograft model. Bioluminescence (BLI) imaging of whole body myeloma burden demonstrated a reduction in total flux in one of two experiments (Figure 7E and 7F). However, flow cytometric analysis of MM1.S-GFP⁺ cells in the bone marrow showed no difference between anti-sclerostin treatment and control treatment (Figure 7F and 7F). The reason for the discrepancy between the BLI analysis and the direct bone marrow flow cytometric assessment of myeloma burden in the MM1S model may reflect a reduction in extra-skeletal tumor growth; however, at least in the spleen tumor burden was unaltered in the MM1S model (data not shown). This more likely reflects the increase in cortical bone thickness, caused by anti-sclerostin treatment, attenuating the bioluminescence signal. Together these data demonstrate that despite profoundly effecting the bone environment, inhibiting sclerostin with the anti-sclerostin antibody does not affect myeloma burden.

Discussion

Cancers that develop in bone such as multiple myeloma, result in devastating bone disease and fractures. This is an important cause of morbidity and mortality, and a major clinical problem. Therapies that inhibit osteoclasts do not restore bone mass and thus patients continue to suffer fractures. Inhibiting tumor-derived products is also likely to have limited efficacy due to the heterogeneous behavior of tumor cells. In contrast, anabolic agents that stimulate bone formation by targeting bone-cell specific pathways offer a potential new treatment modality to treat myeloma-induced bone disease. We demonstrated that sclerostin is an osteocyte specific protein that is not expressed by myeloma cells. Treatment

of myeloma-bearing mice with an anti-sclerostin antibody (i) prevented myeloma-induced suppression of osteoblastic bone formation, (ii) prevented myeloma-induced bone loss, (iii) reduced development of osteolytic lesions and (iv) increased bone strength and resistance to fracture. Importantly, the combination of anti-sclerostin antibody and the anti-resorptive agent, zoledronic acid, increased bone mass, bone strength and resistance to fracture more than zoledronic acid treatment alone.

Soluble Wnt antagonists are pivotal regulators of bone formation and have been implicated in suppressing bone formation in myeloma⁵³. We demonstrated that the Wnt antagonists, *Dkk1* and sclerostin were strongly expressed in osteocytes, which are master regulators of bone homeostasis. *Dkk1* was also shown to be expressed by plasma cells isolated from a large cohort of patients with myeloma, supporting previous observations^{18,54}. However, sclerostin was not expressed by myeloma cells isolated from the same cohort of patients, those undergoing relapse or were refractory to disease, nor was it expressed by an extensive panel of myeloma cell lines. This contrasts with reports of sclerostin expression, detected by RT-PCR, in plasma cells isolated from a small cohort of patients with myeloma^{55,56}, the reason for which is unclear but likely reflects technical differences between studies. We also showed that both global *Dkk1* and *Sost* expression was decreased in bones isolated from mice bearing myeloma. The mechanism for this is unclear but might reflect alterations in load detected by osteocytes in response to the presence of tumor cells. Interestingly, local osteocyte production of sclerostin has been shown to be increased when osteocytes interact directly with cancer cells⁵⁷. This demonstrates that regulation of sclerostin expression in bone in the presence of tumor is complex, with both local and generalized responses, suggesting that sclerostin expression may be more heterogeneous in the

presence of myeloma. However, the strong expression of *Sost* in both myeloma-bearing and control bones provided the rationale for investigating the ability of anti-sclerostin antibody to prevent myeloma bone disease.

To establish whether inhibiting sclerostin and thus enhancing Wnt signaling would increase bone formation we treated mice bearing 5TGM1-eGFP cells with an anti-sclerostin antibody. Anti-sclerostin treatment prevented osteoblast suppression, ensuring bone formation was maintained. Inhibiting sclerostin had no effect on the myeloma-induced increase in osteoclastic bone resorption suggesting that treatment retained the coupling between bone resorption and formation. The stimulation of bone formation caused an increase in bone mass in the three models of myeloma bone disease. This was associated with increases in trabecular bone thickness demonstrating that inhibiting sclerostin resulted in new bone formation on existing bone rather than stopping further bone loss, which is seen with anti-resorptive treatments. This is supported by the recent demonstration that an alternative anti-sclerostin antibody treatment can increase bone mass in mice bearing MM1.S human myeloma cells⁵⁸. Importantly our data is consistent with the anti-sclerostin antibody induced increase in bone mass seen in models of osteoporosis^{30,31} and bone repair^{32,33}. Since osteolytic bone lesions are a hallmark of multiple myeloma we also investigated whether anti-sclerostin antibody treatment could prevent development of osteolytic lesions. We discovered that anti-sclerostin antibody treatment reduced development of osteolytic bone lesions. This has also been observed when inhibiting DKK1 and activin signaling^{20,23}. These data argue that retaining the coupling between resorption and formation, even in the presence of increased osteoclastic resorption, is sufficient to prevent development of both generalized myeloma bone disease and osteolytic lesions.

Whilst anti-sclerostin antibody treatment could offer a new approach to treating myeloma bone disease this must to be considered in the context of current gold-standard therapy, which is the anti-resorptive bisphosphonate, zoledronic acid. We showed that anti-sclerostin antibody and zoledronic acid treatment alone both prevented myeloma-induced bone loss and that anti-sclerostin antibody alone increased bone strength. Further, anti-sclerostin antibody treatment was superior to zoledronic acid at improving bone strength. The addition of anti-sclerostin antibody treatment to zoledronic acid increased bone mass over treatment with either agent alone. Importantly, the combination of both treatments also increased resistance to fracture when compared to zoledronic acid treatment alone. These findings suggest that addition of anti-sclerostin antibody treatment to zoledronic acid therapy will result in additional clinically relevant benefits above and beyond the current gold-standard of treatment.

Before anti-sclerostin antibody treatment can be evaluated in a clinical program, consideration needs to be given to a possible impact on tumor growth. Inhibiting a soluble Wnt antagonist may increase local Wnt signaling and promote tumor growth. Whilst Wnt signaling has been shown to stimulate myeloma cell proliferation *in vitro*⁵³, anti-sclerostin antibody treatment had no effect on myeloma burden in the skeleton or spleen of either the 5TGM1-eGFP, 5T2MM or MM1.S models. The absence of effects across multiple models suggests that sclerostin does not regulate pathways that control tumor growth. These data reinforce the hypothesis that sclerostin represents a tractable new therapeutic target in the treatment of myeloma bone disease.

The data presented here provide the rationale for a new approach to treating the bone disease that occurs in multiple myeloma. Inhibiting sclerostin with an anti-sclerostin antibody increases bone mass and resistance to fracture, and when combined with zoledronic acid, is superior to the current standard of care. This supports the rationale for evaluating the efficacy of anti-sclerostin treatment in clinical studies in patients with myeloma. The prevention of fractures would transform prognosis for patients with myeloma, decreasing morbidity, enhancing quality of life and ultimately reducing mortality. This new understanding has implications for treating not only patients with multiple myeloma but also other cancers that develop in the skeleton.

Acknowledgements

We thank the Biological Testing Facility for animal husbandry at the Garvan Institute of Medical Research, The Australian BioResources centre, Mossvale Australia for animal housing and the TKCC Centre for RNA sequencing. We thank Aldo Roccaro, Yawara Kawano and Shokichi Tsukamoto and Antonio Sacco from Dana-Farber Cancer Institute for assistance with sample harvests. We thank Dr Andrew Kung from Columbia University Medical Centre, New York for supplying us with the MM1.S-luc-eGFP cell line and Shruti Shah and Jenny Down, the Garvan Institute of Medical Research for histology sample preparation and immunohistochemistry staining. We thank Novartis Pharma, Basel Switzerland for supply of anti-sclerostin antibody and vehicle buffer.

P.I.C. is funded by Mrs. Janice Gibson and the Ernest Heine Family Foundation, P.A.B. by an Australian Research Council Future Fellowship and M.R.R. by the NIH/NIGMS (GM106391),

NIH/NIDDK (R24 DK092759-01 and start-up funds (MMCRI). This work was supported The Kay Stubbs Cancer Research Grant, Cancer Council NSW and by the Deutsche Forschungsgemeinschaft (SFB/TRR79, TP B1, B12, M9) and the German Federal Ministry of Education (“CLIOMMICS” (01ZX1309), “CAMPSIMM” (01ES1103)).

Data availability

Myeloma patient sample gene expression data are deposited in ArrayExpress under accession numbers E-MTAB-4715, E-MTAB-4717, E-TABM-937, and E-TABM-1088. The osteocyte-enrichment gene expression data is available in the Array Express repository (E-MTAB-5533). Data for gene expression analysis of osteocytes from tumor burdened bones relative to naive samples is available in the Array Express repository (E-MTAB-5555).

Author Contributions

P.I.C, T.G.P and M.M.M conceived the study. M.R.R, M.M.M., S.E. Y., S.T.M and A.S and D.H, designed and performed experiments, and analyzed data. R.L.T., J.A.P., M.K.S., T.L.C., A.M., L.M.T.L, D.A. and C.F and H.F performed experiments. P.A.B., D.G.L., K.V., I.K., I.M.G. , M.K., J.H.D.B., G.R.W., O.O.B., and T.G.P. analyzed and interpreted data. P.A.B., J.H.D.B., G.R.W. revised the manuscript and M.M.M., and P.I.C., wrote the manuscript. All authors have read and approved the manuscript.

Conflict of Interest Statement:

Michaela Kneissel and Ina Kramer are employees of Novartis Pharma. The other authors have no conflicts of interest.

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Figure legends

Figure 1. Sclerostin is expressed by osteocytes but not myeloma cells. (A) Gene expression profiling using DNA-microarray analysis of memory B-cells (MBC, n=5), *in vitro* generated polyclonal plasmablastic cells (PPC, n=5), normal bone marrow plasma cells (BMPC, n=10), malignant plasma cells from patients with newly-diagnosed (MM, n=630) and relapsed/refractory myeloma (MMR, n=82), as well as human myeloma cell lines (HMCL, n=54). Grey dots represent the absence of expression and black dots the presence of expression according to the PANP-algorithm. (B) RNA-sequence analysis of *DKK1* and *SOST* of PPC (n=4), MBC (n=4), BMPC (n=10) and MM (n=263), as well as HMCL (n=19). *DKK1* (204602_at) is found expressed by the majority of malignant plasma cell samples from previously untreated and relapsed myeloma patients. In contrast, *SOST* (223869_at) expression is absent in all normal and malignant plasma cells. (C) Immunohistochemical staining for CD138 (top panel) and Sclerostin (bottom panel) in bone marrow of naïve and mice bearing 5TGM1-eGFP myeloma cells. Slides were scanned on Scanscope CS2 (Aperio) up to X40 original magnification and images captured using Aperio Imagescope at X20 digital magnification. (D) Sclerostin protein measured in media conditioned by MM1.S, and OPM2 human myeloma cells lines, 5TGM1 murine myeloma cells and primary osteocytes, (n=4, **** p<0.0001, data are mean ± 1 SEM). (E) Density plot highlighting the osteocyte-enriched expression of secreted Wnt-signaling antagonists (N=6). (F) Tumor burden significantly alters the expression of the secreted Wnt-signaling pathway antagonists *Sost*, *Dkk1* and *Frzb*

(Multiple-comparison adjusted p-value * < 0.05, ** < 0.01, **** < 0.0001). N= 6, Data are mean $\pm$ 1 SD. (G) Immunohistochemical staining for Sclerostin in osteocytes from naïve and 5TGM1-bearing mice, arrow heads denote positive staining osteocytes with positive stained canaliculi (inset) . Slides were scanned on Scanscope CS2 (Aperio) up to X40 original magnification and images captured using Aperio Imagescope at X40 digital magnification. The scale bar is 50 μ m.

Figure 2. Anti-sclerostin treatment prevents myeloma induced bone loss in the 5TGM1 murine model of myeloma. (A) Schematic depicting study design for 5TGM1 studies. (B) 3D microCT reconstructions of distal femora representing each treatment group in experiment 2. BM- bone marrow, CB- cortical bone, TB – trabecular bone, GP- growth plate. (C) Dot plot of Trabecular bone volume/total volume ratio (BV/TV) from distal femora of experiment 1 (n=5-6) and experiment 2 (n=8), data are mean $\pm$ 1 SEM, **** p<0.0001, * p<0.05, ** p<0.01. (D) 3D microCT reconstructions of L4 vertebrae representing L4 from each treatment group in experiment 2. BM- bone marrow, CB- cortical bone, TB – trabecular bone, GP- growth plate. (E) Dot plot of Trabecular bone volume/total volume ratio (BV/TV) from lumbar vertebra 2 (L2) from experiment 1 (n=6) and lumbar vertebra 4 (L4) from experiment 2 (n=8), data are mean $\pm$ 1 SEM, * p<0.05, ** p<0.01, *** p<0.001.

Figure 3. Anti-Scl treatment prevents 5TGM1 myeloma-induced suppression of bone formation (A) TRAP/Hematoxylin stained histological sections from representative mice from each treatment group showing osteoblasts on the endocortical bone surface (inset,

blue arrows) and osteoclasts reacted for TRAP and stained red (white arrows). BM – bone marrow, CB – cortical bone, scale bar is 200 μ m. Slides scanned on Scanscope CS2 (Aperio) up to X40 original magnification and images captured using Aperio Imagescope at X20 digital magnification. (B) Dot plots of osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone surface (N.Oc/BS), data are mean $\pm$ 1 SEM, * p <0.05. ** p <0.001. (C) Dot plots of osteoblast surface/bone surface (Ob.S/BS) and osteoblast number/bone surface (N.Ob/BS), N= 8, data are mean $\pm$ 1 SEM, * p <0.05. ** p <0.001. (D) Representative sections from each group showing mineralized bone surfaces labelled with calcein. Images captured on Leica DMI 5500 at X2.5, using LAS X software (Leica), scale bar 500 μ m. Double label bone surfaces were used to measure mineral apposition rate (inset - red arrows show the distance between two labels separated by a fixed time interval, images captured on Leica DMI 5500 at X40 using LAS X software (Leica), scale bar 20 μ m). BM – bone marrow, CB – cortical bone, GP - growth plate, ES – endosteal bone surface, PS – periosteal bone surface. (E) Dot plots of mineral apposition rate (MAR), mineralizing surface (MS) and bone formation rate (BFR). N= 5, data are mean $\pm$ 1 SEM ** p <0.01, * p <0.05, ## p <0.01 (students ttest), # p <0.05 (students ttest).

Figure 4. Anti-Scl treatment reduces formation of osteolytic bone lesions. (A) Schematic describing the study design for investigations of bone lesions in 5T2MM-bearing mice. (B) (i) TRAP/Hematoxylin stained histological section of a tibia from a 5T2MM bearing mouse. Slides scanned on Scanscope CS2 (Aperio) up to X40 original magnification and images captured using Aperio Imagescope X2 digital magnification. Scale bar is 500 μ m, black arrows point to cortical lesions. (ii) Higher magnification (X12 and X30) images which demonstrate the temporal development of cortical bone lesions in the 5T2MM model. 1. Initial erosion of

the endosteal surface of the cortical bone (CB) by red TRAP positive osteoclasts (black arrows) adjacent to tumor cells in the bone marrow (BM), 2. Formation of a cortical bone lesion (blue dotted line) by osteoclasts (black arrows). 3. A cortical lesion which has penetrated the cortex with the tumor (blue dotted line) extending into the surrounding soft tissue. Top panel scale bar is 100µm, bottom panel scale bar is 50 µm. (C) Representative 3D reconstruction of tibia from each treatment group showing full cortex penetration lesions (white arrows) in the 5T2MM + control and 5T2MM + Anti-Scl groups (D) Dot plots of Lesion number per bone for femur and tibia. N= 7-8 per group, data are mean $\pm$ 1 SEM, *p<0.05.

Figure 5. Anti-Scl treatment prevents loss of bone mechanical strength in myeloma burdened bones. (A) Images demonstrating the positioning of L4 vertebrae for compression testing between the load cell and the custom designed jig in the Instron 5944. (B) Representative load displacement curve of a L4 vertebra from the naïve control group showing the outcomes of stiffness, yield point and maximum load at the first point of failure (C) Dot plots of maximum load for L4 vertebrae from the 5TGM1-eGFP (i) MM1.S (ii) and 5T2MM (iii) models of myeloma. N= 8 5TGM1, N=5-9 MM1.S, N=6-8 5T2MM, data are mean $\pm$ 1 SEM *p<0.05. ** p<0.01, *** p<0.001, **** p<0.0001, # p<0.05 Students t-test.

Figure 6. Combination treatment with Zoledronic Acid (ZA) and Anti-Sclerostin (Anti-Scl) treatment is superior in preventing bone loss in the 5TGM1 murine model of myeloma. (a) (A) Schematic describing the study design for investigations of bone loss in 5TGM1-eGFP bearing mice treated with both Anti-Scl and ZA. (B) Examples of three-dimensional (3D) microCT reconstructions of distal femora from each treatment group. (C) Dot plots of the ratio of trabecular bone volume to tissue volume (BV/TV), trabecular thickness (Tb.th), trabecular number (Tb.N) and cortical bone thickness (Ct.Th) in femora for each treatment

group. N=8 per group, data are mean $\pm$ 1 SEM, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001. (D) Dot plot of L4 vertebra ratio of bone volume to tissue volume (BV/TV) for each treatment group, data are mean $\pm$ 1 SEM # p <0.05 (t-test), ** p <0.01, *** p <0.001, **** p <0.0001. Table IV. Structural parameters from microCT scans of L4 vertebrae, N=8 per group, data are mean $\pm$ 1 SEM, ^a p <0.001, ^d p <0.05 compared to naïve + control. ^b p <0.01, ^e p <0.001, ^f p <0.05 compared to 5TGM1 + control, ^g p <0.001 compared to 5TGM1 +ZA and ^h p <0.001 compared to 5TGM1 + Anti Scl. (E) Dot plot of maximum load from L4 vertebra for each treatment group, N=8 per group, data are mean $\pm$ 1 SEM, # p <0.05 (t-test), ** p <0.01, *** p <0.001, **** p <0.0001.

Figure 7. Anti-Scl treatment does not impact tumor burden. (A) Representative flow cytometry plots of 5TGM1-eGFP control treated bone marrow and 5TGM1-eGFP Anti-Scl treated bone marrow showing the percentage of 5TGM1-eGFP⁺ cells in the sample (black box). (B) Dot plots of bone marrow tumor burden as a percent of total bone marrow cells (% GFP⁺) in experiments 1 and 2 of the 5TGM1 study, N=6 expt 1 and N=8 Expt 2, data are mean $\pm$ 1 SEM. (C) Dot plots of spleen tumor burden as a percent of total cells (% GFP⁺) in experiments 1 and 2 of the 5TGM1 study, N=6 expt 1 and N=8 Expt 2, data are mean $\pm$ 1 SEM. (D) Dot plots of bone marrow and spleen tumor burden (% Idiotyp⁺) in the 5T2MM study, N=6-7, data are mean $\pm$ 1 SEM. (E) Dot plots of whole body tumor burden (Total flux p/s) and bone marrow percent GFP⁺ cells in MM1.S experiment 1. N= 9 per group, data are mean $\pm$ 1 SEM. * p <0.05. (F) Dot plots of whole body tumor burden (Total flux p/s) at 3 weeks and bone marrow percent GFP⁺ cells in MM1.S experiment 2. N= 8-9 for whole body tumor and N=5-9 for bone marrow tumor, data are mean $\pm$ 1 SEM.

Figure 1

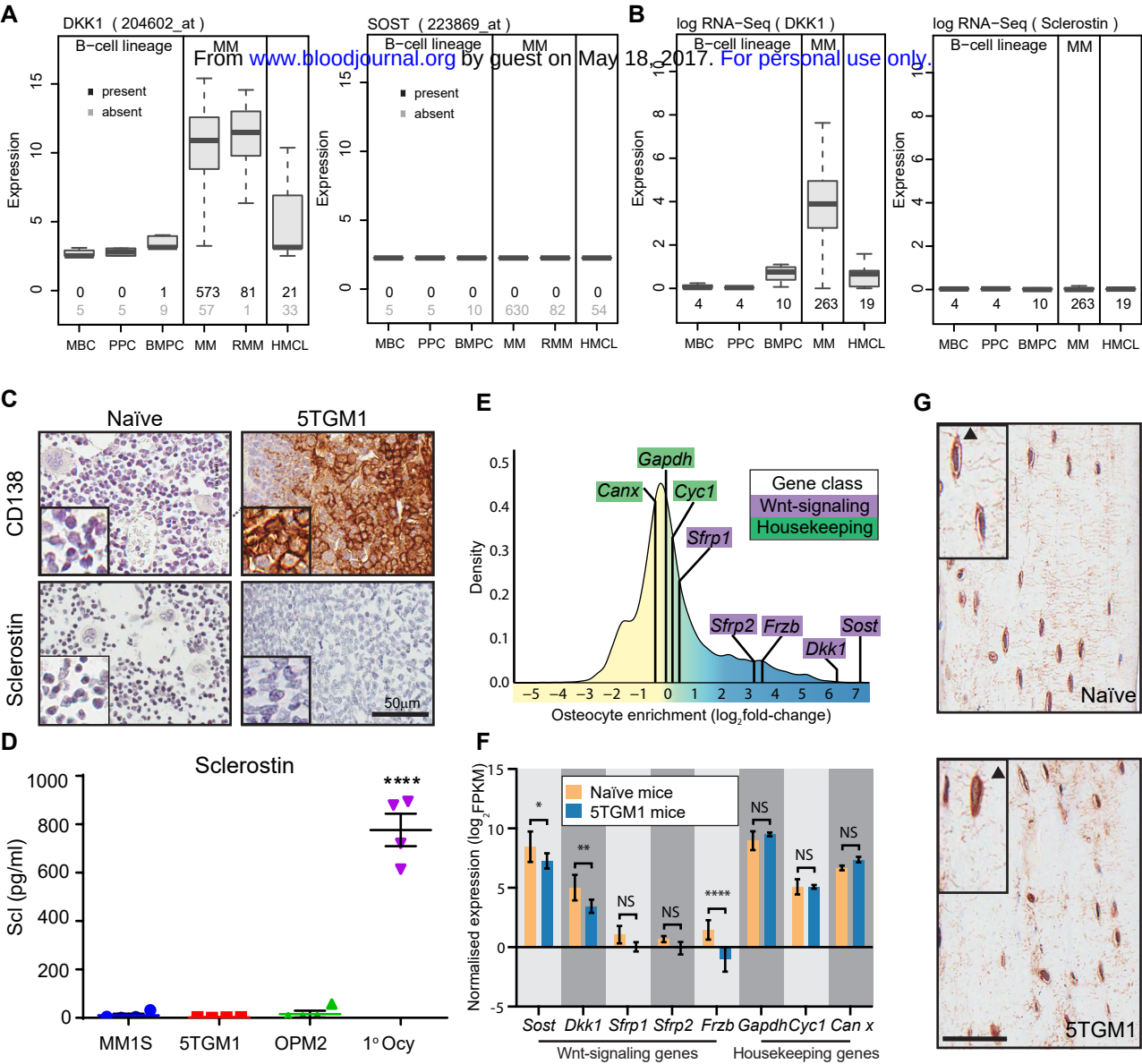


Figure 2

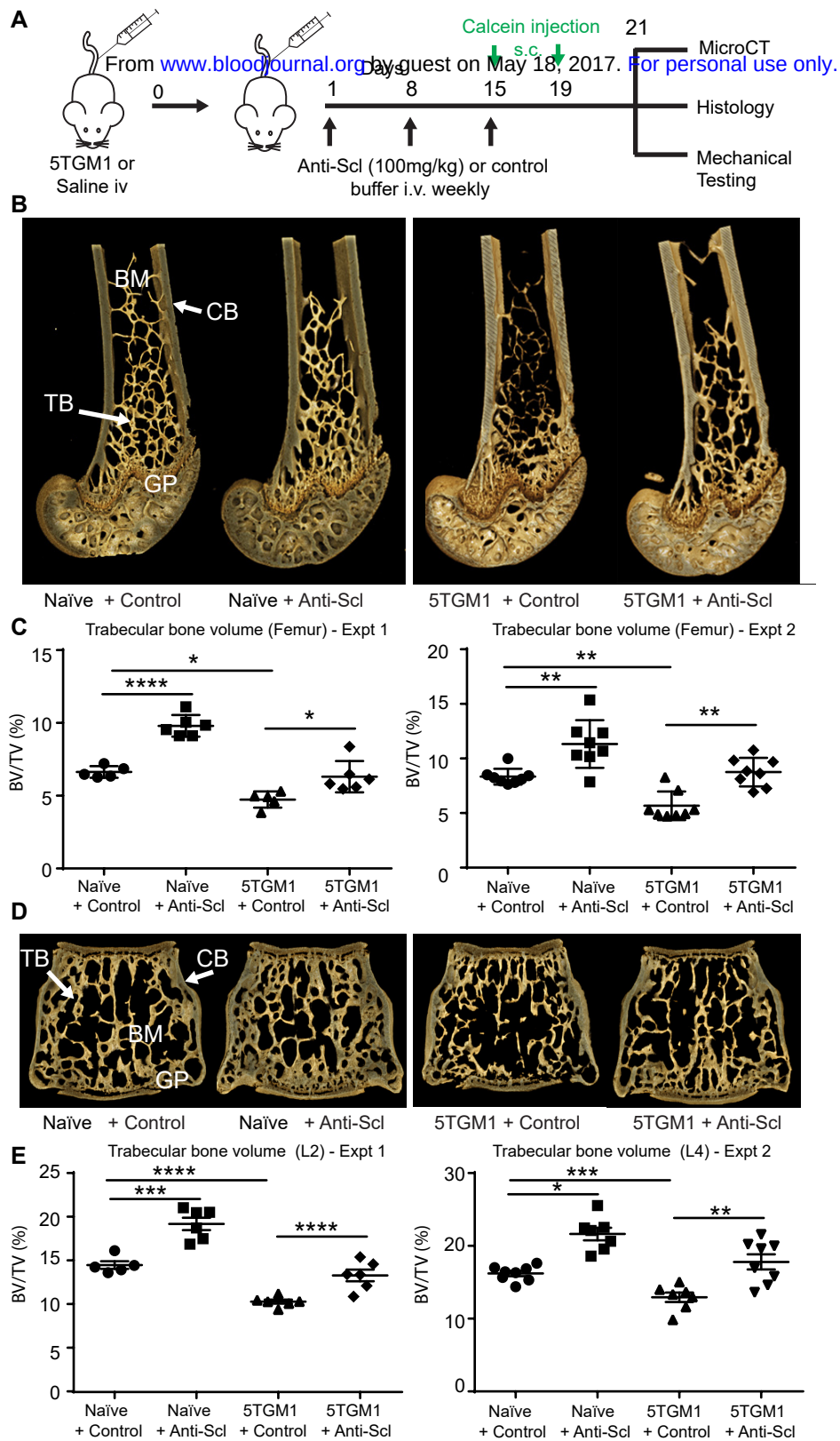


Figure 3

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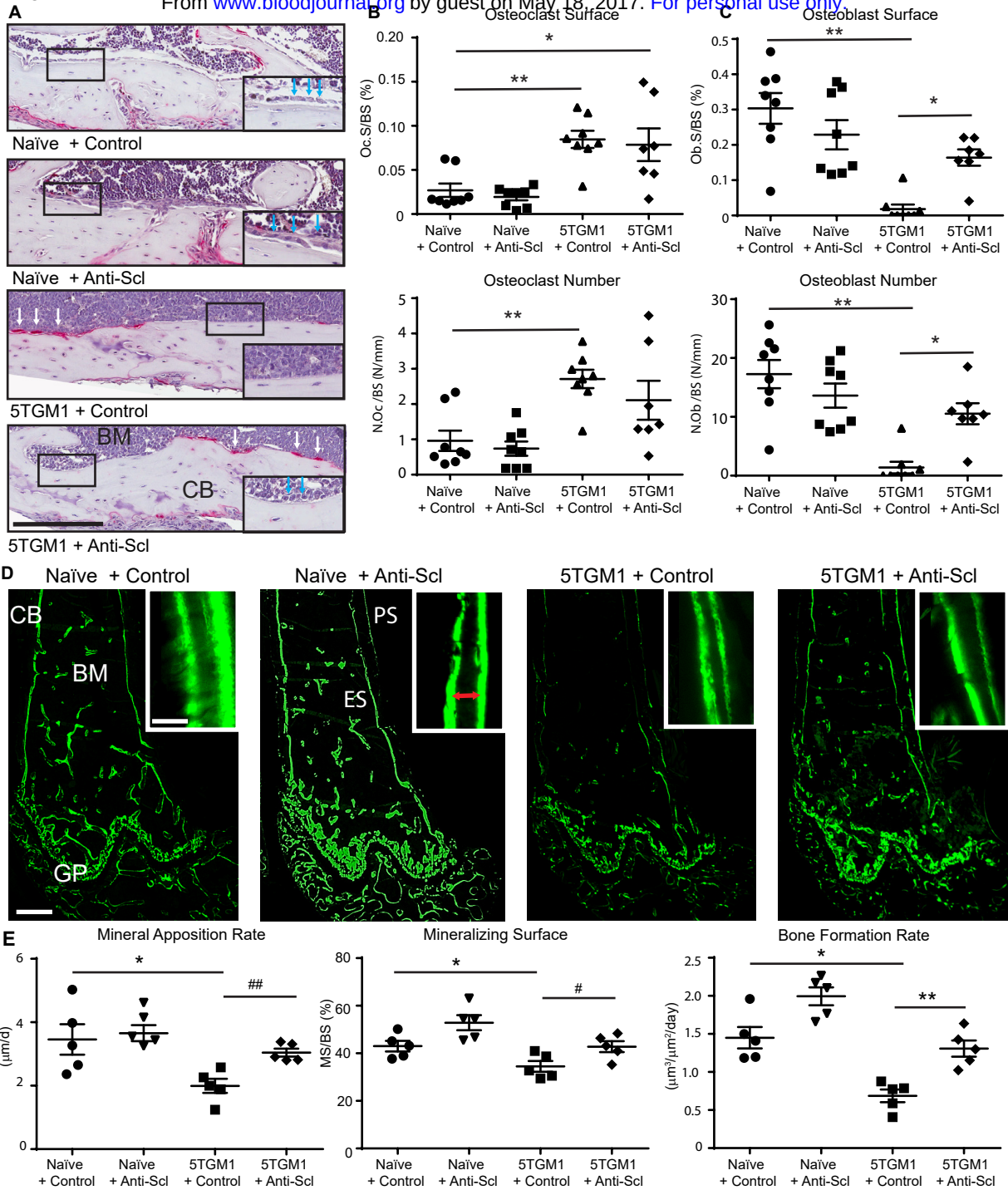


Figure 4

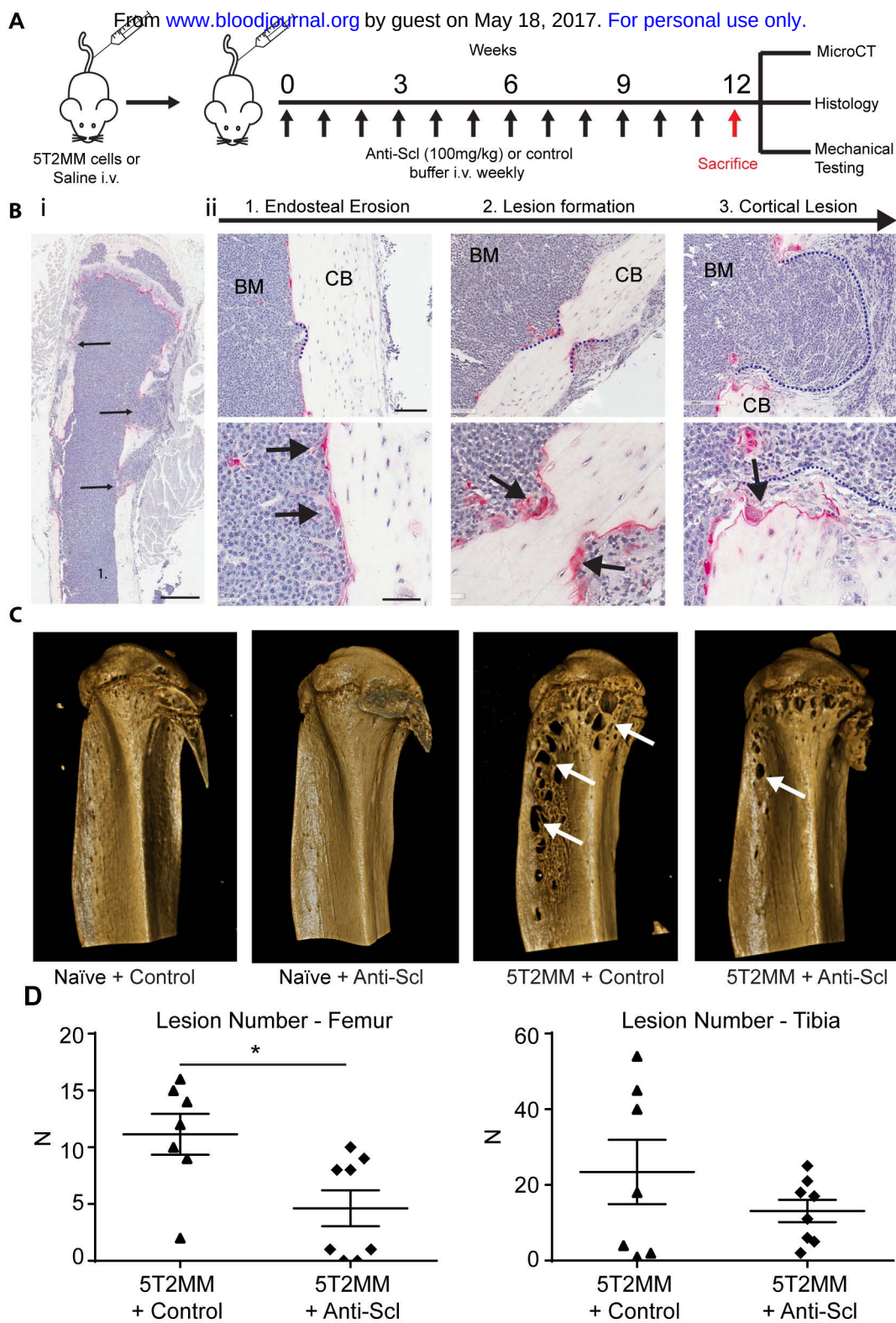


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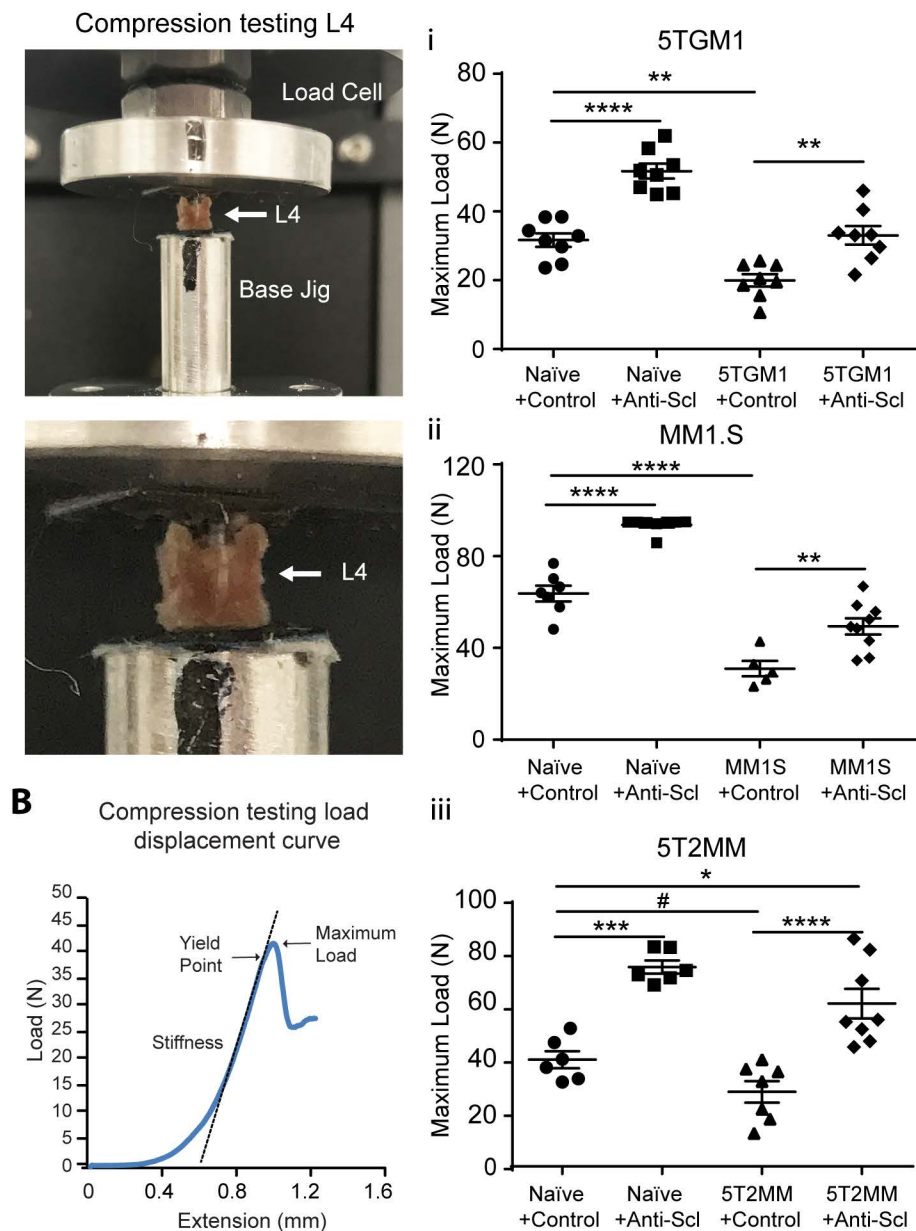


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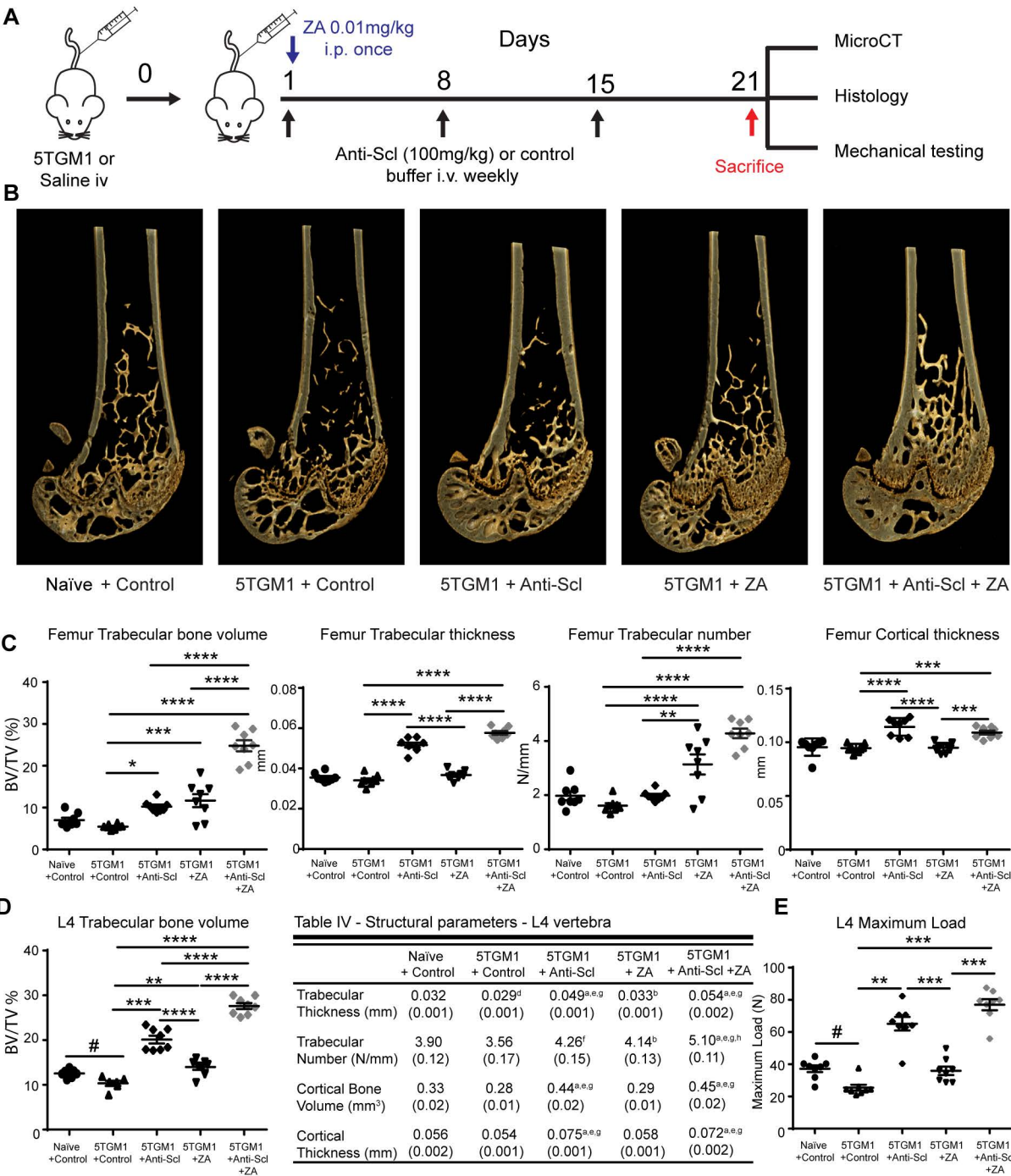
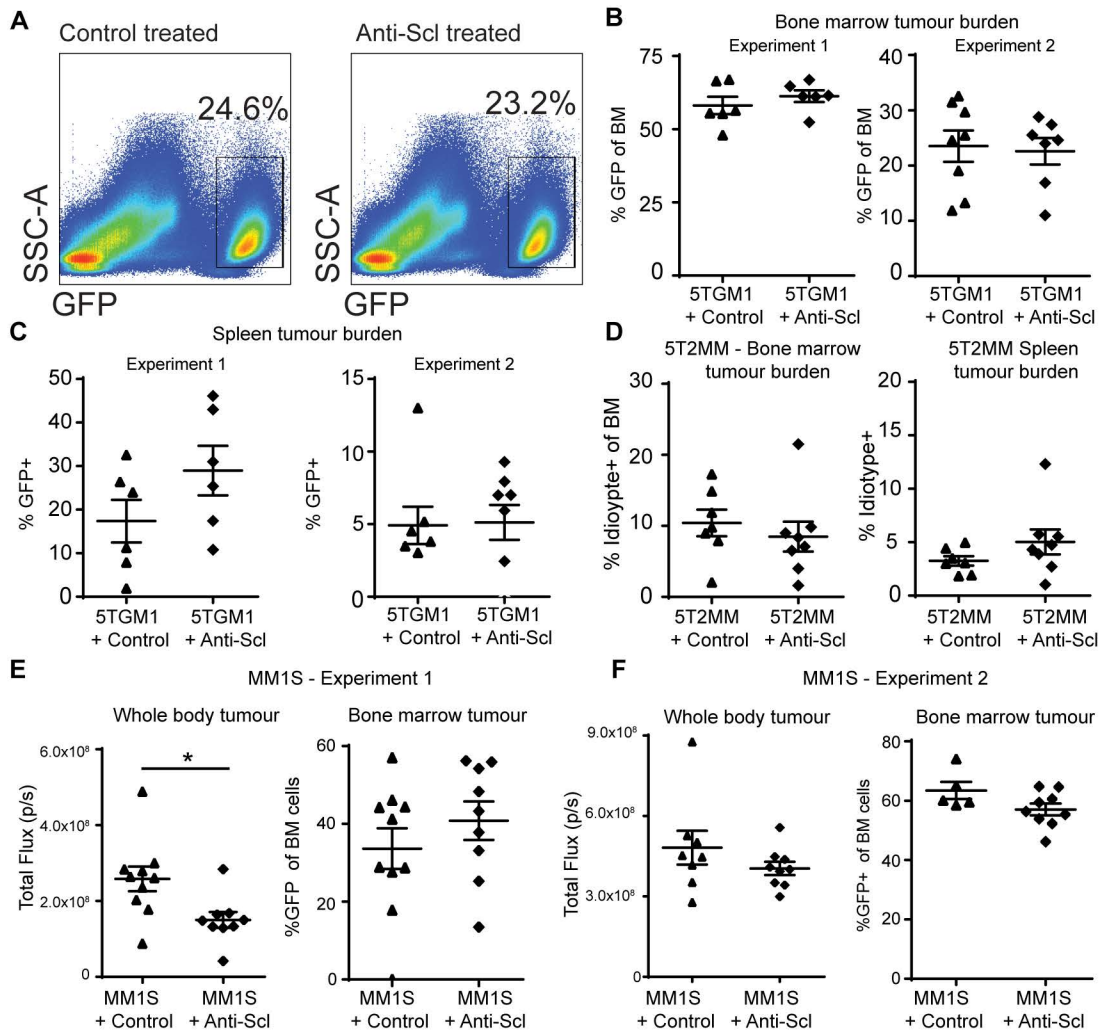


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Prepublished online May 17, 2017;
doi:10.1182/blood-2017-03-773341

Inhibiting the osteocyte specific protein sclerostin increases bone mass and fracture resistance in multiple myeloma

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